other than hepatitis C, autoimmune liver disease, alcoholic or drug-induced liver injury, malignant tumour, biliary disorder, fulminant hepatitis or peptic ulcer; ii) required hospitalisation for cardiac, renal or pancreatic disease; iii) were pregnant or lactating; iv) alcohol dependent or drinking more than approximately 22 g/day alcohol; v) were participants in another clinical study within 4 weeks before the observation period; or vi) were sensitive to UDCA or other bile acid preparations.

The protocol was approved by the ethics committee of each institution participating in the study. Patients were informed of the details of the clinical study and agreed to participate. We conducted this clinical study in accordance with the Declaration of Helsinki and good clinical practice.

Study design

After the 8-week observation period patients were treated with oral (prandial) UDCA (Urso, Mitsubishi Pharma, Osaka, Japan) for 24 weeks at 150, 600 or 900 mg/day, divided into three doses, under double-blind conditions. Double blinding used placebo, 50 and 100 mg tablets identical in appearance to the test drug. The UDCA doses were established from a previous clinical study of UDCA in patients with CH-C.[9] Concomitant use of drugs and therapies included in the exclusion criteria were prohibited throughout the observation and treatment periods.

Changes in serum ALT levels were previously reported to be −26% and −25.5% with 600 and 900 mg/day of UDCA, respectively, compared to untreated controls and no significant changes were observed with 150 mg/day.[9] Based on these data, we assumed a standard deviation of 30% for per cent changes in ALT, and the necessary sample size was calculated to be 200 in each group to detect any superiority of the 600 and 900 mg/day doses over 150 mg/day at a significance level of 0.05 and a power of 0.9.

We enrolled patients who met all criteria and gave written informed consent between July 2002 and May 2004 in 62 institutions with liver clinics throughout Japan. Each patient was assigned randomly to one of the three dose groups by using numbered containers provided based on a permuted block method (block size: 6).

When treatment or evaluation was discontinued because of patient request, aggravation of symptoms, adverse events or other reasons, prior data were included in the evaluation as final observation data.

To investigate the long-term effects of UDCA, the protocol included an option for additional UDCA administration for a minimum of 28 weeks and a maximum of 80 weeks (total 52–104 weeks including the initial 24 weeks) if the ALT level had decreased by at least 15% at week 20 compared to the baseline. In the additional period, the double-blind setting was discontinued and the dose of 600 mg/day was adopted, which could be increased to 900 mg/day by the decision of each patient and the physician responsible. Patients who entered the additional phase could discontinue UDCA administration anytime after week 52.

Laboratory tests

Blood was collected every 4 weeks from the start of the observation period to the end of drug administration. Serum ALT was measured as a primary endpoint of liver function, and AST and GGT as secondary endpoints, using conventional methods. Blood samples taken at the start of observation, at 0, 4 and 12 weeks of treatment, and at the final observation were analysed to determine leukocyte and erythrocyte counts, haemoglobin, haematocrit, thrombocyte count, and the levels of ALT, AST, GGT, alkaline phosphatase, lactate dehydrogenase, total protein, albumin, cholinesterase, total bilirubin, direct bilirubin, total cholesterol, urea nitrogen, creatinine, Na, K and Cl.

For bile acid composition analysis, blood was collected at the start of treatment and at the final observation in a fasted condition. Serum total bile acid was measured by the 3α-hydroxysteroid dehydrogenase method. Bile acid fractions were determined by a specific liquid chromatography-electrospray mass spectrometry, using an HPLC system (Agilent 1100 series, Agilent Technologies, CA, USA) equipped with a C18 cartridge (CAPCELL PAK C18 UG120A, Shiseido, Tokyo, Japan) and a mass spectrometer (Quattro Ultima, Micromass Technologies, Manchester, UK).

Serum HCV-RNA level was measured prior to treatment and at the final observation by a reverse transcriptional polymerase-chain-reaction method.

All analyses and measurements were performed in a single contract laboratory (SRL, Tokyo, Japan).

Statistical analysis

Patients' backgrounds were compared among the three dose groups by χ^2 test and ANOVA. Changes in serum ALT, AST and GGT levels due to UDCA administration were compared among the groups by repeated-measure ANOVA. Differences between groups were tested by using linear contrasts. Subgroup analyses of median changes in serum ALT at the final observation, relative to the pre-treatment levels, were performed according to gender, body weight and pre-treatment serum GGT level with Wilcoxon signed-ranks tests. Changes in bile acid and serum HCV-RNA levels were analysed by paired Student's t test. Fischer's exact probability test was applied to the incidences of adverse reactions. A p value <0.05 in a two-tailed test was considered significant. Analyses were done on the full analysis set. This study is registered at ClinicalTrial.gov, number NCT00200343, and is compliant with the published CONSORT guidelines for performance and publication of clinical trials.[12]

RESULTS

Patients

We enrolled 596 patients; 199 received UDCA at 150 mg/day, 200 at 600 mg/day, and 197 at 900 mg/day. Safety was evaluated in all patients as adverse events based on signs and symptoms and abnormal laboratory test results. Efficacy was evaluated in 586 patients (195, 150 mg/day; 198, 600 mg/day; and 193 at 900 mg/day), excluding 10 who lacked sufficient data. At the end of 24 weeks' administration, 392 patients were eligible for additional long-term administration. Of these patients, 280 chose to participate in the study and others refused mainly because of lack of time. Twenty three patients discontinued before week 52, one of them for biochemical relapse, and other 10 patients violated protocol. The effects of long-term administration were evaluated among the remaining 247 patients (fig 1).

Patients' backgrounds are summarised in table 1. Differences observed in gender, body weight and history of treatment with interferon between the three groups are indicated ($p<0.15$).

Changes in ALT, AST and GGT

Serum ALT, AST and GGT levels before and during treatment are shown in figs 2–4. The responses of ALT, AST and GGT over time were greater for 600 and 900 mg/day administration compared to 150 mg/day (ALT, $p<0.001$ and $p=0.021$; AST, $p<0.001$ and $p<0.001$; GGT, $p<0.001$ and $p<0.001$, respectively). No difference was observed between the 600 and 900 mg/day groups in ALT ($p=0.926$) or AST ($p=0.429$), but GGT differed significantly ($p<0.001$). Serum ALT, AST and GGT levels decreased by 4 weeks into treatment and remained

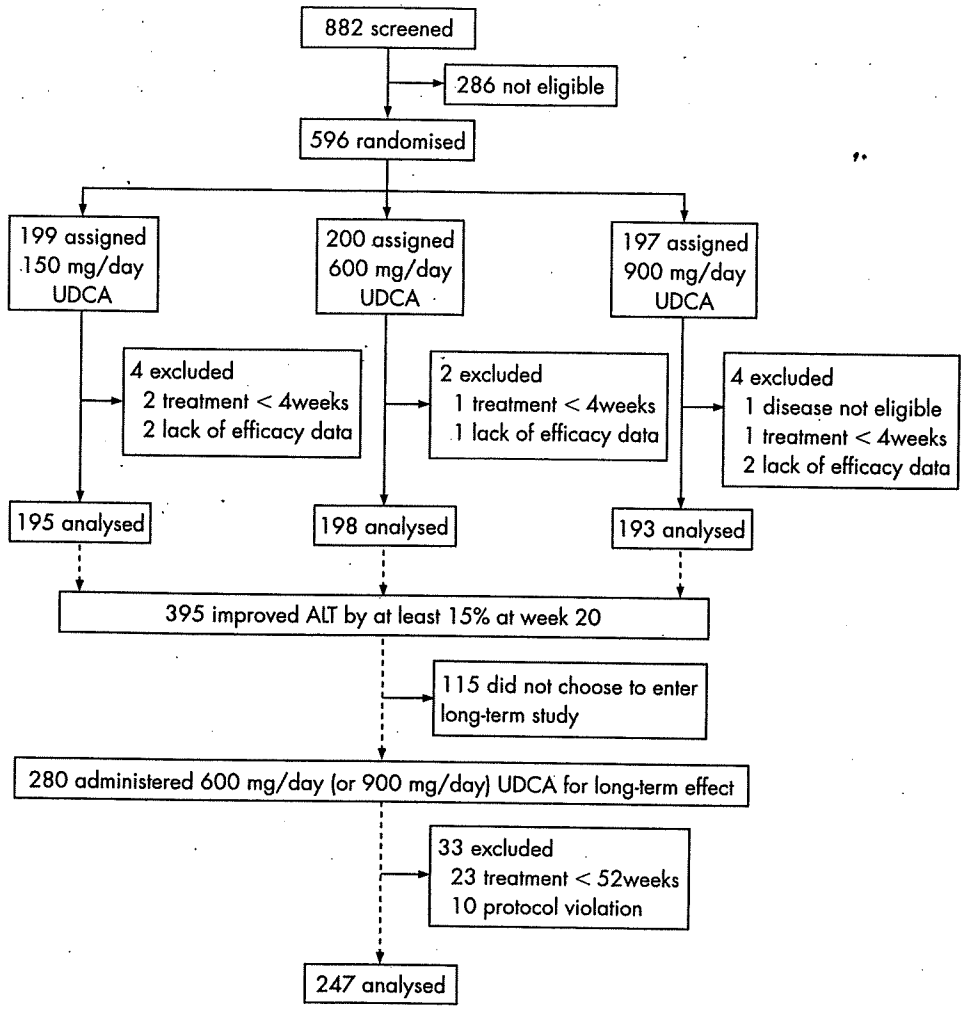

Figure 1 Trial profile.

constant. Serum ALT, AST and GGT levels at the final observation, together with median changes relative to 0 week (baseline), are shown in table 2. The mean decreases in serum ALT levels from the baseline value were 13.4, 30.6 and 29.3 IU/l in the 150, 600 and 900 mg/day groups, respectively. The median changes in ALT at the final observation were −15.3%, −29.2% and −36.2% in the corresponding groups (table 2).

