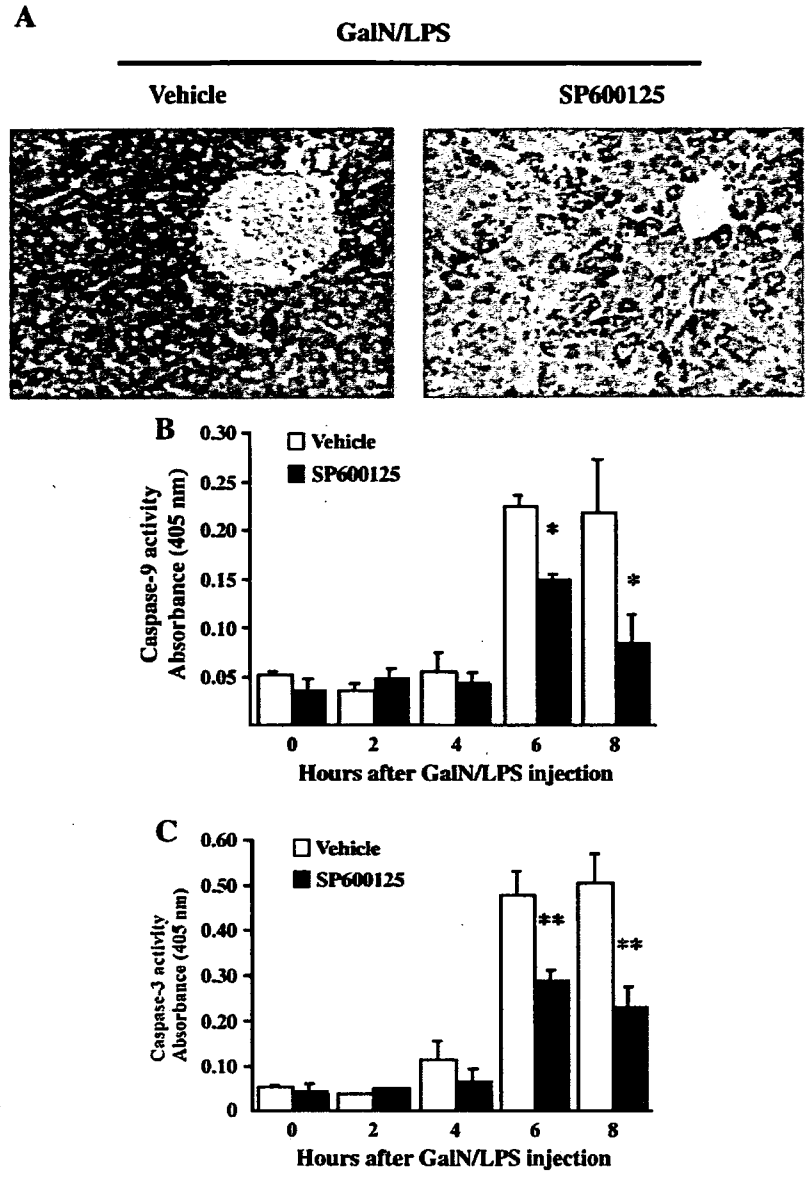

Fig. 6. (A) Immunohistochemistry for cytochrome *c* in the livers of SP600125-treated mice and vehicle-treated mice at 6 h after GalN/LPS administration (×200). Effect of SP600125 on caspase-9 (B) and -3 (C) activities in the livers of mice after GalN/LPS injection. The results are presented as the mean ± SD for 5 samples. $^{*}P<0.05$, $^{**}P<0.01$, vs vehicle-treated mice.

administration. To determine whether SP600125 altered the expression of Bad mRNA, we examined the expression of the mRNA by reverse-transcription-PCR in the livers of SP600125- and vehicle-treated mice after GalN/LPS administration (Fig. 7B). Consistent with the alteration in the protein levels of Bad, the expression of Bad mRNA was also markedly downregulated in the livers of SP600125-treated mice beginning at 2 h after GalN/LPS administration.

Discussion

As apoptosis of hepatocytes is the major underlying cause of FHF, a better understanding of this molecular mechanism has important implications in devising strategies for treating FHF. The JNK signaling pathway is one of the most important apoptosis-signaling pathways, and is activated by various forms of liver injury (Trautwein et al., 1998; Bendinelli et al., 1996; Bradham et al., 1997; Schattenberg et al., 2006; Chang et al., 2006). Consistent with previous report (Wang et al., 2006), we observed that GalN/LPS administration led to prolonged hepatic JNK activation. The duration of JNK activation is thought to be a critical factor determining cell proliferation or apoptosis; that is, transient JNK activation leads to cell proliferation, whereas sustained JNK activation causes cell apoptosis (Chen et al., 1996). In the liver, transient and modest JNK activation is required for liver regeneration (Schwabe et al., 2003), whereas sustained JNK activation promotes cell death (Schattenberg et al., 2006; Maeda et al., 2003). The sustained

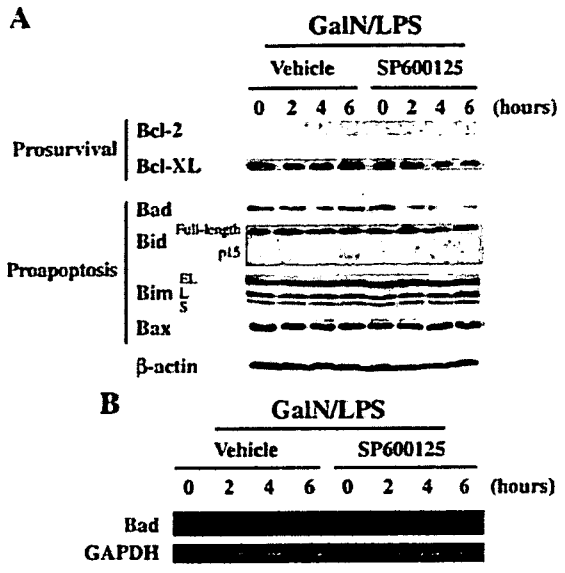

Fig. 7. Effect of SP600125 on Bcl-2 protein family expression in the livers of mice after GalN/LPS administration by Western blot analysis (A) and reverse-transcription PCR (B). All experiments were performed at least 3 times, and representative results are shown.

activation of JNK in mice with GalN/LPS-induced FHF may have similarly triggered a cell death response in hepatocytes in vivo.

Our study demonstrated that SP600125 blocked p-c-*Jun* in the livers of GalN/LPS-treated mice. One possible target for p-c-*Jun* in the AP-1 complex is the TNF-α gene itself, which contains an AP-1 binding site on its promoter (Becker et al., 1999). In the hepatic I/R model (Uehara et al., 2005) and liver transplantation model (Uehara et al., 2004), SP600125 decreased hepatocyte apoptosis, resulting in improved survival. In the former, SP600125 decreased the hepatic TNF-α level, whereas in the latter it did not, although TNF-α was a critical cytokine in the liver injury produced in both models. In the present study, SP600125 was able to suppress hepatocyte apoptosis and prolong the survival of mice with GalN/LPS-induced FHF without suppressing the increased level of serum TNF-α. This difference observed among the studies may have been due to differences in experimental design. This finding in GalN/LPS-induced FHF suggests that SP600125 suppresses GalN/LPS-induced hepatocyte apoptosis via its interaction with the downstream targets of TNF-α.

Our results strongly suggest that JNK activity is required for activation of the mitochondria-mediated apoptosis pathway. Supporting evidence included clearly demonstrated prevention of cytochrome *c* release, and caspase-9 and caspase-3 activation by SP600125 after GalN/LPS administration. To further elucidate the underlying mechanisms, we examined the effects of JNK on the expression of Bcl-2 family proteins, which govern the mitochondria-dependent pathway of apoptosis. Among these proteins, there is direct evidence that hepatocyte-specific Bcl-XL-deficient mice develop spontaneous and continuous apoptosis in hepatocytes (Takehara et al., 2004); that is, hepatocytes cannot survive without expressing Bcl-XL, and this molecule must be a crucial apoptosis antagonist of hepatocytes since these cells do not express Bcl-2. Consistent with a recent study using GalN/LPS-treated *jnk2* null mice (Wang et al., 2006), in the present study SP600125 had no effect on the steady-state expression of Bcl-XL after GalN/LPS administration. The influence and function of prosurvival Bcl-2 family proteins, including Bcl-XL, are regulated by BH3-only proteins, which are proapoptotic members of the Bcl-2 family (Huang and Strasser, 2000). To date, eight members of the BH3-only protein family have been characterized: Bad, Bid, Bik, Bim, Bmf, Hrk, Noxa, and Puma. BH3-only proteins, except for Noxa, are capable of binding to Bcl-XL and neutralizing its function (Baskin-Bey and Gores, 2005). A recent study using knockout mice has shown that ablation of *jnk2*, but not *jnk1*, inhibits Bid cleavage, but has no effect on the steady-state

