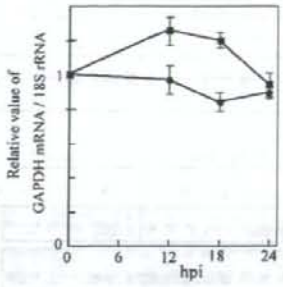

Fig. 3. The mRNA levels of GAPDH in B95a cells infected with MeV-HL. The expression levels of GAPDH mRNA in mock- (closed circle) or MeV-HL- (closed square) infected B95a cells were determined using one-step real-time RT-PCR and shown as means of three experiments.

mRNA to 18S rRNA was not suppressed by MeV-HL infection. Since the transcription level of GAPDH did not decrease during the period of shut-off, the inhibition of host protein synthesis in MeV-HL-infected B95a cells is suggested to occur at a post-transcription stage.

3.3. Modification of eIF4G, eIF4E and 4E-BP1 by MeV-HL infection

Previous reports on other viruses indicated that cap-binding proteins such as eIF4G, eIF4E, and 4E-BP1 are major targets for the virus-induced shut-off of host protein synthesis [6]. Picornavirus (except for cardiovirus) cleaved the eIF4G, which is one of the subunits of cap-binding complex eIF4F, by viral protease, $2A^{pro}$ [15–17]. This cleavage results in inhibition of binding of eIF4F to cap of host mRNA. In adenovirus- or influenza virus-infected cells dephosphorylation of eIF4E, which is a cap-binding protein, is observed [18–20]. Phosphorylation of eIF4E increases its affinity for the cap of mRNA [21]. Therefore, dephosphorylation of eIF4E by viral infection results in decrease of the affinity for the cap and may inhibit cap-dependent translation. The eIF4E is also regulated by eIF4E-binding protein-1 (4E-BP1). Encephalomyocarditis virus (EMCV), poliovirus and VSV dephosphorylate 4E-BP1 [22,23]. Dephosphorylated 4E-BP1 binds to eIF4E strongly, resulting in the suppression of cap-dependent translation. Considering these functions of cap-binding proteins, we first examined the characteristics of these three proteins in B95a cells at intervals after inoculation with MeV-HL. As shown in Fig. 4a, eIF4G was not cleaved throughout the course of MeV-HL-infection. Moreover, dephosphorylation was not observed for eIF4E and 4E-BP1 until 36 hpi (Fig. 4b and c). These results indicate that eIF4G and eIF4E are not involved in MeV-HL-induced shut-off of host protein synthesis as their function appear to be intact.

3.4. Accumulation of phosphorylated eIF2α in MeV-HL-infected B95a cells

Given that the modification of eIF4F was not detected in MeV-HL-infected B95a cells, we then focused on phosphorylation of eIF2α. It was reported that the interferon-inducible

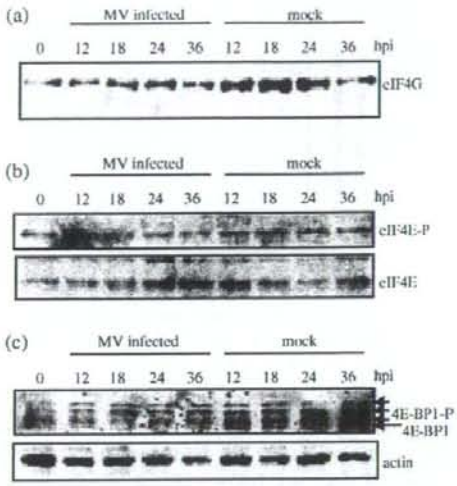

Fig. 4. Modification of the components of the eIF4F complex in MeV-HL-infected B95a cells. Expression levels of eIF4G and the phosphorylated states of eIF4E and 4E-BP1 in mock- or MeV-HL-infected B95a cells were determined by Western blotting assay. (a) Cell lysates were subjected to 6% SDS-PAGE and the proteins were transferred onto nitrocellulose membrane. The eIF4G was detected by Western blotting assay using rabbit antibody against eIF4G. (b) Detection of phosphorylated eIF4E by Western blotting assay using antibodies against phospho-eIF4E at serine 209 (upper panel) or eIF4E (lower panel) antibody. (c) The phosphorylation state of 4E-BP1 in the mock- or MeV-HL-infected B95a cells was examined by Western blotting assay using goat antibody against 4E-BP1. The quantity of protein was normalized to that of β-actin determined by goat antibody against β-actin.

PKR, known as a kinase that phosphorylate eIF2α at serine 51 [24], is activated by dsRNA during the infection with RNA viruses and involved as a host defense in preventing the translation of viral transcripts, concomitantly with the inhibition of host mRNA translation [25]. Considering such function of eIF2α, we analyzed the phosphorylation state of eIF2α by Western blotting assay with an antibody against phospho-eIF2α or eIF2α (Fig. 5). The ratio of phospho-eIF2α in MeV-HL-infected B95a cells increased after 12 hpi and reached a maximum (3.9-fold increase) at 18 hpi, although the effect was lower than that observed in the control with thapsigargin that induces eIF2α phosphorylation through ER-stress [26]. Thereafter, the ratio was sustained until 36 hpi. Phosphorylation of eIF2α occurred at a relatively early stage of infection, prior to the clear inhibition of host protein synthesis. The acceleration of host shut-off was accompanied by an increase in phosphorylation of eIF2α.

3.5. Suppression of MeV-HL-induced phosphorylation of eIF2α in B95a cells stably expressing S51A mutated human eIF2α

Involvement of phosphorylation of eIF2α in shut-off of host protein synthesis in MeV-HL-infected B95a cells was examined using B95a cells that stably express

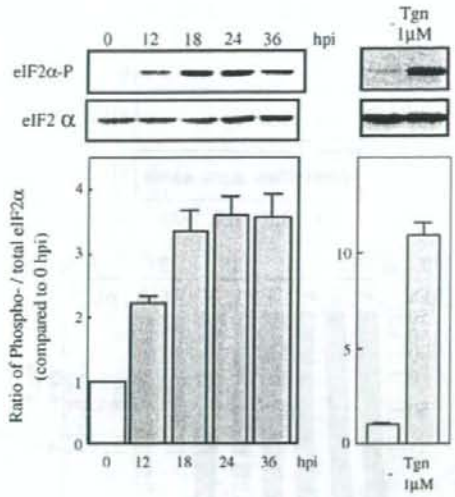

Fig. 5. Phosphorylation of eIF2α in MeV-HL-infected B95a cells. Lysates of mock- or MeV-HL-infected B95a cells were analyzed by Western blotting assay using antibodies against eIF2α-P (phosphorylated form at serine 51) or eIF2α on the same membrane (left, top). Quantitation of the relative amounts of phospho-eIF2α and the total eIF2α was measured and the ratio of phosphorylated eIF2α to total eIF2α (vs. 0 hpi) is shown as a bar graph (left, bottom). As a control experiment, B95a cells were treated with 1 μM thapsigargin for 1 h and shown as the same way as left column (right). The values are means ± standard errors of triplicate determinations.

eIF2α mutant, of which phosphorylation site serine 51, was replaced to alanine (B95a-2αS51A) and is able to inhibit the phosphorylation of endogenous eIF2α [27]. As a control experiment, the B95a cells that stably express wild type of eIF2α (B95a-2αWT) were used. The phosphorylation rate of total eIF2α in B95a-2αWT cells apparently increased at 18 hpi (Fig. 6a), whereas that in B95a-2αS51A cells was significantly inhibited. Shut-off of host protein synthesis was noted from 12 hpi in B95a-2αWT cells similar to the parental B95a cells. In B95a-2αS51A cells, shut-off of host protein synthesis was suppressed until 18 hpi (Fig. 6b and c) and the rate of host protein synthesis was higher than that of B95a-2αWT cells throughout the test period. These results indicate that the phosphorylation of eIF2α involved in shut-off of host protein synthesis in MeV-HL-infected B95a cells.

4. Discussion

In the present study, we showed that MeV-HL induces the shut-off of host protein synthesis in B95a cells. This shut-off is not specific feature of MeV-HL because other field isolates, 9106 and 9301 strain, also induce the shut-off in B95a cells. On the other hand, MeV-Ed that has been reported not to induce the shut-off in epithelial or epithelial-like cells did not induce the shut-off of host protein synthesis in B95a cells as well. Therefore, the inability of MeV-Ed to induce shut-off is suggested to be a characteristic of this strain

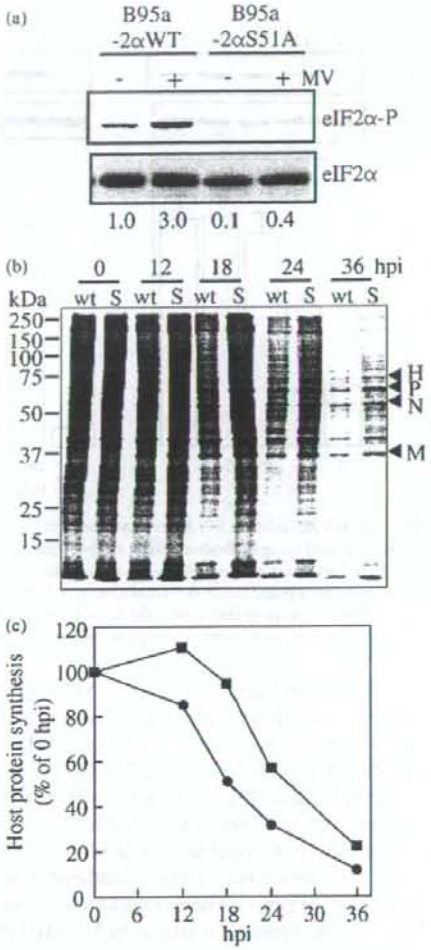

Fig. 6. MeV-HL-induced shut-off in eIF2αWT and S51A expressing cells. (a) eIF2α was detected by Western blotting assay using antibodies against phospho-eIF2α (upper panel), or eIF2α (lower panel) on the same membrane. The ratio of phosphorylated eIF2α (vs. mock infected B95a-2αWT) is shown under each lane. (b) Protein synthesis in MeV-HL-infected B95a-2αWT (wt) and B95a-2αS51A (S) cells was examined similar to Fig. 1a. Viral proteins are indicated to the right of the image. (c) The rates of host protein synthesis in B95a-2αWT cells (closed circle) or B95a-2αS51A (closed square) were determined from Fig. 6b by quantitation similar to Fig. 1b.

and independent of cell type. Similarly to MeV, Smith et al. reported that ability of reovirus to induce the shut-off of host protein synthesis is dependent of the viral strain [28].

