Conclusions: Our multicenter cooperative survey revealed that medical procedure was the most frequent source of infection in acute hepatitis C. As concerns the therapy, interferon treatment should be initiated within 24 weeks after onset of symptoms.

Keywords: Hepatitis C virus (HCV); Acute hepatitis; Medical procedure; Interferon

1. Introduction

There are about 170 million people infected with the hepatitis C virus (HCV) worldwide [1], and the infection progresses to hepatic cirrhosis in 10–30% [1,2]. Since patients often lack subjective symptoms even in acute hepatitis C [3], infection is often realized by patients when the pathology progresses to hepatic cirrhosis and hepatocellular carcinoma. There are a variety of sources of infection, such as medical procedure, intravenous drug use, and sexual behavior [4,5]. In addition, vertical transmission of HCV has been reported, and it seems that maternal viral load is significant for infection to fetus [6]. On the other hand, as a therapy for acute hepatitis C, interferon (IFN) administration has been established to be effective [4,5,7–13].

Although the initial prevention of hepatitis C virus (HCV) infection is ideal, the most effective method of preventing progression to the chronic hepatitis C is still controversial in the acute phase. In Japan, the development of acute hepatitis C due to blood transfusion has markedly decreased after introduction of the HCV antibody test for screening of blood donors [14]. However, infection from intravenous (i.v.) drug use and incidences due to accidental contamination of medical staff are still important problems [15,16]. Investigation for the sources of infection in acute hepatitis C is very important for the prevention. In this study, we investigated a national survey on the route of infection of acute hepatitis C and the therapeutic effectiveness according to the timing of IFN therapy. This survey consists of the largest number of case reports and may reflect the current situation of acute hepatitis C in Japan.

2. Patients and methods

2.1. Patients

A retrospective study was performed in patients of 12 facilities nationwide who developed acute hepatitis C after 1990. The total number of patients at the facilities was 102. Informed written consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Age, gender, source of infection, HCV serotype or genotype, HCV-RNA level, histology of liver biopsy, fluctuation in alanine aminotransferase (ALT) level, presence or absence of IFN therapy, course when not treated with IFN, duration between onset of symptoms and IFN therapy, type of IFN, total dose of IFN, administration method, total duration of administration, and therapeutic results were investigated in each patient.

2.2. Diagnosis of acute hepatitis C

The diagnostic criteria of acute hepatitis C were HCV-RNA detectable at the time of an elevated ALT level, followed by development conversion of HCV antibody. Patients in whom HCV antibody was already positive at the onset were excluded.

2.3. Natural course

In patients who followed the natural course without any treatments, the chronic hepatitis was defined as persistence of HCV-RNA positivity for 6 months or longer, and resolution was defined as a disappearance of serum HCV-RNA within 6 months followed by persistent negativity for 6 months or longer.

2.4. Definition of fluctuation of ALT

In patients diagnosed with acute hepatitis C, when one peak of the serum ALT level was observed, the fluctuation was designated as monophasic, and when two or more peaks were observed, the fluctuation was designated as bi- or multiphasic.

2.5. Serologic tests

Anti-HCV antibody was determined using a second-generation or third-generation enzyme-linked immunosorbent assay (Ortho Diagnostics Systems, Tokyo, Japan). Hepatitis C virus RNA was quantified by using the bDNA signal amplification assay (Chiron Corp.) or the Cobas Amplicor HCV Monitor test ver1.0 or 2.0 (Roche Diagnostic Systems, Tokyo, Japan). The data were represented as Meq/ml, K copies/ml, and KIU/ml, respectively. Detection of HCV-RNA to determine the response of IFN treatment was used by Amplicor HCV (Roche Diagnostics K.K., Japan). Hepatitis C virus serotype was determined using the genotyping enzyme-linked immunosorbent assay (International Reagents Corporation, Tokyo, Japan) to be type 1 or 2 [17].

2.6. IFN therapy

For IFN, IFN-α (natural form, gene recombinant, or consensus IFN), or IFN-β was used (Table 4). No concurrent treatment with IFN and ribavirin was administered to any patient. Among patients treated with IFN, the sustained

virological response (SVR) was defined undetectable HCV-RNA in serum at least 6 months after cessation of therapy. Non-response was defined as detectable HCV-RNA for 6 months after cessation of therapy.

2.7. Statistical analysis

Data were expressed as the mean ± standard deviation for continuous variables and as counts for categorical variables. The results were compared using the Chi-square test, Fisher's exact probability test, or Mann–Whitney *U*-test, depending upon the type of data analysed. Logistic regression was used to analyse the factors contributing to SVR with IFN therapy. *P* values <0.05 were considered significant. Statistical analyses were performed by using Stat View software (version 5.0; SAS Institute Inc., Cary, NC).

3. Results

3.1. Patient characteristics

The baseline characteristics of the 102 patients in this study are shown in Table 1. The distribution of patients by gender and age is shown in Table 2.

3.2. Natural course

The natural course of the disease was followed in 21 patients, and the course could be followed to the outcome in 18 patients (the prognosis was unknown in three patients) (Table 3). The disease progressed to chronic hepatitis C in 61.1% of the patients and resolved spontaneously in 38.9% of the patients. The age and the fluctuation pattern of the ALT level were significantly different between the two groups. As for gender, serum HCV-RNA level, and serogroup, no correlation with spontaneous resolution or chronic hepatitis C was observed.

Table 1
Base-line characteristics of 102 patients

Age	38.6 ± 16.2 (16–84)
Male/female (mean age)	46 (39.2 ± 16.0)/56 (38.2 ± 16.5)
Source of infection (%)	
Medical procedure	33 (32.4)
Accidental needle stick	21 (20.6)
Sexual behavior	8 (7.8)
Drug abuse	6 (5.9)
Tattoo	3 (2.9)
Unknown	31(30.4)
Viral load (high[a]/low/N.D.)	46/45/11
HCVserotype(1/2/N.D.)	54/23/25
IFN/without IFN	81/21

N.D., not determined; IFN, interferon. Details of the routes in medical procedure: surgery 14, blood transfusion 5, endoscopy 3, intravenous injection 4, invasive procedure 3, dental therapy 3, dialysis 1.
[a] Viral load (high): more than 100 KIU/ml or 1 Meq/ml.

3.3. IFN therapy

Table 4 shows the backgrounds of the 81 patients treated with IFN. Of 71 patients in whom the effect was clarified,

Table 2
Distribution of patients according to gender and age

Age (years)	Number of patients					
	Medical procedure (M/F)	Accidental needlestick (M/F)	Sexual behavior (M/F)	Drug abuse (M/F)	Tattoo (M/F)	Unknown (M/F)
<19	0/1	0/0	0/0	0/1	0/0	0/1
20–29	5/1	3/8	1/3	2/1	3/0	2/6
30–39	4/3	3/3	2/1	0/1	0/0	3/3
40–49	2/4	0/4	1/0	0/1	0/0	2/3
50–59	4/3	0/0	0/0	0/0	0/0	2/3
60–69	4/1	0/0	0/0	0/0	0/0	2/0
70–79	0/0	0/0	0/0	0/0	0/0	1/1
>80	0/1	0/0	0/0	0/0	0/0	0/2
Total	19/14	6/15	4/4	2/4	3/0	12/19

M, male, F, female.

