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Hepatitis C Virus Expression and Interferon Antiviral Action Is Dependent on PKR Expression

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Interferon (IFN)-inducible double-stranded RNA-activated protein kinase (PKR) is thought to play a key antiviral role against hepatitis C virus (HCV). However, demonstrating the importance of PKR expression on HCV protein synthesis in the presence or absence of IFN has proven difficult in vivo. In the present experiment, full-length HCV constructs were transiently transfected into two cell lines stably expressing T7 RNA polymerase. HCV expression was monitored under conditions of upregulated or downregulated PKR expression. In addition, IFN was monitored during downregulation of PKR. HCV expression effectively increased PKR expression, as well as that of its regulated proteins. PKR was obviously knocked down by PKR-specific siRNA, which resulted in significantly increased HCV core protein levels. Conversely, over-expression of PKR significantly suppressed HCV core levels in both cell lines. Furthermore, IFN induced high levels of PKR, whereas downregulation of PKR reversed IFN's antiviral effects and increased HCV core levels. Based on these results, it appears that HCV protein expression is directly dependent on PKR expression. PKR is antiviral toward HCV and responsible for IFN's effect against HCV. ***J. Med. Virol. 79:1120–1127, 2007.***
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KEY WORDS: small interfering RNA (siRNA); eukaryotic initiation factor-2α (eIF2α); BT7-H; Huh-T7

INTRODUCTION

Interferon (IFN)-inducible double-stranded RNA-activated protein kinase (PKR) appears to play a key antiviral role against hepatitis C virus (HCV). PKR is one of a number of host IFN-stimulated genes (ISGs) [Sen and Ransohoff, 1993]. Nearly all mammalian cells express PKR at low levels [Kaufman, 2000]. Double-stranded-RNA (dsRNA), produced during RNA viral replication, is a potent activator of PKR [Meurs et al., 1993]. Activated PKR in turn induces phosphorylation of PKR and eukaryotic initiation factor-2α (eIF2α), which inhibits protein synthesis, including that of virally encoded proteins [Samuel, 1979]. PKR appears to play multiple roles in cell growth, differentiation, apoptosis, oncogenesis, and responses to cellular stresses, such as infection [Gale et al., 2000]. However, proof-proving inhibition of HCV protein synthesis by PKR is still lacking [Koev et al., 2002; MacQuillan et al., 2002; Vyas et al., 2003]. While HCV structural protein E2 and nonstructural protein NS5A appear to block activation of PKR, it remains unclear whether these proteins contribute to HCV persistence or resistance to IFN in vivo [Francois et al., 2000; Gerotto et al., 2000; Taylor et al., 2001]. Furthermore, it remains controversial whether PKR is required for elimination of HCV in patients treated with IFN [MacQuillan et al., 2003; Giannelli et al., 2004].

To analyze the precise interaction between PKR and HCV proteins, we used a full-length HCV cell-based expression system as described in a previous report [Lin et al., 2005]. This expression system uses a wild-type H77 sequence (genotype 1a), capable of infecting chimpanzees with no adaptive mutations. It can produce HCV negative-strand RNA in host cell lines stably expressing T7 polymerase [Hiasa et al., 2006]. It is suitable to analyze interactions between HCV and PKR since it utilizes full-length wild-type HCV RNA with no

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adaptive mutations. Moreover, it is capable of synthesizing double-stranded HCV-RNA within host cell lines in which particular IFN signaling pathways have been activated. Moreover, cell-based systems are capable of expressing HCV proteins only by plasmid transfection. This expression system results in efficient production of HCV proteins with minimal artificial effects. Using this HCV cell-based expression system, we explored the relationship between PKR and HCV full genome protein expression in two independent cell lines.

Clinically, IFN remains the only drug capable of eliminating HCV. At present, pegylated IFN-α in combination with ribavirin is standard therapy for patients with HCV. Although PKR is thought to play an important role in IFN's control of HCV protein expression, a number of independent studies suggest that HCV can be suppressed by IFN by mechanisms other than activation of PKR [Francois et al., 2000; Guo et al., 2003, 2004]. Thus, it is still not clear whether PKR is solely responsible for IFN's antiviral effects against HCV. In the present study, first we examined the impact of PKR on HCV protein expression using our binary plasmid-based HCV expression system. We then investigated the role of PKR in mediating IFN's antiviral effects against HCV.

MATERIALS AND METHODS

Cells

Huh-T7 cells were generated by stably transfecting bacteriophage T7 RNA polymerase into Huh-7 cells [Schultz et al., 1996], and BT7-H cells were generated by stably transfecting T7 into African green monkey kidney cell lines BS-C-1 [Whetter et al., 1994] (both gifts of Dr. Stanley M. Lemon, University of Texas, Galveston). Both cell lines were grown in Dulbecco's modified Eagle's medium (D-MEM) (Gibco-BRL, Gaithersburg, MD). For BT7-H cells, we added 500 μg/ml gentamicin sulfate, and for Huh-T7 cells, we added 250 μg/ml gentamicin sulfate to the culture medium.

Plasmid and Cell Transfection

pH77 is a full-length HCV genotype 1a construct [Chung et al., 2001]. Briefly, a plasmid containing a full-length genotype 1a cDNA sequence corresponding to the H77 prototype strain [Yanagi et al., 1997] was adapted at its 5′ and 3′ termini with a T7 promoter and a hepatitis delta virus ribozyme sequence, respectively, to yield pT7-flHCV-Rz (hereafter referred to as pH77). Plasmid pcDNA-PKRwt (pPKR) expressing wild-type PKR was a kind gift from Dr. Michael Gale, Jr. (University of Texas Southwestern) [Meurs et al., 1993]. In order to induce over-expression of PKR, we co-transfected pPKR and pH77 into cells. The plasmid pOS8, expressing β-galactosidase under control of the T7 promoter, was used as control plasmid [Chung et al., 2001]. Transfection of each plasmid was performed using Lipofectamine Reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. In a 6-well tissue culture plate, cells were seeded in 2 ml of medium. For each transfection, 3 μg of plasmid DNA was used. The transfected cells were harvested at different time points.

Synthesis and Transfection of PKR-Specific siRNA

We designed a PKR-specific siRNA (PKRsi-1: GAA CUG CCU AAU UCA GGA C, nt. 520–540) from a PKR sequence template (accession number NM002759) purchased from Dharmacon Research, Inc. (Lafayette, CO). To design PKR-specific siRNA, the mRNA sequence of PKR was screened using the National Center for Biotechnology Information database and the BLAST search algorithm. Cy3 labeled luciferase GL2 duplex (Dharmacon) was used as a control siRNA (Control-si). In order to induce downregulation of PKR, we transfected siRNA 48 hr prior to transfection with pH77. Transfection with 50 pmol of siRNA was performed using siFECTOR (B-bridge International, Sunnyvale, CA). pH77 was transfected using Lipofectamine reagent (Invitrogen).

Cell Culture With or Without IFN

For assays using IFN, cells were cultured in the presence of 100 IU/ml IFNα-2b (Schering-Plough, Kenilworth, NJ) 4 hr after transfection. Medium with or without IFN was changed at Day 1 post-infection and every 2 days thereafter.

Western Blotting

Cells were washed twice with phosphate buffered saline (PBS) and lysed with 100 μl of RIPA buffer (0.5% Nonidet P-40, 10 mM Tris-HCl, pH 7.4, 150 mM of NaCl, 1% sodium dodecyl sulfate). Thirty micrograms of lysate protein were used, and separated by electrophoresis on 4–12% Bis–Tris gradient gel (Invitrogen), then blotted onto Immobilon-P membranes (Millipore, Bedford, MA). Each membrane was then incubated with the relevant antibody. An ECL plus kit (Amersham Pharmacia, Buckinghamshire, UK) was used for detection. Monoclonal antibody to human PKR and polyclonal antibody to eIF2α were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), while polyclonal antibody to phosphorylated PKR was obtained from BioSource (Camarillo, CA), and polyclonal antibody to phosphorylated eIF2α peptide was obtained from ResGen (Invitrogen). Monoclonal antibody to actin was obtained from Chemicon International (Temecula, CA). Appropriate species-specific conjugated secondary antibodies were obtained from commercial kits (Amersham Pharmacia). Autoradiograms were scanned, and the signal intensity of each band analyzed using Scion Image software (Scion Corporation, Frederick, MD).

ELISA Assay for HCV Core Antigen

Cell culture lysates were adjusted to 20 μg/ml. HCV core antigen concentrations were quantified using the

HCV core antigen ELISA test (Ortho-Clinical Diagnostics, Osaka, Japan) following the manufacturer's instructions. HCV core ELISA data were expressed in terms of fmol of HCV core antigen per 20 μg of total protein.