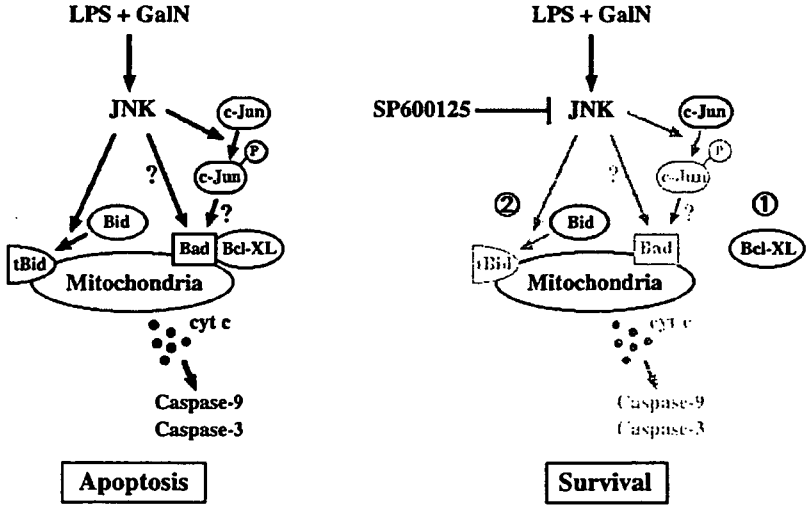

Fig. 8. Schematic model for the mechanism by which SP600125 suppresses GalN/LPS-induced hepatocyte apoptosis. Inhibition of JNK activity by SP600125 induces downregulation of Bad expression, increasing the relative amount of functional Bcl-XL protein (in the early stage; ①), and inhibiting the cleavage of Bid (in the late stage; ②), thereby preventing hepatocyte apoptosis in the liver of mice with GalN/LPS-induced FHF.

expression of Bad, Bax and Bim after GalN/LPS administration (Wang et al., 2006). In the present study, we found that SP600125 downregulated the expression of Bad in the early period following GalN/LPS injection and prevented Bid cleavage in the late period, but had no effect on the steady-state expression of Bax and Bim. Because SP600125 is a potent and specific inhibitor of all JNK isoforms (JNK1, JNK2 and (neural specific) JNK3), our results appear to support the possibility that the functions of both JNK1 and JNK2 are redundant. Indeed, the mortality of GalN/LPS-treated mice administered SP600125 was markedly lower than that of GalN/LPS-treated *jnk1* or *jnk2* null mice (Wang et al., 2006).

There is some evidence for the function of Bad and Bid in the liver; absence of Bad markedly protects primary hepatocytes from glucose-withdrawal-induced apoptosis (Danial et al., 2003), and administration of GalN/LPS to *Bid*-deficient mice offered only partial protection (Zhao et al., 2001). Therefore, it appears that modulation of not only one but also two molecules by SP600125 plays a critical role in GalN/LPS-induced hepatocyte apoptosis. These results permit us to speculate that SP600125 causes an increase in the relative amount of functional Bcl-XL protein, which is freed from Bad (in the early stage) and truncated Bid (in the late stage), thereby preventing hepatocyte apoptosis in the liver of mice with GalN/LPS-induced FHF (Fig. 8). The mechanism involved in the modulation of these BH3-only proteins by JNK blockade requires further study.

In summary, this study has confirmed the critical role of JNK signaling in liver injury induced by GalN/LPS administration in vivo. It is also suggested that SP600125, which inhibits hepatocyte apoptosis by modulating BH3-only proteins, may be a useful therapeutic tool for FHF.

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INTERNAL MEDICINE

□ CASE REPORT □

Decompensated Lamivudine-resistant Hepatitis B Virus-related Cirrhosis Treated Successfully with Adefovir Dipivoxil Allowing Surgery for Hepatocellular Carcinoma

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Abstract

We describe a 64-year-old man with decompensated hepatitis B virus (HBV)-related cirrhosis who became resistant to lamivudine. He was started on adefovir at 10 mg daily while continuing lamivudine therapy. Several months later, his liver function improved and subsequently his ascites disappeared. The serum HBV-DNA level became undetectable 11 months later. Twenty months after the start of additional treatment with adefovir, one hepatocellular carcinoma (HCC) was detected, and the patient underwent a successful hepatectomy. Our findings suggest that the addition of adefovir to ongoing lamivudine therapy is useful for improving liver function in patients with decompensated lamivudine-resistant HBV-related cirrhosis, allowing surgery for HCC.

Key words: adefovir dipivoxil, lamivudine, YMDD mutant, hepatocellular carcinoma, hepatectomy

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Introduction

Hepatitis B virus (HBV) infection is one of the major causes of cirrhosis and hepatocellular carcinoma (HCC), and interferon is one of several therapeutic options for chronic HBV infection. Several studies of interferon therapy have demonstrated that it can induce a sustained loss of hepatitis B e antigen (HBeAg) and reduce HBV-DNA to an undetectable level in about 40% of patients with chronic hepatitis B (CHB) (1, 2). However, this agent is generally contraindicated in patients with severely decompensated HBV infection (3). Recently, lamivudine, a nucleoside analogue, has been used to treat chronic HBV infection. Lai et al reported that one year of treatment with lamivudine induced HBeAg seroconversion in 16% of patients with HBeAg-positive CHB compared to 4% of those receiving placebo, and produced histological improvement (4). Also, Liaw et al reported that lamivudine reduced disease progression (defined as hepatic decompensation, HCC, bacterial peritonitis, bleeding gastroesophageal varices, or death related to liver disease) in about 50% of patients with compensated cirrhosis (5). However, long-term therapy with lamivudine may result in the emergence of genotypic resistance in the form of tyrosine, methionine, aspartate, aspartate (YMDD) mutations, which is occasionally associated with severe, or even fatal, hepatitis flare-up (6-8). Thus, there is a clear need for alternative or additional therapies.

Adefovir dipivoxil (adefovir) is a nucleotide analogue that is converted to an active metabolite, adefovir diphosphate. In large studies of both HBeAg-positive and -negative patients with CHB, adefovir has been shown to significantly reduce HBV-DNA levels in most patients and to improve liver histology and serum alanine aminotransferase (ALT) levels (9,

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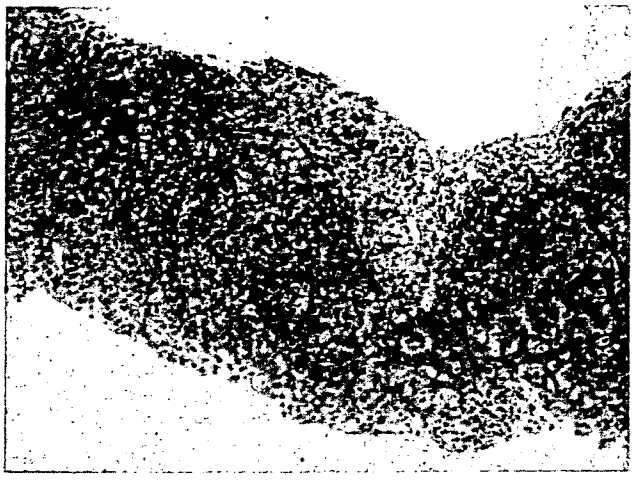

Figure 1. Liver biopsy specimen before lamivudine treatment shows severe necro-inflammation and fibrosis. It was graded as A3F3 according to the New Inuyama Classification (H&E stain, original magnification ×40).

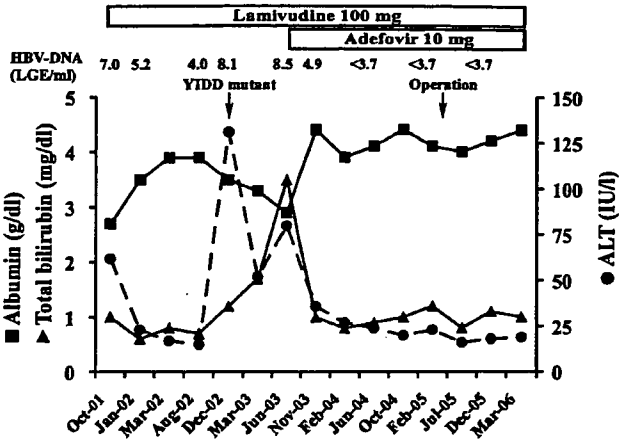

Figure 2. Albumin, total bilirubin, ALT and HBV-DNA levels during the clinical course of the patient from the start of lamivudine treatment.

10). Similarly, the results of long-term administration of adefovir have shown that this agent is effective in reducing the level of HBV-DNA, normalizing the level of ALT, and improving liver histology, and is generally well tolerated (11). Also, adefovir effectively suppresses lamivudine-resistant HBV in CHB patients after liver transplantation, or in patients with decompensated liver disease (12, 13).

In this report, we present a case of decompensated lamivudine-resistant HBV-related cirrhosis that was treated using adefovir. The patient's clinical status and liver function were sufficiently improved to allow surgery for HCC, which developed 20 months after the beginning of adefovir therapy.

Case Report

A 64-year-old Japanese male patient had been followed since 1985 for HBV-related chronic hepatitis. He was positive for HBs and HBe antigens and had no history of elevated serum ALT. In 2001, the serum ALT level fluctuated between 50-80 IU/l (normal 4-41 IU/l), the HBV-DNA level was shown to be more than 7.0 log genome equivalent (LGE)/ml by transcription-mediated amplification, and the alpha-fetoprotein (AFP) level was elevated to 290 ng/ml (normal upper limit 6 ng/ml). However, the ratio of AFP-L3 was 7.5% (normal upper limit 10%), and the desgamma carboxy prothrombin (DCP) level was within normal limits. Abdominal ultrasonography and dynamic computed tomography (CT) revealed no evidence of HCC. A liver biopsy specimen showed severe necro-inflammation and fibrosis, which was graded as A3F3 according to the New Inuyama Classification (Fig. 1) (14).