Table 3
Base-line characteristics of 18 untreated patients

	Resolved group (seven cases)	Chronic group (11 cases)	*P* value
Age	64.4 ± 15.2	45.6 ± 14.3	0.0331[a]
Gender (male/female)	2/5	4/7	>0.9999
HCV RNA level (high[b]/low/N.D.)	2/4/1	6/4/1	0.6084
Serogroup (1/2/N.D.)	4/0/3	4/2/5	0.4667
Fluctuation of ALT level (monophasic/bi- or multiphasic/N.D.)	5/0/2	0/8/3	0.0008[a]

N.D., not determined; ALT, alanine aminotransferase. Fluctuation of ALT level: monophasic; one peak of the serum ALT was observed, bi- or multiphasic; two or more peaks of the serum ALT were observed (N.D. was excluded from statistical comparisons).
[a] Statistically significant.
[b] Viral load (high): more than 100 KIU/ml or 1 Meq/ml.

Table 4
Base-line characteristics of 81 patients treated with interferon

Age	38.6 ± 16.2
Gender (male/female)	43/38
HCV RNA level (high[a]/low/N.D.)	38/36/7
HCV serogroup (1/2/N.D.)	46/21/14
Fluctuation of ALT level (monophasic/bi- or multiphasic/N.D.)	21/53/7
Type of IFN (α/β)	63/18
Total IFN dose (MU)	470 ± 228.1 (52–972)
Duration of IFN administration (w)	17.6 ± 8.9 (4.0–42.0)
Outcome (SVR[b]/NR/N.D.)	57/14/10

N.D., not determined; ALT, alanine aminotransferase; IFN, interferon; MU, million units; SVR, sustained virological response; NR, non-response: detectable HCV RNA in serum for 6 months after cessation of therapy.
[a] HCV RNA level (high): more than 100 KIU/ml or 1 Meq/ml.
[b] Sustained virological response: undetectable HCV RNA in serum at least 6 months after cessation of therapy.

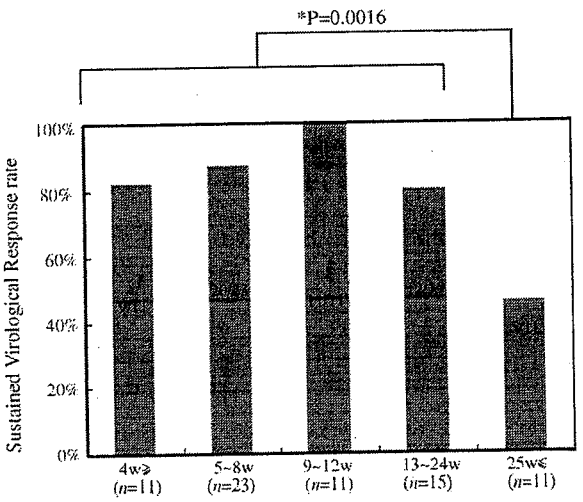

Fig. 1. Sustained virological response rate according to duration between onset of symptoms and initiation of IFN therapy. The groups treated with IFN 0–24 weeks after onset of symptoms and treated after 25 weeks were compared. Comparison by the Chi-square test. (*) Statistically significant; w, week.

57 patients (80.3%) had SVR. Table 5 shows the results of the logistic regression analysis of SVR-related factors. Age, gender, serogroup, HCV-RNA level, fluctuation of ALT, duration between onset and initiation of IFN, type of IFN, total IFN dose, and duration of IFN administration were evaluated statistically by univariate and multivariate analysis. On multivariate analysis as well as univariate analysis, the duration between onset of symptoms and initiation of IFN therapy was the only factor related to SVR.

The SVR rate according to the duration before initiation of IFN therapy was investigated (Fig. 1), and the SVR rate was found to be significantly higher in patients treated before 24 weeks than in patients treated after 25 weeks. However, immediate administration has not been associated with higher SVR rate (0–8 weeks versus 9–24 weeks).

On comparison of the SVR rate by the source of infection, the SVR rate was 100% in the patients infected by accidental needlestick (19/19) (the prognosis was unknown in two of 21 patients infected by needlestick). This was significantly higher than that in patients infected via other routes (19/19 versus 38/52, $P < 0.05$). The duration between onset of symptoms and initiation of IFN therapy was investigated according to the source of infection, and the duration was shortest in the needlestick group (9.7 ± 5.3 weeks).

4. Discussion

We examined the source of infection and optimal timing of therapy in patients with acute hepatitis C at 12 facilities in Japan. Since there has been no study performed in more than 100 patients with acute hepatitis C in Japan, this study may reflect the current situation in Japan. HCV serogroup of 25 patients were not determined (Table 1). Several reasons are considered. Firstly, the study is retrospective. Secondly,

Table 5
Logistic regression analysis of odds ratio for sustained virological response

Variable	Odds ratio	95% CI	*P* value
Univanate			
Age(40>/40≤)	2.48	0.73–8.46	0.147
Gender (female/male)	2.48	0.74–8.33	0.143
Serogroup (1/2)	1.03	0.23–4.54	0.969
HCV RNA level (high[a]/low)	1.75	0.46–6.68	0.413
Fluctuation of ALT (monophasic/bi-or multiphasic)	1.57	0.38–6.45	0.531
Duration between onset and initiation of IFN (≤24w/≥25w)	7.50	1.85–30.48	0.005[b]
Type of IFN (alpha/beta)	4.33	0.52–36.18	0.176
Total IFN dose (>300MU/≤300MU)	2.27	0.63–8.15	0.208
Duration of IFN administration (≥24w/<24w)	1.43	0.44–4.67	0.551
Multivariate			
Duration between onset and initiation of IFN (≤24w/≥25w)	15.78	1.37–181.61	0.027[b]

ALT, alanine aminotransferase; IFN, interferon; MU, million units; 95% CI, 95% confidence interval.
[a] HCV RNA level high: More than 100 KIU/ml or 1 Meq/ml.
[b] Statistically significant.

titer of anti-HCV is often low in early phase of acute hepatitis C. Many patients were considered to be infected during a medical procedure. Studies on risk of surgery for the development of acute hepatitis C have been reported previously [18]. Alfonso et al. performed a large-scale surveillance in Italy and found that 25.5% of patients (261/1023) with acute hepatitis C had undergone an invasive procedure. Therefore, medical care should be recognized as an important source of infection in the sporadic incidence of acute hepatitis C. On the other hand, in blood donors of Western Mexico, the most frequent risk factors for HCV transmission were transfusion (42%) and household exposure (14.8%) [19]. Therefore, the main risk factors for infection may differ with countries.