Cellular RNA Extraction and Real-Time RT-PCR for PKR and GAPDH mRNA

Total RNA was extracted with TRIzol reagent (Invitrogen) and treated two times for 4 hr with DNase I using a DNase-free kit (Ambion, Austin, TX). RNA was adjusted to 0.3 μg/μl. Reverse transcription was performed as described previously [Hiasa et al., 2003]. PKR and GAPDH cDNA were then quantified by real-time PCR using LightCycler technology and SYBR green I dye (Roche Diagnostics, Mannheim, Germany). Real-time PCR for PKR was performed with 2 μl of purified cDNA in a reaction SYBR green I mixture containing 4 mM $MgCl_2$, and 5 pM each of forward primer (5′-AGCACACTCGCTTCTGAATC-3′) and reverse primer (5′-CTGGTCTCAGGATCATAATC-3′) [Hiasa et al., 2003]. The PCR consisted of an initial denaturation step for 10 min at 95°C, then 40 cycles under the following conditions: 10 sec at 95°C, 10 sec at 58°C, and 15 sec at 72°C. For PCR amplification of GAPDH, we used a commercial GAPDH primer set (Roche Search LC, Mannheim, Germany), with conditions as recommended by the manufacturer. For analysis of PKR mRNA, we determined PKR mRNA copy numbers, which were normalized to the GAPDH copy number, to provide standardized values.

Statistical Analysis

Each analysis was performed in at least quadruplicate to arrive at a mean value and SE. Data were analyzed statistically using SPSS 10.0J software (SPSS, Chicago, IL). Mean value differences were analyzed by the Mann–Whitney U test. P-values < 0.05 were considered to be significant.

RESULTS

Expression of HCV Activates PKR in This Cell-Based System

We first examined the level of HCV core protein expression in our cell-based system, and monitored the time course of HCV core protein expression in BT7-H and Huh-T7 cell lines stably expressing T7 polymerase. We have previously demonstrated that transfection of pH77 (containing full-length HCV cDNA of genotype 1a) leads to successful HCV replication in these cell lines [Hiasa et al., 2006]. The basal level of HCV core protein expression in BT7-H cells was approximately 10 times greater than in Huh-T7 cells, however, expression quickly fell by Day 7 (Fig. 1A). As determined by Western blot analysis, an increase in phosphorylated PKR with HCV expression was observed, and increased amounts of PKR and phosphorylated eIF2α (indicative of PKR activity) were also observed from Days 2 to 5 in BT7-H cells (Fig. 1C). In Huh-T7 cells, less significant increases in PKR protein, as well as phosphorylated PKR and phosphorylated eIF2α, were observed than in BT7-H cells. A slight increase in phosphorylated eIF2α was observed on Day 5. The initial peak in HCV protein expression in BT7-H cells appeared to activate PKR by phosphorylation. In contrast, transfection with pOS8 did not increase phosphorylated PKR and eIF2α levels in either cell line. These results suggest that HCV actually enhances PKR function in this model. These results led us to investigate the effect of PKR over-expression on HCV expression.

Over-Expression of PKR Suppresses HCV Core Protein Expression

To determine whether PKR inhibits HCV protein expression, we over-expressed PKR in cell lines using a PKR expression plasmid (pPKR) [Pflugheber et al., 2002]. We co-transfected pH77 with either pPKR or pOS8 (a control plasmid expressing β-galactosidase). As demonstrated by Western blot analysis, pPKR efficiently expressed both PKR and phosphorylated PKR protein in excess of control levels in BT7-H cells (Fig. 2A). Strong expression of PKR continued until Day 5. pPKR also strongly expressed PKR protein in Huh-T7 cells (data not shown). Over-expression of PKR was capable of inhibiting HCV core protein in both cell lines (Fig. 2B). PKR significantly inhibited HCV core protein expression in BT7-H cells from Days 1 to 5 ($P < 0.05$), and in Huh-T7 cells from Days 2 to 4 ($P < 0.05$). These results suggest that PKR can efficiently inhibit HCV protein expression and overcome functional inhibition by HCV proteins, such as E2 and NS5A.

Maintenance of HCV Core Protein Expression Occurs in Cells With Downregulated PKR

We next explored the time course of HCV core protein expression with regard to downregulation of PKR expression. We sought to determine whether HCV replication might be sustained by inhibiting PKR. To silence PKR expression, we designed siRNA targeting the open reading frame of PKR (PKRsi-1), and evaluated the extent of PKR downregulation in both cell lines (Fig. 3A). Transfection with PKRsi-1 resulted in downregulation of PKR mRNA for 7 days (Fig. 3A). When HCV plasmid (pH77) was introduced after downregulation of PKR, the overall increase in subsequent expression of PKR was partially decreased by PKR siRNA (Fig. 3B).

After confirming downregulation of PKR by siRNA, we evaluated the effect of PKR siRNA on HCV protein expression in cell culture to determine whether PKR influences HCV replication. Compared with control siRNA (Control-si), PKRsi-1 significantly increased HCV expression in both cell lines from Days 3 to 5 (Fig. 4). The level of upregulation of HCV core expression was significant in both cell lines (PKRsi-1: $3{,}770.1 \pm 442.0$ vs. Control-si: $2{,}057.1 \pm 329.5$ fmol/

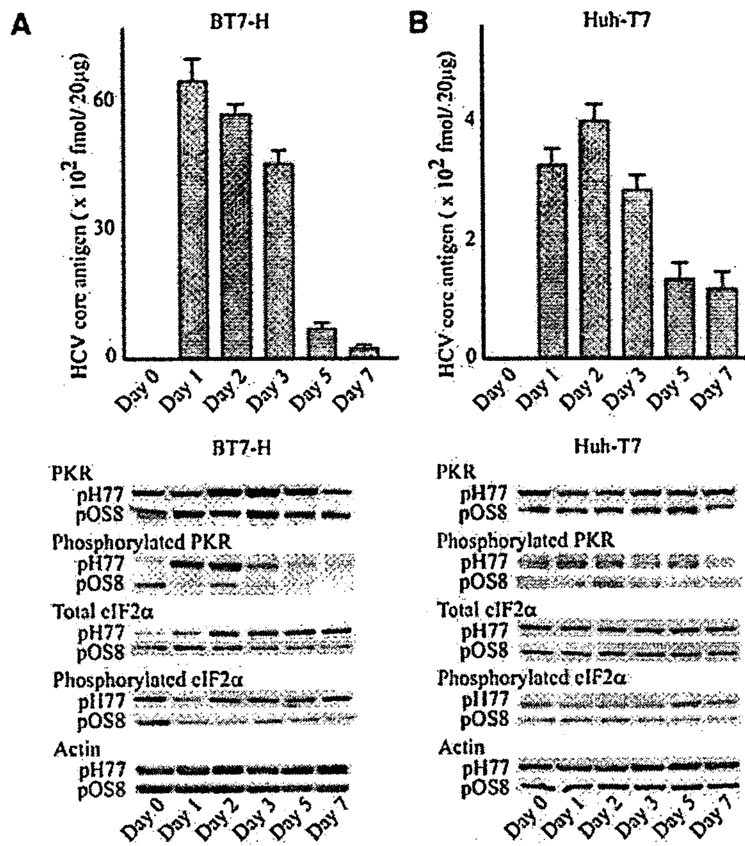

Fig. 1. HCV core protein was efficiently expressed using the binary replication system. HCV expression induced and activated PKR. **A**: A plasmid containing full-genome HCV cDNA (pH77) was transfected into BT7-H and Huh-T7 cell lines. Each line was stably transfected with a T7 polymerase gene. Following transfection of pH77, HCV core protein was expressed for at least 7 days. Results are expressed as the means ± SE of six experiments. **B**: The plasmid pH77 or pOS8 was transfected into BT7-H and Huh-T7 cells. Protein levels of PKR, phosphorylated PKR, total eIF2α, phosphorylated eIF2α, and actin were analyzed by Western blot. In BT7-H cells, PKR was stimulated and activated by HCV expression. In Huh-T7 cells, PKR was partially activated, however, activation was minimal compared to BT7-H cells.

20 μg, on Day 4 in BT7-H cells ($P < 0.01$); PKRsi-1: 448.2 ± 126.4 vs. Control-si: 274.0 ± 14.2 fmol/20 μg, on Day 4 in Huh-T7 cells ($P < 0.01$)) (Fig. 4). These results indicate that PKR directly influences on HCV replication in these cell systems.

Interferon's Anti-HCV Effects are Dependent on PKR Expression

IFN induces PKR expression, however, it is still controversial whether PKR is required for elimination of HCV in infected patients treated with IFN [MacQuillan et al., 2003; Giannelli et al., 2004]. Based on our observations, over-expression of PKR inhibits HCV protein expression. To determine whether PKR is essential to IFN's antiviral activity, the effect of IFN-α on HCV-infected cells with downregulated PKR was examined. We transfected PKRsi-1 into each cell line to inhibit the expression of PKR. After 48 hr, we transfected pH77 to induce expression of HCV protein, then added 100 IU/ml of IFN to the culture medium of selected wells. The cells were harvested from Days 1 to 5 following pH77 transfection. IFN stimulated PKR expression in both cell lines, and strong expression of PKR was observed in cells transfected with control siRNA (Control-si) by Western blot (Fig. 5A). As expected, the cells treated with IFN demonstrated reduced HCV core protein levels compared with IFN untreated, control si-transfected cells. On the other hand, in cells transfected with PKRsi-1, PKR expression was markedly reduced in the presence of IFN, especially prior to Day 3 (Fig. 5A). In cells transfected with control-si, HCV core protein levels were reduced by IFN (Fig. 5B). In contrast, in cells with PKR downregulation, significant increases in HCV core protein expression were observed compared with Control-si transfection at each time point in both cell lines ($P < 0.05$). These data confirm that PKR is an important mediator of IFN's antiviral effects against HCV.