The shut-off of host protein synthesis by virus infection was reported to be caused by a number of mechanisms such as inhibition of transcription, degradation of host mRNA and inhibition of translation. As the level of GAPDH mRNA was unaltered in MeV-HL-infected B95a cells, the shut-off by MeV-HL is suggested to be caused by inhibition of translation.

The shut-off of host translation is caused mainly by inhibition of the cap-dependent mechanism [6]. Contrary to many other virus-infected cells in which the components of the eIF4F complex including eIF4G, eIF4E and 4E-BP1 are involved in cap-dependent translation, they were not modified by MeV-HL infection. Therefore, the cap-binding activity of eIF4F complex appears to be intact. Instead, phosphorylation of eIF2α in MeV-HL-infected B95a cells was noted (Fig. 5). The phosphorylation rate of eIF2α correlated with the inhibition of host protein synthesis after MV infection. In addition, in B95a-2αS51A cells that stably expressed the eIF2α-S51A mutant, the shut-off phenomenon appeared to be suppressed compared with those in B95a and B95a-2αWT cells (Fig. 6). Therefore, phosphorylation of eIF2α is suggested as one of the mechanisms particularly at the early stage for the induction of host shut-off by MeV-HL infection.

Conner and Lyles reported that phosphorylation of eIF2α in VSV-infected cells suppressed viral translation rather than host translation [22]. In the case of MeV-HL infection, the suppression effect on host proteins was obviously much greater than that on viral proteins (Figs. 1a and 6b). MeV-HL mRNA may be more resistant to the effect of phosphorylated eIF2α than cellular mRNA. The mechanisms of the selective synthesis of viral protein in the shutoff stage of MeV-HL-infected cells are currently under investigation.

Recently, we also reported that the N protein of MeV-HL inhibits host translation by the binding to eIF3-p40 [13]. In our report, the inhibitory effect of the N protein is partial and inhibitory rate reaches a plateau at approximately 50–60%. On the other hand, MeV-HL-infection suppressed about 90% of the host translation (Fig. 1b). Experiment using eIF2αS51A mutant in this study, in which the inhibition of eIF2α phosphorylation observed in18 hpi lasted 24 hpi (data not shown) showed that the shut-off was inhibited at 18 hpi but became partial after 24 hpi (Fig. 6c). The expression level of the N protein increases rapidly after 18 hpi and reaches a peak at 24 hpi (data not shown). Taken together, we hypothesize that in MeV-HL-infected B95a cells the accumulation of phosphorylated eIF2α probably resulting from the replication of viral genome occurs at a relatively early stage of infection initiating the shut-off and then binding of increased N protein binds to eIF3-p40 and enhance the shut-off of host translation at later stage of infection.

Acknowledgement

This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the Bio-oriented Technology Research Advance Institution.

References

[1] Lyles DS. Cytopathogenesis and inhibition of host gene expression by RNA viruses. Microbiol Mol Biol Rev 2000;64(4):709–24.

[2] Abreu SL, Lucas-Lenard J. Cellular protein synthesis shutoff by mengovirus: translation of nonviral and viral mRNA's in extracts from uninfected and infected Ehrlich ascites tumor cells. J Virol 1976;18(1):182–94.

[3] Kaufmann Y, Coldstein E, Penman S. Poliovirus-induced inhibition of polypeptide initiation in vitro on native polyribosomes. Proc Natl Acad Sci USA 1976;73(6):1834–8.

[4] Svitkin YV, Ginevskaya VA, Ugarova TY, Agol VI. A cell-free model of the encephalomyocarditis virus-induced inhibition of host cell protein synthesis. Virology 1978;87(1):199–203.

[5] Hiremath LS, Webb NR, Rhoads RE. Immunological detection of the messenger RNA cap-binding protein. J Biol Chem 1985;260(13):7843–9.

[6] Gale Jr M, Tan SL, Katze MG. Translational control of viral gene expression in eukaryotes. Microbiol Mol Biol Rev 2000;64(2):239–80.

[7] Yoshikawa Y, Mizumoto K, Yamanouchi K. Characterization of messenger RNAs of measles virus. J Gen Virol 1986;67(Pt 12):2807–12.

[8] Wechsler SL, Fields BN. Intracellular synthesis of measles virus-specified polypeptides. J Virol 1978;25(1):285–97.

[9] Graves MC. Measles virus polypeptides in infected cells studied by immune precipitation and one-dimensional peptide mapping. J Virol 1981;38(1):224–30.

[10] Kobune F, Takahashi H, Terao K, Ohkawa T, Ami Y, Suzaki Y, et al. Nonhuman primate models of measles. Lab Anim Sci 1996;46(3):315–20.

[11] Kobune F, Sakata H, Sugiura A. Marmoset lymphoblastoid cells as a sensitive host for isolation of measles virus. J Virol 1990;64(2):700–5.

[12] Kai C, Yamanouchi K, Sakata H, Miyashita N, Takahashi H, Kobune F. Nucleotide sequences of the M gene of prevailing wild measles viruses and a comparison with subacute sclerosing panencephalitis virus. Virus Genes 1996;12(1):85–7.

[13] Sato H, Masuda M, Kanai M, Tsukiyama-Kohara K, Yoneda M, Kai C. Measles virus N protein inhibits host translation by binding to eIF3-p40. J Virol 2007;81(21):11569–76.

[14] Tsukiyama-Kohara K, Poulin F, Kohara M, DeMaria CT, Cheng A, Wu Z, et al. Adipose tissue reduction in mice lacking the translational inhibitor 4E-BP1. Nat Med 2001;7(10):1128–32.

[15] Devaney MA, Vakharia VN, Lloyd RE, Ehrenfeld E, Grubman MJ. Leader protein of foot-and-mouth disease virus is required for cleavage of the p220 component of the cap-binding protein complex. J Virol 1988;62(11):4407–9.

[16] Etchison D, Milburn S, Edery I, Sonenberg N, Hershey JW. Inhibition of HeLa cell protein synthesis following poliovirus infection correlates with the proteolysis of a 220,000-dalton polypeptide associated with eukaryotic initiation factor 3 and a cap binding protein complex. J Biol Chem 1982;257(24):14806–10.

[17] Etchison D, Fout S. Human rhinovirus 14 infection of HeLa cells results in the proteolytic cleavage of the p220 cap-binding complex subunit and inactivates globin mRNA translation in vitro. J Virol 1985;54(2):634–8.

[18] Feigenblum D, Schneider RJ. Modification of eukaryotic initiation factor 4F during infection by influenza virus. J Virol 1993;67(6):3027–35.

[19] Hershey Jr WB, Mathews MB, Sonenberg N. Translational control. Plainview, NY: Cold Spring Harbor Laboratory Press; 1996.

[20] Zhang Y, Feigenblum D, Schneider RJ. A late adenovirus factor induces eIF-4E dephosphorylation and inhibition of cell protein synthesis. J Virol 1994;68(11):7040–50.

[21] Mahalingam M, Cooper JA. Phosphorylation of mammalian eIF4E by Mnk1 and Mnk2: tantalizing prospects for a role in translation. Prog Mol Subcell Biol 2001;27:132–42.

[22] Connor JH, Lyles DS. Inhibition of host and viral translation during vesicular stomatitis virus infection. J Biol Chem 2005;280(14):13512–9.

[23] Gingras AC, Svitkin Y, Belsham GJ, Pause A, Sonenberg N. Activation of the translational suppressor 4E-BP1 following infection with encephalomyocarditis virus and poliovirus. Proc Natl Acad Sci USA 1996;93(11):5578–83.

[24] Clemens MJ. Regulation of eukaryotic protein synthesis by protein kinases that phosphorylate initiation factor eIF-2. Mol Biol Rep 1994;19(3):201–10.
[25] Williams BR. PKR; a sentinel kinase for cellular stress. Oncogene 1999;18(45):6112–20.
[26] Treiman M, Caspersen C, Christensen SB. A tool coming of age: thapsigargin as an inhibitor of sarco-endoplasmic reticulum Ca^{2+}-ATPases. Trends Pharmacol Sci 1998;19(4):131–5.
[27] Srivastava SP, Kumar KU, Kaufman RJ. Phosphorylation of eukaryotic translation initiation factor 2 mediates apoptosis in response to activation of the double-stranded RNA-dependent protein kinase. J Biol Chem 1998;273(4):2416–23.
[28] Smith JA, Schmechel SC, Williams BR, Silverman RH, Sciff LA. Involvement of the interferon-regulated antiviral proteins PKR and RNase L in reovirus-induced shutoff of cellular translation. J Virol 2005;79(4):2240–50.