From October 2001, the patient started to receive lamivudine at a dose of 100 mg daily. The subsequent clinical course is shown in Fig. 2. The serum ALT and AFP levels decreased to the normal range. The HBV-DNA level decreased gradually, but did not become undetectable. The patient did not achieve HBeAg seroconversion during lamivudine monotherapy. After 15 months of lamivudine treatment, the serum HBV-DNA level increased rapidly to 8.1 LGE/ml along with serum ALT elevation (131 IU/l). At the same time, the lamivudine-resistant YIDD mutant strain of HBV was detected in the serum. Six months later, the patient complained of dark urine and abdominal fullness. Physical examination showed jaundice of the skin and conjunctivae, and abdominal distention due to ascites. The ALT, total bilirubin, HBV-DNA level and prothrombin time were 80 IU/l, 3.5 mg/dl, 8.5 LGE/ml and 36%, respectively. Abdominal CT revealed massive ascites, an atrophic liver with an irregular surface, and splenomegaly (Fig. 3a). The patient was diagnosed as having decompensated lamivudine-resistant HBV-related cirrhosis, and his liver function did not improve sufficiently despite administration of furosemide, spironolactone, human serum albumin and fresh-frozen plasma.

From July 2003, the patient was treated with adefovir at a dose of 10 mg daily in addition to lamivudine. In November 2003, his liver function parameters improved: albumin 4.4 g/dl, total bilirubin 1.0 mg/dl, ALT 36 IU/l, and subsequently his ascites disappeared. Dynamic CT showed that the liver volume had increased and that the ascites had disappeared (Fig. 3b). The serum HBV-DNA level decreased, and became undetectable 11 months later.

After a follow-up of 20 months, dynamic CT and magnetic resonance imaging showed an enhanced lesion, 12 mm in diameter, in segment 6 of the liver. At that time, the serum total bilirubin and albumin levels, and prothrombin time, were 1.2 mg/dl, 4.1 g/dl and 72%, respectively, but the AFP and DCP levels were within normal limits. For further examination, the patient was admitted in February 2005. CT during arterial portography showed a perfusion defect in segment 6, and CT during hepatic arteriography showed a hyperdense lesion in the corresponding region (Fig. 3c). We

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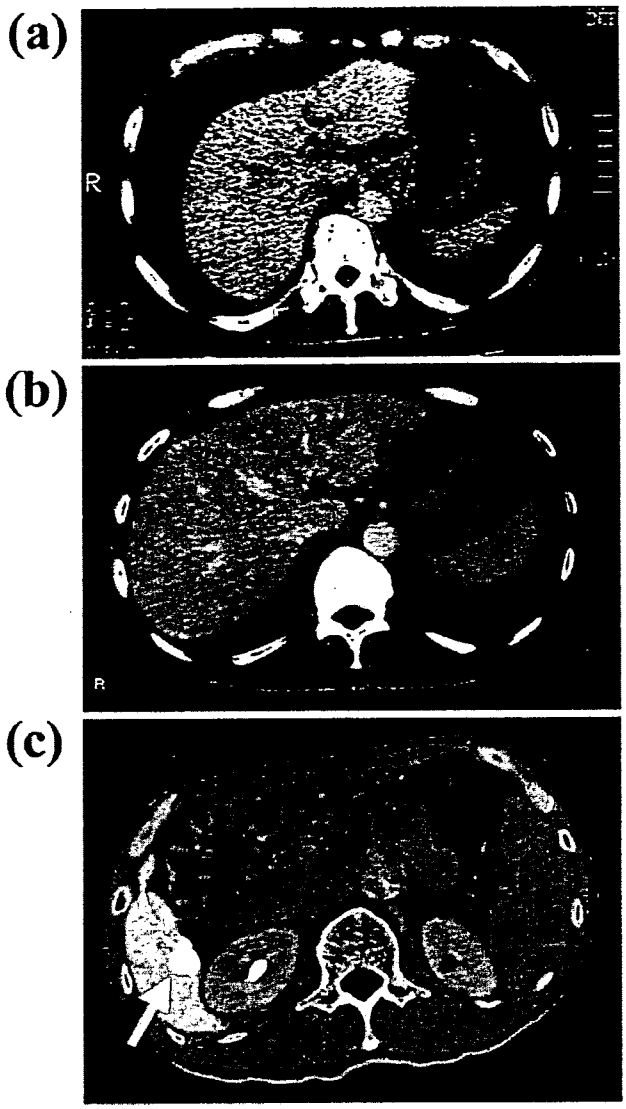

Figure 3. (a)Dynamic CT before adefovir therapy shows **liver cirrhosis with massive ascites. (b) Dynamic CT after 10 months of adefovir therapy shows that the liver volume had increased and that the ascites had disappeared. (c) CT during hepatic arteriography in the first phase showed a hyperdense lesion in segment 6 (white arrow).**

therefore suspected that the lesion in segment 6 was a typical HCC at stage I (T1N0M0) according to the International Union Against Cancer (UICC) stage grouping (15).

After receiving an explanation about treatment, including surgery, percutaneous ethanol injection and radiofrequency ablation, the patient elected to undergo surgery, and subsegmentectomy was performed successfully on March 2005. Grossly, the tumor was a nodular lesion measuring 14 × 14 × 12 mm. Microscopically, the tumor was a moderately differentiated HCC with a trabecular pattern (Fig. 4a). The nontumorous liver tissue showed cirrhosis, diagnosed as A1 F4 (Fig. 4b). There was no evidence of nephrotoxicity, which is a known side effect of adefovir, during this treatment.

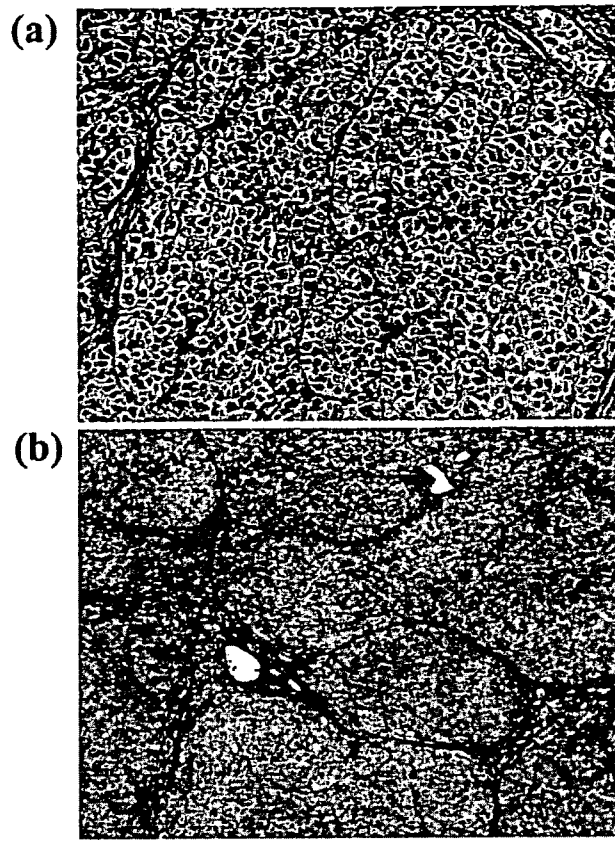

Figure 4. (a and b) Histological findings of specimens obtained by subsegmentectomy in March 2005. (a) The tumor is a moderately differentiated HCC (H&E stain, original magnification ×100). (b) The nontumorous liver tissue around the tumor shows cirrhosis with mild inflammation, which was graded as A1F4 (H&E stain, original magnification ×40).

Discussion

Until recently, the only treatment option for chronic HBV infection has been interferon. However, the development of orally administered nucleoside and nucleotide analogues has revolutionized the treatment of this disease. The first of these agents, lamivudine, has a well established safety and efficacy profile in the treatment of chronic HBV infection (4, 5). With regard to HBV-related HCC, Nakanishi et al reported that liver function in a patient with decompensated HBV-related cirrhosis was sufficiently improved by lamivudine to allow surgery for HCC (16), and Nagamatsu et al reported that prophylactic lamivudine treatment was effective in preventing exacerbation of liver damage in HBeAg-positive patients throughout chemotherapy for HCC (17). However, a major problem complicating the long-term use of lamivudine is a progressive increase in drug resistance in the form of YMDD mutations. In a review, Liaw et al stated that the proportion of patients in whom YMDD mutations are detectable increases with time during lamivudine treatment, from 14% after 1 year to 69% by 5 years (18). Despite the emergence of YMDD mutations, many patients

maintain improvements in the levels of markers of HBV infection and liver injury (7, 19). However, there are several reports of hepatic decompensation and death due to liver failure after breakthrough infection with YMDD mutations (6, 20-22). Similarly, in the present case, the YIDD mutant strain emerged after 15 months of lamivudine administration, and thereafter the patient developed decompensated liver cirrhosis. He was treated with adefovir in addition to lamivudine because his liver function was not sufficiently improved by conventional therapy.

In comparison with lamivudine, the emergence of resistance to adefovir is less common and occurs later (11, 23, 24). Data from a recent study of 125 patients undergoing long-term adefovir treatment indicated that 0% of patients had resistance in year 1, 11% in year 3, and 28% in year 5. Interstingly, all patients who developed adefovir resistance were not receiving lamivudine and adefovir combination therapy, but adefovir monotherapy (24, 25). Moreover, based on *in vitro* studies and limited clinical data, lamivudine has been shown to be effective in patients with adefovir-resistant HBV (25-28). For these reasons, we consider that lamivudine and adefovir combination therapy may be better than adefovir monotherapy. However, as cross-resistance between lamivudine and adefovir has been reported (25, 29), this patient had to be followed carefully during lamivudine and adefovir combination therapy.