Since IFN therapy for acute hepatitis C is not covered by the health care insurance, the therapy could not be administered to all patients. The progression to the chronic hepatitis C in the 18 patients with natural courses without IFN therapy was almost consistent with previous reports [20,21]. As shown in Table 3, a significant difference was observed in age, but this may have been due to the two patients in their 80s in the spontaneous resolution group (data not shown). The important point is that the ALT fluctuation was monophasic in all patients in the spontaneous resolution group. In contrast, the fluctuation was bi- or multiphasic in patients who progressed to chronic hepatitis C. As a characteristic of acute hepatitis C in which spontaneous elimination of the virus is likely to occur, it has been reported that many cases are accompanied by subjective symptoms, such as jaundice and influenza-like symptoms [22,23]. Subjective symptoms are sometimes influenced by the patient's subjective sense. In contrast, the fluctuation of the ALT level may be a more objective index. Hofer et al. observed the natural course for at least 30 days after onset, and when serum HCV-RNA became negative during this period, the disease was resolved at a high rate, suggesting that IFN therapy should be administered to patients in whom negative conversion of HCV-RNA did not occur within 30 days [22]. Combined with our results, it might be likely that the disease resolves spontaneously in patients in whom the ALT level followed the monophasic course, as well as in those in whom the disease is symptomatic and negative conversion of HCV-RNA occurs in the early stage.

As the results of IFN therapy, the SVR rate was 80.3% (57/71) as shown in Table 4. Our present study, albeit retrospective analysis, revealed that therapy initiated within 24 weeks was the only factor related to the SVR in both univariate and multivariate analysis (Table 5). In the randomized controlled study by Hwang et al., the factor related to SVR was the HCV-RNA level before initiation of therapy [9]. However, there were only 33 patients, which may have led to a result different from our results. On the other hand, Nomura et al. recently performed a randomized controlled trial in patients with acute hepatitis C, and their results demonstrate that the SVR rate was significantly higher in the early-intervention group (IFN therapy was initiated 8 weeks after the onset) than in the late-intervention group (IFN therapy was initiated after 1 year observation from the onset) (87% versus 40%) [24]. Otherwise, Gruner et al. prospectively investigated the T-cell dynamics in patients with acute hepatitis C, and found that activity of HCV-specific IFN-γ-producing T cells started to decrease 24 weeks after onset [25]. In addition, T cell actions have been reported to be important for elimination of HCV in the early stage of infection [26–30], and the defective functions of HCV-specific T cells might contribute to viral persistence in chronically infected patients [31]. It is interesting that our results support their reports.

Next, we evaluated the optimal timing of initiation of therapy within 24 weeks. In our previous study, we administered therapy after observation of the course for about 4 weeks when signs of the chronic hepatitis began to appear, not immediately after the onset, and obtained good results [32,33]. Licata et al. investigated the optimum timing of IFN therapy by meta-analysis [34]. Their analysis shows that delaying therapy 2 months after the onset of the disease does not affect the efficacy of treatment, therefore, they suggest that patients should be treated within 60 days from the onset to avoid the unnecessary treatment of affected patients who would spontaneously recover. In our study, the highest SVR rate was obtained in the group treated 9–12 weeks after onset of symptoms as shown in Fig. 1, which was consistent with their analytical results.

The SVR rate obtained by combination therapy with Pegylated-IFN (Peg-IFN) and ribavirin for chronic hepatitis C was 30–54% [35–37], but for acute hepatitis C, the therapeutic result was good even when IFN was administered alone. To elucidate this difference, it may be important to investigate not only the T-cell dynamics but also viral genome in various aspects [7]. In our present study, no patients were treated with Peg-IFN. Recently, the efficacy of Peg-IFN monotherapy with acute hepatitis C has been reported. Santantonio et al. evaluated the delaying Peg-IFN therapy, targeting sixteen patients who failed to spontaneously clear the virus within 12 weeks from the onset. They reported that 15/16 patients (94%) showed SVR [38]. Since the highest SVR was obtained in the group treated 9–12 weeks after onset in our study, it is important to start the IFN therapy in optimal timing regardless of the kind of IFN. The high SVR has been obtained by IFN monotherapy, so that, it is necessary to investigate whether ribavirin should be administered concurrently with IFN.

In conclusion, the major sources of infection of acute hepatitis C in Japan were the medical procedure and accidental needlestick. The disease may be likely to resolve spontaneously in patients in whom fluctuation of the ALT level follows the monophasic course. The SVR rate was significantly higher in the group treated with IFN within 24 weeks after the onset of symptoms than in the group treated after 25 weeks. In cases of acute hepatitis C, it is desirable to administer IFN at least within 24 weeks when the ALT level starts to follow a multiphasic course.

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特集 肝細胞癌の予防治療

研究の現状

ラミブジンによる発癌予防とその理論的背景

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索引用語：HBV，ラミブジン，肝細胞癌，発癌抑制

1 はじめに

B型肝炎ウイルスに対するラミブジンの治療が導入され，その抗ウイルス効果による臨床症状の改善はめざましいものがある．肝硬変患者における黄疸や腹水など肝不全症状の改善のみならず，B型肝炎における主な死亡原因であった肝細胞癌の発生が抑制されることが報告されている．しかしながらラミブジンによる肝細胞癌抑制の機序については明らかではない．肝癌発生の機序自体も特定されていないため明確な回答を求めるのは難しいが，これまでの知見をふまえて考察してみたい．

2 B型肝炎における発癌の特徴

1．B型慢性肝疾患における発癌のパターン

わが国における肝細胞癌の原因としてはC型肝炎によるものが80％を占め，B型肝炎が関与する例は15～20％である．B型肝炎とC型肝炎からの肝細胞癌の発症は肝硬変の存在や，高齢，男性，飲酒などと関連が深いが，両者には発症の仕方に違いが見られる．C型肝炎における発癌は，ほとんどの例が肝硬変症からの発癌であり，年齢と共に発癌率が上昇していくのに対し，B型肝炎では，慢性肝炎からの発癌も多く，C型肝炎に比べ40歳以下の低年齢で発癌する例も見られる[1,2]．また，肝硬変症からの累積肝細胞癌発症率をみると，C型肝炎では直線的に増加していくのに対し，B型肝炎では，いったん上昇するものの，途中でplateau phaseが見られ，その後また上昇に転じるという3相構造を示している[3]．このことはB型肝炎の発癌には，C型肝炎と共通する因子とB型肝炎に特徴的な因子が混在していることを示している．また，C型肝炎ではウイルス型による発癌の違いは見られないが，B型肝炎では遺伝子型や，DNA量の違いで発癌の仕方が異なっており[4～7]．C型肝炎に比べてB型肝炎ウイルスの肝細胞癌発症に関わる程度が強いことを