Table 1 Characteristics of patients with chronic hepatitis C treated with UDCA (full analysis set)

	150 mg/day (n=195)	600 mg/day (n=198)	900 mg/day (n=193)	p Value
Gender				
Male	97 (49.7%)	117 (59.1%)	123 (63.7%)	0.018
Female	98 (50.3%)	81 (40.9%)	70 (36.3%)	
Age (years)	58.0±12.2	57.7±12.0	59.8±10.1	0.152
Height (cm)	160.1±9.5	161.9±9.2	160.8±8.7	0.163
Weight (kg)	58.8±11.4	61.8±11.2	61.6±11.9	0.017
ALT (IU/l)	109.2±49.7	106.3±59.4	110.6±57.3	0.745
AST (IU/l)	84.0±39.1	82.4±41.8	85.2±45.0	0.796
GGT (IU/l)	87.5±73.0	82.4±62.2	85.9±66.3	0.744
Interferon*				
Absent	119 (61.0%)	100 (50.5%)	96 (49.7%)	0.044
Present	76 (39.0%)	98 (49.5%)	97 (50.3%)	

Data represent the number of patients or mean±SD.
*Previous interferon treatment.

The mean decreases in serum AST levels from the baseline value were 8.5, 19.3 and 19.7 IU/l in the 150, 600 and 900 mg/day groups, respectively. The mean decreases in serum GGT levels from the baseline value were 17.1, 32.7 and 42.1 IU/l in the 150, 600 and 900 mg/day groups, respectively.

Long-term effects

The decreases in ALT, AST, GGT levels from the baseline value were maintained during long-term administration of UDCA, as shown in table 3.

Subgroup analyses

The decrease in serum ALT was significantly greater in the 600 and 900 mg/day groups than in the 150 mg/day group for most subgroups by gender, body weight or baseline serum GGT levels (table 4). Although the difference between the 600 and 900 mg/day groups as a whole was not significant, the subgroup of baseline GGT≥80 IU/l showed a significantly lower level of GGT with 900 mg/day administration ($p = 0.004$).

Bile acid in serum

Total bile acid concentration in serum increased in a dose-dependent manner from the start of drug administration to the final observation, as shown in table 5. The ratio of UDCA to total bile acid was increased significantly in all groups at the final observation compared to baseline. The ratio of UDCA at the final observation was similar in the 600 and 900 mg/day groups. The proportion of less hydrophilic bile acids was

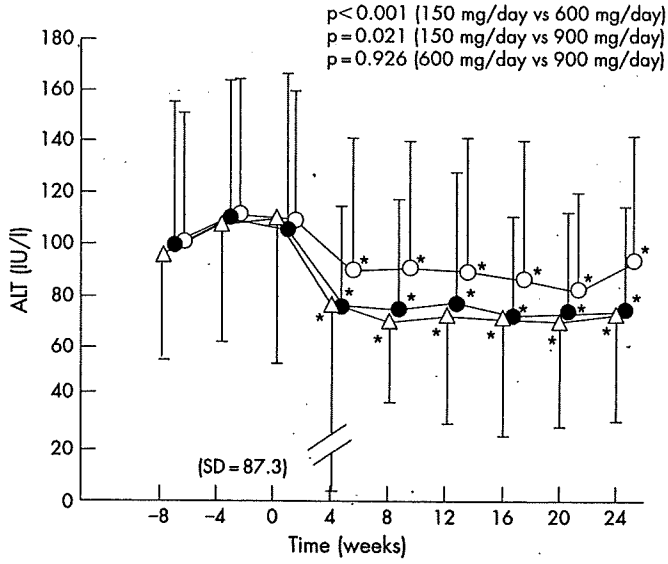

Figure 2 Changes in serum ALT levels in patients with chronic hepatitis C before and during the treatment period. Data are expressed as mean±SD. Open circles, 150 mg/day; filled circles, 600 mg/day; open triangles, 900 mg/day; *p<0.01, paired t test (vs week 0). The p values refer to repeated measures ANOVA.

decreased accordingly. The proportion of chenodeoxycholic acid at the final observation was decreased significantly in all groups, and was similar in the 600 and 900 mg/day groups. The proportions of cholic acid and deoxycholic acid were also decreased significantly compared to baseline.

Virus load

HCV-RNA levels (mean±SD) changed from the baseline of 1477±1280 to 1366±1224 kIU/ml in the 150 mg/day group, from 1463±1299 to 1358±1233 kIU/ml in the 600 mg/day group, and from 1553±1318 to 1552±1398 kIU/ml in the 900 mg/day group. None of these changes was significant.

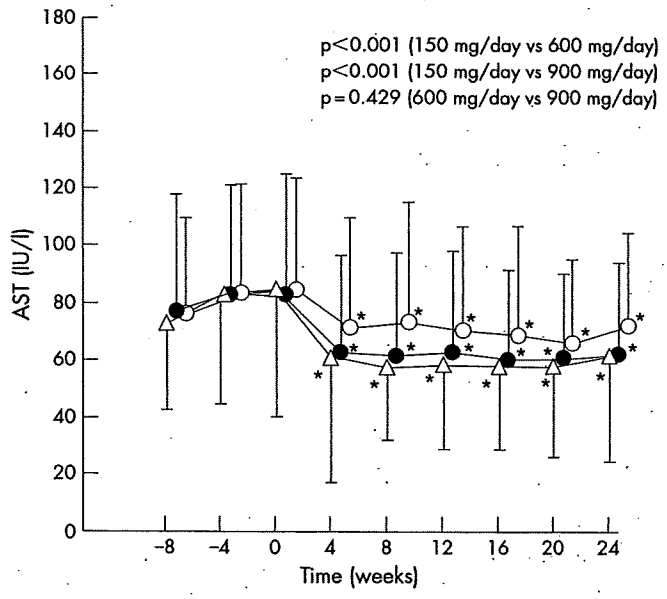

Figure 3 Changes in serum AST levels in patients with chronic hepatitis C before and during the treatment period. Data are expressed as mean±SD. Open circles, 150 mg/day; filled circles, 600 mg/day; open triangles, 900 mg/day; *p<0.01, paired t test (vs week 0). The p values refer to repeated measures ANOVA.

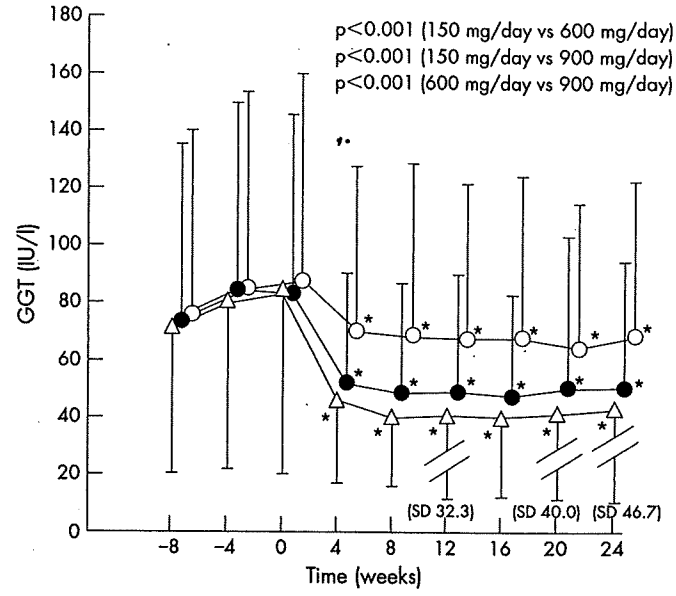

Figure 4 Changes in serum GGT levels in patients with chronic hepatitis C before and during the treatment period. Data are expressed as mean±SD. Open circles, 150 mg/day; filled circles, 600 mg/day; open triangles, 900 mg/day; *p<0.01, paired t test (vs week 0). The p values refer to repeated measures ANOVA.

