and nontumourigenic rat fibroblasts, and to repress p21 promoter activity through modulation of the activity of p53. Moreover, the NS3 protein suppresses actinomycin D-induced apoptosis of NIH3T3 cells.

The function of the HCV NS4B protein is unknown, although it may function as part of the replication complex. NS4B protein also transforms NIH3T3 cells in cooperation with H-ras. The HCV NS5A protein may play an important role in regulating HCV replication as it is part of the replication complex, but its function remains largely unclear. The NS5A protein represses transcription of p21, while it activates the human proliferating cell nuclear antigen gene. Moreover, introduction of NS5A into NIH3T3 cells promoted anchorage-independent growth and tumour formation in nude mice. The NS5A protein reportedly interacts with p53 and inhibits p53-mediated apoptosis. It has also been reported that the NS5A protein interacts with Bax and inhibits apoptosis to protect p53-negative HCC cells from sodium phenylbutyrate-induced apoptosis. In addition, the NS5A protein interacts with Bin-1, a tumour suppressor with pro-apoptotic properties. Apoptosis induced by Bin-1 is inhibited by NS5A expression. These functions of NS5A that suppress apoptosis may contribute to hepatocarcinogenesis, in addition to the effects of the core protein. It has also been reported that the NS5A protein physically associates with p53 and represses p21 gene expression. Moreover, the NS5A protein is involved in the induction of chromosome instability via mitotic cell cycle dysregulation.

The HCV NS5B protein is an RNA-dependent RNA polymerase and appears to downregulate the retinoblastoma tumour suppressor, resulting in activation of E2F-responsive promoters and cell proliferation. Recently, it has been demonstrated that HCV increases DNA damage and mutation of cellular genes including proto-oncogenes, and it is known that core protein expression impairs DNA repair in human hepatoma cells. Such an accumulation of mutations in cellular genes may lead to cellular transformation. In addition, iron overload induces mitochondrial injury and increases the risk of HCC development in HCV polyprotein-expressing transgenic mice.

HCV proteins regulate transcription of cellular genes including p53 and p21, activate signal transduction pathways and suppress apoptosis. These functions of HCV proteins may lead to hepatocyte proliferation and transformation. However, to clarify the molecular mechanisms of hepatocarcinogenesis by HCV, comprehensive analyses of the function of HCV proteins may be needed in the future. The recently developed HCV subgenomic replicon and robust HCV infection systems are expected to help analyse the effect of not only the HCV proteins but also the replicative cycle of the virus in this process.

Prevention of HCC

HCC is quite unique in that known acquired factors are the main cause of carcinogenesis – in Japan, 80% of cases are due to HCV and 10% due to HBV. HCV is also the dominant cause of HCC in several European countries and the United States. This has two important implications. Firstly, it enables us to distinguish patients at high risk of HCC and perform efficient surveillance. Secondly, HCC is preventable, at least theoretically, by controlling virus infection. This section will focus on the prevention of HCV-related HCC but similar strategies may be applicable to HBV.

Primary prevention of HCV-related HCC includes strategies for the prevention of HCV infection and those for viral eradication (Table 5.3). As for the former, HCV transmission in

Table 5.3 Prevention of HBV- or HCV-related HCC

Virus	HBV	HCV
Primary, new infection	Neonate vaccination	General infection control
Primary, existing infection	Antiviral therapy (?)	Antiviral therapy
	Nucleos(t)ide analogues	Interferon plus ribavirin
Secondary	Early diagnosis and curative treatment	
Tertiary	Transplantation Antiviral therapy (?)	

the general population has been declining for decades in Japan, as evidenced by the reverse proportionality between age and the prevalence of HCV infection. HCV transmission through blood transfusion, once the main route of infection in Japan, has been virtually eliminated by the improved HCV detection systems established in the early 1990s. Neonatal and sexual transmissions are infrequent, although injecting drug use with shared needles will remain a threat. Thus, we can concentrate our efforts on viral eradication in patients with chronic hepatitis C (CHC).

The effect of interferon therapy on HCC occurrence was once controversial. However, abundant clinical studies performed in Japan clearly demonstrated that HCC incidence was reduced among patients with CHC treated with interferon, especially those showing sustained virological response. Resolution of cirrhosis was also noted following sustained virological response. As regards interferon therapy, CHC in Japan can be characterized by two features: the predominance of HCV genotype 1b and the age of patients. In Japan, 70% of cases are caused by genotype 1b and the remaining by genotype 2a/2b. Conventional interferon monotherapy was not effective in the majority of patients, and therapy used to be given selectively to patients with genotype 2a/2b infection. Today, combination therapy with pegylated interferon and ribavirin gives sustained virological rates of about 50% in patients with genotype 1b infection, which is much improved but not yet satisfactory. In addition, those with CHC in present-day Japan are relatively old as the peak of HCV occurred half a century ago. This may increase susceptibility to untoward effects. There is therefore still plenty of room for improvement in the effectiveness and safety of antiviral treatment.

As stated elsewhere, there have been great advances in secondary prevention of HCC, that is early diagnosis and treatment, with the result that short-term prognosis of patients with HCC has been much improved. However, long-term survival is not proportionally extended. The major reason for this is the very frequent recurrence of HCC after apparently complete treatment, either medical ablation or surgical resection. This appears to be mainly due to *de novo* carcinogenesis in the liver. This is quite likely because the liver of HCC patients is often cirrhotic, and the sustained inflammation may further increase the risk of carcinogenesis. Theoretically, strategies similar to those of primary prevention may be applicable to patients with HCV-related HCC, although efficacy and safety of antiviral therapy can be compromised by pre-existing liver dysfunction. Some studies have shown beneficial effects of interferon therapy in HCC treatment. Needless to say, early diagnosis and complete treatment of primary HCC lesions are prerequisites for antiviral therapies. In other cases, safe and effective chemotherapeutic agents would be useful as adjuvant therapy to be used along with ablation or resection for relatively advanced HCC where microscopic intrahepatic metastases are suspected. However, conventional chemotherapeutic agents are neither sufficiently effective for HCC nor safe enough for protective long-term use. Some new agents appear promising but their effects need to be confirmed. Tertiary prevention of HCC is one of the most challenging tasks in current hepatology.

Treatment of HCC

Prevention of HCV-related HCC is one of the most important issues in current hepatology. We conducted two cohort studies to examine the potential efficacy of interferon in prevention of HCC. One of the studies was among patients with CHC mostly without cirrhosis and the other among those with compensated cirrhosis. With the CHC cohort, we showed that the risk of HCC development that was strongly associated with the stage of liver fibrosis, age and gender was reduced by interferon therapy to one-fifth among sustained virological responders compared with untreated patients. Life expectancy was also significantly prolonged. The benefit of interferon therapy was greater among those with the higher risk of HCC. The second study also confirmed the benefits of interferon therapy on HCC prevention among patients with compensated cirrhosis.

The prognosis of HCC is related not only to tumour stage but also to underlying liver function (Figure 5.2), so surgery has a limited role in treatment of HCC. Only 20–30% of patients are candidates for hepatectomy because of underlying cirrhosis or multiple lesions. Recently, surgeons have refined both selection criteria and surgical techniques, and treatment

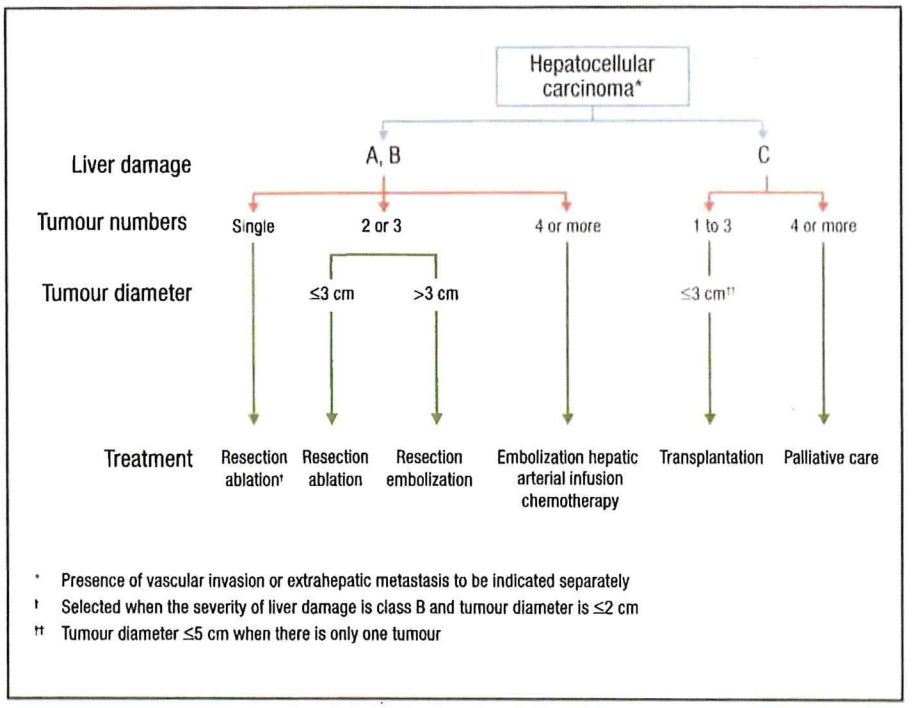

Figure 5.2 Treatment algorithm for HCC

morbidity can be less than 1%. Even after apparently curative resection, however, 80% of patients develop recurrence within 5 years because of latent metastasis or metachronous multicentric carcinogenesis.