In this case, HCC was detected 20 months after the start of additional treatment with adefovir. The patient's clinical status and liver function were sufficiently improved when HCC was detected, allowing surgical resection to be performed successfully. Histologically, the nontumorous liver tissue showed improvement of necro-inflammation, but not fibrosis. Some clinical trials have shown that adefovir, as well as lamivudine, contributes to the histological improvement (9-11). Among them, Hadziyannis et al reported that necro-inflammation showed a high improvement rate (over 70 %) from the first year of adefovir therapy, whereas liver fibrosis showed increasing improvement, reaching more than 70% at 5 years (11). In the present case, also, it is expected that long-term therapy with adefovir will help to improve the patient's liver fibrosis.

In conclusion, our experience suggests that adefovir therapy is effective for reversing hepatic decompensation due to replicating lamivudine-resistant HBV and that continuation of treatment with lamivudine and adefovir maintains stable liver function, allowing subsequent surgery for HCC.

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Altered expression of TLR homolog RP105 on monocytes hypersensitive to LPS in patients with primary biliary cirrhosis ☆,☆☆

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***Backgrounds/Aims*: Toll-like receptors (TLRs) have emerged as a key component of the innate immune system that triggers antimicrobial responses. Altered monocyte responses to ligands for TLRs have been reported in patients with primary biliary cirrhosis (PBC), yet the precise mechanism remains unknown.**

***Methods*: We investigated *in vitro* responses to a TLR4 ligand, lipopolysaccharide (LPS), using peripheral blood mononuclear cells and monocytes from 25 patients with PBC, 10 patients with chronic viral hepatitis (CVH), and 20 healthy individuals.**

***Results*: After stimulation with LPS, we found significantly higher amounts of IL-1β, IL-6, and IL-8 production in PBC patients. Through the TLR4 signaling pathway, activation of NF-κB and expression of MyD88 mRNA were significantly increased in PBC patients, and the level of TLR4 expression was significantly increased on PBC monocytes as compared with CVH patients and controls. Of significance, the surface expression of RP105, which has recently been shown to be involved in negative regulation of TLR4 signaling, on PBC monocytes was significantly decreased in comparison with CVH patients ($P = 0.016$) and controls ($P < 0.001$).**

***Conclusions*: These results suggest that expression of RP105 and TLR4 is altered on PBC monocytes, which appear to be hypersensitive to LPS, resulting in increased secretion of pro-inflammatory cytokines.**

***Keywords*: Innate immunity; Toll-like receptors; Pro-inflammatory cytokines; RP105/MD-2; NF-κB**

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Abbreviations: PBC, primary biliary cirrhosis; TLR, Toll-like receptor; LPS, lipopolysaccharide; PGN, peptidoglycan; PBMCs, peripheral blood mononuclear cells; CBA, Cytometric Bead Array; IL, interleukin; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

1. Introduction

Primary biliary cirrhosis (PBC) is a progressive autoimmune liver disease characterized by destruction of the small intrahepatic bile ducts, portal inflammation, and the presence of antimitochondrial antibodies (AMA) [1]. Although the role of adaptive immune responses has been extensively studied, the pathogenesis of PBC remains obscure. Molecular mimicry, or the resemblance of pathogen- and host antigen-derived epitopes recognized by immune cells, has been suggested to be one mechanism for the induction of PBC [2,3], and therefore infectious agents have been proposed as triggers for PBC [4,5]. As it is becoming increasingly clear

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that the innate immune response plays a prominent role in regulating the quality of the adaptive immune response that ensues [6], the importance of innate immunity in the pathogenesis of PBC is now becoming apparent [7].

Toll-like receptor (TLR) family has recently emerged as a key component of the innate immune system that detects microbial infection and triggers antimicrobial host defense responses [8–10]. The role of immune responses *via* TLR signaling in the pathogenesis of PBC has been investigated in only a few studies. Takii et al. reported that the mRNA expression of TLR3, which is known to induce type I interferon, was significantly higher in the portal tract and liver parenchyma of patients with early stage PBC than in patients with autoimmune hepatitis and chronic hepatitis [11]. Kikuchi et al. showed that peripheral blood mononuclear cells (PBMCs) from patients with PBC produced significantly higher levels of polyclonal IgM when stimulated with unmethylated oligonucleotide (CpG)-B, a natural ligand for TLR9 [12,13]. These IgM were produced predominantly by $CD27^+$ memory B cells, which express high levels of TLR9 [12]. These results suggest that increased levels of IgM and hyper-responsiveness to CpG are a universal and fundamental feature of the immune dysregulation that leads to PBC.

Binding of pathogen-associated molecular patterns (PAMPs) to TLRs triggers a complex series of events leading to increased expression of pro-inflammatory genes [8,9]. Mao et al. recently reported altered monocyte responses to defined ligands for TLRs in patients with PBC, and speculated that such altered monocyte responses resulting in increased secretion of pro-inflammatory cytokines may be critical in the breakdown of self-tolerance [14]. Although their findings may provide new insight into the pathogenesis of PBC, they did not indicate the precise mechanism involved. Besides several previously reported negative regulators of TLR signaling [8], RP105, a TLR4 homolog, has recently been shown to be involved in negative regulation of TLR4 signaling [15]. The principal objective of the present study was to investigate the mechanism responsible for alterations of monocyte responses through TLR signaling, especially that of TLR2 and TLR4, which are critically involved in the response to Gram-positive and -negative microbial stimuli, in patients with PBC. Concerning the role of TLRs in the development of autoimmune diseases, TLR2 and TLR4 seem to be important because dead cells release nuclear molecules that can bind to TLR2 and TLR4 on macrophages and trigger them to clear up apoptotic bodies, thus maintaining self-tolerance [16]. We therefore investigated *in vitro* responses to defined ligands for TLR2 (peptidoglycan; PGN) and TLR4 (lipopolysaccharide; LPS) using PBMC and peripheral blood monocytes from patients with PBC.

2. Patients and methods

2.1. Patients

After obtaining appropriate informed consent in writing under Institutional Review Board-approved protocols at Niigata University Medical and Dental Hospital, heparinized peripheral blood was collected from 25 patients with PBC, 10 patients with chronic viral hepatitis (CVH), and 20 healthy individuals. All the patients with PBC were positive for AMA, and showed increased serum levels of IgM (265.6 ± 172.0 mg/dl). Twenty-four patients were female and one was male, with a mean age of 64.6 years (range; 44–75 years). All the patients were diagnosed to have early stage disease (18 patients at stage 1 and 7 at stage 2, according to Scheuer's classification [17]) and receiving ursodeoxycholic acid (UDCA) as the sole treatment for PBC. Ten patients with CVH (8 women and 2 men, age 65.7 ± 16.8 years) and 20 healthy subjects (15 women and 5 men, age 46.8 ± 14.2 years) served as disease and healthy controls, respectively. As expected, none of the controls was AMA positive, and showed increased serum levels of IgM.

2.2. Reagents

Monoclonal antibodies (mAb) used were anti-human CD14 (clone: M5E2), anti-human CD45 (2D1), anti-human CD83 (HB15e), and anti-human CD180 (RP105) (G28-2) (BD PharMingen, San Diego, CA). Anti-human TLR2 (TL2.1) and anti-human TLR4 (HTA125) mAb were purchased from eBioscience, San Diego, CA. Control murine IgG2a and IgG1 were purchased from BD PharMingen.

2.3. Cell isolation

PBMCs were isolated using Ficoll-Paque Plus (Amersham Biosciences Co., St. Louis, MO) density gradient centrifugation. After centrifugation, cells were washed with phosphate-buffered saline (PBS) containing 0.5% heat-inactivated fetal calf serum and counted in a hemocytometer; the viability of cells was determined using trypan blue exclusion. An aliquot containing 5×10^5 PBMCs was used for flow cytometry. $CD14^+$ cells were purified from PBMCs by isolation with anti-human CD14 immunomagnetic beads (Miltenyi Biotech, Auburn, CA) (purity of $CD14^+$ population: >92%).

2.4. Stimulation of monocytes

PBMCs or purified $CD14^+$ cells were resuspended at 1×10^6/ml in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal calf serum, penicillin (50 U/ml), and streptomycin (50 μg/ml). Cells were then dispensed in a volume of 2 ml per well into individual wells of 24-well flat-bottomed microtiter plates (Corning, Acton, MA). Equal volume of media containing the appropriate TLR ligands was added to triplicate wells. The final concentrations for each TLR ligand stimulus are as follows: 10 μg/ml PGN (*Staphylococcus aureus*, Sigma, St. Louis, MO), 1 μg/ml LPS (*Escherichia coli* serotype 0111:B4; Sigma). In addition, a baseline control containing only media was included. According to preliminary experiments for determining the culture durations, the cultures were then incubated for 1 h at 37 °C in 5% CO_2 for flow cytometric analysis and nuclear extraction analysis, for 3 h for quantitative RT-PCR, for 6 and 24 h for Cytometric Bead Array (CBA) analysis.

2.5. Cytometric Bead Array (CBA)

The quantitation of IL-1β, IL-6, IL-8, IL-12p70, and TNF-α in the supernatants of cultured PBMCs with or without TLR ligands was determined using a human CBA kit (BD Biosciences, San Jose, CA) as described previously [14].

2.6. NF-κB subunit p65 specific ELISA

Activated nuclear factor-kappa B (NF-κB) subunit p65 was quantified by a sensitive multi-well colorimetric assay using Trans-AM™ transcription factor assay kit (Active Motif, Carlsbad, CA). Briefly, nuclear extracts (3 μg) were collected from 5×10^6 cells using Nuclear Extraction Kit (Active Motif), and incubated in 96-well plates coated with oligonucleotide containing the NF-κB consensus site (5'-GGGACTTTCC-3') for 1 h, and then assayed for p65 binding according to the manufacturer's instructions.