DISCUSSION

In the present study, we used a full-length HCV cell-based expression system to demonstrate that PKR influences HCV expression. Sufficient HCV protein expression for at least 7 days was achieved using this

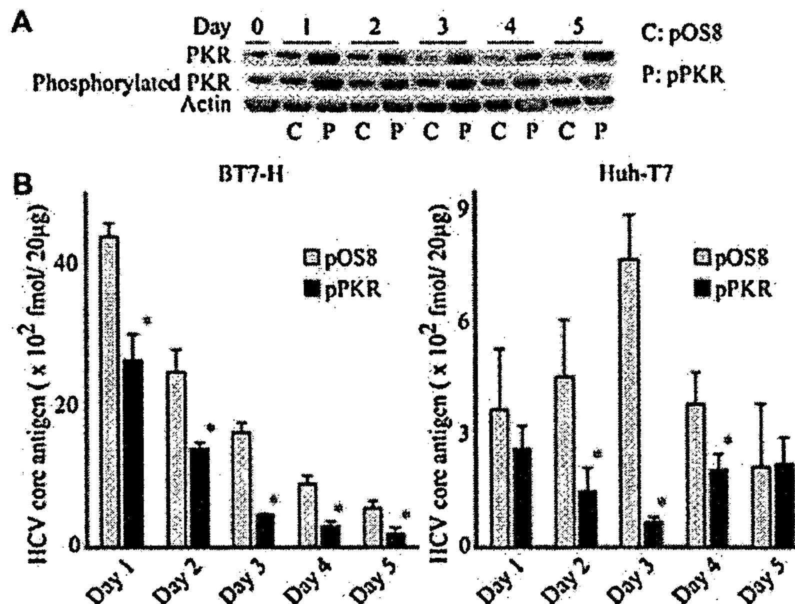

Fig. 2. Over-expression of PKR inhibited HCV core expression. **A**: pH77 and a PKR expression vector (pPKR) were co-transfected into BT7-H cells. A plasmid expressing β-galactosidase (pOS8) under the T7 promoter was used as a control. pPKR markedly upregulated PKR and phosphorylated PKR expression. **B**: After over-expression of PKR, significant inhibition of HCV core protein expression was observed. Results are expressed as the means ± SE of four experiments. *$P < 0.05$ compared with control. C, pOS8; P, pPKR.

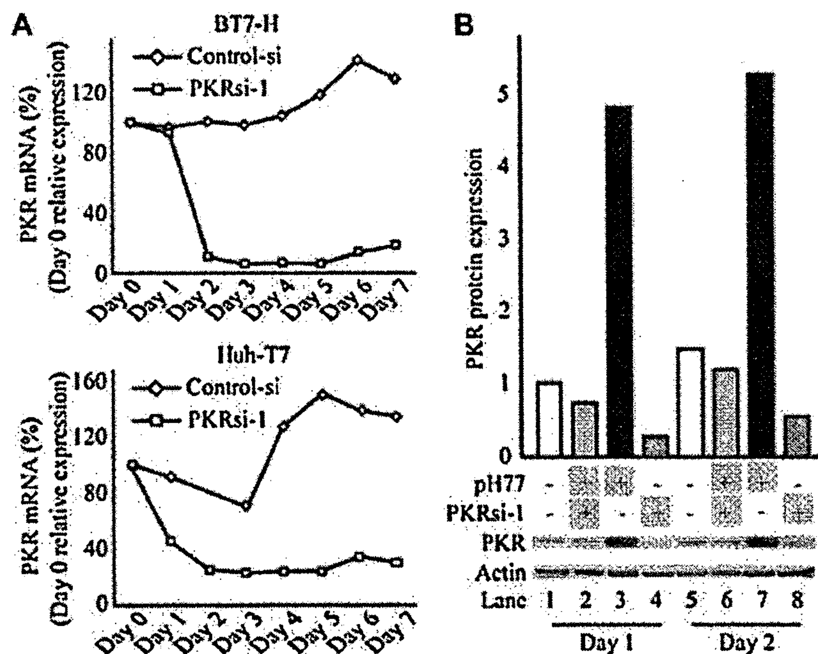

Fig. 3. PKR-specific siRNA efficiently downregulated PKR. **A**: When BT7-H and Huh-T7 cells were transfected with PKR-specific siRNA (PKRsi-1), marked downregulation of PKR mRNA was observed, compared with use of control siRNA (Control-si). This downregulation of PKR mRNA continued until Day 7. PKR mRNA was expressed as a ratio of GAPDH mRNA. Each value shown indicates relative expression from Day 0. Results are expressed as the mean of two experiments. **B**: Transfection of BT7-H cells with PKRsi-1 was followed 48 hr later by pH77 transfection. Expression of PKR was evaluated by Western blot, then analyzed by Scion image software, and plotted as a bar graph. pOS8 was used as a control plasmid for pH77, and Control-si was used as a control for PKRsi-1 (both are indicated by (-)). PKRsi-1 downregulated PKR expression effectively (**lane 1** vs. **lane 4**, **lane 5** vs. **lane 8**). In the presence of HCV expression, PKR was stimulated (**lane 1** vs. **lane 3**, **lane 5** vs. **lane 7**). Nevertheless, PKRsi-1 still efficiently inhibited PKR expression, compared to control siRNA (**lane 2**, **lane 6**).

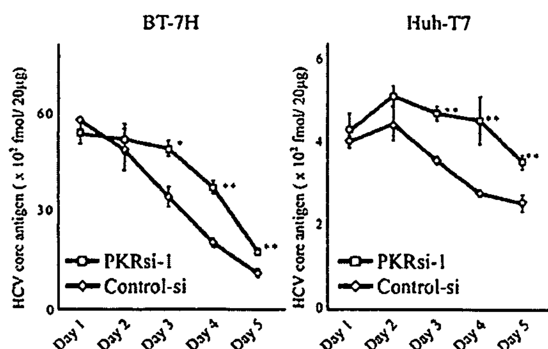

Fig. 4. PKR-silenced cells demonstrated prolonged HCV replication. PKR was downregulated by PKR-specific siRNA (PKRsi-1). Forty-eight hours after transfection with siRNA, we transfected pH77 and evaluated HCV core expression by ELISA assay. The cell lysates were harvested on Days 0–5 after transfection with pH77, and equal quantities of lysate were assayed for HCV core antigen. Data of PKR knockdown BT7-H or Huh-T7 cells using PKRsi-1 were compared with BT7-H or Huh-T7 cells transfected with control siRNA (Control-si). Error bars indicate the means ± SE of five experiments. $^*P < 0.05$, $^{**}P < 0.01$.

expression system by transfection with pH77. HCV replication activated PKR, especially within BT7-H cells. Over-expression of PKR inhibited HCV protein expression, and downregulation of PKR enhanced HCV protein expression. When we added IFN, HCV protein

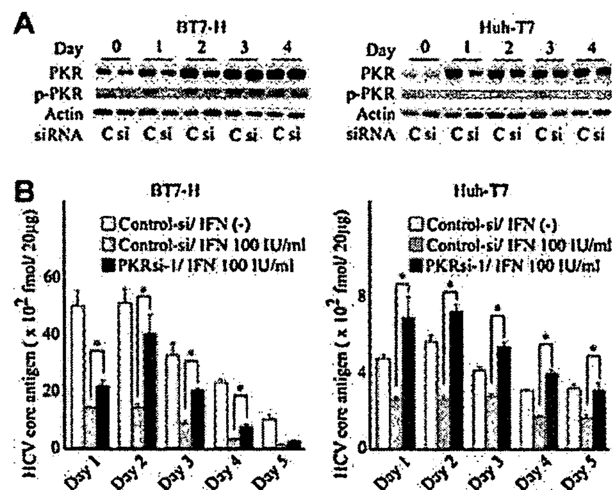

Fig. 5. Inhibition of HCV expression by IFN was PKR-dependent. A: Interferon (IFN) upregulated PKR in pH77 transfected cells. Nevertheless, PKR-specific siRNA (PKRsi-1) efficiently downregulated PKR expression and PKR phosphorylation in both cell lines, especially in the first 3 days. C, Control-si; si, PKRsi-1; p-PKR, phosphorylated PKR. B: Silencing of PKR by PKRsi-1 contributed to a diminished IFN effect on HCV protein expression. Cells were harvested daily, and HCV core protein measured by ELISA assay. Inhibition of HCV core protein by IFN was observed in control-si transfected cells compared with IFN untreated, control siRNA-treated cells. In contrast, despite treatment with IFN, the expression of HCV core protein was significantly increased at all time points in PKRsi-1 transfected cells compared with control-si transfected cells. These results indicate that IFN's antiviral effects against HCV are PKR-dependent. Results are expressed as the means ± SE of four experiments. $^*P < 0.05$; PKRsi-1 cells were compared with Control-si cells with IFN.

expression was inhibited in a dose-dependent manner within this system. Downregulation of PKR partially overcame this inhibition by IFN, resulting in increased HCV protein synthesis. These results indicate that HCV expression is directly dependent on PKR, and also indicates that PKR is a key player in IFN's sustained effects against HCV.