Liver transplantation is an effective option for patients with HCC fulfilling the Milan criteria: solitary tumour of 5 cm or less in diameter or three or fewer lesions each 3 cm or less in diameter. However, feasibility of transplantation is limited by a shortage of organ donors, although living donor transplantation can be offered for HCC. Consequently, various non-surgical therapies have been developed for HCC. Among them, image-guided local ablation therapies (such as percutaneous ethanol injection and radiofrequency ablation) have important roles as they are potentially curative, minimally invasive and easily repeatable. At our institute, we have treated 90% of previously untreated patients with HCC by local ablation therapies. We have performed ethanol injection on a total of 2000 cases since 1985, and microwave coagulation on a total of 200 cases since 1995, with satisfactory long-term results. However, since the introduction of radiofrequency ablation into clinical practice in 1999, there has been a drastic shift from ethanol injection and microwave coagulation to radiofrequency ablation. Furthermore, recent randomized controlled trials have proved that radiofrequency ablation is superior to ethanol injection in the treatment of HCC.

We recommend the following general requirements for radiofrequency ablation.

1) Lesions are unresectable or the patient refuses surgery.

2) Three or fewer lesions, each ≤3 cm in diameter.

3) No extrahepatic metastasis or vascular invasion.

4) No excessive bleeding tendency (platelet count >50 x 10^9/l and prothrombin activity >50%).

5) No refractory ascites.

6) Total bilirubin level <3.0 mg/dl.

The rate of complete tumour necrosis has been reported to range from 80 to 100%. By the end of 2005, we performed radiofrequency ablation on a total of 2350 cases (1219 patients) of HCC at the University of Tokyo Hospital. Although we put no restriction on lesion location, the final computed tomography scan after radiofrequency ablation showed no residual cancer

in 99.4% of the cases. Apparent viable cancer tissue remained in only 14 cases (0.6%). Using an artificial pleural effusion technique, artificial ascites technique and guided-needle method, we could ablate lesions on the surface of the liver, beneath the diaphragm, near the large vessels, adjacent to other organs and those that were detected by computed tomography but could not be identified by ultrasound. Among 1219 patients, 652 received radiofrequency ablation for initially diagnosed lesions and the remaining 567 had radiofrequency ablation for recurrence. The 1, 2, 3, 4, 5 and 6–year survival rates of the 652 patients were 96%, 88%, 80%, 69%, 58% and 53%, respectively. Image-guided local ablation therapies can be used as a bridge to transplantation.

We also conducted a randomized controlled trial to examine the effect of interferon on prognosis of patients with HCV-related HCC who had received complete tumour ablation. Interferon therapy was associated with better survival primarily as a result of the preservation of liver function and also probably prevention of recurrence. Beneficial effects on HCC prevention and liver function preservation were most marked in sustained virological responders. Further improvement in prognosis may be expected in the future because the current combination therapy of pegylated interferon and ribavirin shows higher efficacy than interferon alone.

Transcatheter arterial chemoembolization (TACE) has been widely performed for unresectable HCC. TACE is efficacious for multiple or large lesions and can be performed in cases of impaired liver function. However, it is not effective for capsular invasion, extracapsular growth or vascular invasion. It is rarely a curative treatment and has some adverse effects on noncancerous liver tissue. Thus, TACE should be performed for advanced HCC that cannot be treated by resection or image-guided percutaneous tumour ablation. At present, other options such as radiolabelled yttrium glass beads, radiolabelled lipiodol and immunotherapy cannot be recommended as standard therapy for advanced HCC outside clinical trials.

Chemotherapy has long been used for highly advanced HCC and its outcome has been dismal. However, we performed a combination therapy of interferon with repeated intra-arterial infusion of fluorouracil (5-FU) on 116 patients with portal vein invasion. Nineteen (16%) patients showed complete response and another 42 (36%) showed partial response. Adverse events were limited to nausea and appetite loss. The survival rates at 12 and 24 months among the overall treated patients were 34% and 18%, respectively, in contrast to 15% and 5% among the historical controls. Survival rates at 12 and 24 months were 81% and 59% among complete responders, respectively, and 43% and 18% among partial responders.

Further Reading

Chang MH, Shau WY, Chen CJ, *et al.* Hepatitis B vaccination and hepatocellular carcinoma rates in boys and girls. *JAMA* 2000; **284:**3040–3042.

Pybus OG, Charleston MA, Gupta S, Rambaut A, Holmes EC, Harvey PH. The epidemic behavior of the hepatitis C virus. *Science* 2001; **292:**2323–2325.

Omata M, Yoshida H, Shiratori Y. Prevention of hepatocellular carcinoma and its recurrence in chronic hepatitis C patients by interferon therapy. *Clin Gastroenterol Hepatol* 2005; **3:**S141–S143.

Tanaka Y, Hanada K, Mizokami M, *et al.* A comparison of the molecular clock of hepatitis C virus in the United States and Japan predicts that hepatocellular carcinoma incidence in the United States will increase over the next two decades. *Proc Natl Acad Sci USA* 2002; **99:**15584–15589.

El-Serag HB, Davila JA, Petersen NJ, McGlynn KA. The continuing increase in the incidence of hepatocellular carcinoma in the United States: an update. *Ann Intern Med* 2003; **139:**817–823.

Choo QL, Richman KH, Han JH, *et al.* Genetic organization and diversity of the hepatitis C virus. *Proc Natl Acad Sci U S A* 1991; **88:**2451–2455.

Moriya K, Fujie H, Shintani Y, *et al.* The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. *Nat Med* 1998; **4:**1065–1067.

Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* 1999; **285:**110–113.

Wakita T, Pietschmann T, Kato T, *et al.* Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* 2005; **11:**791–796.

Kato N, Yoshida H, Ono-Nita SK, *et al.* Activation of intracellular signaling by hepatitis B and C viruses: C-viral core is the most potent signal inducer. *Hepatology* 2000; **32:**405–412.

Yoshida H, Shiratori Y, Moriyama M, *et al.* Interferon therapy reduces the risk for hepatocellular carcinoma: national surveillance program of cirrhotic and noncirrhotic patients with chronic hepatitis C in Japan. IHIT Study Group. Inhibition of hepatocarcinogenesis by interferon therapy. *Ann Intern Med* 1999; **131:**174–181.

Shiratori Y, Imazeki F, Moriyama M, *et al.* Histologic improvement of fibrosis in patients with hepatitis C who have sustained response to interferon therapy. *Ann Intern Med* 2000; **132:**517–524.

Sakon M, Umeshita K, Nagano H, *et al.* Clinical significance of hepatic resection in hepatocellular carcinoma: analysis by disease-free survival curves. *Arch Surg* 2000; **135:**1456–1459.

Kubo S, Nishiguchi S, Hirohashi K, *et al.* Effects of long-term postoperative interferon-alpha therapy on intrahepatic recurrence after resection of hepatitis C virus-related hepatocellular carcinoma. A randomized, controlled trial. *Ann Intern Med* 2001; **134:**963–967.

Shiratori Y, Shiina S, Teratani T, *et al.* Interferon therapy after tumor ablation improves prognosis in patients with hepatocellular carcinoma associated with hepatitis C virus. *Ann Intern Med* 2003; **138:**299–306.

Shiratori Y, Ito Y, Yokosuka O, *et al.* and the Tokyo-Chiba Hepatitis Research Group. Antiviral therapy for cirrhotic hepatitis C: association with reduced hepatocellular carcinoma development and improved survival. *Ann Intern Med* 2005; **142:**105–114.

Balsells J, Charco R, Lazaro JL, *et al.* Resection of hepatocellular carcinoma in patients with cirrhosis. *Br J Surg* 1996; **83:**758–761.

Mazzaferro V, Regalia E, Doci R, *et al.* Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. *N Engl J Med* 1996; **334:**693–699.

Shiina S, Teratani T, Obi S, *et al.* A randomized controlled trial of radiofrequency ablation with ethanol injection for small hepatocellular carcinoma. *Gastroenterology* 2005; **129:**122–130.

Obi S, Yoshida H, Toune R, *et al.* Combination therapy of intraarterial 5-fluorouracil and systemic interferon-alpha for advanced hepatocellular carcinoma with portal venous invasion. *Cancer* 2006; **106:**1990–1997.