2.7. Immunofluorescence analysis

Cell surface antigen expression was determined by a three-color immunofluorescence assay. Cells (10^5) were incubated with the appropriate FITC-, phycoerythrin- or CyChrome-conjugated mAbs for 30 min at 4 °C in PBS with 0.1% FCS and 0.02 mM NaN_3. After wash, the labeled cell samples were analyzed on FACScan (Becton Dickinson, San Jose, CA). The data acquired were analyzed with CellQuest Pro software (BD Immunocytometry Systems).

2.8. Quantitative PCR

Total cellular RNA was extracted from 2×10^6 purified $CD14^+$ monocytes by RNeasy Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. Reverse transcription of the RNA samples to cDNA was performed with Transcriptor First Strand cDNA Synthesis Kit (Roche, Penzberg, Germany) with anchored-oligo$(dT)_{18}$ Primer. Real-time PCR was performed on a lightcycler (Roche) using the primers as described previously [18,19]. Expression levels of mRNA were normalized to β-actin mRNA levels.

2.9. Statistical analysis

The significance of differences was analyzed statistically by the unpaired *t* test with Welch's correction or Mann–Whitney *U* test using GraphPad software (GraphPad Software Inc., San Diego, CA). The level of significance was set at $P < 0.05$.

3. Results

3.1. Cytokine production after stimulation with LPS or PGN

Fig. 1 illustrates the production of pro-inflammatory cytokines after stimulation with LPS or PGN as measured using the CBA kit. LPS- or PGN-stimulated PBMCs from patients with PBC were found to produce significantly higher amounts of IL-1β (mean ± standard deviation; 1434 ± 591 pg/ml, range; 777–3022 vs. 528 ± 297 pg/ml, range; 310–1117 in controls; $P < 0.001$, and 2930 ± 1095, 1357–5235 vs. 2249 ± 862, 682–3,763; $P = 0.042$, respectively), TNF-α (362 ± 320, 52–1230 vs. 162 ± 157, 7–462; $P = 0.017$, and 1324 ± 841, 82–2750 vs. 777 ± 720, 37–1735; $P = 0.041$, respectively), and IL-8 (64,503 ± 28,628, 30,820–125,000 vs. 32,852 ± 12,700, 23,270–64,070; $P < 0.001$, and 67,930 ± 33,084, 33,510–115,910 vs. 30,680 ± 7801, 24,150–48,447; $P < 0.001$, respectively) after 24 h culture than those from controls (Fig. 1a–c). Production of IL-12p70 was also significantly higher in the patients with PBC than in the controls (85 ± 52 vs. 45 ± 55; $P = 0.035$, and 89 ± 71, vs. 39 ± 49; $P = 0.016$, respectively), although the values were relatively low (Fig. 1d). These results indicate that PBMCs from patients with PBC appear to be more sensitive to signaling *via* TLR2 and TLR4, resulting in increased secretion of pro-inflammatory cytokines.

3.2. LPS-induced NF-κB activation

It has been shown that upon ligand binding, TLR2 and TLR4 recruit various protein kinases *via* several adaptor molecules, such as myeloid differentiation factor 88 (MyD88), leading to the activation of NF-κB [8,9]. The level of activated NF-κB p65 in freshly isolated PBMCs was similar in both PBC patients and controls. However, nuclear translocation of NF-κB was significantly increased in PBC patients after LPS stimulation (0.279 ± 0.134 ng/μl, range; 0.129–0.489 vs. 0.158 ± 0.064, 0.072–0.287 in controls; $P < 0.001$) (Fig. 2). Although pro-inflammatory cytokine production was increased after stimulation with PGN, we were unable to detect any significant difference in NF-κB activation between patients with PBC and controls.

3.3. Flow cytometric analysis of CD83 surface expression on $CD14^+$ monocytes

Mean fluorescence intensity (MFI) values of CD83, an activation marker, on $CD14^+$ monocytes were studied by flow cytometry. After stimulation with LPS, monocytes from patients with PBC showed significantly increased expression of CD83 in comparison with those from patients with CVH (73.4 ± 29.3 vs. 43.2 ± 38.8; $P = 0.044$) and controls (vs. 40.6 ± 14.7; $P < 0.001$), while there was no difference in CD83 expression on freshly isolated monocytes. Monocytes from PBC patients also showed increased expression of CD83 after stimulation with PGN, but not to a significant degree (Fig. 3).

3.4. Expression of surface TLR2 and TLR4 on $CD14^+$ monocytes and MyD88 mRNA

In order to determine the effects of PGN or LPS on the expression of their respective receptors, TLR2 and TLR4, their surface expression was evaluated by flow cytometry. On freshly isolated $CD14^+$ monocytes, the surface expression levels of TLR2 and TLR4 were similar in both patients and controls. The expression of TLR2 was up-regulated after 1 h culture with PGN, but did not differ significantly between patients and controls (Fig. 4a).

In contrast, TLR4 expression was down-regulated after 1 h culture with LPS. The level of TLR4 expression after 1 h stimulation was significantly increased on $CD14^+$ monocytes from patients with PBC as compared to those from patients with CVH (18.0 ± 3.5 vs. 13.9 ± 5.4; $P = 0.046$) and controls (vs. 11.4 ± 4.5; $P < 0.001$) (Fig. 4b). Although TLR4 expression recov-

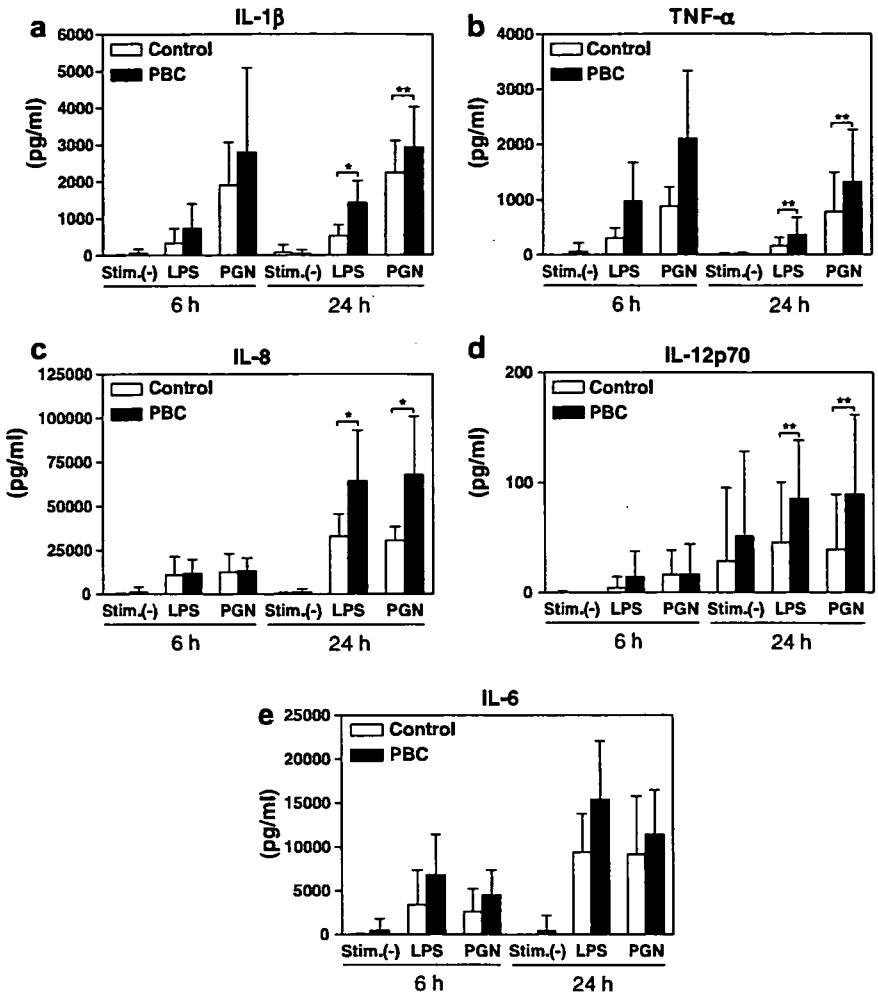

Fig. 1. Significantly increased production of pro-inflammatory cytokines by PBMCs from patients with PBC after stimulation with LPS and PGN. PBMCs from patients with PBC ($n = 22$) and controls ($n = 15$) were cultured with LPS (1 μg/ml) or PGN (10 μg/ml), or without any stimulation (Stim.(−)), for 6 (6 h) or 24 h (24 h). Production of pro-inflammatory cytokines including IL-1β (a), TNF-α (b), IL-8 (c), IL-12p70 (d), and IL-6 (e) was measured using Cytometric Bead Array (CBA) kit. The values are presented as means + SD. $^{*}P < 0.01$, $^{**}P < 0.05$.