Safety

The observed adverse reactions possibly associated with UDCA administration are shown in table 6. The overall incidences of adverse reactions were 18.1%, 21.5% and 17.8% in the 150, 600 and 900 mg/day groups, respectively, with no significant difference between the groups. Diarrhoea was reported most often. No severe adverse reactions were seen.

DISCUSSION

UDCA is frequently used for cholestatic liver diseases, primary biliary cirrhosis in particular. UDCA improves biochemical indices such as serum GGT, ALT and bilirubin. Histopathological improvements have been shown[13] and prolonged survival reported.[14 15] Although its effect on survival remains controversial,[16 17] UDCA is the only approved medication for primary biliary cirrhosis. Suggested mechanisms for UDCA include reducing the cytotoxicity of hydrophobic bile acids, stimulating hepatobiliary secretion and anti-apoptosis.[18]

UDCA was used to decrease serum aminotransferase levels for so-called non-A non-B chronic hepatitis before the discovery of HCV.[8 19 20] Takano *et al* restricted their study to patients with CH-C and found the optimal dose of UDCA to be 600 mg/day.[9] There was a greater reduction in GGT (40.5%) than in ALT (26.0%), as also observed in the current study. The reported effect of UDCA was stronger among CH-C patients with morphological bile duct injury,[21] and UDCA administration was accompanied by histological improvement of biliary lesions but not of hepatitis.[22] These data suggest that UDCA may act on the biliary system in CH-C through enhanced bile formation and/or modification of bile acid composition. In fact, bile duct injury is characteristic of CH-C, although not specific.[23] In this study, the changes in bile acid composition were similar in the 600 and 900 mg/day groups but smaller in the 150 mg/day group, and this may have been associated with the changes in serum biomarkers.

Nakamura *et al* reported that UDCA had a greater effect in CH-C patients with autoimmune characteristics, that is high immunoglobulin G concentration or positive anti-nuclear or anti-smooth muscle antibodies,[24] which suggests involvement

Table 2 Serum ALT, AST and GGT levels in patients with chronic hepatitis C after treatment with UDCA

	Dose (mg/day)	Pre-treatment, mean±SD	Post-treatment, mean±SD	Change (%), median (range)
ALT (IU/l)	150	109.2±49.7	95.8±60.2	−15.3 (−80.7 to +375.9)
	600	106.3±59.4	75.7±41.9	−29.2 (−88.3 to +95.2)
	900	110.6±57.3	81.3±90.5	−36.2 (−81.4 to +1696.9)
AST (IU/l)	150	84.0±39.1	75.5±43.6	−13.6 (−74.2 to +347.2)
	600	82.4±41.8	63.1±32.9	−25.0 (−82.7 to +72.5)
	900	85.2±45.0	65.5±49.6	−29.8 (−79.0 to +1026.1)
GGT (IU/l)	150	87.5±73.0	70.4±58.3	−22.4 (−74.6 to +145.9)
	600	82.4±62.2	49.7±43.0	−41.0 (−81.1 to +153.1)
	900	85.9±66.3	43.8±44.8	−50.0 (−80.1 to +213.9)

Table 3 Serum ALT, AST and GGT levels in patients with chronic hepatitis C during long-term administration of UDCA

	Pre-treatment	Treatment period		
	Week 0	Week 24	Week 48	Week 104
Patients (n)	247	242*	243†	149‡
ALT (IU/l)	114.8±54.1	70.7±37.4	67.9±36.3	63.5±31.9
AST (IU/l)	86.6±41.7	59.0±31.5	56.6±27.4	54.1±23.7
GGT (IU/l)	87.3±67.6	49.5±42.6	47.3±40.5	41.8±30.1

Data are expressed as mean±SD.
*Corresponding data missing in five patients; †corresponding data missing in four patients; ‡administration between week 52 and week 104 was optional and 149 patients opted for the maximum term.

of immunomodulatory mechanisms. Indeed, studies in vitro have shown that UDCA suppresses NF-κB-dependent transcription by binding to the glucocorticoid receptor[25] and decreases proinflammatory cytokine-induced transcription of phospholipase A2.[26] These mechanisms may act cytoprotectively in vivo. The choleretic and cytoprotective mechanisms are not necessarily mutually exclusive.

We examined the effect of UDCA on CH-C in terms of serum biochemical markers in a large-scale, double-blind investigation. We confirmed that a dose of 600 mg/day, that is 10 mg/kg body weight on average, was more effective than 150 mg/day, while adverse effects remained similar and minimal. The doses of 600 and 900 mg/day induced similar decreases in serum ALT and AST. Consequently, it appears that 600 mg/day is the preferred dose of UDCA, assuming that serum transaminase levels reflect the degree of hepatocellular damage.

The decrease in serum GGT differed significantly between the 600 and 900 mg/day groups. In contrast to the decrease in ALT or AST, that of serum GGT may represent improved cholestasis from biliary injury in CH-C. Although the importance of biliary injury in CH-C is unclear, it is possible that a 900 mg/day dose has additional benefits compared to 600 mg/day, as the incidence of adverse effects did not differ between the two doses. It is of interest that the decrease in ALT was significantly different between the two doses in patients with high baseline GGT levels (table 4).

The long-term effects of UDCA therapy in CH-C patients are yet to be elucidated. Changes in liver histology following UDCA administration are not evident from short-term observation. However, it is possible that delayed progression of fibrosis by UDCA can be revealed only by much longer-term observation,

Table 4 Subgroup analyses of change in serum ALT in patients with chronic hepatitis C after treatment with UDCA

	Dose (mg/day)	No. of patients	Change (%), median (range)	p Value vs 150 mg	p Value vs 600 mg
Gender					
Male	150	97	−14.9 (−80.7 to +375.9)		
	600	117	−33.1 (−88.3 to +93.1)	<0.001	
	900	123	−36.4 (−79.1 to +1696.9)	<0.001	0.430
Female	150	98	−18.0 (−79.0 to +175)		
	600	81	−25.0 (−74.7 to +95.2)	0.058	
	900	70	−35.8 (−81.4 to +315.3)	0.002	0.076
Body weight (kg)					
<60	150	115	−14.9 (−80.7 to +375.9)		
	600	82	−28.6 (−74.7 to +95.2)	0.002	
	900	91	−35.2 (−81.4 to +315.3)	0.001	0.356
≥60	150	80	−16.7 (−73.4 to +166.1)		
	600	116	−30.3 (−88.3 to +93.1)	0.003	
	900	102	−36.6 (−77.1 to +1696.9)	<0.001	0.096
GGT (IU/l)					
≤39	150	45	−14.5 (−73.4 to +71.4)		
	600	39	−32.7 (−62.9 to +93.1)	0.049	
	900	45	−26.6 (−81.4 to +1696.9)	0.112	0.616
40–79	150	79	−15.2 (−69.1 to +175)		
	600	90	−30.3 (−74.7 to +95.2)	0.001	
	900	70	−36.3 (−77.7 to +200)	<0.001	0.633
≥80	150	71	−18.2 (−80.7 to +375.9)		
	600	69	−28.6 (−88.3 to +53.8)	0.057	
	900	78	−41.2 (−79.1 to +119.3)	<0.001	0.004

The p values refer to Wilcoxon signed-ranks tests.

Table 5 Composition of serum bile acid in patients with chronic hepatitis C treated with UDCA

	Dose (mg/day)	Before treatment	After treatment	p Value
Total bile acid concentration (μmol/l)	150	8.63±9.76	13.69±19.28	<0.001
	600	9.42±12.04	21.89±24.20	<0.001
	900	9.17±9.30	28.74±39.78	<0.001
Cholic acid (%)	150	17.69±10.33	11.35±7.08	<0.001
	600	17.75±10.35	5.93±4.53	<0.001
	900	18.15±9.54	5.14±4.19	<0.001
Deoxycholic acid (%)	150	21.62±16.24	13.84±11.39	<0.001
	600	19.86±16.84	6.50±7.06	<0.001
	900	18.74±15.29	5.68±6.58	<0.001
Chenodeoxycholic acid (%)	150	54.46±14.12	39.93±11.61	<0.001
	600	55.37±13.95	24.66±10.01	<0.001
	900	55.95±13.65	23.31±12.72	<0.001
Ursodeoxycholic acid (%)	150	5.93±8.72	34.25±13.75	<0.001
	600	6.70±9.72	62.26±13.69	<0.001
	900	6.83±10.6	65.12±16.84	<0.001
Lithocholic acid (%)	150	0.30±0.99	0.62±1.66	0.010
	600	0.33±1.23	0.66±1.35	0.010
	900	0.33±1.12	0.75±1.49	0.001

Data are expressed as mean±SD. The p values refer to paired t test (before vs after treatment).

because the natural progression of fibrosis in CH-C is usually slow, taking decades to establish cirrhosis.[27 28] The effect of UDCA lasted for at least 104 weeks without attenuation (table 3).