Liver INTERNATIONAL
OFFICIAL JOURNAL OF THE INTERNATIONAL ASSOCIATION FOR THE STUDY OF THE LIVER

Liver International ISSN 1478-3223

BASIC STUDIES

Double-stranded RNA-activated protein kinase inhibits hepatitis C virus replication but may be not essential in interferon treatment

Jin-Hai Chang[1], Naoya Kato[1,2], Ryosuke Muroyama[1,2], Hiroyoshi Taniguchi[1], Bayasi Guleng[1], Narayan Dharel[1], Run-Xuan Shao[1], Keisuke Tateishi[1], Amarsanaa Jazag[1], Takao Kawabe[1] and Masao Omata[1]

1 Department of Gastroenterology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan
2 Unit of Disease Control Genome Medicine, Institute of Medical Science, University of Tokyo, Tokyo, Japan

Keywords
double-stranded RNA-activated protein kinase – hepatitis C virus – interferon – interferon-stimulated gene – RNA interference

Abbreviations
DMEM, Dulbecco's modified Eagle's medium; dsRNA, double-stranded RNA; eIF, eukaryotic translation initiation factor; FBS, fetal bovine serum; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IRES, internal ribosomal entry site; ISG(s), interferon-stimulated gene(s); MEF(s), mouse embryonic fibroblast(s); MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; PKR, double-stranded RNA-activated protein kinase; RLU, relative light units; SD, standard deviation; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; shRNA, short hairpin RNA; siRNA, short interfering RNA; UTR, untranslated region.

Correspondence
Dr Naoya Kato, Unit of Disease Control Genome Medicine, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan
Tel: +81 3 6409 2335
Fax: +81 3 6409 2336
e-mail: kato-2im@ims.u-tokyo.ac.jp

Received 21 May 2008
Accepted 4 September 2009

DOI:10.1111/j.1478-3231.2009.02144.x

Abstract

Background: Double-stranded RNA-activated protein kinase (PKR), an interferon (IFN)-stimulated gene, is activated by binding with double-stranded RNA, a putative replicative intermediate of the hepatitis C virus (HCV). Activated PKR phosphorylates the α subunit of eukaryotic initiation factor-2 to inhibit the translation of viral protein. *Aims/methods:* We established stable PKR knockdown Huh7 cells using RNA interference and investigated the effect of PKR against HCV replication using a subgenomic replicon that expressed luciferase reporter protein and the JFH1 full-length HCV genome. *Results:* In stable PKR knockdown cells that harboured a subgenomic replicon, luciferase activity was approximately three times higher than that of control cells, indicating that the subgenomic replicon replicated with a higher efficiency in stable PKR knockdown cells than that in control cells. Furthermore, stable PKR knockdown cells secreted significantly more HCV particles than did control cells after transfection with the full-length HCV genome. The replication of the subgenomic replicon was suppressed by the addition of IFN-α in both cells. Although the extent of suppression was significantly lower in stable PKR knockdown than control cells using a low concentration (2.5–5 U/ml) of IFN-α, even 10 U/ml IFN-α suppressed the replication of subgenomic replicon by $> 98\%$ in both cells. *Conclusions:* Double-stranded RNA-activated protein kinase plays an important role in suppressing HCV replication in an innate state, but may not be essential in IFN therapy.

The hepatitis C virus (HCV) is a positive-stranded RNA virus that causes chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) worldwide (1–3). Type-1-interferon (IFN)-induced antiviral signals are regarded as the major defence against HCV infection, and anti-HCV therapy is primarily based on IFNs (4, 5). IFNs allow cells to become innately primed for defence against an eventual virus attack by inducing the transcription of IFN-stimulated genes (ISGs) that exert antiviral, anti-tumour and immunomodulatory actions by producing a complex set of proteins. Some ISGs have an important role in controlling viral replication.

Double-stranded RNA-activated protein kinase (PKR), one of the best-known ISGs, is a serine–threonine-type phosphorylation enzyme that combines with double-stranded RNA (dsRNA) and is then activated.

Given that the replication of the HCV genome is catalysed by its RNA-dependent RNA polymerase NS5B, dsRNA may be formed during its life cycle (6, 7). These HCV-replicative intermediates may be targeted by the PKR-based antiviral response (8). Activated PKR inhibits protein synthesis by phosphorylating the eukaryotic translation initiation factor (eIF)2α regulatory site, Ser51 (9). This triggers the general shutdown of protein synthesis and inhibition of viral propagation. In fact, the HCV internal ribosome entry site forms a binary complex with the 40S ribosomal subunit, recruits initiation factor eIF3 and the ternary eIF2/GTP/Met-tRNA(i)(Met) complex and joins 60S subunits to assemble translation-competent 80S ribosomes (10–12).

However, the exact role of PKR in the regulation of HCV replication in an innate state of liver cells has not been well documented. Furthermore, it remains unknown whether PKR plays an important role in IFN treatment against HCV infection. We evaluated whether PKR has anti-HCV activity using an HCV subgenomic replicon (13) and recently developed full-length HCV genome JFH1 (14) in human hepatoma cells.

Materials and methods

Cell lines

Human hepatoma cells (Huh7) that allow high replication of the HCV subgenomic replicon were a gift from Dr N. Sakamoto (Tokyo Medical and Dental University, Japan) [Tanabe *et al.* (15)]. Human cervical carcinoma cells (HeLa) were obtained from the Riken cell bank (Tsukuba, Japan). These cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma, St Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS).

Establishment of the Huh7 cell line with a stable knockdown expression of double-stranded RNA-activated protein kinase

A sequence that targets the PKR gene was selected, and sense and antisense oligonucleotides (5′-CACCGCGGGAAATTAGATAAAGTACGTGTGCTGTCCGTACT TTGTCTAGTTTCTCGCTTTTT-3′ and 5′-GCATAAAAAGCGAGAAACTAGACAAAGTACGGACAGCACACGTACTTTATCTAATTTCCCGC-3′ respectively) were designed to generate a short hairpin RNA (shRNA). The short interference RNA (siRNA) expression vector for PKR, pcPUR+U6-PKRi, was designed and constructed according to the manufacturer's instructions using the pcPUR+U6i cassette vector (iGENE Therapeutics, Tsukuba, Japan) (16, 17). These vectors were introduced into Huh7 cells to establish stable PKR knockdown cells and control cells. Briefly, the targeting (pcPUR+U6PKRi) or control (pcPUR+U6i) vector was transfected into Huh7 cells using FuGene6 (Roche, Basel, Switzerland), and the puromycin (Sigma)-resistant clones were selected as stable transfectants. The knockdown of PKR was confirmed by Western blotting.

Western blotting

To determine the expression level of PKR, immunoblotting was performed. Cell extracts were adjusted to the same protein concentration using a Micro BCA protein assay reagent kit (Pierce, Rockford, IL, USA), resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes (Hybond-P; Amersham, Buckinghamshire, UK). Rabbit anti-PKR antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and rabbit polyclonal anti-PKR[pT^{451}] antibody (Invitrogen, Carlsbad, CA, USA) were used as a primary antibody. The HeLa cell lysate was used as a positive control for PKR and p-PKR bands, according to the manufacturer's instructions. β-actin protein was detected using the anti-β-actin antibody (Sigma) as a primary antibody at a dilution of 1:2500. Horseradish peroxidase-conjugated secondary antibody was purchased from Amersham. Bound antigens were detected using the ECL-Plus system (Amersham).

In vitro transcription and transfection of the hepatitis C virus subgenomic replicon and the full-length genome

The pRep-Feo, an HCV subgenomic replicon plasmid that expresses a chimeric protein that consists of neomycin phosphotransferase and firefly luciferase, was kindly provided by N. Sakamoto (13, 15). The pJFH1 that contained the full-length HCV JFH1 cDNA downstream of the T7 RNA promoter was a gift from T. Wakita (National Institute of Infectious Diseases, Japan) (14). *In-vitro*-transcribed HCV subgenomic RNA and full-length JFH1 RNA were prepared as described previously (13, 14). Briefly, after linearizing pRep-Feo or pJFH1 with *Xba*I, RNA transcripts were synthesized from 2 μg of the linearized plasmid using the MEGAscript T7 system (Ambion, Austin, TX, USA), according to the manufacturer's instructions.

To examine the effect of PKR knockdown on the luciferase activity generated by the subgenomic replicon, high replication permissive Huh7 cells grown under optimum conditions were trypsinized and collected by centrifugation (100*g*, 5 min). The cells were washed three times in ice-cold RNAse-free phosphate-buffered saline (PBS) and resuspended at 1×10^7 cells/ml in PBS. A transfection mixture that contained 10 μg of RNA transcripts made from pRep-Feo was prepared. We mixed 0.42 ml of washed Huh7 cells with the transfection mixture, added this mixture electroporation cuvettes (Bio-Rad Laboratories, Hercules, CA, USA), and immediately pulsed (0.270 kV, pulse-length 30 μs, one pulse) samples using a Bio-Rad electroporation system. After pulsed cells were allowed to recover for 10 min at room temperature, they were plated onto five 10 cm culture

Liver International (2009)

dishes. At 24 h after transfection, we replaced the medium with DMEM that contained 10% FBS and 250 μg/ml G418. Approximately 3 weeks later, surviving colonies were picked up, and a luciferase assay was performed. The luciferase activity of subgenomic replicon-harbouring stable PKR knockdown cells and control cells was measured and compared. In addition, the effect of IFN-α 2b (Schering-Plough K.K., Osaka, Japan) against the replication of the subgenomic replicon was investigated in these subgenomic replicon-harbouring cells. Similarly, RNA transcripts made from pJFH1 were transfected into Huh7 cells to examine the effect of PKR knockdown on the amount of core protein secreted from the replicating JFH1 genome.