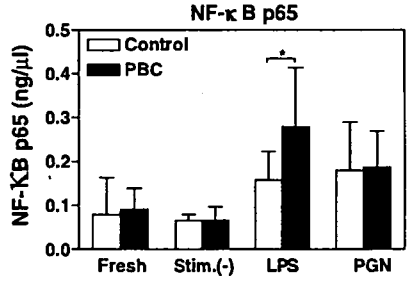

Fig. 2. Significantly increased nuclear translocation of NF-κB in PBMCs from patients with PBC after stimulation with LPS. PBMCs from patients with PBC ($n = 22$), and from controls ($n = 15$), were stimulated with PGN (10 μg/ml) or LPS (1 μg/ml), or without any stimulation (Stim.(−)), for 1 h. Nuclear translocation of activated NF-κB subunit p65 was quantified using DNA-binding capture of NF-κB with a consensus NF-κB-binding oligonucleotide in the nuclear extracts collected from freshly isolated (Fresh), or cultured PBMCs. The values are presented as means + SD. $^{*}P < 0.01$.

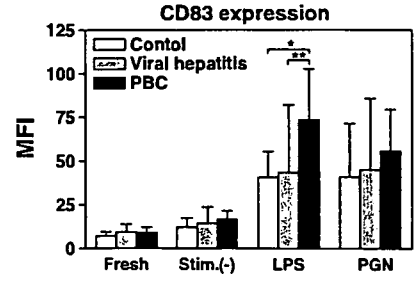

Fig. 3. Significant increase of CD83 surface expression on $CD14^{+}$ monocytes from patients with PBC after stimulation with LPS. Surface expression of CD83 on freshly isolated (Fresh) or cultured $CD14^{+}$ cells was analyzed. PBMCs from patients with PBC ($n = 25$) and chronic viral hepatitis ($n = 10$), and from controls ($n = 20$), were cultured with LPS (1 μg/ml) or PGN (10 μg/ml), or without any stimulation (Stim.(−)), for 1 h. Values were quantified as mean fluorescence intensity (MFI) by flow cytometry. The values are presented as means + SD. $^{*}P < 0.01$, $^{**}P = 0.044$.

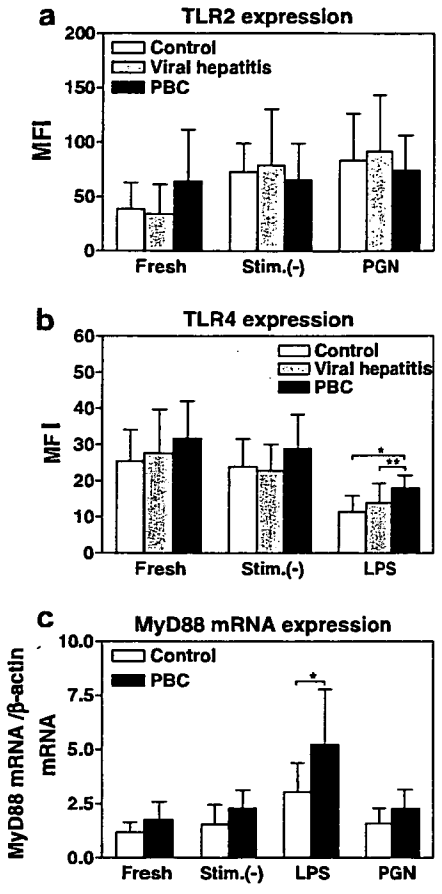

Fig. 4. Significantly increased expression of surface TLR4 and MyD88 mRNA in CD14$^+$ cells from patients with PBC after stimulation with LPS. Surface expression of TLR2 (a) and TLR4 (b) on freshly isolated (Fresh) or cultured CD14$^+$ cells was analyzed. PBMCs from patients with PBC ($n = 25$) and chronic viral hepatitis ($n = 10$), and from controls ($n = 20$), were stimulated with PGN (10 μg/ml) or LPS (1 μg/ml), or without any stimulation (Stim.(−)), for 1 h. Values were quantified as mean fluorescence intensity (MFI) by flow cytometry. (c) Expression of MyD88 mRNA in freshly isolated (Fresh) or cultured CD14$^+$ cells from patients with PBC ($n = 16$) and controls ($n = 15$) was quantified by real-time PCR. Steady-state levels of mRNAs were normalized relative to β-actin mRNA levels. The values are presented as means + SD. $^*P < 0.01$, $^{**}P = 0.046$.

ered to the pre-culture level after 24 h stimulation with LPS, TLR4 expression before and after 24 h stimulation with LPS did not differ significantly between patients with PBC and controls (data not shown). Real-time PCR showed that LPS-stimulated CD14$^+$ monocytes from patients with PBC had a significantly higher level of mRNA expression of MyD88, a TLR adaptor molecule, than in the controls (5.23 ± 2.54, range; 2.95–11.0 vs. 3.02 ± 1.36, 1.60–6.26; $P < 0.001$) (Fig. 4c).

3.5. Expression of surface RP105 on CD14$^+$ monocytes and RP105 mRNA

We then investigated the effect of LPS on surface expression of RP105 using flow cytometry before and after 1 h culture with LPS. On freshly isolated CD14$^+$ monocytes, the level of surface expression of RP105 was similar in both patients with PBC and controls (253.1 ± 147.8 vs. 288.3 ± 157.1). Representative data for RP105 expression on CD14$^+$ monocytes from healthy individuals and PBC patients after 1 h culture with LPS are shown in Fig. 5a. An interesting finding was that the surface expression of RP105 on CD14$^+$ monocytes from PBC patients after LPS stimulation was significantly decreased in comparison with CVH patients (187.6 ± 88.1, range; 91.2–346.3 vs. 335.2 ± 155.1, 111.0–565.8; $P = 0.016$) and controls (vs. 344.9 ± 137.3, 131.1–568.5; $P < 0.001$) (Fig. 5b). Interestingly, the level of RP105 expression on PBC monocytes decreased significantly after culture without any stimulation, as compared to monocytes from CVH patients (131.3 ± 77.1, 42.8–248.2 vs. 233.8 ± 113.3, 91.4–439.6; $P = 0.022$) and controls (vs. 242.3 ± 128.3, 93.8–487.5; $P = 0.007$). The mRNA of RP105 in purified CD14$^+$ monocytes was also significantly decreased in PBC patients after stimulation with LPS (0.012 ± 0.006, 0.003–0.023 vs. 0.025 ± 0.010, 0.010–0.041; $P < 0.001$) (Fig. 5c). Taken together, these results suggest that a significant decrease of the negative regulator of TLR4 signaling may be responsible for the increased response to LPS in patients with PBC.

4. Discussion

This study revealed altered expression of TLR4 and the TLR4 homolog RP105 on monocytes from patients with PBC, further confirming that the disease involves increased sensitivity to the defined TLR4 ligand, LPS. It was also revealed that expression of the mRNA for the TLR adaptor molecule MyD88 was significantly increased in monocytes from PBC patients after stimulation with LPS, suggesting that the alteration in immediate processes such as recruitment of the adaptor molecule to the TLR4 molecule may arise from altered interaction between the receptor and its ligand. Mammalian TLRs are characterized structurally by an extracellular leucine-rich repeat domain and an intracytoplasmic signaling domain (Toll-IL-1 receptor domain) that is highly conserved across the TLR family [8]. The TLR-like molecule RP105 has a conserved extracellular leucine-rich repeat, but unlike TLRs, lacks a Toll-IL-1 receptor domain [19–21]. Divancovic et al. recently reported widespread expression of RP105, directly mirroring that of TLR4, on antigen-presenting cells [15]. They also reported that RP105 and its helper molecule, MD-1, interacted directly with the TLR4 signaling complex, inhibiting its ability to bind microbial ligand [15]. Therefore, the increased expression of TLR4 itself and the decrease in the negative regulator of TLR4 signaling, RP105, after stimulation with LPS

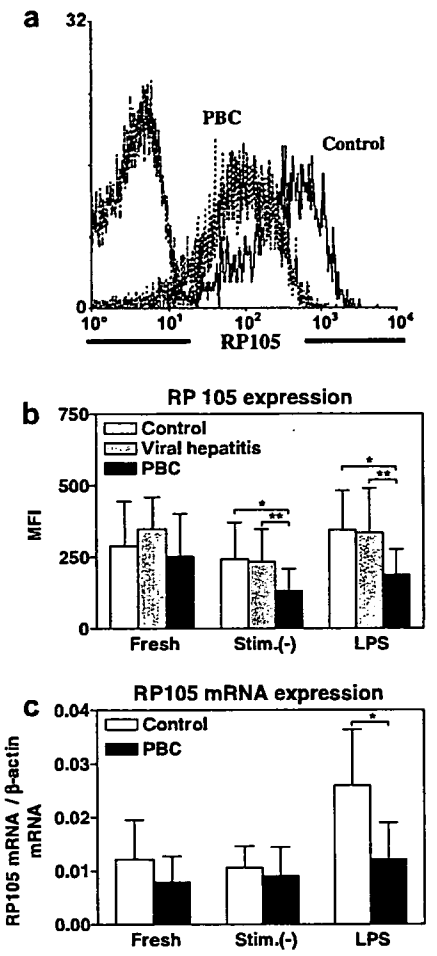

Fig. 5. Significant decrease in surface expression of RP105 and its mRNA in CD14$^+$ cells from patients with PBC after stimulation with LPS. (a) Results of flow cytometric analysis of PR105 on CD14$^+$ cells from a representative patient with PBC and a control subject after stimulation with LPS. (b) PBMCs from patients with PBC ($n = 20$) and chronic viral hepatitis ($n = 10$), and from controls ($n = 15$), were cultured with or without LPS (1 μg/ml) for 1 h, then surface expression of RP105 on CD14$^+$ cells was analyzed. Values were quantified as MFI by flow cytometry. (c) Expression level of RP105 mRNA in freshly isolated (Fresh) or cultured CD14$^+$ cells from patients with PBC ($n = 16$) and controls ($n = 15$) analyzed by real-time PCR. Steady-state levels of mRNAs were normalized relative to the level of β-actin mRNA. The values are presented as means + SD. $^*P < 0.01$, $^{**}P < 0.05$.

appears to be one of the mechanisms responsible for the hypersensitivity of PBC monocytes to LPS.