Several reports have described a role of PKR in HCV infection, as well as in mediating IFN's antiviral effects. PKR mRNA is significantly over-expressed in the hepatic tissue of patients with chronic hepatitis C, compared with other causes of liver damage [Yu et al., 2000]. However, other reports conclude that PKR protein expression is not upregulated in viral liver disease, including chronic hepatitis C [MacQuillan et al., 2002]. In addition, IFN is the only drug that can eliminate HCV, and it stimulates a number of IFN-stimulated genes, including PKR, 2′5′-oligoadenylate synthetase, and MxA [Sen and Ransohoff, 1993]. Among these, PKR is thought to play an important antiviral role against HCV. Determining the precise role of PKR in HCV infection, as well as the role of PKR in IFN treatment aimed at eliminating HCV, is important to better understand the nature of HCV persistence and to establish a suitable therapeutic protocol for IFN. Thus, we used a recently established full-length HCV cell-based expression system. This system expresses all HCV proteins from wild-type HCV sequences capable of infecting chimpanzees [Lin et al., 2005] without any adaptive mutations. Several reports using HCV replicon models suggest that PKR may mediate viral replication [Pflugheber et al., 2002; Rivas-Estilla et al., 2002; Chang et al., 2006]. However, replicon systems are subgenomic and cannot assess the effects of an entire HCV polyprotein, including E2 and other structural proteins. Since HCV E2 has been reported to impair PKR function [Taylor et al., 1999], and since HCV core protein has also been reported to activate PKR [Theodore and Fried, 2000], a system expressing the full-genome HCV sequence is better suited to analyzing interactions between HCV proteins and PKR. Our system expresses all proteins of the full-length HCV genotype 1a sequence, and therefore incorporates the effects of HCV core and E2 proteins. Recently, an infectious model of HCV genotype 2a capable of producing virus particles has been established [Wakita et al., 2005]. HCVpp or HCVcc are other infectious clones capable of replicating in culture [Lindenbach et al., 2005]. These HCV replication systems can produce virus particles, which are released into the culture medium and re-infect host cells during culture. Analysis of the effects of these systems on PKR expression is also of interest, although it should be noted that the affect by re-infection of HCV may influence outcomes.

It appears that HCV expression and replication are regulated by a number of cellular pathways. In particular, host defense against viruses involves a number of dsRNA-induced proteins, including PKR, TLR-3, and RIG-I. Huh-7 cells harboring HCV replicons respond poorly to dsRNA signaling [Lanford et al.,

2003]. In Huh-7 cells, very limited expression of the Toll like receptor-3 (TLR-3) is observed [Li et al., 2005]. Furthermore, induction of IFN regulatory factor-1 (IRF-1) results in a loss of permissiveness among Huh-7 cells harboring replicons [Kanazawa et al., 2004]. Huh-7.5 cells, known as cured cells, are derived from Huh-7 cells and harbor a single point mutation in RIG-I, the retinoic acid-inducible gene-I, which is thought to participate in sensing the presence of dsRNA molecules [Sumpter et al., 2005]. This Huh-7 derived cell line is highly permissive for HCV replication, however, there may also be disruption of normal antiviral signaling pathways. We therefore examined a BT7-H cell line in addition to a Huh-7 derived cell line. Using our expression system, the response of PKR to HCV replication in HuhT7 cells was less than that of BT7-H cells. Cell lines, such as BT7-H, which supported a higher level of HCV replication appeared to activate PKR more robustly, suggesting that PKR activation by HCV requires adequate HCV expression in the host cell. We therefore speculate that this is due to the formation of HCV dsRNA during replication, along with direct activation of PKR by HCV core protein. Within the Huh-T7 cell line, decreased HCV expression was observed, which appeared to result in decreased PKR activity. As a result, a more dramatic decrease in HCV protein was observed in BT7-H cells, compared to Huh-7 cells. This phenomenon may explain why HCV persists in hepatocytes. Lower levels of HCV replication may result in less PKR activation, thus enabling viral persistence.

In the present study, over-expression of PKR significantly inhibited HCV protein expression in both cell lines. These findings are consistent with an antiviral action of PKR. In Huh-T7 cells, strong PKR expression significantly inhibited HCV expression, while reduced PKR expression permitted HCV expression. To further characterize the relationship between PKR and HCV, we examined the effect of downregulation of PKR. Recently, post-transcriptional gene silencing using siRNA in mammalian cells has been proven an effective means of gene-specific silencing [Elbashir et al., 2001]. We used this method to specifically downregulate PKR. Of note, dsRNA molecules exceeding 30 nucleotides generally induce stimulation of PKR and activate the IFN signaling pathway [Balachandran et al., 2000]. However, siRNA molecules do not because of their short length (19–24 nucleotides). The PKR-specific siRNA used in this study downregulated PKR mRNA, resulting in a 93% reduction of PKR protein expression. Cells in which PKR was downregulated using siRNA demonstrated persistent HCV expression, indicating that PKR is a key molecule in the control of HCV replication and expression.

PKR is thought to be a key antiviral molecule of the IFN signaling pathway [Clemens and Elia, 1997]. However, other reports suggest that translation of HCV is independent of PKR activation by IFN in vitro [Koev et al., 2002]. Recently, several PKR-independent antiviral pathways have been discovered and are believed to play an important role in cellular defense against HCV infection, including 2′5′-oligoadenylate synthetase, MxA, as well as ISG6-16 and ISG15 [Zhu et al., 2003]. Clinically, the level of PKR expression prior to IFN treatment is not predictive of IFN's antiviral effects [MacQuillan et al., 2003; Giannelli et al., 2004]. Thus, it remains a matter of debate whether inhibition of HCV expression by IFN is PKR-dependent. Our results from PKR-silenced cells demonstrating reduced IFN-mediated antiviral effects indicate that IFN's antiviral effects against HCV are at least partially PKR-dependent. These findings suggest that strategies to enhance PKR activity may be a rational therapeutic approach to the management of HCV infection.

Based on these results, we conclude that both HCV expression and IFN effectively induce PKR, and that PKR can influence HCV expression in the presence or absence of IFN. PKR has direct antiviral activity against HCV and is a key mediator of IFN's effects against HCV.

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Prevalence of Diabetes and Incidence of Angiopathy in Patients with Chronic Viral Liver Disease

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Summary **Patients with chronic liver disease (CLD) often develops glucose intolerance. We explored the prevalence of diabetes mellitus in viral CLD, and analyzed factors profoundly affecting the diabetic angiopathies. 229 CLD patients (124 chronic hepatitis and 105 liver cirrhosis) entered the study. The diagnosis of diabetes was made with the criteria by World Health Organization. Laboratory investigation included serum asparate aminotransferase, alanine aminotransferase, albumin, fasting blood sugar, hemoglobin A1c (HbA1c), fasting immunoreactive insulin, and HOMA-R (FBS*IRI/405). The incidence of macro- and micro-angiopathy were also examined. Forty (17.5%) CLD patients were diagnosed diabetes, giving a significantly higher incidence than that of general cohort (5.3%) ($p<0.001$). Among them, 12 (30%) had the triopathy, significantly lower than that in a matched group of diabetic patients without CLD (65%) ($p<0.001$). Significantly increased levels of HbA1c and HOMA-R were observed in diabetic CLD with angiopathy compared with diabetic CLD without. Incidence of diabetes was increased in viral CLD patients. The rate of diabetic angiopathies in CLD, however, was relatively low, this could be explained by low coagulability in these patients. Poor control of hyperglycemia, partly due to insulin resistance, might explain the onset of angiopathy in diabetic CLD.**

***Key Words*: hepatogenous diabetes, chronic liver disease, diabetes mellitus, insulin resistance, glucose intolerance**

Introduction

Patients with chronic liver disease (CLD) are characterized by impaired glucose tolerance [*1–4*]. In particular, 50–80% of cirrhotic patients are glucose intolerant, and 10–20% of them develop diabetes mellitus (DM) [*2, 5–11*]. Insulin resistance has been suggested as the major cause of such glucose intolerance in cirrhotic patients [*12, 13*]. The impact of hepatogenous DM [*1, 3*] has been recently reported to worsen long-term prognosis caused by hepatic failure [*3, 6, 14*]. Diabetic macroangiopathy (cardiac events, stroke, and peripheral arterial disease) as well as microangiopathy (retinopathy, neuropathy, and nephropathy) in cirrhotics were, however, relatively rare as compared to those in DM alone [*14*].

Reported studies as to the occurrence of diabetes in liver disease, however, employed mostly the alcoholic CLD [*3, 5,*

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15–18]. Alcohol damages the pancreas directly, leading to insufficient secretion of insulin. The association of hepatitis C virus infection with the development of DM has been also suggested [*5*, *7–9*, *19*]. However, incidence and significance of diabetic angiopathies in chronic viral liver disease have not yet been clarified.