In the natural course of CH-C, those patients with normal serum aminotransferase levels show slow fibrosis progression[29] and a low incidence of hepatocellular carcinoma.[30 31] By multivariate analysis, the risk of hepatocellular carcinoma after interferon treatment without virological response was shown to be 0.26, 0.36 and 0.91 in patients whose ALT levels were normal, moderately elevated (less than twice the upper normal limit) and highly elevated, respectively, compared to untreated patients. It may be that when UDCA lowers serum ALT levels the risk of hepatocellular carcinoma is decreased. A retrospective study showed that hepatocellular carcinoma developed within 5 years from the onset of HCV-related early cirrhosis in 10 of 56 patients (18%) who took UDCA and 18 of 46 patients (39%) who did not.[32] Interestingly, ALT levels were similar in the two groups, possibly because UDCA was likely to be prescribed to those patients with high baseline ALT levels. Although these data were obtained from a non-randomised, retrospective study, they suggest that UDCA may provide cancer protective effects independent of decreasing ALT.

In summary, we confirmed, in a large-scale, double-blind study, that a UDCA dose of 600 mg/day was optimal to decrease serum ALT and AST levels in CH-C patients without serious adverse effects. A dose of 900 mg/day resulted in additional decreases in serum GGT levels, and may be preferred in patients with prevailing biliary injuries. The long-term effects of UDCA administration on prognosis, hepatocarcinogenesis in particular, remain to be investigated in future studies.

Table 6 Summary of adverse reactions

	150 mg/day	600 mg/day	900 mg/day
Overall incidence	18.1% (36/199)	21.5% (43/200)	17.8% (35/197)
Total adverse reactions, n	44	62	45
Common adverse reactions, n (%)*			
Abdominal distension	2 (1.0)	2 (1.0)	2 (1.0)
Upper abdominal pain	2 (1.0)	4 (2.0)	2 (1.0)
Constipation	3 (1.5)	4 (2.0)	2 (1.0)
Diarrhoea	7 (3.5)	8 (4.0)	8 (4.1)
Dyspepsia	3 (1.5)	2 (1.0)	2 (1.0)
Loose stool	1 (0.5)	6 (3.0)	5 (2.5)
Stomach discomfort	2 (1.0)	2 (1.0)	3 (1.5)
Pruritus	3 (1.5)	3 (1.5)	2 (1.0)

*The adverse reactions which were observed in 1% or more of the patients.

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BASIC STUDIES

New ablation procedure for a radiofrequency liver tissue coagulation system using an expandable needle

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Keywords
expandable needle – liver cancer – radiofrequency ablation (RFA) – stepwise expansion

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Abstract

Objective: The stepwise hook extension technique for an expandable needle, which we reported previously, allowed roll-off in short time with low power. The aim of this study was to investigate experimentally the efficacy of a modified extension procedure. *Methods:* Three pigs underwent 10 radiofrequency ablation (RFA) procedures using the 10-hook electrode of LeVeen needle. The conventional technique was used in five RFA (group 1; the electrode was deployed in four steps to full extension), while the new technique was used in the other five RFA (group 2; the electrode was closed after the same three steps as group 1 and then fully extended). *Results:* The shape of the RFA-induced zone was cone-like or irregular in group 1 and oval-like in group 2. The diameter vertical to the shaft was larger in group 2 (37, range 33–42 mm) than in group 1 (23, range 20–29 mm). The median ablation time was longer in group 2 (10 min 13 s) than in group 1 (3 min 56 s). Although the required energy was higher in group 2 than in group 1, that per volume was comparable between the groups (median 0.9 vs. 1.4 kJ/mm^3). *Conclusions:* Our new procedure requires a longer session but produces larger necrosis of a uniform ellipsoid volume, making it potentially suitable for tumours more than 3 cm in diameter.

Percutaneous treatment including radiofrequency ablation (RFA) and percutaneous ethanol injection (PEI) is often used for small-size hepatocellular carcinoma (HCC) as it is less invasive than surgical therapy. RFA has become the first-choice local treatment because of the excellent outcome; the efficacy of RFA in HCC tumours measuring < 2 cm in diameter is similar to that of PEI but it requires fewer treatment sessions, and the efficacy in HCC tumours > 2 cm in diameter is better than with PEI (1). In addition, RFA is also more cost-effective than surgical resection of small HCC (2). Because the volume ablated during one RFA session is of a diameter < 3.0–4.0 cm in most cases, RFA therapy is now restricted to tumour < 3 cm. In this regard, previous studies reported that the necrotic area could be enlarged by a saline injection before RFA (3, 4), combination of RFA with PEI (5, 6), RFA with an ethanol–lipiodol injection (7), RFA with transcatheter arterial embolization (8) and RFA with transient arterial obliteration (9–11).

Among the three commercially available RFA apparatuses, the radiofrequency tumour coagulation system (RTC system; Boston Scientific, Natick, MA, USA), radiofrequency interstitial tumour ablation system (RITA System, RITA Medical Systems Inc., Mountain View, CA, USA) and cool-tip RF system (Radionics Inc., Burlington, VT, USA), the first two types have adopted the expandable needle. We reported previously the efficacy of the stepwise hook extension technique for RFA therapy of HCC (12). The technique allows rapid roll-off at a lower power and reduces any possible increase in intratissue pressure that may cause scattering of intrahepatic metastasis (13–15). Additionally, we have designed a new technique involving full re-expansion after stepwise extension, that may ensure full expansion of the needle to enlarge the ablated zone. The aim of this study was to investigate experimentally the new expansion technique and to compare it with the conventional stepwise extension technique.

Materials and methods

We used the RTC system comprising the RF3000 generator and a slim expandable needle (30 mm,

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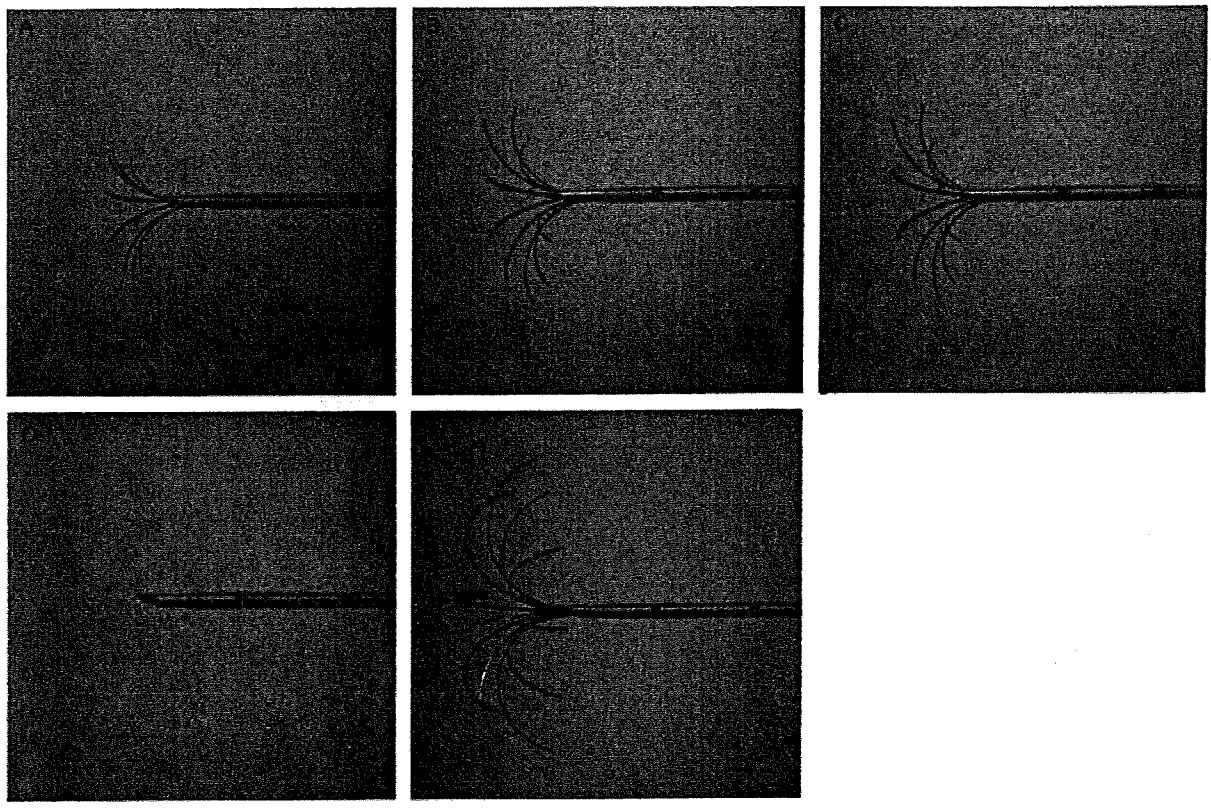

Fig. 2. The new stepwise procedure. (A) A quarter-length of the electrode tines is expanded in the first step. (B) A half-length is expanded in the second step. (C) A three-quarter length is expanded in the third step. (D) The electrode tines are closed in the shaft. (E) Tines are expanded to full length in the final step.