Cell growth assay

To examine the growth of control and stable PKR knockdown cells, 3×10^4 cells/well were seeded onto 24-well plates. After 24 or 48 h, the number of viable cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Sigma) (18). The data were reported as the mean ± standard deviation determined from triplicate wells.

Transfection of plasmid DNA that expresses double-stranded RNA-activated protein kinase

Three days after transfection, JFH1 RNA-transfected Huh7 cells, which were grown in six-well culture plates to ~60% confluency, were transfected with 0.4 μg of plasmid DNA using the Effectene Transfection Reagent (Qiagen, Hilden, Germany). Expression plasmid pRc-PKR, which contained the PKR gene, was provided by B. R. G. Williams (The Cleveland Clinic, Cleveland, OH, USA). The pRc-CMV (empty vector) plasmid was used as a control.

Luciferase assay and quantification of the hepatitis C virus core protein

Subgenomic replicon-harbouring cells were harvested after G418 selection, and the luciferase assay was performed using the PicaGene Dual SeaPansy system (Toyo Ink, Tokyo, Japan) with a luminometer (Lumat LB9507; EG&G Berthold, Bad Wildbad, Germany) to measure firefly luciferase activity as relative light units. Firefly luciferase activity was normalized for cell quantity based on the MTT assay. The luciferase assay was performed at least three times. The HCV core protein in the culture supernatant of JFH1 RNA-transfected cells was quantified using an immunoassay as described previously (19).

Statistical analyses

Statistical analyses were performed using the *t*-test (StatView J; Abacus Concepts, Berkeley, CA, USA), and $P < 0.05$ was deemed statistically significant.

Results

Establishing stable knockdown Huh7 cells

The expression of PKR and activated PKR (p-PKR) proteins in Huh7 cells was detected by Western blotting. Although PKR and p-PKR were expressed weakly in control cells without IFN, their expression was weaker in stable knockdown cells than that in control cells (Fig. 1A). The expression of PKR and p-PKR in control cells increased after adding 10 U/ml IFN-α 2b, whereas the expression in stable knockdown cells was much weaker, even after the addition of IFN-α 2b, compared with control cells (Fig. 1A). This indicated successful knockdown of PKR mRNA expression. Cell proliferation was analysed using the MTT assay, and no difference was observed between stable knockdown cells and control cells (Fig. 1B).

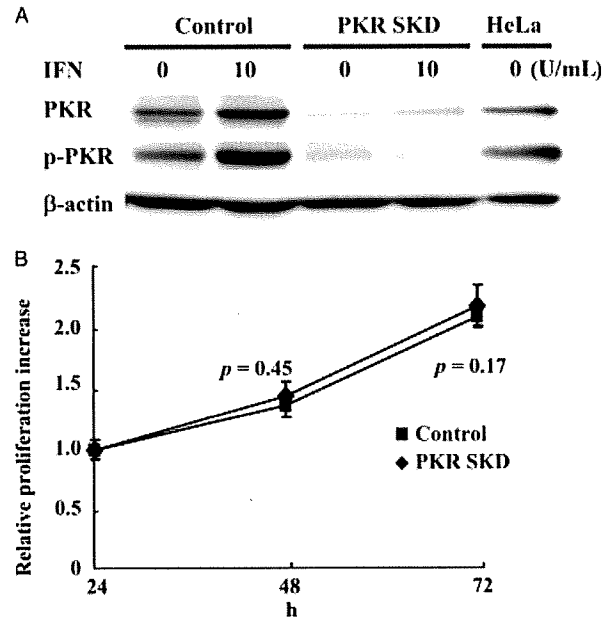

Fig. 1. Establishing SKD Huh7 cells. Huh7 cells were transfected with pcPUR+U6i or pcPUR+U6i-PKRi and incubated with puromycin to select stably transfected cells. The total cell lysates of single clones were subjected to Western blotting to monitor PKR and β-actin protein expression. (A) The endogenous expression of PKR and p-PKR in control and stable PKR knockdown cells and 24 h after the addition of IFN-α 2b. HeLa cell lysates were used as a positive control. (B) The proliferation of SKD and control cells. Stable PKR knockdown and control cells were seeded in 24-well plates, and the number of viable cells was determined using the MTT assay at 24, 48 and 72 h after passage. The error bars indicate the standard deviation of triplicate samples. IFN, interferon; MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; PKR, double-stranded RNA-activated protein kinase; SKD, stable knockdown.

Double-stranded RNA-activated protein kinase knockdown increases hepatitis C virus subgenomic replicon replication in Huh7 cells

To evaluate whether PKR affects HCV replication, HCV subgenomic replicon RNA was transfected into stable knockdown Huh7 cells and control Huh7 cells. High levels of luciferase activity were detected from G418-resistant clones after a few weeks of G418 selection. Relative luciferase activity was significantly higher in lysates from stable knockdown cells compared with lysates from control cells, indicating that the subgenomic replicon replicated better in stable knockdown cells (Fig. 2). Although Figure 2 shows the relative luciferase activities obtained from monoclonal G418-resistant cells, similar results were obtained for polyclonal G418-resistant cells (data not shown). The expression of PKR and p-PKR protein in cells with or without the HCV subgenomic replicon was detected by Western blotting, which revealed much weaker PKR and p-PKR expression in stable PKR knockdown cells compared with control cells (Fig. 3A). No difference in proliferation was observed between stable knockdown cells and control cells harbouring the subgenomic replicon (Fig. 3B), suggesting that cell growth did not contribute to the difference in the relative luciferase activity between stable knockdown cells and control cells harbouring the subgenomic replicon.

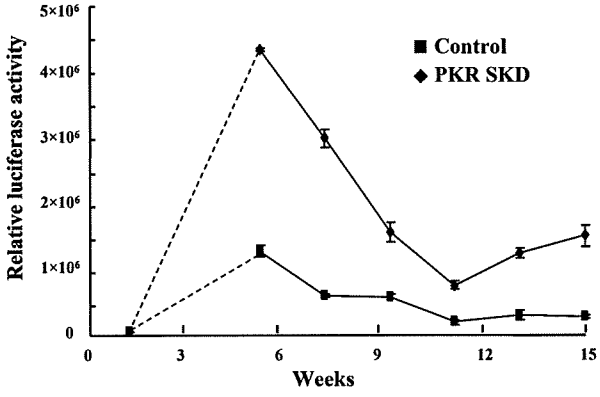

Fig. 2. Subgenomic replicon-related luciferase activities in SKD and control cells. A subgenomic replicon expressing a chimeric protein that consisted of neomycin phosphotransferase and firefly luciferase was transfected into SKD and control cells. The subgenomic replicon-related luciferase activity in G418-resistant cells was measured for 15 weeks. The MTT assay was performed at the same time, and firefly luciferase activity was normalized against cell quantity based on the MTT assay. The results are expressed as the mean of three wells. The error bars indicate the standard deviation of triplicate samples. MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; PKR, double-stranded RNA-activated protein kinase; SKD, stable knockdown.

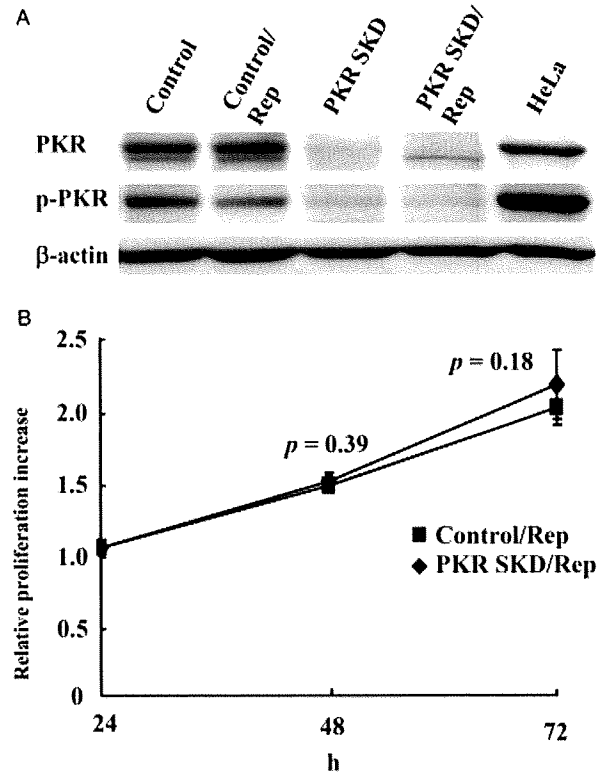

Fig. 3. (A) Expression of endogenous PKR and p-PKR in control cells and SKD cells with or without the HCV subgenomic replicon. HeLa cell lysates were used as a positive control. (B) The proliferation of control and SKD cells with the HCV subgenomic replicon. The number of viable cells was determined using the MTT assay at 24, 48 and 72 h after passage. The error bars indicate the standard deviation of triplicate samples. HCV, hepatitis C virus; MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; PKR, double-stranded RNA-activated protein kinase; Rep, pRep-Feo HCV subgenomic replicon; SKD, stable knockdown.