We here conducted a study to elucidate the prevalence of DM and the incidence of diabetic macro- and microangiopathy in chronic viral liver disease, and to characterize the clinical background of those patients.

Materials and Methods

Patients

The subjects consisted of 229 patients with CLD aged 61 years (median, range 24–87 years) who were either hospitalized or followed-up at the outpatient clinic between January 2000 and March 2001. Of those patients, 124 (90 males and 34 females) were diagnosed as chronic hepatitis, and 105 (69 males and 36 females) were liver cirrhosis. The etiologies of liver disease were hepatitis B virus in 51 patients and hepatitis C in 178 patients. Clinical characteristics of the patients are given in Table 1.

Three hundred and forty five non-insulin dependent DM patients without CLD from our hospital were also included in this study as a disease-control group against diabetic CLD patients. Informed consent was obtained from each subject.

Methods

Diagnosis of chronic hepatitis or liver cirrhosis was based on liver biopsy, blood examination, and imagings such as ultrasound or computed tomography. The demographic data including age, sex, alcohol drinking, duration of liver disease, duration of diabetes, and family history of diabetes were collected. Body mass index (BMI) was calculated as body weight in kilograms divided by the square of height in meters (kg/m^2). Patients with autoimmune hepatitis, alcoholic hepatitis, Wilson's disease, hemochromatosis, drug-induced hepatitis, or hepatitis of unknown cause were excluded from the study.

Serum total bilirubin (T-Bil), aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin (Alb), fasting blood sugar (FBS), platelet count (Plt), triglyceride (TG), total cholesterol (T-cho), and urine protein were measured by standard methods. Severity of liver cirrhosis was graded by Child-Pugh classification [*20*]. Diabetes was diagnosed according to the current World Health Organization (WHO) criteria [*21*]. Mild glucose intolerance was not included in the diagnosis of diabetes. The presence of hypertension, or the development of hepatocellular carcinoma (HCC) was also recorded.

For CLD patients with diabetes, glycosylated hemoglobin (HbA1c) and fasting immunoreactive insulin (IRI) were measured. The insulin resistance was calculated on the basis of fasting plasma levels of glucose and insulin according to the HOMA model as follows [*22*]:

Insulin resistance (HOMA-R)=fasting insulin (μU/ml)×

Table 1. Clinical characteristics of the 229 patients with chronic liver damage

Characteristic	Chronic hepatitis	Liver cirrhosis	*p* value*
No. of patients	124	105	
Age (years)	57 (24–78)	64 (32–87)	$p<0.001$
Gender (male/female)	90/34	69/36	ns
Etiology (HBV/HCV)	30/94	21/84	ns
No. with HCC	7 (6%)	49 (47%)	$p<0.001$
No. with Hypertension	15 (12%)	21 (20%)	ns
BMI (kg/m^2)	22.4 (16.7–32.4)	22.3 (14.5–32.2)	ns
T-Bil (mg/dl)	0.8 (0.4–1.1)	1.2 (0.4–2.6)	$p<0.05$
AST (IU/l)	49 (15–186)	68 (14–346)	$p<0.001$
ALT (IU/l)	57 (7–274)	56 (9–195)	ns
FBS (mg/dl)	102 (72–225)	92 (69–124)	$p<0.01$
Alb (g/dl)	4.1 (2.6–4.8)	3.6 (2.3–4.7)	$p<0.001$
PT (%)	84 (55–100)	70 (41–100)	ns
Plt ($\times 10^4/\mu l$)	17.0 (5.0–34.2)	11.8 (2.4–33.2)	$p<0.001$

Values are expressed as median (range). *by Mann-Whitney's *U* test or Fischer's exact probability test. Abbreviations used are; HBV, hepatitis B virus; HCV, hepatitis C virus; BMI, body mass index; HCC, hepatocellular carcinoma; T-Bil, total bilirubin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; FBS, fasting blood sugar; Alb, serum albumin; PT, prothrombin time; Plt, platelet count; ns, not significant.

fasting glucose (mg/dl)/405

In order to determine the presence of triopathy, the patients were further examined by ophthalmologists for retinopathic complications, and by the measurement of urinary trace albumin, blood urea nitrogen (BUN) and creatinine (Cr) for nephropathy. Neurological examinations on patellar tendon reflex and Achilles tendon reflex, and on paralysis of the feet were carried out for neuropathy. The CLD patients with diabetes were categorized into two groups: those with either of thriopathy and those without triopathy.

The study protocol conformed to the ethical guideline of the 1975 Declaration of Helsinki as reflected in a prior approval by the institution's review board for human research [*23*].

Statistical analysis

The results are expressed as median and range. For statistical comparison between median values, Mann-Whitney's *U* test was used. Fisher's exact test was used to compare incidences. All statistical tests performed were two-tailed. Difference was considered significant when *p* value was less than 0.05. All analyses were performed using Stat View 5.0 for Macintosh (SAS Institute, Cary, NC).

Results

Forty patients with chronic liver disease (11 chronic hepatitis and 29 liver cirrhosis) were diagnosed diabetes (Table 2). This incidence of diabetes in CLD (40/229, 17.5%) was significantly higher than that in an age- and sex-matched general cohort (5.3%) obtained from reference 1 ($p<0.001$ by chi-square test). The incidence of DM in LC (29/105, 27.6%) was significantly higher than that in CH (11/124, 8.9%) ($p<0.001$) (Table 2). In CH, all patients with DM were HCV-positive while no HBV(+)-CH patient presented DM ($p<0.05$, Table 2). In contrast, such difference between HCV and HBV was not found in LC. There was no significant difference in clinical parameters, except FBS and HbA1c, between CLD patients with DM and those without DM.

Among 40 CLD patients with DM, 12 (30%) were complicated with diabetic triopathy (Table 3). This incidence in diabetic CLD was significantly lower than that in DM without CLD (225/345, 65%) ($p<0.001$). Such difference was due to a significantly lower incidence of retinopathy in diabetics with CLD ($p<0.05$). The incidence of neuropathy in diabetics with CLD tended to be also lower than those without CLD ($p=0.06$), while the incidence of nephropathy was not different between both groups (Table 3). Only one patient (1/40, 2.5%) developed a major cardio-vascular event and cerebral infarction that is considered to result from hepatogenous diabetes.

Clinical characteristics of 40 diabetic CLD patients were compared with those of 345 diabetic patients without liver disease (Table 3). There was no significant difference in age, gender, BMI, and treatment modalities between the

Table 2. Incidence of diabetes mellitus in chronic liver damage

Characteristic	DM(−)	DM(+)	*p* value*
No. of patients	189 (82.5%)	40 (17.5%)	
Age	64 (24–87)	65 (53–77)	ns
Gender (male/female)	132/57	27/13	ns
CH/LC	113/76	11/29	$p<0.001$
CH (HBV/HCV)	30/83	0/11	$p<0.05$
LC (HBV/HCV)	16/60	5/24	ns
Child-Pugh classification (A/B/C)	44/25/7	16/9/4	ns
BMI (kg/m^2)	22.0 (14.5–32.2)	22.3 (16.9–32.4)	ns
No. with HCC	51 (27%)	13 (33%)	ns
AST (IU/l)	68 (15–346)	62 (21–154)	ns
ALT (IU/l)	56 (7–274)	51 (9–166)	ns
FBS (mg/dl)	92 (72–125)	148 (69–225)	$p<0.01$
HbA1c (%)	4.8 (3.5–6.3)	6.8 (4.3–10.8)	$p<0.01$
Alb (g/dl)	3.6 (2.3–4.8)	3.5 (2.3–4.7)	ns
Plt (×10^4/μl)	11.8 (2.5–34.2)	9.9 (2.4–28.6)	ns

Values are expressed as median (range). *by Mann-Whitney's *U* test or chi-square test. Abbreviations used are; DM, diabetes mellitus; CH, chronic hepatitis; LC, liver cirrhosis; HBV, hepatitis B virus; HCV, hepatitis C virus; BMI, body mass index; HCC, hepatocellular carcinoma; AST, aspartate aminotransferase; ALT, alanine aminotransferase; FBS, fasting blood sugar; HbA1c, glycosylated hemoglobin; Alb, serum albumin; Plt, platelet count; ns, not significant.