Akihiro MATSUMOTO et al: Efficacy of lamivudine for preventing hepatocellular carcinoma in chronic hepatitis B: it's theoretical back ground
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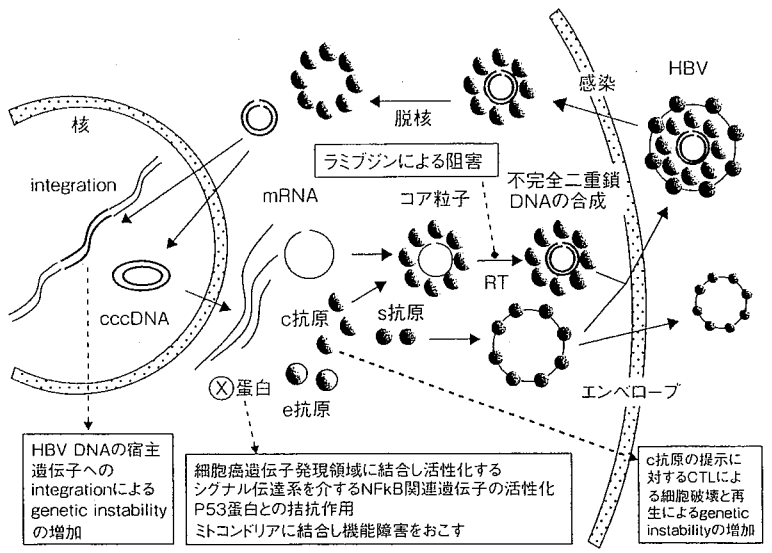

図1　B型肝炎の増殖と癌化との関連

示している．

2．B型肝炎の増殖と発癌に関わる因子

B型肝炎ウイルスと発癌の関係を考える上で，その増殖の過程(図1)を知ることは重要である．B型肝炎のウイルス粒子は，外側をS抗原を含む脂質膜のエンベロープに覆われている．その中に，コア蛋白に包まれた不完全二重鎖DNAであるB型肝炎の遺伝子がある．B型肝炎ウイルスが肝細胞に感染すると，ウイルスDNAが細胞質中へ放出され，肝細胞の核へと移行する．核に移ったB型肝炎ウイルスDNAは閉環二重鎖のcccDNAとなる．cccDNAを鋳型として4種類のmRNAが転写され，小胞体へ輸送されB型肝炎ウイルスの蛋白が翻訳される．mRNAのうち最も長いmRNAはウイルス遺伝子の全長を含み，新しいウイルスの遺伝子となるprogenomic RNAと呼ばれ，コア蛋白によって形成されるコア粒子に内包される．progenomic RNAはコア粒子の中でウイルスの逆転写酵素によりDNAに転写され，ゴルジ体においてエンベロープに包まれ，細胞外へ分泌される．これまで検討された中で，この中のいくつかの過程において癌化との関連が報告されている．これらは直接的な関連と，間接的な関連に分けられる．直接癌化との関連が指摘されているのはB型肝炎ウイルス遺伝子DNAが宿主である肝細胞の遺伝子へのintegrationを起こすことと，発現されたX蛋白による癌化の促進である．また，間接的に関連するものとしては，B型肝炎ウイルス感染細胞を宿主の免疫系が攻撃することによって，肝の破壊と再生が起こる中でgenetic instabilityが高まっていくことである．

3．B型肝炎ウイルスの直接癌化作用

B型肝炎とC型肝炎の大きな違いは，その遺伝子がDNAか，RNAであるかの違いである．これまで判明しているC型肝炎の生活史ではC型肝炎はその増殖の場は細胞質であり，遺伝子RNAは増殖に際し核へ移行することはない[8]．それに対しB型肝炎では遺伝子であるDNAは核内で二本鎖DNAとして存在し，複製されたRNAが細胞質に運ばれて

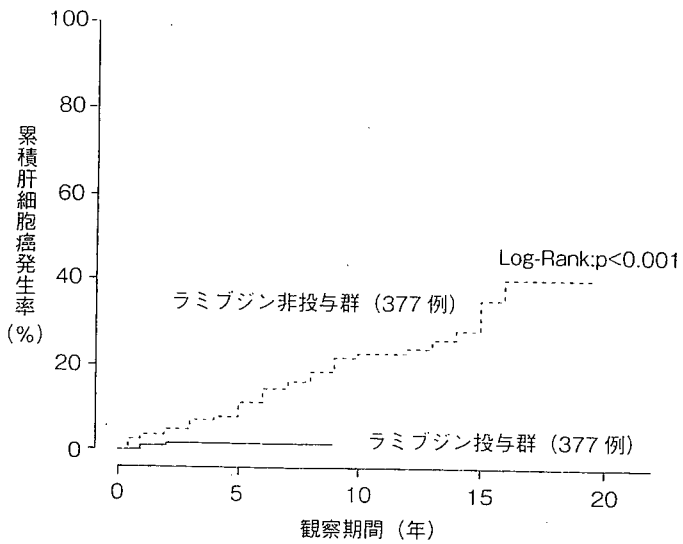

図2　ラミブジン投与群と非投与群における累積肝細胞癌発生率の比較（2003年）（文献25より改変）

B型肝炎ウイルスの持つ逆転写酵素によりDNAへ転写され，新しいウイルスとして放出される[9]．核内に存在するB型肝炎ウイルスのDNAは高率に宿主のDNAにintegrationすることが知られている[10]．特にX遺伝子を含む領域が高頻度に組み込まれることが報告されている．X遺伝子領域から翻訳されるX蛋白には細胞内のシグナル伝達を通じて発癌遺伝子を誘導したり，ミトコンドリアを障害することによって酸化ストレスを増加させる細胞障害活性を有することが報告されている[11～15]．

4．B型肝硬変と発癌

B型肝炎およびC型肝炎ではウイルスの持続感染と肝線維化の進行，肝細胞癌の発生が密接に関連している．このような慢性炎症を母体とした発癌に関してOxyradical overload diseaseという概念が提唱されている[16]．これまで行われてきた抗ウイルス療法であるインターフェロン治療では短期ではあまり効果は見られないが，1年以上の長期投与ではHBVのウイルス量を減らし，肝炎を抑えることにより肝細胞癌の発症を低下させることができるという報告がある[17～19]．ただ，インターフェロン治療においてはインターフェロン治療自体に抗癌薬のモジュレーター効果が認められるため[20]，炎症抑制以上の効果が得られていると思われる．

3　ラミブジンによる発癌抑制効果

1．長期経過における発癌抑制

ラミブジンの治療により肝発癌が抑制されるかどうかは大きな命題であった．近年それに関する報告がいくつかなされている．LiawらはB型慢性肝疾患651例に対し二重盲検法による予後調査を行い，ラミブジン投与群とプラセボ群での発癌率を比較した．その結果，肝細胞癌発生率はラミブジン投与群で3.9%，プラセボ群で7.4%（hazard ratio 0.49, $p = 0.047$）で，ラミブジン群が有意に発癌抑制していることを示した[21]．その他の報告においてもラミブジン治療は肝細胞癌の発生を

表1　Matched case control studyにおけるラミブジン投与群とコントロール群の肝線維化別発癌率

線維化 staging	ラミブジン投与群 (n = 377) HCC/ n (%/年)	コントロール群 (n = 377) HCC/ n (%年)
F0	0/ 7 (0.0)	6/ 0 (0.0)
F1	1/103 (0.4)	7/117 (1.1)
F2	1/ 95 (0.4)	8/ 97 (1.6)
F3	2/107 (0.7)	12/ 90 (2.6)
F4	0/ 65 (0.0)	23/ 67 (6.6)