Double-stranded RNA-activated protein kinase limits the secretion of hepatitis C virus particles from the JFH1 full-length hepatitis C virus genome

To determine whether PKR is involved in regulating HCV replication, the amount of core protein secreted into the culture medium was measured for up to 16 days after transfection with JFH1 full-length HCV RNA. Significantly more core protein was secreted from JFH1 replicating within stable knockdown cells than within control cells (Fig. 4). In addition, the amount of core protein secreted into the culture medium was measured 48 h after the transfection of a PKR-expressing or control plasmid. Significantly less core protein was secreted from PKR-overexpressing cells harbouring JFH1 compared with control cells (Fig. 5). Because PKR knockdown significantly upregulated the replication of not only the HCV subgenomic replicon but also the full-length HCV JFH1 genome, these results confirmed that PKR is one of the factors limiting HCV replication.

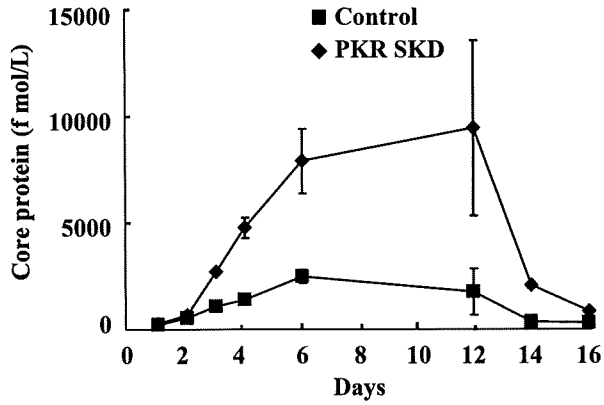

Fig. 4. Amount of core protein secreted from SKD and control cells harbouring JFH1 HCV. The JFH1 full-length HCV genome was transfected into SKD and control cells, and secreted core protein in the culture supernatant was quantified using an immunoassay. Core protein was measured up to 16 days after transfection. The error bars indicate the standard deviation of triplicate samples. HCV, hepatitis C virus; PKR, double-stranded RNA-activated protein kinase; SKD, stable knockdown.

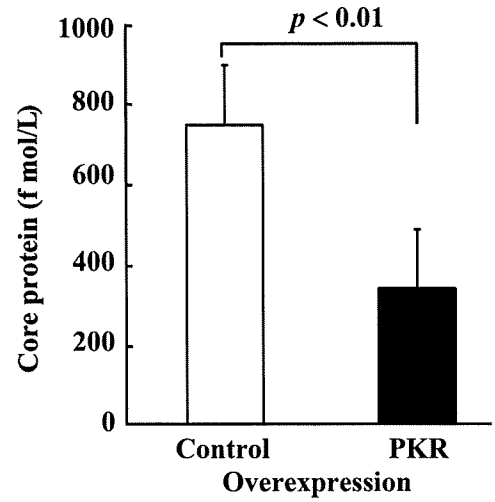

Fig. 5. The amount of core protein secreted from Huh7 cells harbouring the JFH1 full-length hepatitis C virus genome, with or without PKR overexpression. The core protein was measured 48 h post-transfection with pRc-PKR or pRc-CMV (control). The error bars indicate the standard deviation of triplicate samples. PKR, double-stranded RNA-activated protein kinase.

Interferon is fully effective against the hepatitis C virus subgenomic replicon in stable knockdown cells

To evaluate whether PKR knockdown had any effect on the HCV response to IFN treatment, control and stable knockdown cells harbouring the subgenomic replicon were treated with IFN-α 2b at a concentration of 2.5, 5 or 10 U/ml. Replication of the subgenomic replicon was suppressed and luciferase activity was reduced in both control and stable knockdown cells after adding IFN-α 2b (Fig. 6A). The extent of suppression was significantly lower in stable knockdown cells than that in control cells in the presence of a relatively lower concentration of IFN-α 2b ($P < 0.01$; Fig. 6B), whereas 10 U/ml IFN-α 2b suppressed replication of the subgenomic replicon by $> 98\%$ in both stable knockdown cells and control cells. This confirmed that the expression of PKR and p-PKR protein was not induced in stable knockdown cells harbouring the subgenomic replicon (Fig. 6C). Because patients with hepatitis C receiving IFN therapy show a maximum serum concentration of 50 U/ml, PKR may not be essential in IFN therapy for HCV.

Discussion

Hepatitis C virus infects approximately 170 million individuals worldwide and is a major aetiological agent of chronic liver disease and HCC (20). Although knowledge of hepatitis C has increased since HCV was identified in 1989 (21), the fundamental aspects of HCV biology still remain unclear. The study of HCV replication and virus–host interaction during HCV infection had been hampered by the lack of efficient cell culture systems for HCV replication. However, the HCV subgenomic replicon system has been developed (13, 22), and several groups have successfully developed reliable and robust cell culture systems for infectious HCV production and propagation in human hepatoma cells using cDNA derived from genotype 2a HCV, JFH1 (13, 14, 23–25). The development of these HCV replication systems has allowed various molecular studies of HCV replication, host–cell interactions and antiviral strategies.

Here, we provided a detailed description of how PKR suppresses HCV replication, using recently developed HCV replication systems for human liver cells, especially a system for the production of infectious HCV particles, and the RNA interference technique to make stable PKR knockdown liver cells. Both PKR and p-PKR were induced by addition of IFN in control cells but not in stable PKR knockdown cells, indicating successful knockdown of PKR. There was no difference in the levels of PKR and p-PKR between replicon-naïve and replicon-transfected control cells, and almost no PKR and p-PKR were detected in stable PKR knockdown cells regardless of the existence of a replicon as was expected. This indicates that replicon (HCV-derived) RNA hardly induces PKR and p-PKR in these cells. We provided evidence that a PKR-based antiviral response does control HCV replication. It was previously shown that an HCV subgenomic replicon replicates more efficiently in PKR knockout mouse embryonic fibroblasts (MEFs) than in wild-type MEFs; however, data from human liver cells and the full-length HCV genome are lacking (26). Recently, it was shown that the overexpression of PKR transiently suppresses the replication of the HCV subgenomic replicon in liver cells; however, data from the full-length HCV genome and PKR knockdown are lacking (27). Moreover, it was shown in liver cells transfected with genotype 1a full-length HCV cDNA that the

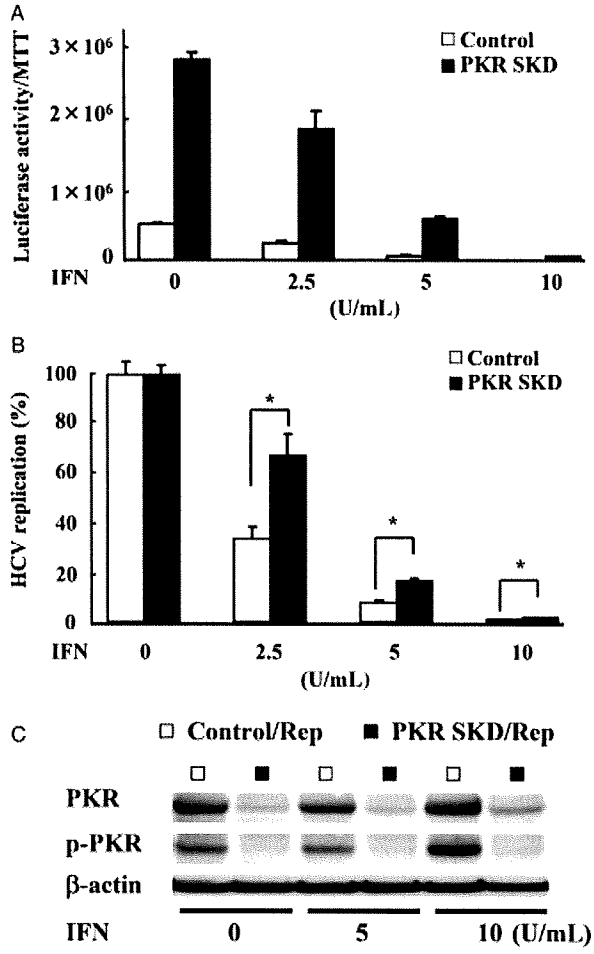

Fig. 6. (A) Subgenomic replicon-related luciferase activities in SKD and control cells that were treated with IFN-α 2b for 48 h. The luciferase activities were normalized against cell quantity based on the MTT assay. (B) Subgenomic replicon-related luciferase activities in SKD and control cells treated with IFN-α 2b. Stable PKR knockdown and control cells were treated with IFN-α 2b for 48 h, and luciferase activity was expressed as a percentage of that without IFN. The error bars indicate the standard deviation of triplicate samples. Asterisks indicate $P < 0.01$. (C) The expression of PKR and p-PKR during IFN treatment. At 24 h after IFN treatment, extracts of cells with the HCV subgenomic replicon were subjected to Western blotting. PKR and p-PKR were detected. HCV, hepatitis C virus; IFN, interferon; MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; PKR, double-stranded RNA-activated protein kinase; Rep, pRep-Feo HCV subgenomic replicon; SKD, stable knockdown.

transient overexpression of PKR results in decreased HCV core protein levels. Transient downregulation of PKR expression by siRNA results in increased HCV core protein levels; however, data for stable HCV replication and stable PKR knockdown are lacking (28). In contrast, our results from the stable knockdown and transient overexpression of PKR show that PKR suppresses the replication of the HCV subgenomic replicon and the full-length genome in human liver cells.