Antibody to hepatitis B core antigen is associated with the development of hepatocellular carcinoma in hepatitis C virus-infected persons: A 12-year prospective study

KAZUO TANAKA[1], YUMIKO NAGAO[1,2], TATSUYA IDE[1,2], RYUKICHI KUMASHIRO[1] and MICHIO SATA[1,2]

[1]Second Department of Medicine and [2]Department of Digestive Disease Information and Research, Kurume University School of Medicine, Asahi-machi, Kurume, Fukuoka 830-0011, Japan

Received December 9, 2005; Accepted January 26, 2006

Abstract. Several studies have reported that antibody to hepatitis B core antigen (anti-HBc) positivity may influence the development of hepatocellular carcinoma (HCC) in chronic hepatitis C patients, but the evidence is still not conclusive. In this study, we examined whether the presence of anti-HBc positive was associated with the development of HCC in hepatitis C virus (HCV)-infected subjects among the residents in an HCV hyperepidemic area who were followed up for 12 years. In an HCV hyperendemic area (positive rate of anti-HCV: 23.4%), 509 residents were examined by health screening in 1990. After 12 years of follow-up, we evaluated the risk factors for HCC. The incidence of HCC was compared between anti-HBc positive and anti-HBc negative subjects after 12 years of prospective observation. Univariate and multivariate analyses were conducted to determine risk factors for the development of HCC. The incidence of HCC was significantly higher in the anti-HBc positive group (13 subjects) than in the anti-HBc negative group (0 subjects) (P=0.012). Multivariate analysis identified positivity for anti-HBc and HCV RNA, history of icterus, and female gender as independent determinants of the development of HCC. Our findings provide clear evidence in a prospective study that presence of anti-HBc, that is, past hepatitis B virus (HBV) infection, is a risk factor for the development of HCC in HCV-infected people.

Introduction

The number of hepatitis B virus (HBV) and hepatitis C virus (HCV) infection carriers worldwide is estimated at 350 million (1) and 170 million (2), respectively. HBV and HCV infections include substantial proportions of cases with past infection, asymptomatic carriers, acute hepatitis and chronic hepatitis, and HBV infections may cause fulminant hepatitis. Especially, chronic HBV and HCV infections may lead to cirrhosis and hepatocellular carcinoma (HCC) (1,3). It was reported that the frequency of HCC due to chronic HCV infection is higher in Japan than in any other country (4). Several studies have reported that occult HBV infection may also be one of the causative factors of HCC (5,6). The presence of occult HBV infection is diagnosed based on the fact that HBV DNA still exists in serum and liver tissue after hepatitis B surface antigen (HBsAg) disappears in acute or chronic HBV infection (7-9), or even after antiviral treatment is successful. Although some studies reported that occult HBV infection is associated with HCV-related liver dysfunction (10) or the development of HCC (11-13), these associations have still not been clearly demonstrated in a prospective study.

A higher incidence of HBV DNA is commonly seen in patients with anti-HBc-positive serology than in those with anti-HBc negative serology in coinfections with HBV and HCV (10), and using PCR amplification, most studies have demonstrated the presence of the HBV DNA genome in 22% to 87% of the patients who are HBsAg negative and HCV RNA positive (10,14-18). Some studies showed that HBV infection could occur in recipients of livers donated from subjects with anti-HBc but without HBsAg (19,20). That is, anti-HBc, which was initially considered to be an index for the past HBV infection in which all HBV had been cleared, has emerged as a convincing marker of occult hepatitis B (19,21-23). Also, several studies showed that the anti-HBc positivity was associated with the development of HCC in patients with HCV-associated chronic liver disease (11,24-26), but these associations have not been clearly demonstrated.

Since 1990, we have conducted health screenings of the residents of H town (adult population: 7,389), Fukuoka prefecture in northern Kyushu, Japan (27). This town is known for its high prevalence of liver disease. We previously reported that the town had a high prevalence of HCV carriers, 120/509 (23.6%) in 1990, and that HCV infection was the principal cause of liver dysfunction and HCC (27,28).

In the present study, we analyzed the influence of anti-HBc positivity on the development of HCC in HCV-infected people in the same town during 12 years in a prospective manner.

Correspondence to: Dr Kazuo Tanaka, Second Department of Internal Medicine, Kurume University School of Medicine, 67 Asahimachi, Kurume 830-0011, Japan
E-mail: wada.isi@alto.ocn.ne.jp

Key words: antibody to hepatitis B core antigen, occult hepatitis B virus, hepatitis C virus, hepatocellular carcinoma, HCV hyper epidemic area

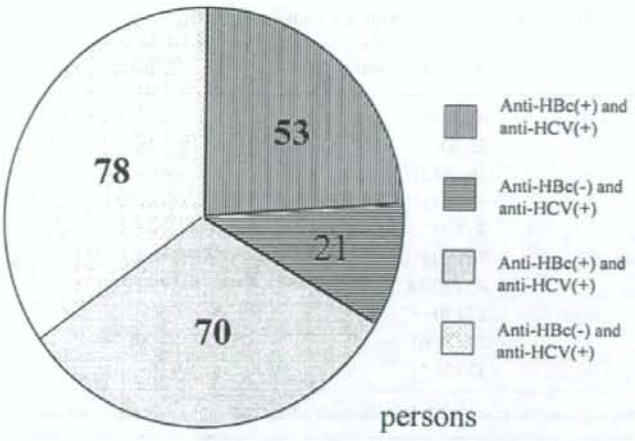

Figure 1. Diagram showing incident of hepatitis virus markers (anti-HCV and anti-HBc) among the 222 inhabitants 12 years ago. Fifty-three inhabitants were anti-HBc positive and anti-HCVpositive, 21 were anti-HBc negative and anti-HCVpositive, 70 were anti-HBc positive and anti-HCV negative, and 78 were anti-HBc negative and anti-HCV negative.

Subjects and methods

Subjects. In 1990, of a total 9,799 inhabitants, 739 (10%) of the 7,389 inhabitants >20 years old were randomly selected as follows: the names of the residents (as they appeared on their resident cards) were arranged in order according to the Japanese phonetic syllabary. Then every tenth resident was selected. As a result, 509 subjects (6.9% of H town residents) gave their informed consent to participate in the study.

Of 509 participants initially screened in 1990, 69 people had died and 55 people had moved to other regions as of 2002. Thus, 385 of the original 509 residents survived in the area and 139 residents agreed to participate in the medical follow-up survey, while 26 did not agree to participate, and the remaining 220 residents did not declare their intention either way in 2002. For 14 of these remaining 220 inhabitants, the records were obtained from the primary physicians. Consequently, we analyzed the outcome in terms of the liver disease in 222 inhabitants (69+139+14) in 2002.

Information on cigarette smoking, alcohol consumption, and history of icterus, and blood transfusion was obtained at the time of enrollment through interviews by the doctors in charge and experienced public health nurses. Smoking was defined as >10 cigarettes per day for >10 years. Alcohol consumption was defined as a daily intake of ≥75 g of ethanol per day for >10 years.

Serological assay. In 1990, sera were collected from all the participants, and conventional liver function tests were performed: serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), gammaglutamyl transpeptidase (γ-GTP), total protein (TP), albumin (Alb), total cholesterol (TC), total bilirubin (TB), zinc turbidity (ZTT) were measured. Anti-HCV was measured using HCV PHA 2nd generation kits (Dainabot Co. Ltd., Tokyo, Japan). These results were confirmed using a second generation recombinant immunoblot assay (RIBA II) (Ortho Diagnostic System, Tokyo, Japan). Measurement of HBsAg and anti-HBc was performed with an enzyme immunoassay kit (Mizuho Medy Co. Ltd., Tosu, Saga, Japan). Titers of anti-HBc yielding >70% inhibition were assessed as positive.

Detection of HCV RNA by RT-PCR. All subjects who were anti-HCV-positive were tested for the presence of serum HCV RNA, which was detected by reverse transcription-nested polymerase chain reaction (RT-nested PCR) using primers based on the sequences of the 5'UTR (untranslated region) of the HCV genome, as described previously (29).

Statistical analysis. Continuous data were expressed as mean ± SD, minimum and maximum. Categorical data were expressed as frequency and/or percentage. For comparing the background between anti-HBc positive and negative, the χ^2 and Wilcoxon's test were used to analyze quantitative data. Univariate and multivariate analysis were performed by logistic regression to calculate odds ratio and its 95% confidence interval. The SAS (statistical analysis system) computer program (release 8.2; SAS Institute Inc., Cary, NC, USA) was used for the logistic regression. A P-value of <0.05 was considered statistically significant.

Results

In 2002, anti-HCV was detected in 74 of the 222 inhabitants (Fig. 1). HCV RNA was detected in 53 (71.6%), HBsAg in 1 (1.4%), and anti-HBc in 53 (71.6%) of these 74 people. We asked the primary physician of these 74 inhabitants about the diagnosis of liver disease, and found thereby that 8 inhabitants had died of HCC and 5 inhabitants had been treated for HCC (total 13 inhabitants).

The 74 inhabitants were divided into two groups: 53 who were positive and 21 who were negative for anti-HBc, and the clinical characteristics observed in the screening were compared between the two groups. No significant differences

Table I. Characteristics of anti-HCV positive patients with and without anti-HBc.

Characteristics	Anti-HBc positive (N=53)	Anti-HBc negative (N=21)	P-value
Age (year)	62.3±10.9	58.0±16.4	NS
Sex: M:F	23:30	05:16	NS
Smoking (%)	16 (30.2)	4 (19.0)	NS
History of icterus (%)	8 (15.1)	3 (14.3)	NS
Alcohol consumption (%)	3 (5.7)	2 (9.5)	NS
History of blood transfusion (%)	8 (15.1)	4 (19.0)	NS
ALT level (IU/l)	40.6±30.8	27.5±17.9	NS
HBsAg (%)	1 (1.9)	0 (0)	NS
HCV RNA (%)	39 (73.6)	14 (66.7)	NS
HCC (%)	13 (24.5)	0 (0)	0.012

Age and serum ALT level were expressed as mean ± SD. HCC, hepatocellular carcinoma; NS, not significant.

Table II. Univariate analysis of risk factors that influence the development of HCC.