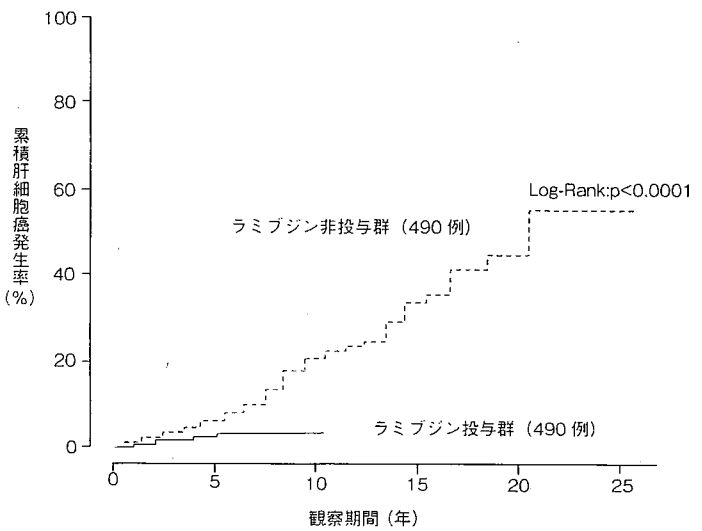

図3　ラミブジン投与群と非投与群における累積肝細胞癌発生率の比較 (2005年)

抑制することが報告されている[22]．ラミブジンの治療はB型肝炎の長期予後に良い影響を及ぼすものと思われる．われわれは，2003年の第24回犬山シンポジウムにて行われた大規模なretrospectiveな全国調査においてラミブジン投与群では非投与群に比べ有意に肝癌発症率が抑制されていることを報告した[23]．ラミブジン治療群377例と年齢，性，肝線維化，ALTおよびHBe抗原の状態をマッチさせた非治療群377例の累積肝細胞癌発生率を比較したところ，Log-rank testで有意差を認めた(図2)．肝線維化と肝細胞癌の発生の関係を見てみると，F0, F1, F2からの発癌はラミブジン治療群でも起こっているのに対し，非治療群では肝硬変からの発癌が多い(表1)．症例数は少ないものの，ラミブジン治療群で肝硬変からの発癌がより強く抑制されているのが特徴である．さらに，2005年の第25回犬山シンポジウムにおいて，追跡調査を行ったところ，同様の解析でp<0.0001という有意差が示された(図3)．年率の肝細胞癌発生率をみると，2003年の調査ではラミブジン治療群で0.4%/人/年，非治療群では2.5%/人/年であり，2005年の調査では各々0.9%/人/年，4.9%/人/年とより顕著な差が見られ，ラミブジンに発癌

抑制効果があることが示された．

4 ラミブジンによるHBV病態の変化と発癌抑制

1．ラミブジンによる病態の変化

それでは，ラミブジンの投与がどのように発癌率に影響を与えるのであろうか．ラミブジン治療を行うと，血液中のHBV DNA量が低下しそれに伴ってALTが改善される[24,25]．さらにその状態が続くと肝の線維化が改善されることが報告されている[26,27]．しかしながらラミブジンのB型肝炎ウイルスの増殖阻害作用はコア粒子中のHBV RNAをDNAに変換するB型肝炎ウイルス由来の逆転写酵素の阻害であり，肝細胞中のcccDNAは残存しており，ウイルス蛋白産生能は維持されていると思われる(図1)．われわれの開発したHBコア関連抗原測定系を用いて，血中のウイルス蛋白の動態を見てみると，血液中のHBV DNAがラミブジン治療開始とともに速やかに減少するのに対してコア関連抗原の下がりは緩徐である[28]．したがって，ラミブジン投与後もウイルス増殖能は投与開始前よりは低いが保たれており，ラミブジンの投与を中止するとHBV DNAは再上昇し，肝炎が再燃する[24]．YMDD変異株が出現し，ラミブジン耐性となった場合もHBV DNAが上昇し，肝炎の再燃が見られる[29]．この現象から考えると，HBV DNAの低下に伴ってALTが低下するのは，肝細胞への再感染が減少するためと思われるが，ウイルスの再感染と肝障害の出現の関係については現在のところ不明であり今後の検討が必要である．ただ，Ikedaら[7]が報告しているように，インターフェロン長期療法でも発癌抑制効果が認められており，この場合，非発癌例では発癌例に比べて明らかに血中HBV DNAレベルが低いという．このことから，HBVの発癌抑制にはHBV DNAの抑制が重要であることが示唆される．

2．ラミブジンの発癌抑制に関わる因子

ラミブジンは宿主の遺伝子複製に対する影響はなく，直接的な抗腫瘍効果は期待できない．また，ラミブジンはintegrationされたHBVには影響を与えないことが報告されており[30]，B型肝炎ウイルスの直接的な癌化促進作用をとめることはできないと思われる．ラミブジン治療による発癌抑制の最も考えられる機序としては，B型肝炎ウイルスDNAを抑制することにより肝炎を抑制し，肝細胞の破壊と再生によるgenetic instabilityの増加を抑えることにあると思われる．この考え方は，慢性肝炎例からの*de novo*の発癌は抑制できないが，酸化ストレスの蓄積による肝硬変症からの発癌が減少しているという現象をよく説明している．しかしながら肝細胞癌発症機序の解明と合わせて，今後さらなる検討が必要であると思われる．

5 おわりに

B型肝炎の治療は経口の抗ウイルス剤であるラミブジンの登場により大きく変貌した．耐性株の出現や，胎児に対する安全性などの問題が残っているが，他の抗ウイルス剤の併用などにより，より安全かつ効果的な治療が行われ，将来のB型肝炎患者からの発癌を減少させることができるようになることを願ってやまない．

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Cell-based models of sustained, interferon-sensitive hepatitis C virus genotype 1 replication

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Abstract

We have previously reported hepatitis C virus (HCV) replication using a novel binary expression system in which mammalian cells were transfected with a T7 polymerase-driven full-length genotype 1a HCV cDNA plasmid (pT7-flHCV-Rz) and infected with vaccinia-T7 polymerase. We hypothesized that the use of replication-defective adenoviral vectors expressing T7 (Ad-T7pol) or cell lines stably transfected with T7 (Huh-T7) would alleviate cell toxicity and allow for more sustained HCV replication.

CV-1, Huh7, and Huh-T7 cells were transfected with pT7-flHCV-Rz and treated with Ad-T7pol (CV-1 and Huh7 only). Protein and RNA were harvested from cells on days 1, 2, 3, 5, 7, and 9 post-infection. No cytotoxicity was observed at 9 days post-infection in any cell type. HCV positive- and negative-strand RNA expression were strongest during days 1–3 post-infection; however, HCV RNA remained detectable throughout the 9-day observation period. Furthermore, transfection with a replication-incompetent plasmid suggested that efficient HCV replication is dependent upon NS5B gene expression. Finally, after 1–2 days of IFN treatment, HCV positive-strand levels decreased significantly compared to HCV-infected but untreated samples ($p < 0.05$).