Table 1. Duration of ablation (in minutes seconds) and RF-induced area in groups 1 and 2

	Group 1					Group 2				
	1	2	3	4	5	1	2	3	4	5
Duration										
First step	2′ 55″	3′ 00″	1′ 18″	1′ 24″	0′ 58″	1′ 47″	2′ 15″	2′ 46″	1′ 42″	1′ 10″
Second step	2′ 14″	1′ 28″	0′ 49″	0′ 27″	0′ 32″	1′ 02″	2′ 33″	0′ 18″	0′ 17″	0′ 23″
Third step	0′ 58″	1′ 40″	0′ 35″	0′ 45″	0′ 34″	1′ 48″	1′ 01″	1′ 26″	0′ 22″	0′ 32″
Fourth step	0′ 44″	2′ 28″	1′ 14″	0′ 56″	0′ 52″	5′ 36″	6′ 40″	6′ 19″	6′ 29″	4′ 29″
Total	6′ 51″	8′ 36″	3′ 56″	3′ 32″	2′ 56″	10′ 13″	12′ 29″	10′ 49″	8′ 50″	6′ 34″
RF-induced area										
Transverse diameter, mm	20	28	25	23	22	33	42	38	37	35
Longitudinal length, mm	27	24	30	30	32	20	30	27	27	34
Shape	Irregular	Cone-like	Cone-like	Cone-like	Cone-like	Ellipsoid	Ellipsoid	Ellipsoid	Ellipsoid	Ellipsoid

larger in group 2 than that in group 1 (group 1: 5.5 kJ range 4.0–14.8 kJ, group 2: 25.0 kJ range 13.4–30.6 kJ, $P=0.016$) respectively.

Size and shape of ablated tissue

Table 1 shows the shape and size of the RF-induced areas in groups 1 and 2. In group 1, the shape of the ablated zone was cone-like or was sometimes irregularly shaped. The length along the shaft was longer than the vertical diameter as shown in Figure 3A. In group 2, the ablated zone was near-oval in shape, with the short axis equivalent to the shaft (Fig. 3B). As shown in Table 2, the area perpendicular to the shaft and the ablation volume were larger in group 2 than in group 1: vertical diameter: 23 (range 20–28) mm vs. 37 (range 33–42) mm ($P=0.008$). This indicates that our technique produced a larger area of necrosis following

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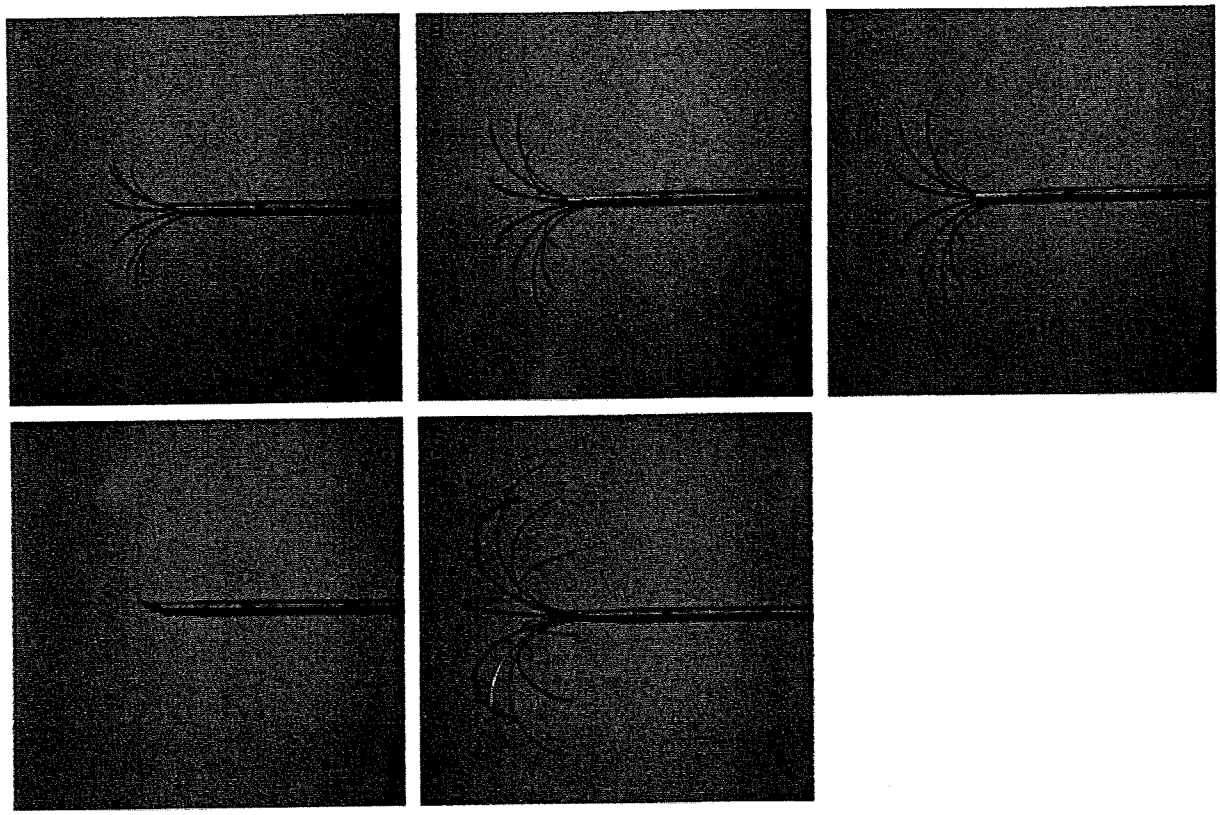

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	1	2	3	4	5	1	2	3	4	5
Duration										
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Second step	2′ 14″	1′ 28″	0′ 49″	0′ 27″	0′ 32″	1′ 02″	2′ 33″	0′ 18″	0′ 17″	0′ 23″
Third step	0′ 58″	1′ 40″	0′ 35″	0′ 45″	0′ 34″	1′ 48″	1′ 01″	1′ 26″	0′ 22″	0′ 32″
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Total	6′ 51″	8′ 36″	3′ 56″	3′ 32″	2′ 56″	10′ 13″	12′ 29″	10′ 49″	8′ 50″	6′ 34″
RF-induced area										
Transverse diameter, mm	20	28	25	23	22	33	42	38	37	35
Longitudinal length, mm	27	24	30	30	32	20	30	27	27	34
Shape	Irregular	Cone-like	Cone-like	Cone-like	Cone-like	Ellipsoid	Ellipsoid	Ellipsoid	Ellipsoid	Ellipsoid

larger in group 2 than that in group 1 (group 1: 5.5 kJ range 4.0–14.8 kJ, group 2: 25.0 kJ range 13.4–30.6 kJ, $P = 0.016$) respectively.