Factors	HCC group (n=13)	non-HCC group (n=61)	Odds ratio	95% CI	P-value
Age (years)	65.3±8.1 (53-82)	60.1±13.4 (23-89)	1.035	0.984-1.088	0.1866
Sex: male (%)	6 (46.2)	22 (36.1)	0.658	0.196-2.205	0.4976
Smoking (%)	4 (30.8)	13 (21.3)	1.641	0.435-6.190	0.4646
Alcohol consumption (%)	5 (38.5)	22 (36.1)	1.108	0.323-3.804	0.8706
History of blood transfusion (%)	3 (23.1)	8 (13.1)	1.988	0.448-8.810	0.3659
History of icterus (%)	5 (38.5)	5 (8.2)	7.000	1.652-29.667	0.0083[a]
AST (IU/l)	65.5±31.1 (28-131)	33.0±21.9 (13-132)	1.041	1.015-1.068	0.0016[a]
ALT (IU/l)	57.5±24.8 (20-108)	32.6±27.1 (9-160)	1.028	1.006-1.050	0.0119[a]
γ-GTP (IU/l)	127.1±195.3 (17-720)	32.4±34.2 (7-196)	1.015	1.003-1.027	0.0158[a]
Total protein (IU/l)	7.97±0.88 (6.6-10.0)	8.05±0.58 (6.6-9.8)	0.808	0.309-2.107	0.6622
Albumin (g/dl)	3.98±0.49 (3.0-4.9)	4.33±0.31 (3.2-4.8)	0.094	0.017-0.507	0.0060[a]
Total cholesterol (mg/dl)	160.5±33.1 (111-224)	173.8±32.5 (111-257)	0.987	0.967-1.007	0.1851
Total bilirubin (mg/dl)	1.01±0.50 (0.5-2.3)	0.77±0.27 (0.4-1.3)	7.537	1.170-48.533	0.0335[a]
ZTT (KU)	15.35±5.76 (1.1-21.7)	11.40±4.86 (2.5-27.4)	1.161	1.026-1.314	0.0183[a]
Anti-HBc (%)	13 (100)	40 (65.6)	9.150	1.407-	0.0161[a]
HCV RNA (%)	13 (100)	40 (65.6)	9.150	1.407-	0.0161[a]

[a]P<0.05; HCC, hepatocellular carcinoma; CI, confidence interval. Age, AST, ALT, γ-GTP, total protein, albumin, total bilirubin and ZTT were expressed as mean ± SD (range).

were observed between the two groups in age, sex, smoking, history of icterus or blood transfusion, alcohol consumption, ALT level, HBsAg, or HCV RNA. Significant differences were observed for the incidence of HCC (13 versus 0) between these two groups (P=0.012) (Table I).

Univariate and multivariate analyses of factors that influenced the incidence of HCC. The influence of age, sex, smoking, history of icterus, history of blood transfusion, alcohol consumption, AST, ALT, γ-GTP, TP, Alb, TC, TB, ZTT, anti-HBc and HCV RNA on the development of HCC was analyzed by univariate and multivariate analyses.

Table II shows the basic characteristics of the 74 inhabitants with anti-HCV divided into two groups: a group with HCC (HCC group) and a non-HCC group, and shows the results of univariate analyses. The mean age and sex were not significantly different between the HCC group and non-HCC group. Serum levels of AST, ALT, γ-GTP, TB, and ZTT were significantly higher in the HCC group than in the non-HCC group (P<0.05). The serum level of Alb was significantly lower in the HCC group than in the non-HCC group (P<0.05). The frequency of anti-HBc, HCV RNA, and history of icterus were significantly higher in the HCC group than in the non-HCC group (P<0.05). The frequency of smoking, alcohol

Table III. Multivariate analysis of risk factors that influence the development of HCC.

Factors	Odds ratio	95% CI	P-value
Age (years)	0.987	0.852-1.132	0.8428
Sex: female	190.517	2.157- >999.999	0.0188[a]
Smoking	40.580	0.656- >999.999	0.0824
Alcohol consumption	5.051	0.163-3.804	0.3644
History of blood transfusion	0.964	<0.001- >999.999	0.9918
History of icterus	311.186	5.066- >999.999	0.0042[a]
AST (IU/l)	1.013	0.855-1.244	0.8776
ALT (IU/l)	0.974	0.791-1.101	0.7013
γ-GTP (IU/l)	1.006	0.990-1.080	0.6950
Total protein (IU/l)	15.131	0.227- >999.999	0.2035
Albumin (g/dl)	<0.001	<0.001-11.319	0.1236
Total cholesterol (mg/dl)	1.018	0.952-1.106	0.6028
Total bilirubin (mg/dl)	7.911	0.060- >999.999	0.4127
ZTT (KU)	0.695	0.370-1.196	0.1853
Anti-HBc positive	>999.999	1.556-	0.0292[a]
HCV RNA positive	>999.999	3.767-	0.0063[a]

[a]P<0.05; HCC, hepatocellular carcinoma; CI, confidence interval.

consumption, and history of blood transfusion were not significantly different between the HCC group and non-HCC group.

Multivariate logistic regression analyses identified anti-HBc positivity, HCV RNA positivity, history of icterus, and female sex as independent risk factors for the development of HCC (Table III).

Discussion

Several studies have shown that anti-HBc positivity was associated with the development of HCC in patients with HCV-associated chronic liver disease (11,24-26). However, considering the natural history of all HCV infections, the results of those previous studies have some problems, i.e., the observation period was short and the research was performed in a retrospective manner in patients with chronic hepatitis and liver cirrhosis. Our study was a prospective study that investigated the disease progress after 12 years, and was thought to reflect the natural history of HCV infections, because we did not investigate only HCV-associated chronic liver disease but also covered all HCV infections such as past HCV infection and asymptomatic carriers of HCV (30,31). In this study, we obtained clear evidence that anti-HBc-positivity was a risk factor for the development of HCC in HCV-infected people.

It has been suggested that HBV can induce liver tumor formation by at least two distinct mechanisms. First, HBV DNA sequences are frequently found integrated into chromosomes of hepatocytes that have evolved into HCC, and a direct role of HBV in hepatocarcinogenesis has thus been inferred (32,33). Second, HBV DNA sequences may be caused by disruption of tumor suppressor gene function (34). It has been shown that HBV DNA sequences can be detected in some of the liver or serum from anti-HBc-positive patients (9,10), and the presence of anti-HBc does not entirely exclude the possibility of chronic HBV infection. Though the presence of anti-HBc has been used as a marker of past HBV infection, the integration of HBV DNA in hepatocytes may cause carcinogenesis, as noted above. That is, anti-HBc-positivity may represent occult HBV infection. The presence of anti-HBc alone, in the absence of HBV DNA testing, has been used in some studies as a marker of occult hepatitis B (19,21-23). Pollicino *et al* provided clear evidence that occult HBV was a risk factor for the development of HCC and showed that the potential mechanisms whereby HBV might induce tumor formation occur in most cases of occult infection (6).

To detect occult HBV infection, it is necessary to examine whether HBV DNA is present. However, serum HBV DNA levels are frequently below the limits of detection in anti-HBc-positive patients, and there is a pronounced risk of false-positive results from contamination (35) or amplification of non-HBV-DNA targets, and the sensitivity of detection is variable (36,37). In a previous study in which serum HBV DNA was tested in 20 anti-HBc positive patients with HCV-associated HCC, HBV DNA was not detected by a real-time PCR assay with a minimum detection limit of $10^{4.7}$ copies/ml (1.7 log copies/ml) (38,39). Considering these results, it might not be possible to detect serum HBV DNA in some anti-HBc-positive subjects. Therefore, if we could examine liver tissues by PCR to examine whether occult HBV infection is present, we could be more certain of the presence of occult HBV infection.

In contrast to our findings, in some studies anti-HBc positivity was not found to be associated with the development

of HCC in patients with HCV-associated chronic liver disease (9,39,40). One study showed that anti-HBc was detected significantly more frequently in blood donors with than without anti-HCV, but the prevalence of anti-HBc was no different between the patients with HCV-associated HCC and anti-HCV-positive blood donors. Therefore, no epidemiological evidence was obtained for a role of past HBV infection in hepatocarcinogenesis in patients infected with HCV in Japan (40). Also, Yano *et al* showed that the clinical features of HCV-associated HCC were unaffected by anti-HBc-positivity (39). In addition, a study in Taiwan suggested that occult HBV infection might have little influence on the clinicopathologic course of chronic HCV infection (9).

It was reported that the frequency of HCC due to chronic HCV infection is higher in Japan compared with any other country (4). If the frequency of HCC due to chronic HCV infection is high, it is necessary to consider the possibility that anti-HBc positivity may be associated with hepatocarcinogenesis. In addition to HBV, other environmental and host factors might also be associated with the pathogenesis of HCC (4,41-43).

We continued carrying out health screenings of the residents of H town and conducted a cohort study of liver disease among the same residents over a 12-year period. The results of this study showed that anti-HBc is associated with the development of HCC in HCV-infected people.

Acknowledgements

We would like to thank the following institutions for collection of data of the residents' liver disease: Amagi Asakura Medical Association Asakura Hospital, Ishii Surgery Clinic, Shigematsu Medical Office, Tanabe Medical Office, Haki Clinic, Fukuoka Prefectural Hepato-gastroenterological Center, Moriyama Medical Office, Yamaga Medical Office, Wada Surgery Clinic, Chikugo River Onsen Hospital, Harazuru Onsen Hospital, Digestive Tract Internal Medicine of St. Mary's Hospital, Digestive Tract Internal Medicine of Koga Hospital, and Majima Digestive Tract Clinic. Moreover, we thank the following companies for supplying the related inspection reagent kits for the hepatitis virus: Ortho-Clinical Diagnostics, K.K., Roche Diagnostics K.K., and Abbott Laboratories. This study was supported in part by grants as a project for establishing new high technology research centers, by a Grant-in-Aid for Scientific Research (C) (No. 11670548), by a Grant-in-Aid for Encouragement of Young Scientists (No. 14770256) from the Ministry of Education, Science, Sports and Culture of Japan, and the Hepatitis C Research Group (2001-2003) under the auspices of the Ministry of Health, Labor and Welfare.