In conclusion, these refined binary systems offer more durable and authentic models for identification of host cellular processes critical to HCV replication and will permit longer-term analysis of virus–host interactions critical to HCV pathogenesis and the treatment of genotype 1 infections.

Keywords: Hepatitis C virus; HCV; Replication; Genotype 1; Adenovirus vector; Huh-T7

1. Introduction

Hepatitis C virus (HCV) is a leading cause of chronic liver disease, including hepatitis, cirrhosis, and hepatocellular carcinoma (Alter et al., 1999). The combination of interferon (IFN) and ribavirin (RBV) is the standard treatment for chronic HCV infection; however, their effectiveness remains limited (McHutchison and Poynard, 1999). The lack of a full-length HCV tissue culture model has limited not only the ability to screen novel antiviral agents but also the ability to precisely characterize the antiviral effect of IFN, particularly against genotype 1 infections.

We recently reported successful cell-based HCV replication using a novel binary expression system in which mammalian cells were transfected with a T7 polymerase-driven full-length genotype 1a HCV cDNA plasmid and infected with a recombinant vaccinia vector encoding T7 polymerase (Chung et al., 2001). However, HCV replication driven by vaccinia-based vectors is restricted to short-term studies due to the cytotoxic

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effects of vaccinia. Moreover, vaccinia encodes two proteins, E3L (Chang et al., 1992; Watson et al., 1991) and K3L (Carroll et al., 1993; Gale et al., 1996), that act as potent inhibitors of the IFN-induced double-stranded RNA-activated protein kinase (PKR). Due to these limitations, we sought to further refine our HCV replication model using alternative, less disruptive modes of T7 polymerase delivery.

We hypothesized that the use of replication-defective adenoviral vectors expressing T7 or cell lines stably expressing T7 would alleviate cell toxicity and allow for more sustained HCV replication.

Recombinant replication-defective adenoviral vectors have comparable infectivity to vaccinia vectors. These adenoviral vectors cannot replicate inside infected cells, because they lack the E1A and E1B proteins necessary for viral vector replication. Moreover, these vectors lack the E3 gene that inhibits immune responses by interacting with cytoplasmic MHC class-I molecules (Wold and Gooding, 1989). Thus, adenoviral vectors maintain infectivity and protein delivery with minimal cytotoxicity. By transfecting the HCV cDNA construct into Huh7 cell lines stably expressing T7 polymerase (Huh-T7) (Schultz et al., 1996), the need for viral delivery systems was removed altogether. Using these alternative delivery methods, we have established a refined HCV replication model that produces more sustained viral RNA replication, leads to less perturbation of host genes, and represents a more authentic system for studying virus–host interactions relevant to HCV pathogenesis. These refined models were also utilized to characterize the antiviral kinetics of IFN on HCV replication.

2. Materials and methods

2.1. Cell lines

CV-1 cells (American Type Culture Collection, Manassas, VA) and Huh7 (Dr. Robert Lanford, Southwest Foundation for Biomedical Research) and Huh-T7 (Dr. Stanley Lemon, University of Texas) (Schultz et al., 1996) were maintained in Dulbecco's modified Eagle medium containing 10% fetal bovine serum.

2.2. Plasmids and transfection-infection

The binary replication system has been described previously and is capable of successful positive-strand and negative-strand HCV RNA synthesis, efficient HCV protein production, and quasispecies generation (Chung et al., 2001; Contreras et al., 2002). Briefly, a plasmid containing the infectious full-length genotype 1 cDNA sequence corresponding to the H77 prototype strain (Yanagi et al., 1997) was adapted at its 5′ and 3′ termini with the T7 promoter and a hepatitis delta virus ribozyme sequence, respectively, to yield pT7-flHCV-Rz (hereafter referred to as H77). As a negative control, a mutant plasmid in which the GDD active site of the NS5B RNA-dependent RNA polymerase (RdRp) was mutated to AAG (hereafter referred to as $H77_{GDD \rightarrow AAG}$) was generated by site directed mutagenesis (Quick Change; Stratagene; La Jolla, CA). This substitution is associated with replication-incompetence in replicon models (Blight et al., 2000). H77 and $H77_{GDD \rightarrow AAG}$ were used to transfect CV-1, Huh7, or Huh-T7 cells at 70% confluency on 6-well plates with Lipofectamine (Invitrogen, Carlsbad, CA). Plasmids were transfected at concentrations of 1 μg/well for CV1 cells and 3 μg/well for Huh7 and Huh-T7 cells. Transfection efficiency was assessed by co-transfection with 0.1 μg/well of phRL-TK (Int^-) (Promega, Madison, WI) and luciferase activity quantified using the Dual-Luciferase reporter assay system (Promega). For CV-1 and Huh7 cells, T7 polymerase was delivered using a recombinant vaccinia virus vector (vTF7-3) (Fuerst et al., 1986) or a recombinant adenovirus vector (Ad-T7pol) 24 h after H77 transfection. In control experiments, a replication-defective adenovirus vector lacking the T7 polymerase gene (Ad-Psi5) was used. Adenoviral vectors were provided by the Harvard Gene Therapy Initiative's Viral Vector core (Boston, MA).

2.3. X-gal staining of pOS8-transfected cells

To compare the transfection and infection efficiency of the vaccinia and adenovirus vectors, the pOS8 plasmid, which contains a T7 promoter flanking the β-galactosidase gene, was co-transfected into cells. After 48 h, cultured cells were washed with PBS, fixed with 0.25% glutaraldehyde for 1 h at 4 °C, and stained with 0.1% 5-bromo-5-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as described previously (Hiasa et al., 1998; Miyake et al., 1996).

2.4. Interferon experiments

Interferon alpha 2b was obtained from Schering Plough (Kenilworth, NJ). For CV-1 and Huh7 cells, 100–1000 IU/mL of IFN was added 5 h after infection with adenovirus vector. For Huh-T7 cells, 100–1000 IU/mL of IFN was added 5 h after transfection with H77. Medium with or without IFN was changed at day 1 post-infection and every 2 days thereafter.