Size and shape of ablated tissue

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Table 2. Comparison of ablation time (in minutes seconds) and RF-induced areas between groups 1 and 2

	Group 1	Group 2	*P*
Incidence of roll-off	5/5	5/5	1.000
Duration of the first step	1′ 24″ (0′ 58″–3′ 00″)	1′ 47″ (1′ 10″–2′ 46″)	1.000
Second step	49″ (0′ 27″–2′ 14″)	23″ (17″–2′ 33″)	0.421
Third step	45″ (0′ 34″–1′ 40″)	1′ 1″ (22″–1′ 48″)	1.000
Fourth step	56″ (0′ 44″–2′ 28″)	6′ 19″ (4′ 29″–6′ 40″)	0.008
Total ablation time	3′ 56″ (2′ 56″–8′ 36″)	10′ 13″ (6′ 34″–12′ 29″)	0.032
Required energy for ablation, kJ	5.5 (4.0–14.8)	25.0 (13.4–30.6)	0.016
Diameter of the cross-section vertical to the axis, mm	23 (20–28)	37 (33–42)	0.008
Axial length, mm	30 (24–32)	27 (20–34)	0.841
Shape of RF-induced area			
Ellipsoid	0	5	
Cone-like	4	0	
Irregular	1	0	

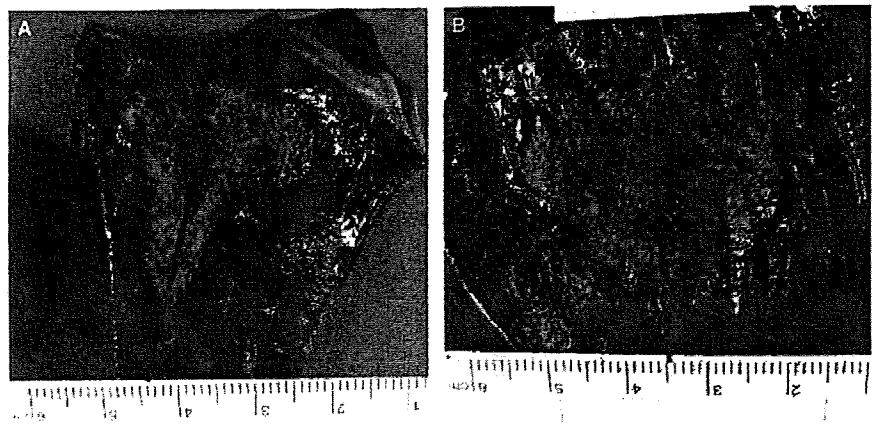

Fig. 3. Photographs of the coagulated area. Arrow shows the direction of the needle shaft. (A) The shape of the area produced by the conventional procedure is cone-like (RFA#3). (B) The shape of the area produced by the new procedure is ellipsoid in shape (RFA#3).

one session of RF. Although the axial length of the ablation zone showed no significant difference between the two groups, that in group 2 seemed a slightly shorter than that in group 1: axial length: 30 (range 24–32) mm vs. 27 (range 20–34) mm ($P=0.841$). Based on the assumption that the shape of the necrotic area was a combination of a hemisphere and a cone in group 1 and an ellipsoid in group 2, the estimated volume of the ablated liver tissue was 5.7 (range 3.8–7.8) μm^3 for group 1 and 20 (range 11–28) μm^3 for group 2. Using this value and the total required energy for ablation, the calculated energy required for ablation per volume was 0.9 (range 0.7–2.5) J/mm^3 for group 1 and 1.4 (range 0.6–1.8) J/mm^3 for group 2 ($P=1.000$).

Needle expansion

Figures 4 and 5 show X-ray images of the electrode tines in the pig liver at each step. Both in the second step and the third step, the progress of the tines' spread was smaller than at the first step. The needle expansion at the third step did not reach three quarters length in both groups 1 and 2. The extent of the expansion at the final step was nearly similar to that at the second and third steps in group 1, while it was nearly complete in group 2.

Discussion

Radiofrequency ablation therapy is one of the curative therapies for HCC measuring < 30 mm in diameter, while surgical resection is the only curative treatment for HCC more than 30 mm and < 50 mm in diameter. However, surgical resection cannot be performed in patients with cirrhotic liver and liver dysfunction. Thus, a technique that widens the RF-ablated area can improve, at least theoretically, the survival of cirrhotic patients with HCC over 30 mm in diameter.

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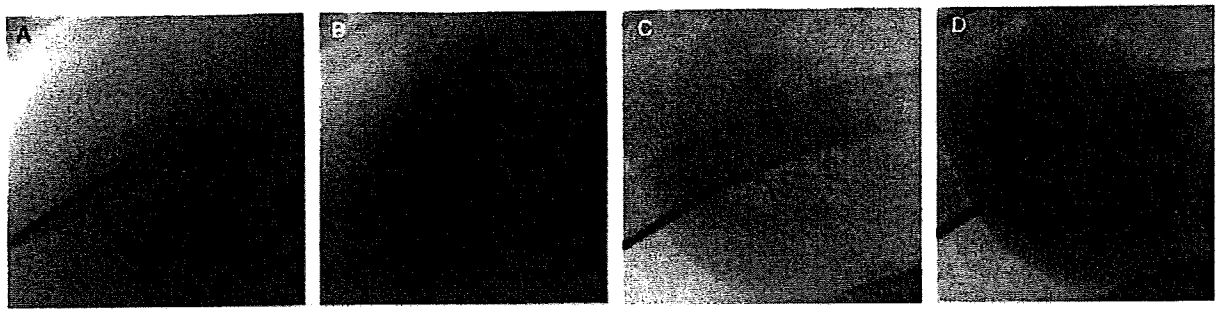

Fig. 4. Electrode tines in a pig liver during the conventional four-stepwise extension procedure. (A) First step. (B) Second step. (C) Third step. (D) Final step. At the second step, the third step and the final step, the progress of the tines' spread is smaller in comparison with that at the first step. The extent of the expansion at the final step was nearly similar to that at the second and third steps.

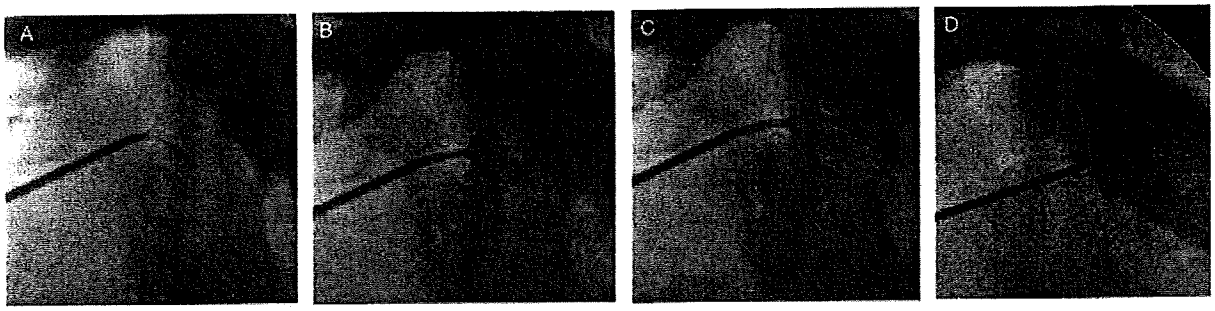

Fig. 5. Electrode tines in a pig liver during the new stepwise extension procedure. (A) First step. (B) Second step. (C) Third step. (D) Final step. At the second step, the third step and the final step, the progress of the tines' spread is smaller in comparison with that at the first step. The extent of the expansion at the final step was nearly complete.

The shape of the ablated zone depends on the needle type (6). For example, the path along the shaft is longer than the transverse diameter when using the cool-tip electrode (Radionics System; Radionics, Burlington, VT, USA), shorter when using the expandable needle of RTC system and compatible with each other when using the LeVeen needle (RTC system). The shorter path is less disadvantageous than the shorter perpendicular diameter, because the ablated zone along with the needle trace can be enlarged by repeating the procedure as the needle is extracted while that perpendicular to the tract cannot be enlarged during one insertion. Although it is often difficult to achieve roll-off during a single-step full expansion procedure using the LeVeen needle, our stepwise procedure has overcome this difficulty and produced an oval ablation zone similar to the single-step procedure.

The LeVeen needle, which had a diameter of 14 G in the first stage, has been made slender for the ease and safety of insertion into the liver. The needle now available in the market has a diameter of 17 G. The slim needle may be easier to deform during insertion and difficult to fully extend within the liver by the conventional stepwise method. The liver tissue resistance consists of resistance acting on the needle tip and that on the side. The strength of the former is proportional to the cross-section and that of the latter is to the surface area. Based on this, the slender shaft is subjected to a large stress and strain resulting in larger deformation, although its resistance is smaller. Thus, the hooks of the slim needle hardly extend as expected; it cannot be fully extended when expanded slowly as shown in Figure 4. This is because the shaft is pushed back as the electrode is inserted towards the liver. To overcome this inconvenience, we investigated a new technique: full re-expansion after stepwise extension, which allows a sharper and definite expansion of the slim needle to full length. Thus, this technique is suggested to be more advantageous in a slimmer needle; this procedure has not been examined in needles 14 or 15 G in diameter.

The additional reason for the larger ablation zone made by the new method is that the tanned tumour or parenchymal tissue would be removed from the surface of the multiple tines when they are once closed in the shaft. The tan was observed on the tip of the shaft

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when the needle was extracted from the liver. The tan adhering on the tine may prevent the uniform electric current, which results in a decrease in the electric efficiency. Thus, the removal of tan can result in an increase in the effectiveness of RF ablation procedure and that in the ablation zone.