References

1. Maynard JE: Hepatitis B: global importance and need for control. Vaccine (Suppl): S18-S20, 1990.
2. WHO: Global surveillance and control of hepatitis C. Report of a WHO consultation organized in collaboration with the viral hepatitis prevention board, Antwerp, Belgium. J Viral Hepat 6: 35-47, 1999.
3. Niederau C, Lange S, Heintges T, *et al*: Prognosis of chronic hepatitis C: result of a large, prospective cohort study. Hepatology 28: 1687-1695, 1998.
4. Yoshizawa H: Hepatocellular carcinoma associated with hepatitis C virus infection in Japan: projection to other countries in the foreseeable future. Oncology 62 (Suppl 1): 8-17, 2002.
5. Yotsuyanagi H, Shintani Y, Moriya K, *et al*: Virologic analysis of non-B, non-C hepatocellular carcinoma in Japan: frequent involvement of hepatitis B virus. J Infect Dis 181: 1920-1928, 2000.
6. Pollicino T, Squadrito G, Cerenzia G, *et al*: Hepatitis B virus maintains its pro-oncogenic properties in the case of occult HBV infection. Gastroenterology 126: 102-110, 2004.
7. Blackberg J and Kidd-Ljunggren K: Occult hepatitis B virus after acute self-limited infection persisting for 30 years without sequence variation. J Hepatol 33: 992-997, 2000.
8. Fong T-L, Di Bisceglie AM, Gerber MA, *et al*: Persistence of hepatitis B. Hepatology 18: 1313-1318, 1993.
9. Kao JH, Chen PJ, Lai MY, *et al*: Occult hepatitis B virus infection and clinical outcomes of patients with chronic hepatitis C. J Clin Microbiol 40: 4068-4071, 2002.
10. Caccoiola I, PollicinoT, Squadrito G, *et al*: Occult hepatitis B virus infection in patient with chronic hepatitis C liver disease. N Engl Med 341: 22-26, 1999.
11. Sheu JC, Huang GT, Shin LN, *et al*: Hepatitis C and B viruses in hepatitis surface antigen-negative hepatocellular carcinoma. Gastroenterology 103: 1322-1327, 1992.
12. Paterlini P, Driss F, Nalpas B, *et al*: Persistence of hepatitis B and hepatitis C viral genomes in primary liver cancers from HBsAg-negative patients study of a low-endemic area. Hepatology 17: 20-29, 1993.
13. Kubo S, Nishiguchi S, Hirohashi K, *et al*: Clinical significance of prior hepatitis B virus infection in patients with chronic hepatitis C virus-related hepatocellular carcinoma. Cancer 86: 793-798, 1999.
14. Fukuda R, Ishimura N, Niigaki M, *et al*: Serologically silent hepatitis B virus confection in patients with chronic hepatitis C virus associated chronic liver disease: clinical and virological significance. J Med Virol 58: 201-207, 1999.
15. Uchida T, Kaneita Y, Gotoh K, *et al*: Hepatitis C virus is frequently coinfected with serum marker negative hepatitis B virus: probable replication promotion of the former by the latter as demonstrated by *in vitro* cotransfection. J Med Virol 52: 399-405, 1997.
16. Gonzalez S, Navas S, madejon A, *et al*: Hepatitis B and D genomes in hepatitis B surface antigen negative patients with chronic hepatitis C. J Med Virol 45: 168-173, 1995.
17. Villa E, Crottola A, Buttafoco P, *et al*: Evidence of hepatitis B virus in patients with chronic hepatitis C with or without serological markers of hepatitis B. Dig Dis Sci 40: 8-13, 1995.
18. Berger A, Doerr HW, Rabenau HF, *et al*: High frequency of HCV infection in individuals with isolated antibody to hepatitis B core antigen. Intervirology 43: 71-76, 2000.
19. Dickson RC, Everhart JE, Lake JR, *et al*: Transmission of hepatitis B by transplantation of livers from donors positive for antibody to hepatitis B core antigen. Gastroenterology 113: 1668-1674, 1997.
20. Uemoto S, Sugiyama K, Marusawa H, *et al*: Transmission of hepatitis B virus from hepatitis B core antibody-positive donors in living related liver transplants. Transplantation 65: 494-499, 1998.
21. Sagnelli E, Coppola N, Scolastico C, *et al*: HCV genotype and 'silent' HBV co-infection: two main risk factors for a more severe liver disease. J Med Virol 64: 350-355, 2001.
22. Joller-Jemelka J, Wicki A and Grob P: Detection of HBs antigen in anti-HBc alone positive sera. J Hepatol 21: 269-272, 1994.
23. Marusawa H, Umemoto S, Higikata, *et al*: Latent hepatitis B virus infection in healthy with antibodies to hepatitis B core antigen. Hepatology 31: 488-495, 2000.
24. Chiba T, Matsuzaki Y, Abei M, *et al*: Multivariate analysis of risk factors for hepatocellular carcinoma in patients with hepatitis C virus-related liver cirrhosis. J Gastroenterol 31: 552-558, 1996.
25. Okada S, Sato T, Okusaka T, *et al*: Past exposure to hepatitis B virus as a risk factor for hepatocellular carcinoma in patients with chronic liver disease. Br J Cancer 77: 2028-2031, 1998.
26. Marusawa H, Osaki Y, Kimura T, *et al*: High prevalence of anti-hepatitis B virus serological markers in patients with hepatitis C virus related liver disease in Japan. Gut 45: 284-288, 1999.

27. Sata M, Nakano H, Suzuki H, *et al*: Sero-epidemilogic study of hepatitis C virus infection in Fukuoka, Japan. J Gastroenterol 33: 218-222, 1998.
28. Nagao Y, Fukuizumi K, Tanaka K, *et al*: The prognosis for life in an HCV hyperendemic area. Gastroenterology 125: 628-629, 2003.
29. Okamoto H, Okada S, Sugiyama Y, *et al*: Detection of hepatitis C virus RNA by a two-stage polymerase chain reaction with two pairs of primers deduced from the 5'-noncoding region. Jpn J Exp Med 60: 215-222, 1990.
30. Nagao Y, Tanaka K, Kobayashi K, *et al*: Analysis of approach to therapy for chronic liver disease in an HCV hyperendemic area of Japan. Hepatol Res 28: 30-35, 2004.
31. Nagao Y, Tanaka K, Kobayashi K, *et al*: A cohort study of chronic liver disease in an HCV hyperendemic area of Japan: a prospective analysis for 12 years. Int J Mol Med 13: 257-265, 2004.
32. Feitelson MA and Duan LX: Hepatitis B virus X antigen in the pathogenesis of chronic infections and development of hepatocellular carcinoma. Am J Pathol 150: 1141-1157, 1997.
33. Tamori A, Nishiguchi S, Kubo S, *et al*: Possible contribution to hepatocarcinogenesis of X transcript hepatitis B virus in Japanese patients with hepatitis C virus. Hepatology 29: 1429-1434, 1999.
34. Zhou YZ, Slagle BL, Donehower LA, *et al*: Structural analysis of a hepatitis B virus genome integrated into chromosome 17p of a human hepatocellular carcinoma. J Virol 62: 4224-4231, 1988.
35. Khristova M, Nainan O, Xia G-L, *et al*: False-positive HBVDNA results among persons with only antibody to hepatitis B core antigen. Antiviral Ther 5 (Suppl 1): B22, 2000.
36. Ke-Qin H: Occult hepatitis B virus infection and its clinical implications. J Viral Hepat 9: 243-257, 2002.
37. Torbenson and Thomas DL: Occult hepatitis B. Lancet Infect Dis 2: 479-486, 2002.
38. Ide T, Kumashiro R, Koga Y, *et al*: A real-time quantitative polymerase chain reaction method for hepatitis B virus in patients with chronic hepatitis B treated with lamivudine. Am J Gastroenterol 98: 2048-2051, 2003.
39. Yano Y, Yamashita F, Sumie S, *et al*: Clinical significance of antibody against hepatitis B virus core antigen in patients infected with hepatitis C virus-related hepatocellular carcinoma. Liver Int 23: 227-231, 2003.
40. Hiraoka T, Katayama K, Tanaka J, *et al*: Lack of epidemiological evidence for a role of resolved hepatitis B virus infection in hepatocarcinogenesis in patients infected with hepatitis C virus in Japan. Intervirology 46: 171-176, 2003.
41. Matsuzaki Y, Chiba T, Hadama T, *et al*: HBV genome integration and genetic instabillity in HBsAg-negative and anti-HCV-positive hepatocellular carcinoma in Japan. Cancer Lett 119: 53-61, 1997.
42. Matsuzaki Y, Sato M, Saito Y, *et al*: The role of previous infection of hepatitis B virus in HBs antigen negative and anti-HCV-positive Japanese patients with hepatocellular carcinoma: etiological and molecular biological study. J Exp Clin Cancer Res 158: 73-84, 1999.
43. Chen P-J and Chen D-S: Hepatitis B virus infection and hepatocellular carcinoma. Molecular genetics and clinical perspectives. Semin Liver Dis 19: 253-262, 1999.