2.5. Cellular RNA extraction and qualitative strand-specific rTth RT-PCR

Cells were washed three times with phosphate-buffered saline. RNA was extracted using TRIzol (Invitrogen; Carlsbad, CA), and treated two times for 4 h with DNase I using the DNA-free kit (Ambion; Austin, TX) following the manufacturer's protocol. RNA was quantified by UV spectrum analysis, and adjusted to 0.3 μg/μL. HCV RNA was detected utilizing a previously described qualitative strand-specific r*Tth* reverse transcription PCR (RT-PCR) assay (Castet et al., 2002; Lanford et al., 1995). For detection of negative-strand HCV RNA, 1 μg of RNA in 10 μL of dietyl pyrocarbonate-treated water was layered with mineral oil and heated at 95 °C for 1 min, and lowered to 70 °C. A 20 μL mixture containing 10 pM of HCV-II sense primer (5′-CAC TCC CCT GTG AGG AAC T-3′, nucleotides [nt] 38–56 of the 5′UTR) (Laskus et al., 1997), 1× RT buffer (Applied Biosystems; Foster City, CA), 1 mM $MnCl_2$, 200 μM (each) deoxynucleoside triphosphate, and 5 U of r*Tth* enzyme

(Applied Biosystems) was then added. The temperature was dropped to 60 °C for 2 min for annealing and then raised to 70 °C for 20 min for the cDNA reaction. To inactivate the RT activity of r*Tth*, chelating buffer (Applied Biosystems) was added. Forty microliters of the prewarmed PCR mixture containing 10 pM of HCV-I antisense primer (5′-TGG ATG CAC GGT CTA CGA GAC CTC-3′, nt 342–320 of the 5′UTR) (Laskus et al., 1997) and 3.75 mM $MgCl_2$ was added. Twenty-five cycles of PCR (94 °C 15 s, 58 °C 30 s, 72 °C 30 s) were performed.

For GAPDH measurements, RT was carried out using an oligo $d(T)_{16}$ primer under standard conditions (Hiasa et al., 2003). The cDNA product was subjected to 25 cycles of PCR (95 °C 1 min, 60 °C 2 min, 73 °C 2 min), using 50 pM of the GAPDH sense and antisense primers (forward primer 5′-GAA GGT GAA GGT CGG AGT-3′, reverse primer 5′-GAA GAT GGT GAT GGG ATT TC-3′), 0.1 mM of each dNTP, 2.5 mM $MgCl_2$, and 0.5 U *Taq* polymerase. Reaction products were separated on 1.5 % agarose gels.

To ensure efficient removal of plasmid DNA after DNase I treatment, a qualitative PCR was performed. The plasmid DNA was completely digested as no PCR products were observed using this approach.

2.6. RNase protection assay

Antigenomic HCV RNA was detected as described previously (Chung et al., 2001). Briefly, utilizing the sense-oriented [α-^{32}P] UTP-labeled probe (corresponding to 98 nucleotides of the 3′ terminal HCV genome), antigenomic RNA was generated by in vitro transcription using T7 polymerase from the vector pHCV-3′T (Chung and Kaplan, 1999). Transcripts were generated using the RPA III kit according to the manufacturer's instructions (Ambion).

2.7. Real-time quantification of HCV positive- and negative-strand RNA

Positive- and negative-strand HCV RNAs were quantified by real-time PCR using LightCycler technology (Roche Diagnostics, Mannheim, Germany) and SYBR green I dye as described previously (Blackard et al., 2005). One microgram of RNA was used for cDNA synthesis in a mixture containing 5 U of r*Tth* and 10 pM of the appropriate RT primer (HCV-I for positive-strand HCV RNA or HCV-II for negative-strand HCV RNA). cDNA was purified with the High Pure PCR product purification kit (Roche Diagnostics).

Positive- and negative-strand HCV PCR amplifications were performed with 2 μL of purified cDNA in a reaction mixture containing 1 μL of LightCycler Fast Start DNA Master SYBR Green I, 4 mM of $MgCl_2$, and 5 pM of antisense primer KY78 (5′-CTC GCA AGC ACC CTA TCA GGC AGT-3′, nt 311–288 of the 5′UTR) and 5 pM of sense KY80 (5′-GCA GAA AGC GTC TAG CCA TGG CGT-3′, nt 68–91 of the 5′UTR). The PCR consisted of an initial denaturation step of 10 min at 95 °C, followed by 40 cycles of the following thermal conditions: 15 s at 95 °C, 5 s at 70 °C, and 15 s at 72 °C. All samples were analyzed in triplicate. The sensitivity of the PCR for HCV was previously determined to be approximately 230 copies HCV/μL.

For quantification of GAPDH mRNA, RT was performed with the same amount of RNA used for HCV positive- and negative-strand analysis, using the oligo $d(T)_{16}$ primer under standard conditions. For real-time PCR amplification of GAPDH, a commercial GAPDH primer set (Roche Search LC, Mannheim, Germany) was used under the recommended conditions. For real-time PCR amplification of LacZ, sense (5′-GCC TGC GAT GTC GGT TTC CGC GAG G-3′) and antisense primers (5′-GCC AGC GCG GAT CAT CGG TCA GAC G-3′) were utilized under the following conditions: 10 s at 95 °C, 10 s at 68 °C, 16 s at 72 °C (Dobson et al., 1990). The sensitivity of detection was approximately 210 copies/μL.

DNA was quantified measuring SYBR green I dye incorporation into PCR products at 530 nm following manufacturer's instructions. An HCV standard curve was generated using a PCR product corresponding to nucleotides 38–342 of the 5′UTR. At the end of each run, a DNA melting curve was performed to control for sample homogeneity and quality. In a subset of samples, electroporation and sequencing were performed to confirm the correct identity of the amplified PCR product. Data were expressed as the copy number of HCV positive-strand (or negative-strand) RNA per molecule of GAPDH. This analysis was done in quadruplicate for each sample and presented as the mean and standard deviation. Each value was analyzed statistically using the SPSS 10.0 software (SPSS, Chicago, IL). Differences in mean values were compared using the Mann–Whitney *U*-test.

2.8. Western blotting analysis

Cells were washed twice with PBS, and lysed with 100 μL of Nonidet P-40 buffer (0.5% Nonidet P-40, 10 mM Tris, pH 7.4, 150 mM NaCl, 1% SDS). Protein lysate concentrations were measured using the DC protein assay Kit (Bio-Rad, Hercules, CA). Forty microliters of protein lysate were utilized. Separated products were then blotted onto Immobilon-P membranes, and each membrane was incubated with the relevant antibody. The ECL Kit (Amersham Pharmacia, Buckinghamshire, UK) was used for detection. Monoclonal antibody to HCV core protein (515s) (Kashiwakuma et al., 1996) was provided by Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan; monoclonal antibody to β-galactosidase was purchased from Promega. Appropriate species-specific conjugated secondary antibodies were obtained commercially (Amersham Pharmacia).

2.9. ELISA for HCV core antigen

Cell culture lysates were adjusted to 0.2 mg/mL. HCV core antigen concentrations were quantified using the HCV core protein ELISA kit (Ortho-Clinical Diagnostics, Raritan, NJ) following the manufacturer's instructions (Bouvier-Alias et al., 2002). Core ELISA data were expressed as fmol of HCV core antigen per μg of total protein. The lower level of detection for this assay was less than 1.5 pg/mL.

3. Results

3.1. Replication-defective adenoviral vectors successfully replicate HCV RNA without cytotoxicity

To compare the transfection and infection efficiency of the vaccinia and adenovirus vectors, the pOS8 plasmid, which contains a T7 promoter flanking the β-galactosidase gene, was transfected into cells that were then infected with either vaccinia-T7 (vTF7-3) or Ad-T7pol at a multiplicity of infection (MOI) of 10. The parental replication-incompetent vector Ad-Psi5 was used as an adenoviral vector control. At an MOI of 10, each viral vector efficiently expressed β-galactosidase in approximately 50% of cells 24 h after infection (Fig. 1A). Using trypan-blue staining, cell injury was observed in cells transfected with vaccinia-T7 but not in cells transfected with the Ad-T7pol or Ad-Psi5 vectors (data not shown).