Therapies against HCV are based on IFN, which primarily acts by enhancing host innate immunity. However, sustained eradication of the virus is achieved only in a fraction of patients, even with the best-available therapy of combined pegylated-IFN and ribavirin (29). Understanding the factors that contribute to the suppression of HCV replication is critical to the development of better therapeutic measures. IFN stimulates the expression of a number of ISGs with antiviral activity, including PKR (27). However, it has been demonstrated previously that IFN-α/β inhibits HCV RNA replication in PKR(− / −) MEFs as efficiently as in PKR(+/+) MEFs (26). Our detailed experiments indicate that PKR plays an important role in suppressing HCV replication by low-concentration IFN-α 2b; however, IFN-α 2b is not essential at high concentrations because even 10 U/ml IFN-α 2b efficiently (> 98%) suppressed HCV replication. It is possible that the level of PKR expression induced by IFN at a dose of 10 U/ml is sufficient for suppressing HCV replication fully, even if the level was lower than that in control cells. For this reason, the expressions of PKR and p-PKR were analysed during IFN treatment. Both PKR and p-PKR were induced by addition of IFN in control cells but not in stable PKR knockdown cells, indicating successful knockdown of PKR. In stable knockdown cells harbouring the HCV subgenomic replicon, treatment with 10 U/ml IFN (1.8% residual HCV replicon) resulted in a much weaker expression of PKR and p-PKR than that in control cells treated with 5 U/ml IFN (7.5% residual HCV replicon). Furthermore, in stable knockdown cells harbouring the HCV subgenomic replicon, on treatment with 10 U/ml IFN, lower luciferase activities were found than that in control cells harbouring the HCV subgenomic replicon, without IFN or with 5 U/ml of IFN treatment. Moreover, suppression of replication of the HCV subgenomic replicon seems to be independent of activated p-PKR, total amount of PKR and their combination in stable PKR knockdown cells. These details clearly show that the suppression of HCV replication by IFN is not mainly PKR and p-PKR dependent. In fact, the maximum IFN concentration in the serum of patients receiving 3 MIU IFN-α thrice weekly is approximately 30–54.9 U/ml (30). Considering this information, PKR plays an important role in suppressing HCV replication in innate liver cells, but may not be essential in IFN treatment.

It was reported previously that U6 shRNA constructs induce an IFN response in mammalian cells (31). Therefore, it is possible that this IFN response influences the replication of the HCV subgenomic replicon and the full-length genome. Even under such conditions, the replication of HCV was upregulated by stable PKR knockdown, strengthening the conclusion that PKR plays an important role in suppressing HCV replication in innate cells.

Interestingly, a recent study has suggested that the anti-HCV action of ribavirin is partly attributable to its ability to upregulate PKR activity (32). PKR may play a more important role in IFN and ribavirin combination treatment than in IFN monotherapy.

Hepatitis C virus is thought to be able to counteract the host response in various ways. This ability of HCV to counter the host defences may contribute to the establishment of persistent HCV infection. HCV envelope protein E2 has been shown to inhibit PKR (33–35). E2 contains a sequence identical to phosphorylation sites of PKR, inhibits the kinase activity of PKR and blocks its inhibitory effect on protein synthesis and cell growth (35). HCV NS5A binds PKR and inhibits its catalytic activity by the disruption of PKR-dependent translational control and signalling actions (36, 37). Although these interactions of HCV E2 and NS5A with PKR have been assumed to be one mechanism by which HCV circumvents the antiviral effect of IFN, their inhibition of PKR seems to be incomplete. This is because the knockdown of PKR still increased the replication of the HCV subgenomic replicon that contained NS5A and the full-length genome that contained E2 and NS5A. Moreover, the knockdown of PKR decreased the efficacy of IFN treatment against HCV replication, which suggests that E2 and NS5A do not fully suppress PKR function.

Interestingly, HCV core proteins encoded by sequences derived from HCC tumour tissues, but not those derived from their non-tumour counterparts in the same liver, have been reported to activate PKR by direct interaction (8). This suggests that PKR plays an important role not only in the antiviral activity against HCV but also in hepatocarcinogenesis.

In summary, we demonstrated that PKR is an important regulator of intracellular HCV replication, especially in innate liver cells. PKR is an important molecule in the host innate defence against HCV infection, and any functional and physical deficiency of PKR can lead to an intracellular environment highly conducive to efficient HCV replication. However, PKR may not be an important determinant of IFN treatment against hepatitis C. This virus–cell interaction may play an important role in the pathogenesis of hepatitis C.

Acknowledgements

This study was supported by Global COE Program 'Center of Education and Research for Advanced Genome-Based Medicine: For personalized medicine and the control of worldwide infectious diseases'; the Ministry of Education, Culture, Sports, Science and Technology of Japan; Research Fellowships of the Japan Society for the Promotion of Science for Young Scientist; grant-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan and Health and Labor Sciences Research Grants for Research on Hepatitis from the Ministry of Health, Labor and Welfare, Japan. We thank Dr N. Sakamoto, Dr B. R. G. Williams and Dr T. Wakita for providing us with plasmids and cells. We also thank Dr F. Kanai for advice on the experiments and M. Tsubouchi for technical assistance.

References

1. Lauer GM, Walker BD. Hepatitis C virus infection. *N Engl J Med* 2001; **345**: 41–52.
2. Saito I, Miyamura T, Ohbayashi A, *et al.* Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. *Proc Natl Acad Sci USA* 1990; **87**: 6547–9.
3. Shiratori Y, Shiina S, Imamura M, *et al.* Characteristic difference of hepatocellular carcinoma between hepatitis B- and C-viral infection in Japan. *Hepatology* 1995; **22**: 1027–33.
4. Omata M, Yokosuka O, Takano S, *et al.* Resolution of acute hepatitis C after therapy with natural beta interferon. *Lancet* 1991; **338**: 914–5.
5. Fried MW, Shiffman ML, Reddy KR, *et al.* Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; **347**: 975–82.
6. Penin F, Dubuisson J, Rey FA, Moradpour D, Pawlotsky JM. Structural biology of hepatitis C virus. *Hepatology* 2004; **39**: 5–19.
7. Butcher SJ, Grimes JM, Makeyev EV, Bamford DH, Stuart DI. A mechanism for initiating RNA-dependent RNA polymerization. *Nature* 2001; **410**: 235–40.
8. Delhem N, Sabile A, Gajardo R, *et al.* Activation of the interferon-inducible protein kinase PKR by hepatocellular carcinoma derived-hepatitis C virus core protein. *Oncogene* 2001; **20**: 5836–45.
9. Dey M, Cao C, Dar AC, *et al.* Mechanistic link between PKR dimerization, autophosphorylation, and eIF2alpha substrate recognition. *Cell* 2005; **122**: 901–13.
10. Fukushi S, Okada M, Kageyama T, Hoshino FB, Katayama K. Specific interaction of a 25-kilodalton cellular protein, a 40S ribosomal subunit protein, with the internal ribosome entry site of hepatitis C virus genome. *Virus Genes* 1999; **19**: 153–61.
11. Otto GA, Puglisi JD. The pathway of HCV IRES-mediated translation initiation. *Cell* 2004; **119**: 369–80.
12. Siridechadilok B, Fraser CS, Hall RJ, Doudna JA, Nogales E. Structural roles for human translation factor eIF3 in initiation of protein synthesis. *Science* 2005; **310**: 1513–5.
13. Yokota T, Sakamoto N, Enomoto N, *et al.* Inhibition of intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs. *EMBO Rep* 2003; **4**: 602–8.
14. Wakita T, Pietschmann T, Kato T, *et al.* Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* 2005; **11**: 791–6.
15. Tanabe Y, Sakamoto N, Enomoto N, *et al.* Synergistic inhibition of intracellular hepatitis C virus replication by combination of ribavirin and interferon-alpha. *J Infect Dis* 2004; **189**: 1129–39.
16. Miyagishi M, Taira K. U6 promoter-driven siRNAs with four uridine 3′ overhangs efficiently suppress targeted gene expression in mammalian cells. *Nat Biotechnol* 2002; **20**: 497–500.
17. Jazag A, Ijichi H, Kanai F, *et al.* Smad4 silencing in pancreatic cancer cell lines using stable RNA interference and gene expression profiles induced by transforming growth factor-beta. *Oncogene* 2005; **24**: 662–71.