Case Report

Acta Haematol 2006;116:266–271
DOI: 10.1159/000095878

Received: September 9, 2005
Accepted after revision: January 11, 2006

Simultaneous Hepatic Relapse of Non-Hodgkin's Lymphoma and Hepatocellular Carcinoma in a Patient with Hepatitis C Virus-Related Cirrhosis

Korenori Ohtsubo[a, b] Eijiro Oku[a, b] Rie Imamura[a, b] Ritsuko Seki[a, b] Michitoshi Hashiguchi[a, b] Koichi Osaki[a, b] Kazuaki Yakushiji[a, b] Kohji Yoshimoto[a, b] Hideaki Ogata[a, b] Hiroaki Nagamatsu[b, c] Eiji Ando[b] Kazuhide Shimamatsu[d] Takashi Okamura[a] Michio Sata[b]

[a]Department of Internal Medicine, Division of Hematology and [b]Second Department of Internal Medicine, Kurume University School of Medicine, and [c]Department of Medicine and [d]Department of Pathology, Yame General Hospital, Fukuoka, Japan

Key Words

Hepatitis C virus · Hepatocellular carcinoma · Non-Hodgkin's lymphoma · Radiofrequency ablation · Rituximab · THP-COP

Abstract

We report a 66-year-old man with hepatitis C virus (HCV)-related cirrhosis and simultaneous hepatic relapse of non-Hodgkin's lymphoma (NHL) and of hepatocellular carcinoma (HCC). Although the liver is frequently involved by NHL, hepatic colocalization of NHL and HCC is rarely detected by imaging techniques. HCV has been suggested to be lymphotrophic as well as hepatotrophic, and therefore has attracted speculation about a causative role in some cases of lymphoma. The patient had a past history of cutaneous diffuse large B cell lymphoma (DLBCL) in concurrence with HCC 32 months previously. Complete remission (CR) had been maintained for both diseases until February 2004, when ultrasonography and computed tomography (CT) showed multiple liver tumors. Two of these, appearing hyperattenuating in the arterial phase of contrast-enhanced CT, were diagnosed histopathologically as HCC, and treated with radiofrequency ablation. The other tumors, hypoattenuating in the portal phase CT, were diagnosed histopathologically as DLBCL, and treated with cyclophosphamide, tetrahydropyranyl-Adriamycin, vincristine and prednisolone (THP-COP) in combination with rituximab. CR was achieved for both DLBCL and HCC. Given the previously demonstrated immune system tropism and perturbation by HCV, the virus might have contributed to the occurrence of the NHL as well as the HCC.

Introduction

An Italian study reported B cell malignant diseases as the most frequent neoplasms associated with hepatocellular carcinoma (HCC) [1], but few such cases have involved colocalization of both within the liver. According to the report on the focal liver lesions detected by imaging techniques in 414 patients with non-Hodgkin's lymphoma (NHL) [2], only 1 case presented with simultaneous coexistence with NHL and HCC. We know of only four previously reported similar cases [3–6], all associated with hepatitis B virus (HBV) infection. How HBV

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

0001–5792/06/1164–0266$23.50/0

Accessible online at:
www.karger.com/aha

Korenori Ohtsubo, MD
Second Department of Internal Medicine, Kurume University School of Medicine
67 Asahi-machi, Kurume
Fukuoka 830-0011 (Japan)
Tel. +81 942 31 7561, Fax +81 942 34 2623, E-Mail ohtsubo_korenori@kurume-u.ac.jp

and development of lymphoma are related is not known, although some reports have suggested a possible pathogenetic role of HBV in the development of hematologic malignant diseases [7, 8].

Hepatitis C virus (HCV) is well known as a causative agent of chronic hepatitis, which often progresses to liver cirrhosis and HCC in the chronically infected patients. On the other hand, HCV has been associated with various extrahepatic autoimmune diseases [9]. HCV, being lymphotrophic as well as hepatotrophic [10], has attracted speculation about a causative role in some cases of lymphoma [11, 12]. In particular, several investigators have reported an association between HCV and B cell NHL [13–16]. Chronic antigenic stimulation by HCV has been suspected to be related to the development of clonal B cell expansion [12, 17], although the mechanism is not clear. We present a patient with HCV-related cirrhosis who showed simultaneous intrahepatic relapses of HCC and of B cell lymphoma without extrahepatic involvement.

Case Report

A 66-year-old man with HCV-related cirrhosis was referred to our hospital in March 2004 for treatment of newly detected liver tumors. The patient had a 16-year history of HCV-related chronic hepatitis and also a history of treatment of cutaneous diffuse large B cell lymphoma (DLBCL) in concurrence with HCC diagnosed in May 2001. At that time the DLBCL additionally involved the bone marrow, and was assigned to stage IV according to the Ann Arbor staging system. Subsequently complete response (CR) had been maintained after chemotherapy. Further, radiofrequency ablation (RFA) of the HCC located in the left lobe of the liver had been performed successfully; no other lesion had been detected until shortly before the present admission.

In February 2004, ultrasonography and computed tomography (CT) showed multiple tumors in the right lobe of the liver. All tumors except two were hypoattenuating in the portal phase of contrast-enhanced CT (fig. 1a). The other two tumors located in segment 8, were hyperattenuating in the arterial phase (fig. 1b). Histologic examination of a percutaneous needle biopsy specimen obtained from a hypoattenuating tumor showed infiltration by abnormal lymphoid cells with large and sometimes irregularly shaped nuclei (fig. 2a). Immunohistochemical staining indicated that the lymphoid cells were positive for CD20 (fig. 2b), and the tumor was diagnosed as DLBCL.

In March 2004 the patient was admitted to our hospital for treatment. Physical examination on admission disclosed pallor, spider angiomata, and ascites. Laboratory data obtained on admission showed decreases of choline esterase (40 IU/l; normal range 107–233), albumin (3.0 g/dl; normal range 4.0–5.0), and total cholesterol (125 mg/dl; normal range 128–256) as well as an increase of total bilirubin (2.46 mg/dl; normal range 0.0–1.5). Serum concentrations of aspartate aminotransferase, alanine aminotransferase, and lactate dehydrogenase were normal, as was prothrombin time. Soluble interleukin 2 receptor was increased in serum (1,313 U/ml; normal range 220–530). The serum concentration of α-fetoprotein was normal (7.3 ng/ml; normal range 0–8.7), but PIVKA-II (protein induced by vitamin K absence or antagonist II) was increased (254 mAU/ml; normal range 0–40). No extrahepatic involvement by DLBCL was detected in CT, ^{67}Ga scintigraphy, or bone marrow examination.

Following the diagnosis of DLBCL, two courses of cyclophosphamide, tetrahydropyranyl-Adriamycin, vincristine and prednisolone (THP-COP) were given combined with a course of rituximab. As a result, all tumors except two disappeared or decreased greatly in size according to CT (fig. 1c). The two nonresponding tumors were those that were shown as hyperattenuating lesions in the arterial phase of contrast CT (fig. 1d). An abdominal angiogram demonstrated that these two tumors were hypervascular (fig. 3). Histologic examination of a percutaneous needle biopsy specimen obtained from one of these two tumors showed moderately differentiated HCC with a trabecular and pseudoglandular growth pattern (fig. 4). Chemotherapy for DLBCL was suspended, as it had compromised the patient's liver function and exacerbated ascites (fig. 1c). After improvement of liver function, RFA of the two HCC was performed successfully. A CR was attained for both DLBCL and HCC.

Discussion

In a Japanese study concerning extrahepatic primary cancers in 384 patients with HCC, no B lymphocyte-derived neoplasms were detected [18]. On the other hand, an Italian study of 317 patients with HCC found B cell-derived neoplasms to represent the most frequent cancers associated with HCC, accounting for 10 of 35 extrahepatic primary neoplasms, or 28.6% [1]. Disagreement between these two reports concerning the frequency of B cell neoplasms in patients with HCC is likely to involve the difference in ethnicity between study subjects. In Italy, B cell NHL is reported to show a frequent association with HCV. Accordingly, patients with HCV-related HCC are likely to be at increased risk for B cell-derived neoplasms.

As mentioned, the Italian study included 10 patients with B lymphocyte-derived neoplasms associated with HCC. These were varied: 7 cases of NHL, 2 cases of multiple myeloma, and 1 chronic lymphocytic leukemia [1]. These cases also showed a relatively nonspecific distribution pattern, that of double cancers with the B cell neoplasms involving essentially any part of the body. In our patient, DLBCL coexisted with HCC within the liver, with no extrahepatic involvement. According to the report on the focal liver lesions detected by imaging techniques in 414 patients with NHL [2], hepatic lymphomatous involvement was observed in 69 cases, and HCC in