Table 3. Clinical characteristics of diabetic CLD patients and diabetic patients without CLD

		DM without CLD	Diabetic CLD	*p* value*
No. of patients		345	40	
Age (years)		60 (15–87)	65 (53–77)	ns
Gender (male/female)		155/190	27/13	ns
BMI (kg/m^2)		22.8 (16.2–31.8)	23.2 (16.9–32.4)	ns
FBS (mg/dl)		120 (41–303)	148 (69–225)	ns
HbA1c (%)		6.7 (4.2–12.0)	6.8 (4.3–10.8)	ns
HOMA-R		8.3 (0.72–17.58)	6.9 (0.88–32.2)	ns
Insulin		28 (3.1–63.0)	26 (4.4–110)	ns
TG (mg/dl)		128 (33–430)	82 (33–233)	$p<0.05$
T-cho (mg/dl)		195 (127–387)	141 (70–221)	$p<0.001$
PT (%)		89 (77–96)	72 (40–100)	$p<0.001$
Plt ($\times 10^4/\mu l$)		23.8 (12.8–33.4)	9.9 (2.4–28.6)	$p<0.001$
Treatment				
	Diet	49 (14%)	10 (25%)	
	Drug	128 (37%)	17 (43%)	ns
	Insulin	168 (49%)	13 (32%)	
Triopathy		225 (65%)	12 (30%)	$p<0.001$
	Retinopathy	133 (39%)	8 (20%)	$p<0.05$
	Neuropathy	121 (35%)	8 (20%)	$p=0.06$
	Nephropathy	57 (17%)	4 (10%)	ns

Values are expressed as median (range). *by Mann-Whitney's *U* test or chi-square test. Abbreviations used are; DM, diabetes mellitus; CLD, chronic liver disease; BMI, body mass index; FBS, fasting blood sugar; HbA1c, glycosylated hemoglobin; HOMA-R, homeostasis model assessment; Alb, serum albumin; TG, triglyceride; T-cho, total-cholesterol; PT, prothrombin time; Plt, platelet count; ns, not significant.

two groups. While blood biochemical parameters such as FBS, HbA1c, HOMA-R, and IRI were similar, Plt, blood coagulation activity indicated by prothrombin time (PT), TG, and T-cho were significantly lower in diabetic CLD than in DM without CLD (Table 3).

Clinical characteristics of diabetic CLD patients with and without triopathy are shown in Table 4. Those with triopathy had a significantly longer morbidity period of diabetes ($p<0.05$) and received insulin injection at a higher rate ($p<0.05$) than diabetic CLD without triopathy.

Blood biochemical data in diabetic CLD patients with and without triopathy are given in Table 5. Significant difference was observed in HbA1c and HOMA-R between the two groups, suggesting hyperglycemia for a longer period and insulin resistance in the former group. No difference in liver function tests was observed between two groups.

Discussion

The incidence of diabetes as a complication of chronic liver disease is reported to be 13% in chronic alcoholic hepatitis and 14 to 63% in alcoholic liver cirrhosis [*5*, *9*, *15*–*17*]. However, there have been few reports as to the incidence of diabetes in viral liver disease [*16*].

Zein reported 25% of cirrhotic patients before transplantation had diabetes [*16*]. In our study, 8.9% of patients with chronic viral hepatitis and 27.6% of patients with viral liver cirrhosis were complicated with diabetes. Thus, significantly higher incidence of diabetes was observed in chronic liver disease.

There were a few differences between diabetic patients with liver disease (diabetic CLD) and authentic diabetic patients. The most pronounced was that, very few patients had a family history of diabetes in diabetic CLD (4/40, 10%), whereas more than 50% of the latter patients showed that. This is because diabetic state in CLD could be acquired secondary to liver disease *per se,* not due to genetic background as in most non-insulin dependent diabetes mellitus (NIDDM).

The pathogenesis of diabetes in liver disease is not fully understood. In alcoholic liver disease, reduced insulin secretion due to pancreatic damage could be the cause of impaired glucose metabolism. However, that may not be the case of diabetes in viral liver disease, because excess insulin secretion is observed after glucose loading in liver cirrhosis [*24*]. Glucose uptake into hepatocytes after glucose absorption is delayed due to decreased hepatocyte mass in CLD, leading to hyperinsulinemia. Subsequent continuous hyperinsulinemic

Table 4. Clinical characteristics of diabetic CLD patients with and without diabetic triopathy

Triopathy		(−)	(+)	*p* value*
No. of patients		28	12	
Age (years)		64 (53–77)	66 (57–77)	ns
Gender (male/female)		19/9	8/4	ns
Etiology	HBV/HCV	3/25	2/10	ns
CH/LC		7/21	4/8	ns
CH	HBV/HCV	0/7	0/4	ns
LC	HBV/HCV	3/18	2/6	ns
Child-Pugh classification	A/B/C	11/6/4	5/3/0	
BMI (kg/m^2)		23.4 (16.9–32.4)	23.1 (19.4–27.0)	ns
Duration of liver disase (years)		9 (2–19)	10 (4–19)	ns
Duration of DM (years)		5 (1–14)	8 (1–16)	$p<0.05$
Treatment	Diet	10 (36%)	0 (0%)	$p<0.05$
	Drug	11 (39%)	6 (50%)	
	Insulin	7 (25%)	6 (50%)	
Hypertension		7 (25%)	4 (33%)	ns
HCC		10 (36%)	4 (33%)	ns
Family history of DM		1 (3.6%)	3 (25%)	$p<0.05$

Values are expressed as median (range). *by Mann-Whitney's *U* test or chi-square test. Abbreviations used are; CLD, chronic liver disease; HBV, hepatitis B virus; HCV, hepatitis C virus; CH, chronic hepatitis; LC, liver cirrhosis; BMI, body mass index; DM, diabetes mellitus; HCC, hepatocellular carcinoma; ns, not significant.

Table 5. Biochemical analysis of diabetic CLD patients with and without diabetic triopathy

Triopathy	(−)	(+)	*p* value*
No. of patients	28	12	
FBS (mg/dl)	127 (69–257)	172 (110–266)	$p<0.001$
HbA1c (%)	6.3 (4.3–9.2)	7.6 (5.7–10.8)	$p<0.05$
Insulin (U/ml)	22 (4.5–33.0)	34 (9.3–69.1)	ns
HOMA-R	4.1 (0.88–9.49)	13.5 (3.1–32.2)	$p<0.05$
TG (mg/dl)	84 (33–205)	80 (39–233)	ns
T-cho	135 (70–221)	151 (94–216)	ns
T-Bil (mg/dl)	1.4 (0.4–4.9)	1.0 (0.7–1.4)	ns
AST (IU/l)	63 (21–154)	51 (24–97)	ns
ALT (IU/l)	54 (9–166)	47 (17–83)	ns
Alb (g/dl)	3.5 (1.8–4.7)	3.4 (2.4–4.3)	ns
PT (%)	72 (51–99)	81 (58–100)	ns
Plt ($\times 10^4/\mu l$)	9.7 (2.4–28.6)	10.1 (3.9–18.8)	ns
CH	12.8 (10.1–28.6)	16 (8.1–18.8)	ns
LC	7.1 (2.4–15)	7.6 (3.9–14.6)	ns

Values are expressed as median (range). *by Mann-Whitney's *U* test or chi-square test. Abbreviations used are; CLD, chronic liver disease; FBS, fasting blood sugar; HbA1c, glycosylated hemoglobin; HOMA-R, homeostasis model assessment; TG, triglyceride; T-cho, total-cholesterol; T-Bil, total bilirubin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; Alb, serum albumin; PT, prothrombin time; Plt, platelet count; CH, chronic hepatitis; LC, liver cirrhosis; ns, not significant.

state could eventually induce insulin resistance in the patients. Previous reports suggested that insulin resistance, characterized by both decreased glucose transport and decreased nonoxidative glucose metabolism in skeletal muscle, could be the cause of diabetes in liver cirrhosis [*25*]. In addition, glucose effectiveness, glucose metabolic pathway independent of insulin secretion, is also reduced in cirrhotic patients [*4, 26*].

A strong association between HCV infection and diabetes has been recently suggested. Mason reported that HCV infection and age were independent predictors of diabetes, and significantly higher prevalence of HCV infection, especially genotype 2a, was observed in diabetic patients as compared to non-diabetic hepatitis C patients [*8*]. Recognized diabetic factors such as age or obesity may increase the risk for type 2 diabetes in HCV-infected persons [*27*]. We have found higher incidence of DM in chronic hepatitis C than in chronic hepatitis B (Table 2), and also obesity tendency in chronic hepatitis C with DM compared to CH(C) without DM (BMI $25 \pm 4 : 22 \pm 3$ kg/m^2, p=0.08). Additionary higher prevalence of diabetes was observed in liver cirrhosis as compared to that in chronic hepatitis. Furthermore, in contrast to CH, no difference was found in the incidence of DM between HBV-positive cirrhotics and HCV-positive ones. These observations suggest that severity of liver disease could increase by itself the risk of diabetes in CLD.

We showed significantly lower incidence of macro- and microangiopathy in patients with diabetic CLD than in authentic diabetic patients (Table 3), in a similar manner to ref [*28*]. As for macroangiopathy, there was only one patient (2.5%) with cerebral infarction who developed cardio-vascular event possibly attributable to diabetes in CLD group. Microangiopathy was observed in 12 patients (30%). Progressive retinopathy was observed in two patients who had a history of diabetes lasting 10 years and 8 years, respectively. There were two patients (5%) with diabetic neuropathy, who were on medication such as Epalrestat (Kinedak®). Positive trace albumin in urine, ophthalmologic abnormality, and neurological impairment were identified in other eight patients without any subjective symptom. Although the incidence of triopathy in diabetic CLD was significantly lower, the proportion of retinopathy, neuropathy, and nephropathy did not differ between diabetic CLD (2:2:1) and DM without CLD (2.3:2.1:1) (Table 3). Thus, the detection (or non-detection) of statistical significance for the difference in the incidence of each of triopathy between 2 groups (Table 3) may have depended solely on the size of the number of each event.