A larger ablation zone at the final step of the new technique required a longer coagulation time and a higher input energy during the final step and during the total session; the ablation zone, ablation time and the required energy by our method were larger than those by the conventional stepwise method. The required energy per volume, on the other hand, was almost identical.

In conclusion, the new extension procedure for the expandable needle allows coagulation of larger and more oval area even when using the slim needle. This method may be useful to expand the application of RFA for hepatic tumours.

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Original Article

Diabetes mellitus reduces the therapeutic effectiveness of interferon-α2b plus ribavirin therapy in patients with chronic hepatitis C

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Aim: Patients with chronic hepatitis C (CHC) often have diabetes mellitus (DM). However, it is unknown whether DM affects patient response to interferon (IFN) plus ribavirin therapy. Therefore, the aim of this study was to examine the influence of DM on the outcome of IFN-α2b plus ribavirin therapy.

Methods: In a cohort of 110 patients with CHC, the outcome of 6 months of IFN-α2b plus ribavirin therapy was evaluated by comparing the patients with and without DM.

Results: There were 46 sustained-responders; 64 patients did not become sustained responders. Higher age ($P = 0.015$), lower platelet counts ($P = 0.036$), hepatitis C virus (HCV) serotype 1 ($P = 0.001$), advanced liver fibrosis ($P = 0.004$), and the presence of DM ($P = 0.007$) were significantly associated with not becoming a sustained-responder. Seventeen CHC (15%) patients had DM. Sex ratio, age, body mass index, alanine aminotransferase levels, HCV-RNA titer, and HCV serotypes did not significantly differ between the patients with and without DM, while fasting plasma glucose, hemoglobin A1c and liver histological staging were significantly different. On multiple logistic regression analysis, HCV serotype 1 (odds ratio 8.743, 95% confidence interval 2.215–34.517; $P = 0.002$) and the presence of DM (odds ratio 8.657, 95% confidence interval 1.462–51.276; $P = 0.014$) were independently associated with not becoming a sustained-responder.

Conclusions: The findings indicate that DM reduces the response to IFN-α2b plus ribavirin therapy in CHC patients.

Key words: chronic hepatitis C, diabetes mellitus, interferon, ribavirin

INTRODUCTION

CHRONIC HEPATITIS C (CHC) has a high prevalence worldwide. Over a period of 20–30 years, CHC progresses to cirrhosis and hepatocellular carcinoma.[1] Interferon (IFN) is often administered to treat chronic hepatitis C virus (HCV) infection, yet many patients do not eliminate the virus. Recently, therapy with IFN combined with ribavirin has been given to patients with a high viral load or who relapse; compared to IFN monotherapy, combination therapy increases the rate of sustained viral response (SVR).[2,3] However, the rate of SVR is still not high enough.

Several factors that contribute to the response to IFN therapy have been identified. A high viral load, viral genotype 1b, and the absence of mutations in the NS5A and NS5B regions in genotype 1b of HCV are associated with a lower rate of HCV clearance in patients receiving antiviral therapy.[4–7] Host factors, including older age, a higher degree of fibrosis, a longer duration of disease, and certain host genetic factors that affect IFN responsiveness, are associated with a poor response to IFN therapy.[8–14] Furthermore, in previous studies, most patients who received combination IFN-α2b plus ribavirin therapy had a high HCV viral load and were serotype 1; thus, there may be other factors that reduce the efficacy of IFN-α2b plus ribavirin therapy.

Diabetes mellitus (DM) has been associated with HCV infection, especially in patients with liver

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cirrhosis.[15] Furthermore, DM has been implicated in insulin resistance and obesity, which affect IFN effectiveness.[16–18] However, there is no evidence that DM affects the outcome of IFN-α2b plus ribavirin therapy. Therefore, we explored whether the presence of DM affects the response to IFN-α2b plus ribavirin therapy in patients with CHC.

METHODS

Patients

WE ENROLLED 110 CHC patients (male, 86; female, 24; median age, 51 years; range, 27–74 years) who were given IFN-α2b plus ribavirin therapy from December 2001 to August 2003 at our hospital. All participants were Japanese and unrelated to each other. All patients were positive for both anti-HCV antibody and serum HCV-RNA by polymerase chain reaction (PCR). Ninety-two of the patients had HCV serotype 1, and 18 had HCV serotype 2. The patients were separated based on their viral load determined by the Amplicor-Monitor assay into three groups: more than 700 KIU/mL ($n = 66$); 100–700 KIU/mL ($n = 41$); and less than 100 KIU/mL ($n = 3$). No patients had hepatitis B virus (HBV) infection, alcohol-induced liver diseases, or autoimmune liver diseases. Sixty-two patients had received prior antiviral treatment, while 48 patients had not.

Patients were classified as having DM according to criteria for the diagnosis of type 2 DM established in 1999 by the Japan Diabetes Society, which are similar to the WHO type 2 DM diagnostic criteria. In 46 patients, we evaluated glucose intolerance and insulin resistance using 75-g oral glucose tolerance test.

Liver biopsy specimens were obtained from 108 patients for histological examination: 39 had mild fibrosis; 34 had moderate fibrosis; 23 had severe fibrosis; and 12 had liver cirrhosis. Histological activity was mild in 51, moderate in 56, and severe in one. Specimens were histologically classified according to the criteria of the International Hepatitis Group.[19]

Informed consent was obtained from all patients.

Estimation of HBV and HCV markers and laboratory investigations

The presence of hepatitis B surface antigen (HBsAg) and anti-HCV antibody was determined using enzyme immunoassay kits (Dainabot, Tokyo, Japan; Kokusai-Shiyaku, Kobe, Japan). HCV-RNA was detected using the nested PCR with primers for the 5′ untranslated region of HCV. The HCV-RNA titers immediately before IFN-α2b plus ribavirin therapy that were determined using Amplicor-Monitor (Roche Diagnostics, Branchburg, NJ, USA) are expressed as kilo-international units/mL (KIU/mL).[20] The HCV serotype was determined using an enzyme immunoassay (Ohtsuka Laboratories, Tokushima, Japan). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as reported previously.[21]

Treatment schedule

All patients were treated with combination recombinant IFN-α2b and ribavirin therapy at the Ehime University Hospital from December 2001 to August 2003. Ribavirin (Schering-Plough, Osaka, Japan) was given at a daily dose of 600 or 800 mg, depending on body weight (<60 or ≥60 kg, respectively), in combination with IFN-α2b (Schering-Plough) intramuscularly every day for the first 1–4 weeks, and then three times a week for the following 20–23 weeks (total duration, 24 weeks). The starting doses of IFN-α2b were 10 MU per day in 104 patients and 6 MU per day in six patients. Ribavirin was started at 800 mg per day in 72 patients and 600 mg per day in 38 patients.

During treatment, the IFN dose was decreased in 19 patients (17%), and both IFN and ribavirin were discontinued in 17 patients (15%) due to side-effects; the ribavirin dose was decreased in 35 patients (32%), and ribavirin was discontinued without stopping IFN in three patients. Fifty-five patients (50%) completed treatment without discontinuing or decreasing the dosage of either drug.

Criteria for IFN effectiveness

All patients were followed for at least 6 months after IFN-α2b plus ribavirin therapy. Serum alanine aminotransferase (ALT) and HCV-RNA were assayed monthly during this period. Patients were categorized into two groups. Patients with an SVR (sustained-responders) were those who maintained normal ALT levels and had no detectable HCV-RNA based on PCR assays done during the follow-up period; non-responders were those patients who remained positive for HCV-RNA after IFN-α2b plus ribavirin therapy, irrespective of the HCV-RNA levels or the occurrence of relapse during follow-up.

Statistical analysis

All data are expressed as the medians. For continuous variables, the Mann–Whitney *U*-test was used. The difference in proportions was evaluated using the chi-squared test or Fisher's exact test. We assessed all

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variables using a logistic regression model. The model was simplified in a stepwise fashion by removing variables with $P > 0.05$. A value of $P < 0.05$ was considered significant. Calculations were performed using SPSS for Windows, Release 14.0 J (SPSS, Chicago, IL, USA).

RESULTS

Clinical and virological characteristics according to IFN response

FORTY-SIX PATIENTS (42%) were sustained-responders, and 64 (58%) were non-responders. The characteristics of the sustained-responders and the non-responders are shown in Table 1. Sex ratio, body mass index, fasting plasma glucose, hemoglobin (HbA1c), ALT levels, HCV-RNA titer, liver histological activity, and past history of IFN therapy were not significantly different between sustained-responders and non-responders. However, the following were significantly associated with non-response: older age ($P = 0.015$), lower platelet count ($P = 0.036$), HCV serotype 1 ($P = 0.001$), advanced fibrosis ($P = 0.004$), and the presence of DM ($P = 0.007$).