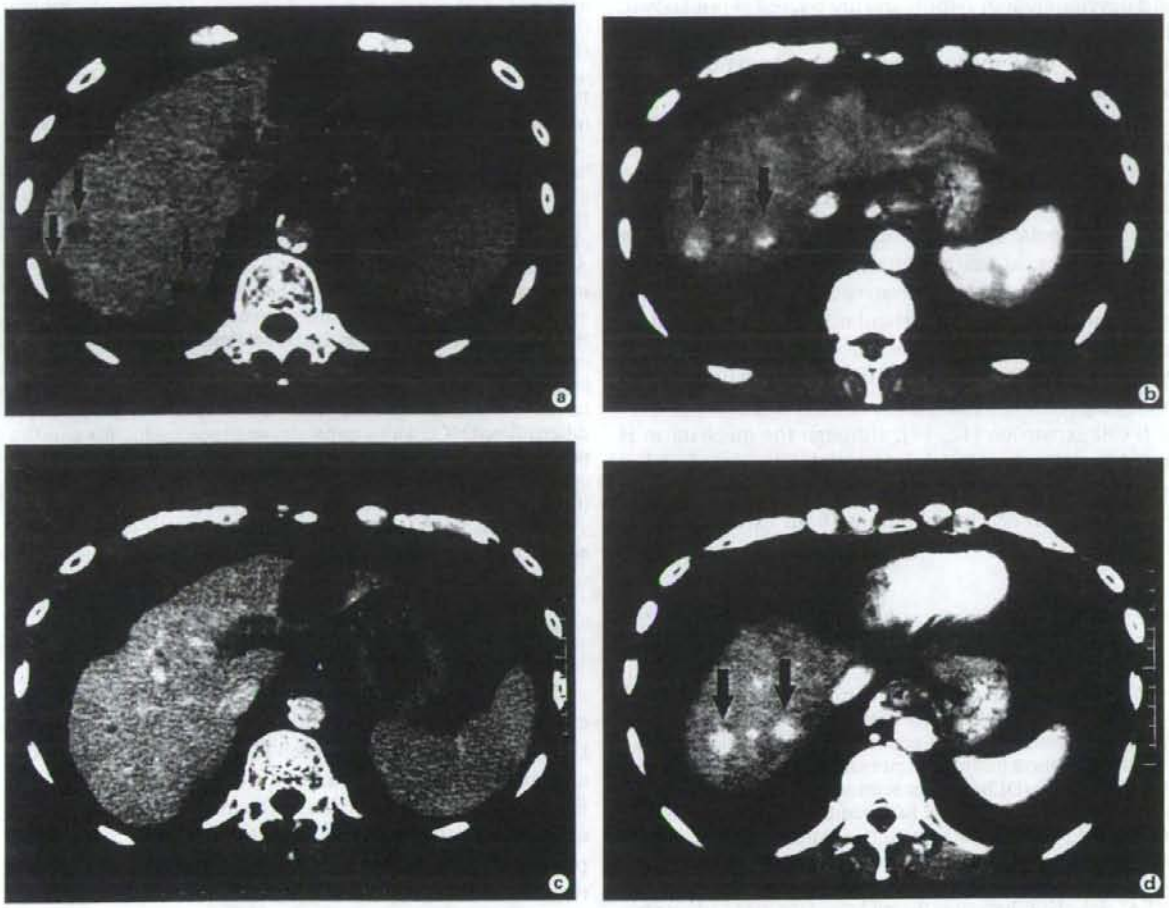

Fig. 1. CT findings during the patient's recent course. **a** Contrast-enhanced CT in the portal phase showed multiple hypoattenuating tumors in the liver (vertical arrows). The horizontal arrow in the left lobe indicates an area of necrosis where RFA had previously been performed. **b** CT in the arterial phase showed two hyperattenuating tumors (arrows) in hepatic segment 8. **c** CT in the portal phase after THP-COP with rituximab showed disappearance or shrinkage of the hypoattenuating tumors shown in **a**. Marked ascites can also be seen. **d** CT in the arterial phase after THP-COP with rituximab showed no shrinkage of the two hyperattenuating tumors shown in **b**.

7 cases, yet only 1 case presented with a simultaneous coexistence of NHL and HCC. That case was described by Cavanna et al. [6]. Although liver is the common site for lymphomatous involvement and occurrence of HCC, we rarely see such a case where the two tumors were simultaneously detected by imaging techniques as distinct hepatic mass lesions without extrahepatic involvement. We know of only 4 previously reported similar cases. Talamo et al. [3] were the first to report a case of simultaneous occurrence of primary hepatic lymphoma and HCC. Takeshima et al. [4] reported a patient with hepatic occurrence of mucosa-associated lymphoid tissue lymphoma together with HCC. These 2 cases showed no evidence of extrahepatic involvement by lymphoma, and they are considered to represent primary hepatic lymphoma, defined as confined to the liver with no evidence of lymphomatous involvement in the spleen, bone marrow, or other lymphoid structures. Shikuwa et al. [5] reported a case

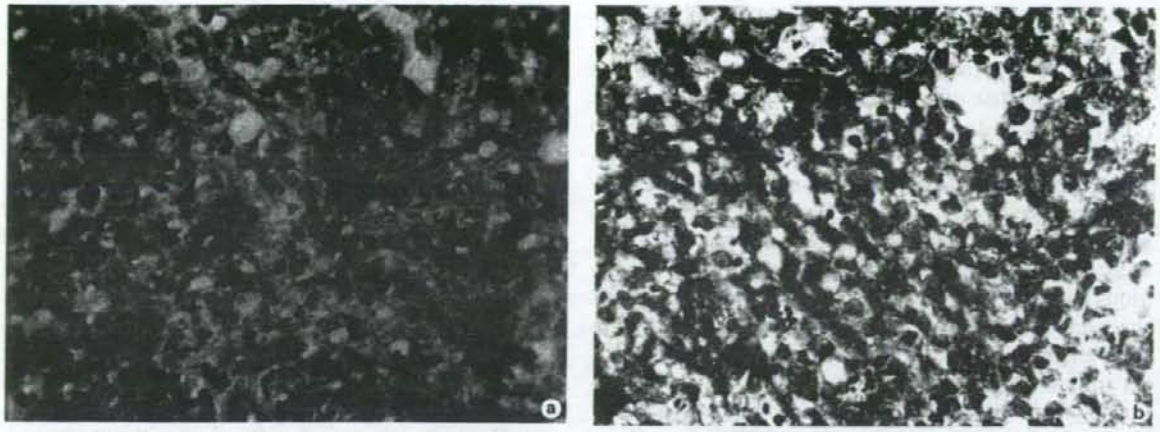

Fig. 2. Microscopic findings in a percutaneous needle biopsy specimen from a hypoattenuating lesion. **a** The liver showed infiltration by abnormal lymphoid cells with large, sometimes irregularly shaped nuclei. Hematoxylin and eosin. ×400. **b** The abnormal lymphoid cells were positive for CD20. Immunohistochemical staining. ×400.

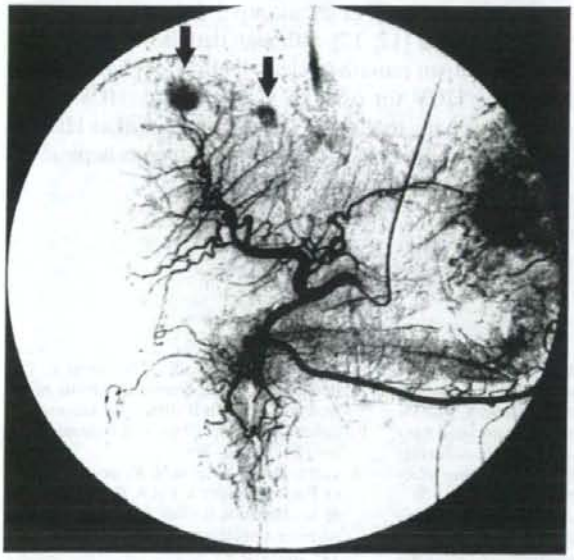

Fig. 3. An abdominal angiogram demonstrated two hypervascular tumors (arrows) in hepatic segment 8.

Fig. 4. Histologic findings in a percutaneous needle biopsy specimen from a hyperattenuating, hypervascular lesion showed moderately differentiated HCC with a trabecular and pseudoglandular growth pattern. Hematoxylin and eosin. ×100.

of colocalized HCC and malignant lymphoma, but autopsy demonstrated that the lymphoma involved the bone marrow in addition to the liver. Cavanna et al. [6] reported a case where NHL relapsed in liver without extrahepatic involvement in a patient with HCC. Our patient also presented with relapse, specifically simultaneous relapse of HCC and DLBCL with no extrahepatic involvement. It has been shown that metastatic cell sub-

populations can outgrow their nonmetastatic counterparts within the primary tumor and it was suggested that the metastatic potential of a primary tumor may increase during the course of its growth [19]. Rowbotham et al. [20] referred to the apparent predilection of tumors to invade the liver in patients with acute liver failure secondary to hepatic infiltration and thus suggested the presence of a phenotypic lymphomatous subtype with selective organ invasion, and additionally reported that over one quarter of patients with lymphoma had a history of previous treatment for the same disease. This fact suggests that chemotherapy might change the behavior of the tumors and enhance the properties which preferentially target and invade the liver. The appearance of such chemotherapy-induced lymphoma cell subpopulations as selectively invading the liver and recurrence of HCC may have resulted in the simultaneous colocalization of the two distinct tumors. Our case report demonstrates that it is important to pay attention to patients with HCV or with a previous history of malignant lymphoma at the diagnosis of hepatic mass lesions.

Importantly, all 4 cases reported prior to ours were associated with HBV infection. Although some reports have suggested a possible pathogenetic role of HBV in the development of hematologic malignant diseases [7, 8], a basis for a relationship between HBV and lymphoma occurrence is not clear. In distinction to the other cases, ours is associated with HCV, not HBV, infection. HCV is a well-known cause of chronic hepatitis, which in these chronically infected patients often progresses to cirrhosis and eventually HCC. On the other hand, HCV has also shown reported associations with various extrahepatic autoimmune diseases, such as mixed cryoglobulinemia, Sjögren's syndrome, renal disease, and neuropathy [9]. As a lymphotropic virus [10], HCV is suspected to contribute to the etiology of B cell NHL [11, 12]. A relationship between HCV and NHL has been demonstrated by many investigators in Italy [13, 14], the United States [15], and Japan [16]. Especially in Italy, a high proportion of HCV positivity has been reported among patients with NHL. Ascoli et al. [21] reported HCV-related extranodal B cell lymphomas of various types. The apparent relationship was supported by a report demonstrating regression in splenic lymphoma with villous lymphocytes in patients with HCV after treatment of the virus with interferon α [22]. In patients with type II mixed cryoglobulinemia, the most common immune disorder related to chronic HCV infection, the paraprotein is a monoclonal IgM rheumatoid factor indicative of clonal B cell proliferation [23]. Thus, chronic B cell stimulation by HCV-related antigens has been proposed as a causative factor in neoplastic transformation [12, 17], although details of the underlying mechanism remain unclear. Our patient had been infected by HCV for over 16 years; indeed, HCV might have caused his malignant lymphoma as well as HCC to result in a unique HCV-related simultaneous hepatic colocalization of HCC and NHL.

References

1 Di Stasi M, Sbolli G, Fornari F, Cavanna L, Rossi S, Buscarini E, Civardi G, Vallisa D, Berte R, Buscarini L: Extrahepatic primary malignant neoplasms associated with hepatocellular carcinoma: high occurrence of B cell tumors. Oncology 1994;51:459–464.

2 Civardi G, Vallisa D, Berte R, Lazzaro A, Moroni CF, Cavanna L: Focal liver lesions in non-Hodgkin's lymphoma: investigation of their prevalence, clinical significance and the role of hepatitis C virus infection. Eur J Cancer 2002; 38:2382–2387.