This study clarified that several factors might affect the occurrence of diabetic macro- and microangiopathy in CLD. First, significantly longer duration and frequent family history of diabetes were observed in CLD patients with diabetic triopathy as compared to those without triopathy. Poor control of blood glucose level, indicated by high FBS and HbA1c, could also be a cause of diabetic triopathy. Interestingly, significantly higher level of HOMA-R was also observed in the group with diabetic triopathy, suggesting insulin resistance might have an impact as a factor for diabetic triopathy. Therefore, improvement of insulin resistance would be important to prevent diabetic angiopathy also in liver disease. Further prospective study is needed to find the relevance between diabetic angiopathy and insulin resistance in CLD patients.

Significant decreases in platelet count and PT were observed in CLD with diabetes as compared with authentic diabetic patients as shown in Table 3. It has been reported that increased platelet aggregation is associated with the development of diabetic complications, and strict blood sugar control could improve platelet aggregation and prevent thriopathy [*29*]. Decreased production of coagulation factors from the liver might also help preventing intravascular coagulation promoted by hyperglycemia in diabetic state [*30, 31*], leading to a low rate of angiopathy in diabetic CLD patients.

It is difficult to avoid some limitations in this kind of clinical study. The limitation in this study is the lack of area under the curve (AUC) analysis of oral glucose tolerance test (OGTT), since AUC better estimates the grade of glucose intolerance in LC than HbA1c. This study was a retrospective cross-sectional study, and we could obtain only the diagnosis in the chart, but not the raw OGTT data to calculate AUC.

In conclusion, high incidence of diabetes was observed in viral CLD. Low intravascular coagulability may explain relatively lower rate of diabetic complication in CLD. However, poor control of hyperglycemia, partly due to insulin resistance, could contribute to the pathogenesis of angiopathy in diabetic CLD. Strict control of blood glucose and improvement of insulin resistance, therefore, should be directed in patients with diabetic CLD to help prevent diabetic angiopathy in those patients.

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Original Article

Oral branched-chain amino acid supplementation improves the oxidized/reduced albumin ratio in patients with liver cirrhosis

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Aim: Branched-chain amino acid (BCAA) supplementation improves hypoalbuminemia in decompensated cirrhotics. Recently, it was clarified that the ratio of oxidized albumin within total albumin rises with progression of liver cirrhosis. We conducted a feasibility study to investigate whether BCAA supplementation might improve this ratio.

Methods: Seven cirrhotic patients (age: 70 ± 6 years; M/F = 4/3; etiology: hepatitis C in six and non-B/non-C hepatitis virus in one; Child–Pugh classification: A in six and B in one) were enrolled consecutively in this study in October 2004 to March 2005. Patients were given 4 g BCAA after each meal for 8 weeks. Serum total, oxidized and reduced albumin, plasma amino acids, glutathione, zinc, selenium, and lipid peroxide concentrations were measured every 2 weeks.

Results: Low total albumin, high oxidized albumin, and low reduced albumin levels were observed at entry. After 8 weeks BCAA supplementation, the ratio of oxidized albumin within total albumin decreased significantly and that of reduced albumin increased significantly ($P < 0.05$, respectively). Total albumin tended to rise and lipid peroxide concentrations tended to fall, but not significantly.

Conclusion: BCAA supplementation improved the oxidized/reduced state of serum albumin. This intervention is effective to maintain the quality of serum albumin in cirrhotic patients.

Key words: branched-chain amino acids (BCAA), human mercaptalbumin (HMA), human non-mercaptalbumin (HNA), liver cirrhosis, oxidized albumin, reduced albumin

INTRODUCTION

PROTEIN-ENERGY MALNUTRITION (PEM) is a common clinical manifestation of patients with liver cirrhosis.[1] PEM per se has a clinical impact in cirrhosis, because it affects the outcome of patients[1] as well as their quality of life (QOL).[2] Recently, it has been reported that long-term oral supplementation with branched-chain amino acids (BCAA) not only increased the serum albumin level, but also improved event-free survival and QOL in patients with decompensated cirrhosis with an adequate daily food intake.[3,4] BCAA has a direct regulatory effect on albumin synthesis and secretion at a cellular level.[5] Serum albumin is an abundant protein with multiple functions such as maintenance of colloid osmotic pressure, ligand-binding and transport properties, enzymatic activities, and antioxidation.[6] Additionally, serum albumin has microheterogeneity including oxidized and reduced forms of albumin.[7] Watanabe *et al.* have previously reported that the serum total albumin level decreased and the ratio of oxidized albumin within total albumin increased with progression of liver cirrhosis.[8]

In chronic hepatitis C virus (HCV) hepatitis, reactive oxygen species (ROS) are produced excessively in the liver due to iron deposition or steatosis, and promote inflammation and fibrosis in the liver.[9,10] Thus, antioxidative molecules may have an important role to

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suppress such disease progression. Among candidate molecules, the most abundant is the reduced albumin in the serum and reduced glutathione in the cytosol.[6] However, no intervention has previously been identified to recover the oxidized/reduced state of serum albumin in cirrhotics. We examined the ratio without any specific intervention for 2 months, but the ratio remained unchanged (Fukushima H, 2006, unpubl. obs). While BCAA supplementation increases serum albumin, its effect on the oxidized/reduced state of albumin has not been elucidated.

We conducted a feasibility study to investigate whether BCAA supplementation might change the ratio of oxidized albumin to reduced albumin, and might affect ROS in patients with liver cirrhosis.

METHODS

WE USED A consecutive entry study design. Liver cirrhosis was diagnosed based on clinical and laboratory profiles. Informed consent was obtained from each subject. The study protocol conformed to the guidelines of the 1975 Declaration of Helsinki as reflected in a prior approval by the institution's review board for human research.

Eligibility and exclusion criteria

Eligibility criteria were viral liver cirrhosis with serum albumin concentration <3.5 g/dL, serum total bilirubin level <3 mg/dL, and sufficient dietary food intake (energy intake of ≥1000 kcal/day and protein intake of ≥40 g/day). Exclusion criteria were the presence of any one of the following: physically detectable ascites and edema, bleeding varices, hepatic encephalopathy of or above grade II, viable liver cancer, and history of albumin infusion or receiving BCAA granules in the preceding 3 months.

Patients

Seven patients participated in this study in October 2004–March 2005, and clinical and laboratory data of these patients are given in Table 1. Patients and their families attended the diet-education course held specifically for this trial by the study dietician. Patients were instructed to take isocaloric (25–35 kcal/kg per day) and isonitrogenous (1.0–1.4 g protein/kg per day) diets and their daily food intake was estimated by self-administered questionnaire throughout the study period.

All subjects were given BCAA granules consisting of BCAA alone (Livact Granules®; Ajinomoto, Tokyo,

Table 1 Clinical and laboratory data of patients with liver cirrhosis at entry to present study

No.	Gender	Age (years)	Etiology	Alb (g/dL)	HMA (%)	HNA-1 (%)	HNA-2 (%)	T-Bil (mg/dL)	PT (%)	Child-Pugh classification
Normal range				3.9–4.9	73.2 ± 2.3*	24.9 ± 2.0*	1.9 ± 0.8*	0.2–1.2	70–120	
1	M	65	HCV	3.4	51.5	45.4	3.1	1.1	84	6A
2	M	64	HCV	3.1	73.1	25.0	1.9	0.8	63	7B
3	F	67	HCV	3.3	43.8	50.3	5.9	0.6	88	6A
4	F	71	HCV	3.1	58.2	38.6	3.2	1.9	78	6A
5	M	82	HCV	3.1	55.4	39.1	5.6	0.9	72	6A
6	M	69	nB/nC	3.6	70.7	27.6	1.7	1.6	79	5A
7	F	72	HCV	3.2	69.3	28.0	2.6	0.6	77	6A
Mean ± SD		70 ± 6		3.3 ± 0.2	60.3 ± 11.0	36.3 ± 9.7	3.4 ± 1.7	1.1 ± 0.5	77.3 ± 8.1	6.0 ± 0.6

*Data in healthy young male subjects.[13]

Alb, albumin; HCV, hepatitis C virus; HMA, human mercaptalbumin; HNA, human non-mercaptalbumin; nB/nC, non-B and non-C hepatitis virus; PT, prothrombin time; T-Bil, total bilirubin.

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Japan; 1 sachet contains 4 g BCAA; L-isoleucine 952 mg, L-leucine 1904 mg, and L-valine 1144 mg, 1 sachet orally after each meal) throughout the 2-month study period. They were instructed to visit their outpatient clinic following an overnight fast at 2, 4, and 8 weeks after BCAA administration, and received blood biochemical tests at each visit.

Outcome markers

Main outcome markers were serum albumin level and the ratio of reduced albumin (human mercaptalbumin, HMA), oxidized albumin (human nonmercaptalbumin, HNA-1), and irreversibly oxidized albumin (HNA-2) within total albumin. Serum total albumin level was measured by the bromcresol green (BCG) method, and HMA, HNA-1, and HNA-2 were determined by high-performance liquid chromatography (HPLC) as reported previously.[11] The HPLC profiles obtained were subjected to numerical curve fitting, and area under each peak was approximated by a Gaussian function (Fig. 1). Glutathione, zinc, selenium, and lipid peroxides (LPO) were measured using respective standard methods. BCAA and BTR (the ratio of BCAA to tyrosine) were determined by HPLC.