Of the 48 patients identified with fasting plasma glucose and immunoreactive insulin levels, the HOMA-IR was not significantly different between the sustained-responders ($n = 18$; median 2.63, range 0.8–13.3) and the non-responders ($n = 28$; median 2.99, range 0.6–9.5).

Background characteristics of patients with and without DM

To assess the influence of DM, we compared the clinical and virological features of patients with and without DM (Table 2). No patient with DM received drugs that improve insulin resistance. Sex ratio, age, body mass index, ALT levels, liver histological staging, HCV-RNA titer, HCV serotypes, and past history of IFN therapy were not statistically significantly different between the two groups. However, fasting plasma glucose and

Table 1 Clinical and virological characteristics of 110 patients with chronic hepatitis C treated with interferon-α2b plus ribavirin therapy based on therapeutic response

Characteristic	Sustained-responders ($n = 46$)	Non-responders ($n = 64$)	*P*-value
Sex (male/female)	35/11	51/13	NS
Age (years)	46 (32–65)	54 (27–74)	0.015
Body mass index (kg/m^2)	24.1 (17.7–31.7)	23.6 (16.6–32.4)	NS
Fastening plasma glucose (mg/dL)	92 (69–140)	96 (73–209)	NS
HbA1c (%)	4.9 (4.2–6.3)	5.0 (4.2–8.7)	NS
Alanine aminotransferase (IU/L)	68 (20–337)	72 (25–247)	NS
Platelet count (×10^4/mm^2)	16.7 (6.4–35.3)	15.0 (5–32.2)	0.036
HCV serotype			
1	32	60	0.001
2	14	4	
HCV-RNA titer			
<100 KIU/mL	3	0	NS
100–700 KIU/mL	19	22	
>700 KIU/mL	24	42	
Histological fibrosis			
Mild	23	16	0.004
Moderate	8	26	
Severe	12	11	
Cirrhosis	2	10	
Histological activity			
Mild	21	30	NS
Moderate	24	32	
Severe	0	1	
Re-treatment (+/–)	26/20	36/28	NS
Presence of DM (non-DM/DM)	44/2	49/15	0.007

Data expressed as median (range). DM, diabetes mellitus; HbA1c, hemoglobin A1c; HCV, hepatitis C virus; NS, not significant.

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Table 2 Clinical and virological characteristics of 110 patients with chronic hepatitis C treated with interferon-α2b plus ribavirin therapy based on the presence of diabetes mellitus

Characteristic	Patients with DM ($n = 17$)	Patients without DM ($n = 93$)	*P*-value
Sex (male/female)	15/2	71/22	NS
Age (years)	55 (39–74)	49 (27–69)	NS
Body mass index (kg/m^2)	23.5 (17.5–32.4)	24.0 (16.6–31.7)	NS
Fastening plasma glucose (mg/dL)	109 (93–209)	92 (69–110)	<0.001
HbA1c (%)	6.7 (5.0–8.7)	4.9 (4.2–5.8)	<0.001
Alanine aminotransferase (IU/L)	93 (39–234)	68 (20–337)	NS
Platelet count (×10^4/mm^2)	14.8 (7.8–32.2)	15.9 (5–35.3)	NS
HCV serotype			
1	14	78	NS
2	3	15	
HCV-RNA titer			
<100 KIU/mL	0	3	NS
100–700 KIU/mL	4	37	
>700 KIU/mL	13	53	
Histological fibrosis			
Mild	3	36	NS
Moderate	4	30	
Severe	6	17	
Cirrhosis	3	9	
Histological activity			
Mild	8	43	NS
Moderate	8	48	
Severe	0	1	
Re-treatment (+/–)	9/8	53/40	NS

Data expressed as median (range). DM, diabetes mellitus; HbA1c, hemoglobin A1c; HCV, hepatitis C virus; NS, not significant.

HbA1c were significantly higher in patients with DM than in those without DM (both $P < 0.001$). Furthermore, SVR was achieved in no patients with an HbA1c ≥6.9%, but was achieved in 11% of patients with an HbA1c <6.9% ($P < 0.05$). The rate of discontinuation of IFN-α2b plus ribavirin therapy due to side-effects did not differ between patients with DM (3/17, 18%) and those without DM (11/93, 12%).

Multiple logistic regression analysis of the factors affecting therapeutic outcome

To evaluate the significance of variables with respect to the outcome of IFN-α2b plus ribavirin therapy, a logistic model to assess factors related to SVR was constructed using all of the variables. The model was refined until it included only the variables independently associated with non-response (Table 3). The two variables were HCV serotype 1 (odds ratio 8.743; 95% confidence interval 2.215–34.517; $P = 0.002$) and the presence of DM (odds ratio 8.657; 95% confidence interval 1.462–51.276; $P = 0.014$).

DISCUSSION

THE PRESENT STUDY offers evidence that DM is one of the independent factors that reduces the effect of 6-month IFN-α2b plus ribavirin therapy in CHC patients. The prevalence of DM is 13–27.6% of CHC patients.[22–26] In some studies, DM patients were shown to have more advanced liver fibrosis than patients without DM,[24,25] and advanced liver fibrosis is a factor

Table 3 Multivariate analysis of the effect of variables on the response to interferon-α2b plus ribavirin therapy

Variable	*P*-value	Multivariate odds ratio (95% CI)†
HCV serotype (1 *vs* 2)	0.002	8.743 (2.215–34.517)
Presence of DM	0.014	8.657 (1.462–51.276)

†Values are the odds of having difficulty becoming a sustained-responder.
CI, confidence interval; DM, diabetes mellitus; HCV, hepatitis C virus.

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that influences the ability to achieve SVR. However, in our patients, the liver histological staging of patients with DM tended to be higher than that of patients without DM, but no significant difference was detected. In addition, the presence of DM did not affect the HCV viral load, ALT levels, or liver histological activity. Multiple logistic regression analysis revealed that the presence of DM and HCV serotype 1, but not liver histological staging, was an independent factor affecting the therapeutic efficacy.

The mechanism by which DM interferes with viral elimination in CHC patients remains unclear. Recently, it was reported that insulin resistance impaired the virological response to peg-IFN plus ribavirin treatment;[18] the insulin resistance index has been found to be an independent factor for achieving a sustained response. Insulin resistance has been shown to be caused by increased production levels of tumor necrosis factor (TNF)-α. In fact, the production of TNF-α is increased in chronic liver injury,[27] and TNF-α is one of the causes of DM in CHC patients.[28–30] A study of a mouse model transgenic for the HCV core gene revealed that HCV caused insulin resistance, and the presence of a high TNF-α level was considered to be one of the factors leading to insulin resistance in the transgenic mice.[31] Furthermore, the baseline TNF-α values in sustained-responders have been found to be significantly lower than in non-responders.[32,33] TNF-α inhibits IFN-α signaling by stimulating the expression of the suppressor of cytokine signaling proteins.[34] In this context, TNF-α is important not only for the development of DM but also for interfering with the elimination of HCV in CHC patients. Thus, in our DM patients, TNF-α could have reduced their response to IFN-α2b plus ribavirin therapy. However HOMA-IR did not differ between sustained-responders and non-responders. Our sample size is too small to evaluate the relationship between insulin resistance and the response to IFN-α2b plus ribavirin therapy. However, fasting blood glucose and HbA1c were significantly higher in DM patients than in patients without DM. In particular, no patient with an HbA1c higher than 6.9% became a sustained-responder. Thus, our results indicate that, in addition to insulin resistance, the level of hyperglycemia is an important factor that interferes with HCV elimination. Hyperglycemia causes increased production of advanced glycation end products, which induce oxidative stress and cytokines, such as TNF-α and interleukin, by combining with their receptors.[35–37] In this manner, hyperglycemia may render IFN-α2b plus ribavirin therapy ineffective.

In conclusion, our study revealed that DM and HCV serotype 1 were independent factors that reduced the rate of viral elimination in CHC patients given combined therapy. However, the mechanism responsible for the reduction of viral elimination in DM patients could not be identified. Further studies are needed to determine how the presence of DM affects viral elimination.

Not only hyperglycemic state of DM but also insulin resistance can be improved by suitable diet therapy, exercise, insulin and other hypoglycemic agents. Hepatologists who initiate antiviral therapy should consult with DM specialists and dietitians before or during antiviral therapy of CHC patients with DM. An improvement of hyperglycemia and insulin resistance can lead to good response of antiviral therapy of CHC patients with DM.

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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