18. Ohashi M, Kanai F, Ueno H, *et al.* Adenovirus mediated p53 tumour suppressor gene therapy for human gastric cancer cells in vitro and in vivo. *Gut* 1999; **44**: 366–71.
19. Aoyagi K, Ohue C, Iida K, *et al.* Development of a simple and highly sensitive enzyme immunoassay for hepatitis C virus core antigen. *J Clin Microbiol* 1999; **37**: 1802–8.
20. Chen SL, Morgan TR. The natural history of hepatitis C virus (HCV) infection. *Int J Med Sci* 2006; **3**: 47–52.
21. Choo QL, Kuo G, Weiner AJ, *et al.* Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* 1989; **244**: 359–62.
22. Lohmann V, Korner F, Koch J, *et al.* Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* 1999; **285**: 110–3.
23. Lindenbach BD, Evans MJ, Syder AJ, *et al.* Complete replication of hepatitis C virus in cell culture. *Science* 2005; **309**: 623–6.
24. Cai Z, Zhang C, Chang KS, *et al.* Robust production of infectious hepatitis C virus (HCV) from stably HCV cDNA-transfected human hepatoma cells. *J Virol* 2005; **79**: 13963–73.
25. Zhong J, Gastaminza P, Cheng G, *et al.* Robust hepatitis C virus infection in vitro. *Proc Natl Acad Sci USA* 2005; **102**: 9294–9.
26. Chang KS, Cai Z, Zhang C, *et al.* Replication of hepatitis C virus (HCV) RNA in mouse embryonic fibroblasts: protein kinase R (PKR)-dependent and PKR-independent mechanisms for controlling HCV RNA replication and mediating interferon activities. *J Virol* 2006; **80**: 7364–74.
27. Itsui Y, Sakamoto N, Kurosaki M, *et al.* Expressional screening of interferon-stimulated genes for antiviral activity against hepatitis C virus replication. *J Viral Hepat* 2006; **13**: 690–700.
28. Tokumoto Y, Hiasa Y, Horiike N, *et al.* Hepatitis C virus expression and interferon antiviral action is dependent on PKR expression. *J Med Virol* 2007; **79**: 1120–7.
29. Hayashi N, Takehara T. Antiviral therapy for chronic hepatitis C: past, present, and future. *J Gastroenterol* 2006; **41**: 17–27.
30. Khakoo S, Glue P, Grellier L, *et al.* Ribavirin and interferon alfa-2b in chronic hepatitis C: assessment of possible pharmacokinetic and pharmacodynamic interactions. *Br J Clin Pharmacol* 1998; **46**: 563–70.
31. Bridge AJ, Pebernard S, Ducraux A, Nicoulaz AL, Iggo R. Induction of an interferon response by RNAi vectors in mammalian cells. *Nat Genet* 2003; **34**: 263–74.
32. Liu WL, Su WC, Cheng CW, *et al.* Ribavirin up-regulates the activity of double-stranded RNA-activated protein kinase and enhances the action of interferon-alpha against hepatitis C virus. *J Infect Dis* 2007; **196**: 425–34.
33. Saunders LR, Barber GN. The dsRNA binding protein family: critical roles, diverse cellular functions. *FASEB J* 2003; **17**: 961–83.
34. Scadden AD, Smith CW. A ribonuclease specific for inosine-containing RNA: a potential role in antiviral defence? *EMBO J* 1997; **16**: 2140–9.
35. Taylor DR, Shi ST, Romano PR, Barber GN, Lai MM. Inhibition of the interferon-inducible protein kinase PKR by HCV E2 protein. *Science* 1999; **285**: 107–10.
36. Gimenez-Barcons M, Wang C, Chen M, *et al.* The oncogenic potential of hepatitis C virus NS5A sequence variants is associated with PKR regulation. *J Interferon Cytokine Res* 2005; **25**: 152–64.
37. Noguchi T, Satoh S, Noshi T, *et al.* Effects of mutation in hepatitis C virus nonstructural protein 5A on interferon resistance mediated by inhibition of PKR kinase activity in mammalian cells. *Microbiol Immunol* 2001; **45**: 829–40.

B型肝炎に対する新たな核酸アナログ療法

加藤直也　室山良介　小俣政男

「肝胆膵」　第58巻　第5号　別刷

(2009年5月)

アークメディア

B型肝炎に対する新たな核酸アナログ療法

加藤直也* 室山良介* 小俣政男**

索引用語：DNAポリメラーゼ阻害剤，逆転写酵素阻害剤，薬剤耐性，ラミブジン，併用療法

1 はじめに

B型肝炎の抗ウイルス療法として，B型肝炎ウイルス(hepatitis B virus: HBV)の逆転写酵素(DNAポリメラーゼ)阻害剤である核酸アナログが使われるようになった．ラミブジン(ゼフィックス)は2000/11から，アデホビル(ヘプセラ)はラミブジン耐性に対するラミブジンとの併用療法が2004/12から，単独療法が2008/9から，エンテカビル(バラクルード)は2006/9から使われている．これらは，HBV DNAの減少，ALT正常化，肝組織像の改善など，慢性肝炎・肝硬変症の治療を劇的に改善し，B型肝炎の治療を大きく変えつつある．しかしながら長期投与における耐性ウイルスの出現，再増殖による肝炎の再燃はこれら核酸アナログを投与する上での最大の問題となっている．そこで，いまだに，より安全で，より抗ウイルス効果が強く，耐性を生じにくい，新たな核酸アナログの登場が待たれている．

2 Telbivudine (LdT)

Telbivudine (L-deoxythymidine: LdT) は，構造はラミブジンに似るが作用点は異なり，ラミブジンが主にRNA依存性DNA合成に作用するのに対してtelbivudineはDNA依存性DNA合成に作用するといわれる．米国ではすでにB型肝炎の治療薬として認可されているが，残念ながらわが国ではまだ開発(臨床試験)が行われていない．

第Ⅲ相試験の結果が報告されている[1]．1,367例(e抗原陽性例921例，e抗原陰性例446例)を対象にtelbivudine 600 mg投与群とラミブジン100 mg投与群に無作為に割付した比較試験であり，104週間の投与を行っている．HBV DNAが5 $\log_{10}$ copies/m*l*未満になったものは，e抗原陽性例ではtelbivudine63％に対しラミブジン48％，e抗原陰性例ではtelbivudine78％に対しラミブジン66％であった．HBV DNAが検出限界以下になったものは，e抗原陽性例ではtelbivu-

Naoya KATO et al: Nucleoside/nucleotide analogues
*東京大学医科学研究所疾患制御ゲノム医学ユニット［〒108–8639 東京都港区白金台 4–6–1］
**山梨県特別顧問

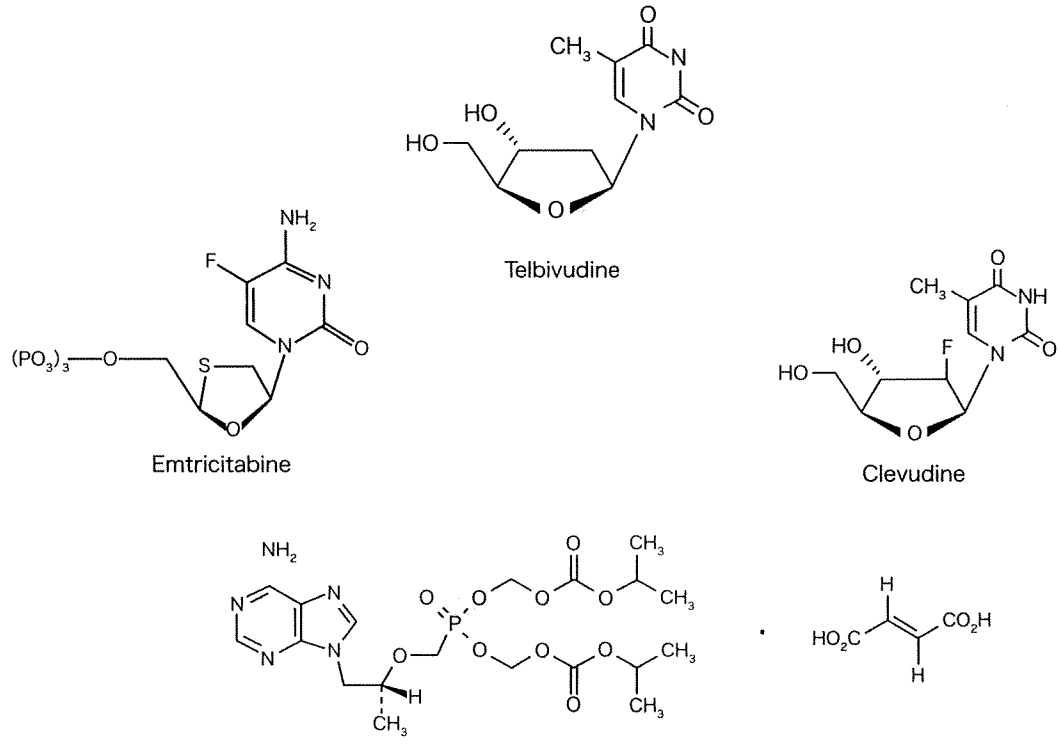

図1　各種抗B型肝炎ウイルス薬の構造

dine56％に対しラミブジン39％，e抗原陰性例ではtelbivudine82％に対しラミブジン57％であった．e抗原陽性例でのe抗原陰性化はtelbivudine35％に対しラミブジン29％であった．抗ウイルス効果に関してはtelbivudineがラミブジンより良好な成績であった．しかも，耐性ウイルスの出現は，e抗原陽性例ではtelbivudine25％に対しラミブジン40％，e抗原陰性例ではtelbivudine11％に対しラミブジン26％であった．耐性ウイルスの出現に関してもtelbivudineがラミブジンより良好な成績であった．ただし，クレアチニンキナーゼ値の上昇は，ラミブジンよりtelbivudineで多く認められた．