Statistical analysis

Data were expressed as the mean and standard deviation (SD) in Tables 1 and 2, while as percentiles (10%, 25%, median, 75%, and 90%) in Figure 2a–c. The differences in blood biochemical data between 0 and 8 weeks after BCAA supplementation were tested by Wilcoxon's test. Differences were considered significant when *P*-values were less than 0.05.

RESULTS

BASELINE DATA REVEALED low serum albumin level, reduced HMA, and increased HNA-1 levels (Table 1).

Eight-week BCAA supplementation did not change the serum total albumin level significantly (Table 2). However, the percentages of HMA within total albumin increased significantly (from 60.3 ± 11.0% to 66.9 ± 9.1%, $P < 0.05$) (Fig. 2a) and those of HNA-1 decreased significantly (from 36.3 ± 9.7% to 30.4 ± 8.4%, $P < 0.05$) (Fig. 2b). HNA-2 did not show significant changes (from 3.4 ± 1.7% to 2.8 ± 1.1%) in this study period (Fig. 2c).

Plasma BCAA concentration and BTR increased significantly at 2 and 8 weeks after BCAA supplementation ($P < 0.05$, respectively) (Table 2). Zinc decreased significantly from 55 ± 7 μg/dL to 42 ± 9 μg/dL ($P < 0.05$) (Table 2) and LPO tended to decrease from 1.5 ± 1.0 nmol/mL to 1.0 ± 0.3 nmol/mL in this study period (Table 2).

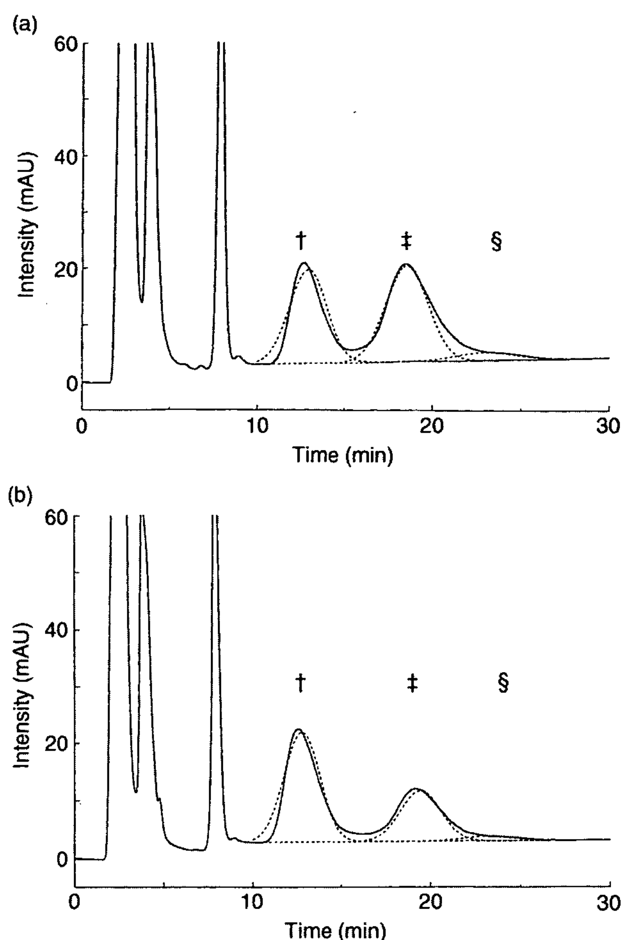

Figure 1 High-performance liquid chromatography (HPLC) profiles of serum albumin from a subject (No. 3) at (a) baseline and (b) 8 weeks after branched-chain amino acid (BCAA) supplementation. HPLC was performed on an ES-502 N column with an increasing ethanol concentration from 0 to 5% in acetatesulfate buffer (pH 4.85). †, HMA; ‡, HNA-1; §, HNA-2. HMA, human mercaptalbumin; HNA, human nonmercaptalbumin. Dotted lines were simulated by Gaussian function.[11]

DISCUSSION

PATIENTS WITH LIVER cirrhosis often suffer from PEM. Our previous research revealed that 75% of cirrhotic patients are in a state of protein malnutrition with serum albumin level below 3.5 g/dL[1]

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Table 2 Changes in serum biochemical markers after BCAA supplementation

	Normal range	Before BCAA supplementation	2 weeks post BCAA supplementation	4 weeks post BCAA supplementation	8 weeks post BCAA supplementation
BCAA (nmol/mL)	270–600	270 ± 101	409 ± 98*	338 ± 148	389 ± 137*
BTR	4.41–10.05	2.32 ± 0.76	3.77 ± 1.80*	2.59 ± 1.47	3.34 ± 1.37*
Serum albumin (g/dL)	3.9–4.9	3.3 ± 0.2	3.4 ± 0.2	3.4 ± 0.4	3.4 ± 0.3
Glutathione (μg/mL)	NA	34 ± 6	34 ± 5	33 ± 4	36 ± 5
Zinc (μg/dL)	65–110	55 ± 7	44 ± 14*	45 ± 6	42 ± 9*
Selenium (μg/dL)	10.6–17.4	11 ± 2	11 ± 1	10 ± 2	11 ± 2
Lipid peroxides (nmol/mL)	1.8–4.7	1.5 ± 1.0	1.5 ± 0.6	1.1 ± 0.3	1.0 ± 0.3

*$P < 0.05$ compared with before BCAA supplementation.
BCAA, branched-chain amino acids; BTR, ratio of BCAA to tyrosine; NA, not available.

Hypoalbuminemia leads to ascites or peripheral edema, and worsens QOL and prognosis. Therefore, improvement of serum albmin level is important in patients with liver cirrhosis. A candidate treatment for this aim is BCAA supplementation. BCAA exerts a protein-sparing effect and increases synthesis and secretion rates of albumin by hepatocytes.[5] L-Leucine in BCAA promotes albumin synthesis *in vitro* through the activation of a transcription factor, mammalian target of rapamycin (mTOR).[12] Recently, it has been demonstrated by randomized controlled trials that BCAA improves not only serum albumin level but also QOL and event-free survival in patients with decompensated liver cirrhosis.[3,4] In these studies, events for the primary end-point were aggravation of hepatic failure (ascites, peripheral edema, hepatic encephalopathy, and jaundice), rupture of esophageal or gastric varices, development of liver cancer, and death due to any cause. The prevention of hepatic failure might be explained in part by the direct effect of BCAA and increased serum albumin,[3,4] but the factors that reduced particular events such as liver cancer remain unclear.

Watanabe *et al.* analyzed the microheterogeneity of serum albumin including oxidized albumin, reduced albumin and glycoalbumin in cirrhotic patients, and found an elevated ratio of oxidized albumin within total albumin whereas total albumin concentration decreased.[8] Human serum albumin (HSA) is a mixture of mercaptalbumin (HMA, reduced albumin) and nonmercaptalbumin (HNA, oxidized albumin), and composes a protein redox couple in the serum.[13] HMA has one free sulfhydryl group in position 34 (Cys-34) and is responsible for the largest fraction of free sulfhydryl in blood sera. The major HNA compound is mixed disulfide with cystine or oxidized glutathione (HNA-1). Others (HNA-2) include oxidation products higher than mixed disulfide, such as sulfenic (-SOH), sulfinic ($-SO_2H$), and sulfonic ($-SO_3H$) states, which comprise a small proportion of the HNA compound.[13] The ratio of oxidized albumin to reduced albumin is usually 1:3 in healthy human sera and means an intravascular redox state. It has been reported previously that such a redox state of serum albumin changes in the elderly, with physical exercise, and various pathophysiological conditions, including renal and hepatic dysfunctions, diabetes mellitus, and patients under anesthesia during surgery.[14]

Reduced albumin might have powerful antioxidant properties against ROS due to both its distinctive sulfhydryl group and its abundance in extracellular fluids.[15] However, there have been only a few studies on the properties of HMA and HNA on a molecular basis. Recently, the functional differences between reduced albumin and oxidized albumin were examined.[16] These results revealed the difference in ligand-binding ability and antioxidant ability. The binding affinities of L-tryptophan and cefazolin to reduced albumin were higher than oxidized albumin and verapamil binding to oxidized albumin was found to be slightly increased. The change of reduced albumin to oxidized albumin could increase free tryptophan, resulting in increased brain serotonin metabolism and causing hepatic encephalopathy in cirrhosis. Although quite speculative, this mechanism may partly explain the inhibition of hepatic failure by BCAA in recent studies.[3,4]

Another difference between reduced albumin and oxidized albumin was the antioxidant capacity. For example, the radical scavenging activity of HSA is decreased by turning to an oxidized form when exposed to hydroxyl radical and UV photolysis of H_2O_2.

In chronic hepatitis C, excessive iron storage or steatosis like non-alcoholic steatohepatitis (NASH)

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