RP105 was originally cloned as a B cell-specific molecule capable of driving B cell proliferation [20,21]. Miura et al. showed that RP105 is expressed on virtually all mature B cells, and is a potential B cell marker in humans as well as mice [22]. Interestingly, however, it has been reported that the number of RP105-negative B cells is significantly increased in patients with systemic lupus erythematosus (SLE) [23], and that these cells are highly activated and may be responsible for the production of autoantibodies as well as polyclonal immunoglobulins [24]. Although the mechanism responsible for the lack of RP105 on B cells in SLE patients remains obscure, altered expression of RP105 may be related to autoimmune responses. In the present study, we also investigated the surface expression of RP105 on CD19$^+$ B cells, but found no increase in the population of RP105-negative B cells in patients with PBC (data not shown). Furthermore, there was no decrease in the level of RP105 expression on freshly isolated monocytes in patients with PBC. However, it was noteworthy that the decreased expression of RP105 on monocytes occurred only after culture with or without LPS. As RP105-negative B cells have been shown to be highly activated, the expression of RP105 might be related to the activation status of cells, and the peripheral blood monocytes in patients with PBC might not be activated *in vivo*. Further investigation remains to be done to clarify the role of RP105 in the production of autoantibodies by B cells and the innate immune responses induced by LPS in PBC.

Previous studies have documented that significant amounts of LPS are absorbed by hepatocytes and secreted into the biliary system [25], and it has become clear that bacterial products play an important role in the pathogenesis of bile duct diseases including PBC [26]. In fact, in PBC patients, it has been reported that LPS accumulates abnormally in the biliary epithelium [27], and that TLR4 is up-regulated in bile duct epithelial cells and periportal hepatocytes [28]. Interestingly, a previous study has demonstrated that treatment of PBC patients with UDCA led to a reduced humoral response against LPS components [29]. As all the studied patients with PBC were treated with UDCA, whereas none of the controls were receiving UDCA treatment, we cannot exclude a possible modulating effect of UDCA on the response to LPS. Although UDCA might have reduced the response to LPS in the present study, we still revealed significantly increased responses to LPS in accordance with the previous study that described the increased response to LPS in PBC patients receiving UDCA [14]. We consider that our findings were not due to the effect of UDCA.

Tolerance to bacterial LPS is an interesting property characterized by a reduced capacity of the host (*in vivo*), or of cultured monocytes (*in vitro*), to respond to LPS activation following initial exposure to this stimulus [30]. Although the molecular mechanism of LPS tolerance remains unclear, it seems to have evolved as a complex orchestrated counter-regulatory response to inflammation [30]. As we showed in the present study, monocytes exposed to LPS showed reduced surface TLR4 expression, which is thought to be one of the mechanisms involved in LPS tolerance [31]. It was noteworthy, however, that the level of TLR4 expression on

PBC monocytes was higher than that on monocytes from patients with CVH and controls when TLR4 expression was reduced following stimulation with LPS, even though there was no significant difference in TLR4 expression on freshly isolated monocytes. Although the present study did not clarify the mechanism responsible for the increased TLR4 expression on PBC monocytes, it is possible that the mechanism of tolerance to LPS could be dysregulated in PBC.

In conclusion, our observations provide additional insight into the mechanism responsible for the hypersensitivity of the innate immune system to LPS in PBC, which may be involved in the initiation of self-tolerance breakdown or in the continuous autoimmune response. As the involvement of TLR4 and RP105 in the pathogenesis of autoimmune diseases, such as SLE, has also been suggested [32], further investigation of the mechanism responsible for altered TLR4 and RP105 expression may provide new insight into the pathogenesis of other autoimmune diseases as well as PBC, and a chance to develop promising and innovative treatments.

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Comprehensive Analysis of the Effects of Ordinary Nutrients on Hepatitis C Virus RNA Replication in Cell Culture[▽]

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To date, only a limited number of studies have reported finding an influence of ordinary nutrients on hepatitis C virus (HCV) RNA replication. However, the effects of other nutrients on HCV RNA replication remain largely unknown. We recently developed a reporter assay system for genome-length HCV RNA replication in hepatoma-derived HuH-7 cells (OR6). Here, using this OR6 assay system, we comprehensively examined 46 nutrients from four nutrient groups: vitamins, amino acids, fatty acids, and salts. We found that three nutrients—β-carotene, vitamin D_2, and linoleic acid—inhibited HCV RNA replication and that their combination caused additive and/or synergistic effects on HCV RNA replication. In addition, combined treatment with each of the three nutrients and interferon alpha or beta or fluvastatin inhibited HCV RNA replication in an additive manner, while combined treatment with cyclosporine synergistically inhibited HCV RNA replication. In contrast, we found that vitamin E enhanced HCV RNA replication and negated the effects of the three anti-HCV nutrients and cyclosporine but not those of interferon or fluvastatin. These results will provide useful information for the treatment of chronic hepatitis C patients who also take anti-HCV nutrients as an adjunctive therapy in combination with interferon. In conclusion, among the ordinary nutrients tested, β-carotene, vitamin D_2, and linoleic acid possessed anti-HCV activity in a cell culture system, and these nutrients are therefore considered to be potential candidates for enhancing the effects of interferon therapy.

Hepatitis C virus (HCV) is a major pathogen of chronic hepatitis (CH) and leads to fatal liver diseases, such as liver cirrhosis and hepatocellular carcinoma (1, 23, 38). Approximately 170 million people worldwide are infected with HCV (40). Therefore, HCV infection is a global health problem. The combination of pegylated interferon (IFN) with ribavirin is currently the most effective therapy for CH C (11, 28), and long-term treatment has been shown to improve the sustained virological response (SVR) rate (37). However, the SVR rate still remains at approximately 55% (11). Combination therapy occasionally also causes adverse effects, such as severe anemia (10) and cerebral vascular lesions (7), and reduction of the dosage leads to insufficient treatment. These adverse effects are more serious in older patients. Therefore, it remains necessary to identify alternative agents that have fewer side effects to couple with IFN. In developing countries, it is difficult to administer expensive IFN therapy. Hence, in such countries, inexpensive anti-HCV reagents are especially desirable (36).

The lack of a small-animal model and a cell culture system to support efficient HCV RNA replication hampered the development of anti-HCV reagents. Since an HCV subgenomic replicon system was developed in 1999 (25), the mechanism of HCV RNA replication has been gradually elucidated by a number of groups (2). However, this subgenomic replicon system may not necessarily reflect actual HCV RNA replication in hepatocyte cell lines, due to the absence of structural proteins. To overcome this problem, a cell culture system for genome-length HCV RNA replication was developed by several groups (4, 15, 33). We also developed a genome-length HCV RNA (strain O of genotype 1b) replication system (OR6) with luciferase as a reporter, which facilitated the prompt and precise monitoring of HCV RNA replication in hepatocyte cell lines (13, 30). In the OR6 assay system, the luciferase activity was well correlated with HCV RNA levels when cells were treated with IFN-α (13). Therefore, we quantified the luciferase activity instead of HCV RNA for the indirect evaluation of HCV RNA replication, although the OR6 assay system doesn't strictly quantify the HCV RNA replication. More recently, several groups have developed cell culture systems that produce infectious viral particles (genotype 2a), which can be used to reconstruct the life cycle of HCV infection in hepatocyte cell lines (24, 39, 45).

Using this OR6 assay system, we demonstrated that mizoribine (30), as an immunosuppressant, and fluvastatin (FLV) (14), as the reagent for hypercholesterolemia, inhibited HCV RNA replication in hepatocyte cell lines. Another immunosuppressant, cyclosporine (CsA), was also identified as an anti-HCV reagent in a subgenomic replicon system (41). These results suggested the expansion of the primary application of the existing therapeutic drugs to new anti-HCV therapy.

Other studies have also revealed that certain ordinary nutrients contained in common foods can influence HCV RNA replication (5, 8, 9, 17, 21, 26). However, in these studies, only a limited number of nutrients were tested. To date, there has

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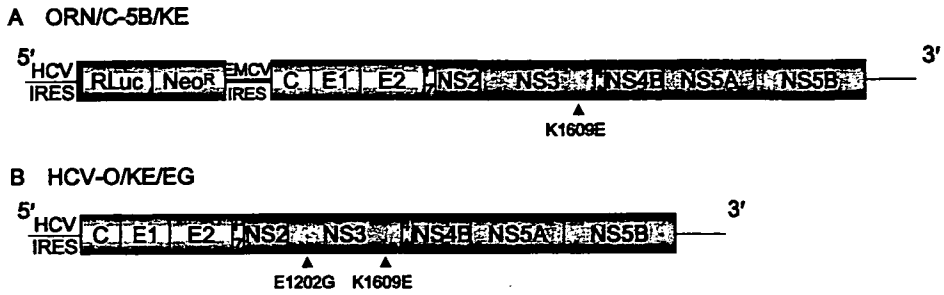


FIG. 1. Schematic gene organization of genome-length HCV RNA. (A) ORN/C-5B/KE RNA replicated in OR6 cells. RL (RLuc) is expressed as a fusion protein with neomycin phosphotransferase (NeoR). The position of an adaptive mutation, K1609E, is indicated by a black triangle. (B) Authentic HCV RNA, HCV-O/KE/EG, was introduced into the OR6c cells by electroporation as previously described (13). The positions of two adaptive mutations, E1202G and K1609E, are indicated by black triangles. IRES, internal ribosome entry site; EMCV, encephalomyocarditis virus.

been no comprehensive analysis of the effects of nutrients on HCV RNA replication. Thus, we inclusively investigated the effects of nutrients on genome-length HCV RNA replication using the OR6 assay system. Here, we report finding that three nutrients, namely, β-carotene (BC), vitamin D_2 (VD2), and linoleic acid (LA), inhibited HCV RNA replication and that combination treatments including each of these nutrients and CsA synergistically inhibited HCV RNA replication. Furthermore, we found that vitamin E (VE) enhanced HCV RNA replication, and that the anti-HCV activities of each of the three nutrients and CsA were abrogated by VE.