600 mgという比較的高用量の投与にもかかわらず，副作用の少ない薬剤であり，安全性は高く，ラミブジンより抗ウイルス効果は強力であるが，長期投与によりラミブジンよりは少ないもののいわゆるYMDD変異ウイルスが高率に出現することが問題である．今後，他の核酸アナログとの併用療法を検討する必要がある．

3　Tenofovir（TDF）

Tenofovir disoproxil fumarate（TDF）はアデホビルに似た構造を持つ．アデホビル同様，Human immunodeficiency virus（HIV）とHBV両者に抗ウイルス活性を有し，HIVに対しては本邦でもすでに保険適用を得ている(2004/4～)．米国では，2008/8にB型肝炎の治療薬として認可されているが，残念ながらわが国ではまだ開発が行われていない．

第Ⅲ相試験の結果が報告されている[2]．慢性B型肝炎患者を無作為にtenofovir投与群(300 mg/日)とアデホビル投与群(10 mg/日)に割り振り，比較検討している．48週投与時点で，HBV DNAが400 copies/m*l*以下になったものは，e抗原陰性群でtenofovirが93%に対しアデホビルが63%，e抗原陽性群でtenofovirが76%に対しアデホビルが13%であった．e抗原陽性群では，ALTの正常化はtenofovirが68%に対しアデホビルが54%であった．すなわち，抗ウイルス効果については，tenofovirがアデホビルに勝ることが明らかになった．安全性についてはtenofovirとアデホビルで差を認めていない．また，48週間投与では耐性ウイルスの出現も認められていない．

長期投与の成績も出てきている[3]．HIVとHBVの共感染があり，平均34か月のtenofovir投与を受けた患者で，52例中9例(17%)でウイルス学的ブレークスルー（耐性ウイルス出現)が認められている．A194T変異によりtenofovirへの感受性が下がることが示されている．

4 Emtricitabine (FTC)

Emtricitabine (FTC)は，シトシンヌクレオシドアナログであり，HBVとHIVの両者に抗ウイルス活性を有する．構造はラミブジンに似ており，*in vitro*ではラミブジンの約2倍強力な抗ウイルス効果を示す[4]．海外では第Ⅲ相試験が行われたが，まだB型肝炎への投与承認には至っていない．また，残念ながらわが国ではまだ臨床試験が行われていない．

プラセボ81例を対照に，167例にemtricitabine 200 mgを48週投与した試験の結果が報告された[5]．治療終了時には167例中103例(62%)に組織学的改善が認められた．HBV DNA減少量の中央値は4.5 $\log_{10}$ copies/m*l*（プラセボ0.4 $\log_{10}$ copies/m*l*)，PCR法でHBV DNAが検出感度以下になった患者の割合は54%（プラセボ2%)，ALT正常化は65%（プラセボ25%)，e抗原セロコンバージョン率は12%（プラセボ12%)であった．耐性株出現は13%で認められている．

また，少数例ながら，長期投与(96週間)によるアデホビル＋Emtricitabineとアデホビル単独投与の無作為比較試験の報告があり，HBV DNAを減少させる効果については，アデホビルとtenofivirの併用投与がアデホビル単独投与に勝ることが明らかとなっている．

Tenofovirとemtricitabineの合剤であるツルバダ(Trubada)という薬剤がHIVの治療に用いられており，HIVとHBVの共感染患者における切り札的な存在となっている．耐性プロファイルが異なり，強力な抗HBV効果を有する両薬剤の併用は，B型肝炎に対しても非常に有効な併用療法となり得ることが期待される．

5 Clevudine (L-FMAU)

Clevudine (L-FMAU)はピリミジンアナログで，*in vitro*でラミブジンの約10倍強力な抗ウイルス効果を示す[2]．HIVには抗ウイルス活性を有さない．主にDNA依存性DNA合成に作用する．海外では臨床試験が行われているが，わが国ではまだ行われていない．

臨床第Ⅱ相試験の成績が報告されている[6]．B型慢性肝炎患者に対して，12週間10 mg/日(10例)，30 mg/日(11例)，50 mg/日(10例)のclevudineを投与し，投与終了時のHBV DNA減少量中央値はそれぞれ3.2, 3.7, 4.2 $\log_{10}$ copies/m*l*であった．明らかな副作用は認められていない．本試験から，至適投

与量は30 mg/日と考えられた.

臨床第Ⅲ相試験の成績も報告されている[7,8]. e抗原陽性の182例のB型慢性肝炎患者に対し, 30 mg/日のclevudineが投与され, 投与24週でのHBV DNA減少は5.1 $\log_{10}$ copies/mlであった. e抗原陰性の63例のB型慢性肝炎患者に対し, 30 mg/日のclevudineが投与され, 投与24週でのHBV DNA減少は, 4.3 $\log_{10}$ copies/mlであった. 興味深いことに, clevudineの抗ウイルス効果は治療終了後も認められている.

また, emtricitabineとの併用療法が報告されている[9]. 163例を, emtricitabine 200 mg + clevudine 10 mg, またはemtricitabine 200 mg単独群に無作為に割付した比較試験である. 24週投与後のHBV DNA量が4,700 copies/ml未満になった患者の割合はemtricitabine + clevudine群74%（emtriditabine群65%）で有意差はなかったが, 投与終了24週後の4,700 copies/ml未満の割合は40%（emtricitabine群23%, p=0.025), ALT正常化は63%（emtricitabine群42%, p=0.002）と, 有意差を認めている. またemtricitabine + clevudine群では, 治療前より投与終了24週後のHBV DNA量で, 1.3 $\log_{10}$ copies/ml（emtricitabine群0.3 $\log_{10}$ copies/ml）の減少を認め, 効果の持続がみられた.

6 おわりに

B型慢性肝炎に対する治療の最終目標は, 肝不全に陥るのを阻止し, また, 発癌を抑止することによりその生命予後を改善することである[10]. 新たな核酸アナログの登場に期待する一方で, それぞれの薬剤に対する耐性株の出現はいまだ最大の問題である. 将来的には, 作用機序あるいは耐性プロファイルの異なる抗HBV薬の多剤併用療法の検討が必須である. しかしながら, わが国での新たな核酸アナログの開発は海外に比べて遅れており, 薬剤の選択肢が少ないことは憂慮される.

文 献

1) Liaw YF, Gane E, Leung N et al : 2-year GLOBE trial results: telbifvudine is superior to lamivudine in patients with chronic hepatitis B. Gastroenterology 136 : 389–392, 2009
2) Marcellin P, Heathcote EJ, Buti M et al : Tenofovir disoproxil fumarate versus adefovir dipivoxil for chronic hepatitis B. N Engl J Med 359 : 2442–2455, 2008
3) Alvarez-Uria G, Ratcliffe L, Vilar JF : Long-term outcome of tenofovir disoproxil fumarate use against hepatitis B in an HIV-coinfected cohort. HIV Med 2009 (Epub ahead of print)
4) Ono SK, Kato N, Shiratori Y et al : The polymerase L528M mutation cooperates with nucleotide binding-site mutations, increasing hepatitis B virus replication and drug resistance. J Clin Invest 107 : 449–455, 2001
5) Lim SG, Ng TM, Kung N et al : A double-blind placebo-controlled study of emtricitabine in chronic hepatitis B. Arch Intern Med 166 : 49–56, 2006
6) Lim SG, Leung N, Hann HW et al : Clinical trial: a phase II, randomized study evaluating the safety, pharmacokinetics and anti-viral activity of clevudine for 12 weeks in patients with chronic hepatitis B. Aliment Pharmacol Ther 27 : 1282–1292, 2008
7) Yoo BC, Kim JH, Chung YH et al : Twenty-four-week clevudine therapy showed potent and sustained antiviral activity in HBeAg-positive chronic hepatitis B. Hepatology 45 : 1172–1178, 2007
8) Yoo BC, Kim JH, Kim TH et al : Clevudine is highly efficacious in hepatitis B e antigen-negative chronic hepatitis B with durable off-therapy viral suppression. Hepatology 46 : 1041–1048, 2007
9) Lim SG, Krastev Z, Ng TM et al : Randomized, double-blind study of emtricitabine (FTC) plus clevudine versus FTC alone in treatment of chronic hepatitis B. Antimicrob Agents Chemother 50 : 1642–1648, 2006
10) Omata M : Treatment of chronic hepatitis B infection. N Engl J Med 339 : 114–115, 1998

ウイルス肝炎の臨床像と遺伝子多型

加藤直也　室山良介

「肝胆膵」　第59巻　第6号　別刷

(2009年12月)

アークメディア