3 Talamo TS, Dekker A, Gurecki J, Singh G: Primary hepatic malignant lymphoma: its occurrence in a patient with chronic active hepatitis, cirrhosis, and hepatocellular carcinoma associated with hepatitis B viral infection. Cancer 1980;46:336–339.

4 Takeshima F, Kunisaki M, Aritomi T, Osabe M, Akama F, Nakasone T, Niino D, Katayama S, Isomoto H, Omagari K, Mizuta Y, Murata I, Kohno S: Hepatic mucosa-associated lymphoid tissue lymphoma and hepatocellular carcinoma in a patient with hepatitis B virus infection. J Clin Gastroenterol 2004;38:823–826.

5 Shikuwa S, Ito M, Nakashima M, Hamasaki K, Naito S, Sekine I, Fujii H: Autopsy case of colocalized tumors of hepatocellular carcinoma and malignant lymphoma. J Gastroenterol 1996;31:129–132.

6 Cavanna L, Civardi G, Fornari F, Vallisa D, Berte R, Buscarini E, Sbolli G, Paties C, Foroni R, Di Stasi M, Buscarini L: Simultaneous relapse of liver cell carcinoma and non-Hodgkin's lymphoma in the liver. Report of a case with diagnosis by ultrasonically guided fine needle aspiration biopsy. Acta Cytol 1994;38: 451–454.

7 Pontisso P, Locasciulli A, Schiavon E, Cattoretti G, Schiro R, Stenico D, Alberti A: Detection of hepatitis B virus DNA sequences in bone marrow of children with leukemia. Cancer 1987;59:292–296.

8 Galun E, Ilan Y, Livni N, Ketzinel M, Nahor O, Pizov G, Nagler A, Eid A, Rivkind A, Laster M: Hepatitis B virus infection associated with hematopoietic tumors. Am J Pathol 1994; 145:1001–1007.

9 Mayo MJ: Extrahepatic manifestations of hepatitis C infection. Am J Med Sci 2003;325: 135–148.

10 Morsica G, Tambussi G, Sitia G, Novati R, Lazzarin A, Lopalco L, Mukenge S: Replication of hepatitis C virus in B lymphocytes (CD19+). Blood 1999;94:1138–1139.

11 Weng WK, Levy S: Hepatitis C virus (HCV) and lymphomagenesis. Leuk Lymphoma 2003; 44:1113–1120.

12 Zuckerman E, Zuckerman T, Sahar D, Streichman S, Attias D, Sabo E, Yeshurun D, Rowe J: Bcl-2 and immunoglobulin gene rearrangement in patients with hepatitis C virus infection. Br J Haematol 2001;112:364–369.
13 Mazzaro C, Zagonel V, Monfardini S, Tulissi P, Pussini E, Fanni M, Sorio R, Bortolus R, Crovatto M, Santini G, Tiribelli C, Sasso F, Masutti R, Pozzato G: Hepatitis C virus and non-Hodgkin's lymphomas. Br J Haematol 1996;94:544–550.
14 Mele A, Pulsoni A, Bianco E, Musto P, Szklo A, Sanpaolo MG, Iannitto E, De Renzo A, Martino B, Liso V, Andrizzi C, Pusterla S, Dore F, Maresca M, Rapicetta M, Marcucci F, Mandelli F, Franceschi S: Hepatitis C virus and B-cell non-Hodgkin lymphomas: an Italian multicenter case-control study. Blood 2003;102:996–999.
15 Zuckerman E, Zuckerman T, Levine AM, Douer D, Gutekunst K, Mizokami M, Qian DG, Velankar M, Nathwani BN, Fong TL: Hepatitis C virus infection in patients with B-cell non-Hodgkin lymphoma. Ann Intern Med 1997;127:423–428.
16 Mizorogi F, Hiramoto J, Nozato A, Takekuma Y, Nagayama K, Tanaka T, Takagi K: Hepatitis C virus infection in patients with B-cell non-Hodgkin's lymphoma. Intern Med 2000;39:112–117.
17 De Re V, De Vita S, Marzotto A, Gloghini A, Pivetta B, Gasparotto D, Cannizzaro R, Carbone A, Boiocchi M: Pre-malignant and malignant lymphoproliferations in an HCV-infected type II mixed cryoglobulinemic patient are sequential phases of an antigen-driven pathological process. Int J Cancer 2000;87:211–216.
18 Takayasu K, Kasugai H, Ikeya S, Muramatsu Y, Moriyama N, Makuuchi M, Yamazaki S, Hirohashi S: A clinical and radiologic study of primary liver cancer associated with extrahepatic primary cancer. Cancer 1992;69:45–51.
19 Kerbel RS, Waghorne C, Korczak B, Lagarde A, Breitman ML: Clonal dominance of primary tumours by metastatic cells: genetic analysis and biological implications. Cancer Surv 1988;7:597–629.
20 Rowbotham D, Wendon J, Williams R: Acute liver failure secondary to hepatic infiltration: a single centre experience of 18 cases. Gut 1998;42:576–580.
21 Ascoli V, Lo Coco F, Artini M, Levrero M, Martelli M, Negro F: Extranodal lymphomas associated with hepatitis C virus infection. Am J Clin Pathol 1998;109:600–609.
22 Hermine O, Lefrere F, Bronowicki JP, Mariette X, Jondeau K, Eclache-Saudreau V, Delmas B, Valensi F, Cacoub P, Brechot C, Varet B, Troussard X: Regression of splenic lymphoma with villous lymphocytes after treatment of hepatitis C virus infection. N Engl J Med 2002;347:89–94.
23 Sasso EH: The rheumatoid factor response in the etiology of mixed cryoglobulins associated with hepatitis C virus infection. Ann Med Interne (Paris) 2000;151:30–40.

Microbiol. Immunol., 50(11), 857–865, 2006

New Epitope Peptides Derived from Hepatitis C Virus (HCV) 2a Which Have the Capacity to Induce Cytotoxic T Lymphocytes in HLA-A2$^+$ HCV-Infected Patients

Yi Wang[1,2,5], Yukari Takao[1,2,3,4], Mamoru Harada[1,3], Shigeru Yutani[3,4], Tatsuya Ide[4], Michio Sata[2,4], Kyogo Itoh[1,2,3], and Akira Yamada*[1,2]

[1]*Cancer Vaccine Development Division, Kurume University Research Center for Innovative Cancer Therapy, Kurume, Fukuoka 830–0011, Japan,* [2]*Center of the 21st Century COE Program for Medical Science, Kurume University, Kurume, Fukuoka 830–0011, Japan,* [3]*Department of Immunology, Kurume University School of Medicine, Kurume, Fukuoka 830–0011, Japan,* [4]*2nd Department of Internal Medicine, Kurume University School of Medicine, Kurume, Fukuoka 830–0011, Japan, and* [5]*Institute of Frontier Medical Science, Jilin University, No.1163, Xinmin Street, Changchun 130021, P. R. China*

Received April 24, 2006; in revised form, August 14, 2006. Accepted August 23, 2006

Abstract: **Because cytotoxic T lymphocytes (CTLs) play an important role in the specific immunotherapy of hepatitis C virus (HCV) infection, a series of CTL epitopes has been defined from HCV genotype 1a or 1b protein. Here, we attempted to identify HCV2a-derived epitopes that are capable of inducing HLA-A2-restricted and peptide-specific CTLs. Peripheral blood mononuclear cells (PBMCs) of HLA-A2$^+$ HCV2a-infected patients or healthy donors were stimulated *in vitro* with each of the HCV2a-derived peptides, which were prepared based on the HLA-A2-binding motif, and their peptide-specific and HLA-A2-restricted cytotoxicities were examined. The HCV2a 432–441, HCV2a 716–724, and HCV2a 2251–2260 peptides were found to efficiently induce peptide-specific CTLs from the PBMCs of HLA-A2$^+$ HCV2a-infected patients. Cytotoxicity was mainly mediated by CD8$^+$ T cells in a HLA class I-restricted manner. These results indicate that the HCV2a 432–441, HCV2a 716–724, and HCV2a 2251–2260 peptides might be applicable for peptide-based immunotherapy of HLA-A2$^+$ HCV2a-infected patients.**

Key words: **HCV, Peptide, CTL, Vaccine**

Hepatitis C virus (HCV) is a leading cause of liver disease and hepatocellular carcinoma throughout the world. Infection with HCV often progresses to chronic hepatitis and thereby to cirrhosis and hepatocellular carcinoma over several decades (2, 16, 27). HCV is a highly variable virus, and at least six known genotypes are found worldwide (3). The genotype of the virus strongly impacts the success of antiviral therapies, and may affect disease progression (19, 32). HCV genotypes 1b and 2a are the most predominant in Italy and some Asian countries, including China, Japan and Korea (12, 31). Patients with the HCV2a infection usually have better responses to interferon (IFN) therapy than those with HCV1b infection. However, about 30–40% of HCV2a-infected patients are resistant to IFN treatment (14, 28). Moreover, there are large numbers of HCV2a-infected patients in developing countries that cannot afford the expensive antiviral therapy. Therefore, it is of great importance to find new therapeutic modalities.

CD8$^+$ cytotoxic T lymphocytes (CTLs) are known to play an important role in the elimination of HCV (7, 24). Virus-specific CTLs recognize viral antigens on infected cells in a human leukocyte antigen (HLA) class I-restricted manner, and then lyse the cells (9). Many research groups have focused on the identification of epitope peptides that can be recognized by CTLs.

**Address correspondence to* Dr. Akira Yamada, Cancer Vaccine Development Division, Kurume University Research Center for Innovative Cancer Therapy, 67 Asahi-machi, Kurume, Fukuoka 830–0011, Japan. Fax: +81–942–31–7745. E-mail: akiymd@med.kurume-u.ac.jp

Abbreviations: CTL, cytotoxic T-lymphocyte; DC, dendritic cells; E/T, effector cells/target cells; HCV, hepatitis C virus; HLA, human leukocyte antigen; IFN, interferon; IL-2, interleukin-2; PBMC, peripheral blood mononuclear cell.