MATERIALS AND METHODS

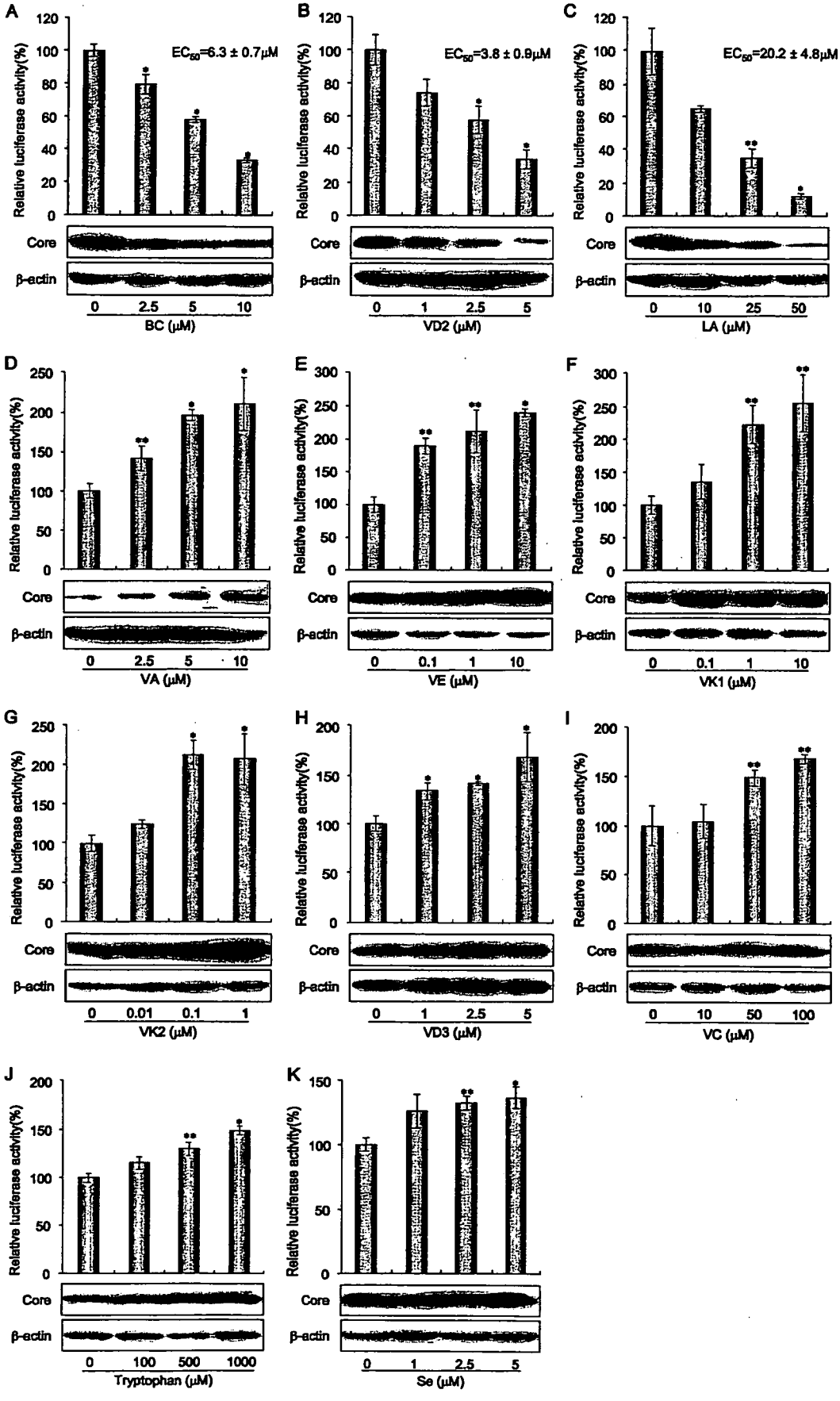
Reagents. Vitamin B_{12}, vitamin K_1 (VK1), vitamin K_3, elaidic acid, and vaccenic acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). BC, vitamin A (VA), vitamin B_1, vitamin B_2, vitamin B_3, vitamin B_6, vitamin C (VC), VD2, vitamin D_3 (VD3), VE, vitamin K_2 (VK2), pantothenic acid, biotin, folic acid, inositol, leucine, isoleucine, valine, tryptophan, phenylalanine, tyrosine, lauric acid, palmitic acid, stearic acid, behenic acid, oleic acid, elaidic acid, LA, arachidonic acid (AA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), $Fe(II)SO_4$, Na_2SeO_4, $Fe(III)(NO_3)_3$, $ZnCl_2$, NaCl, KCl, $CaCl_2$, PCl_3, $MgCl_2$, $CuCl_2$, $MnCl_2$, and IFN-α were purchased from Sigma (St. Louis, MO), and CsA and FLV were purchased from Calbiochem (Los Angeles, CA). IFN-β was kindly provided by Toray Industries, Inc. Fatty acids, except for LA-albumin and oleic acid-albumin compounds (Sigma), were compounded with fatty acid-free bovine serum albumin (Sigma) as described in a previous report (6). We treated the HCV RNA-harboring cells at various concentrations of salts [$Fe(II)SO_4$ at 5, 25, and 50 μM, $Fe(III)(NO_3)_3$ at 10, 100, and 200 μM, $ZnCl_2$ at 20, 50, and 100 μM, Na_2SeO_4 at 1, 2.5, and 5 μM, NaCl at 100, 150, and 300 μM, KCl at 5, 10, and 20 μM, $CaCl_2$ at 2, 4, and 8 μM, PCl_3 at 1, 2.5, and 5 μM, $MgCl_2$ at 0.5, 2.5, and 5 μM, $CuCl_2$ at 20, 50, and 100 μM, and $MnCl_2$ at 30, 60, and 120 μM]. Dimethyl sulfoxide or ethanol was used for the dissolution of the liposoluble nutrients, and the final concentrations (0.1%) of each were equal for the cells with nutrients and those without.

Cell cultures. OR6 cells, polyclonal genome-length HCV RNA (ORN/C-5B/KE)-replicating cells, and subgenomic replicon (ORN/3-5B/KE) cells, which were derived from hepatoma cell line, HuH-7, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, streptomycin (designated as the control medium in this study), and G418 (300 μg/ml) (Geneticin; Invitrogen, Carlsbad, CA), and the cells were passaged twice a week at a 5:1 split ratio. ORN/C-5B/KE and ORN/3-5B/KE are derived from HCV-O (strain O of genotype 1b). OR6c cells are cured OR6 cells from which

TABLE 1. Summary of anti-HCV activities of various nutrients

Nutrient type	Nutrient(s) with the indicated characteristic for HCV[a]		
	Inhibitory	Enhancing	Ineffective
Vitamins			
Liposoluble	BC, VD2	VA (retinol), VE, VK1, VK2, VK3	VD3
Water soluble		VC	*VB1*, *VB2*, *VB3* (*niacin*), *VB6*, VB12, *pantothenic acid*, biotin, *folic acid*, *inositol*
Amino acids			
Branched-chain			*Leucine*, *isoleucine*, *valine*
Aromatic		*Tryptophan*	*Phenylalanine*, *tyrosine*
Fatty acids			
Saturated			Lauric acid (C_{12}), palmitic acid (C_{16}), stearic acid (C_{18}), behenic acid (C_{22})
Mono-unsaturated			Oleic acid (C_{18}; 9-unsaturated), elaidic acid (C_{18}; *trans*-form of oleic acid), vaccenic acid (C_{18}; 11-unsaturated)
Polyunsaturated	LA ($C_{18:2}$, n-6), AA ($C_{20:4}$, n-6), EPA ($C_{20:5}$, n-3), DHA ($C_{22:6}$, n-3)		
Salts	$Fe(II)SO_4$, *$Fe(III)(NO_3)_3$*, $ZnCl_2$	Na_2SeO_4	*NaCl*, *KCl*, *$CaCl_2$*, *PCl_3*, *$MgCl_2$*, $CuCl_2$, $MnCl_2$

[a] Nutrients already contained in the medium are indicated in italics. VB1, vitamin B_1; VB2, vitamin B_2; VB3, vitamin B_3; VB6, vitamin B_6; VB12, vitamin B_{12}; VK3, vitamin K_3.


A
B
C
D
E
F
G
H
I
J
K
Relative luciferase activity(%)
EC50=6.3 ± 0.7μM
EC50=3.8 ± 0.9μM
EC50=20.2 ± 4.8μM
Core
β-actin
BC (μM)
VD2 (μM)
LA (μM)
VA (μM)
VE (μM)
VK1 (μM)
VK2 (μM)
VD3 (μM)
VC (μM)
Tryptophan (μM)
Se (μM)