Ribonuclease protection assay for negative-strand HCV RNA (Fig. 1B) and Western blotting for HCV core protein (Fig. 1C) were performed 24 h after infection. Expression of negative-strand HCV RNA was lower after Ad-T7pol infection compared to vTF7-3 infection, yet was clearly detectable. Similarly, HCV

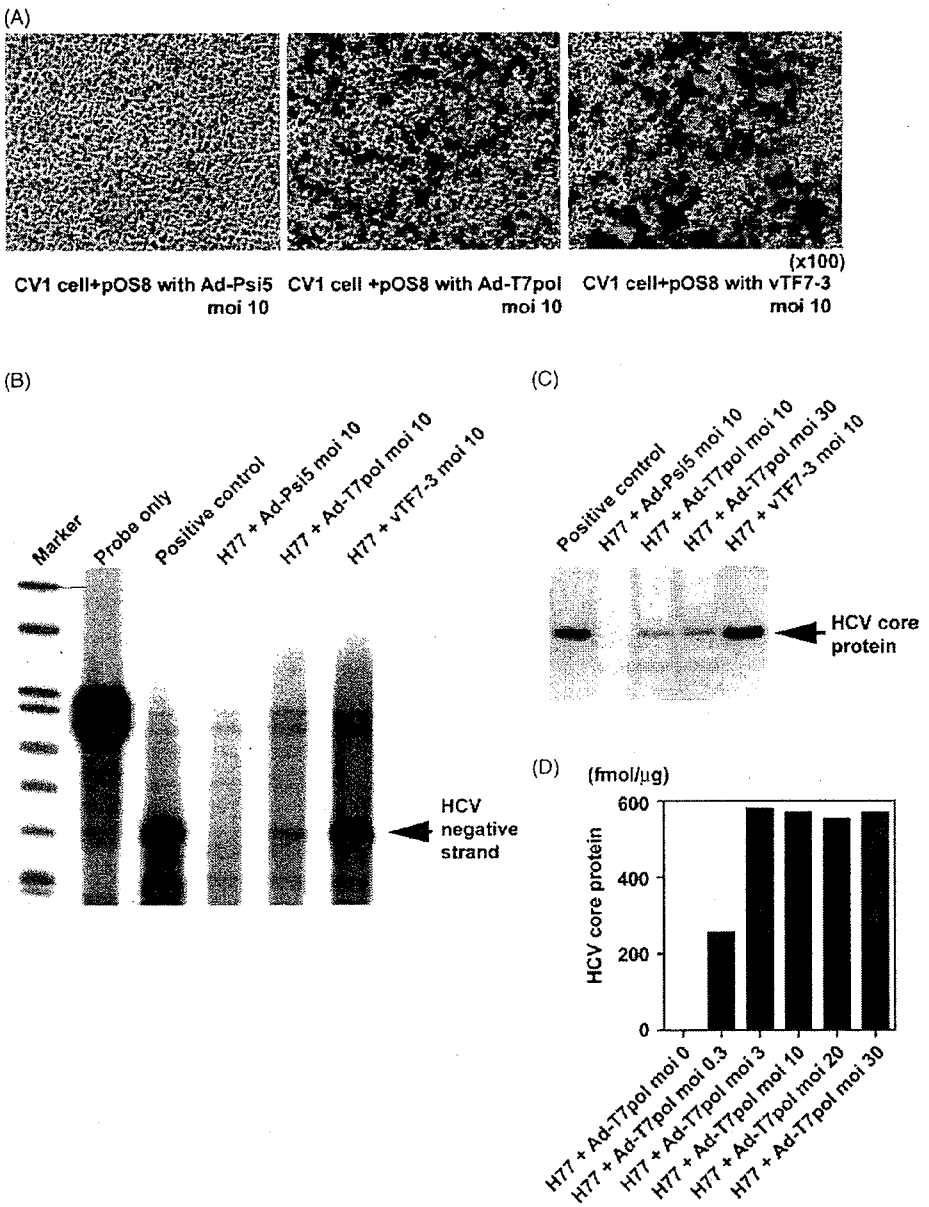

Fig. 1. Comparison of the transfection and infection efficiency of HCV replication system using vaccinia-T7 and adeno-T7 vectors: (A) after transfection with the pOS8 plasmid, cells were infected with virus vectors using control adenovirus (Ad-Psi5), recombinant adeno-T7 polymerase (Ad-T7pol), or vaccinia-T7 polymerase (vTF7-3) at an MOI of 10. (B) RPA for negative-strand HCV RNA was performed with H77 plasmid as a positive control. (C) Western blotting for HCV core protein was performed on CV-1 cell lysates 24 h after infection. (D) Quantitative HCV core ELISA results indicated that an MOI of 10 was optimal for adenoviral-driven HCV protein production.

core protein production was less robust after Ad-T7pol infection; nonetheless, it was clearly detectable. Negative-strand HCV RNA and HCV core protein were not detected when the control Ad-Psi5 vector was used. Quantitative HCV core ELISA results suggested that an MOI of 10 was optimal for adenoviral-driven HCV protein production (Fig. 1D); therefore, an MOI of 10 was selected for all subsequent experiments.

In contrast to increased HCV RNA synthesis and protein production in transfected/infected cells, LacZ mRNA levels decreased rapidly after day 1 and were not detectable after day 7 (data not shown).

3.2. Adenoviral-T7-driven HCV replication is dependent on an intact HCV polymerase gene

The $H77_{GDD \rightarrow AAG}$ mutant (Fig. 2A) was used to assess whether the HCV RNA polymerase gene (NS5B) was necessary for viral replication. By qualitative RT-PCR of the 5′UTR, HCV negative-strand synthesis was detectable only in the presence of both H77 and Ad-T7pol (Fig. 2B) in CV-1 cells. The absence of detectable negative-strand HCV RNA upon transfection of the mutant plasmid ($H77_{GDD \rightarrow AAG}$) indicates that replication was dependent on an intact polymerase sequence. H77 + Ad-T7pol expressed significantly higher core protein levels compared to $H77_{GDD \rightarrow AAG}$ + Ad-T7pol (Fig. 2C), further suggesting an intact polymerase sequence is necessary for robust HCV protein production. Ribonuclease protection assay demonstrated the presence of HCV negative-strand in CV-1, Huh7, and Huh-T7 cell lines on day 2 (Fig. 2D). However, negative-strand HCV RNA was not detected when the $H77_{GDD \rightarrow AAG}$ mutant was transfected, indicating that an intact NS5B sequence was necessary for negative-strand HCV RNA synthesis.

3.3. Kinetic analysis of HCV RNA synthesis and core protein production

The data described above suggest that the Ad-T7pol replication system results in efficient HCV RNA and protein

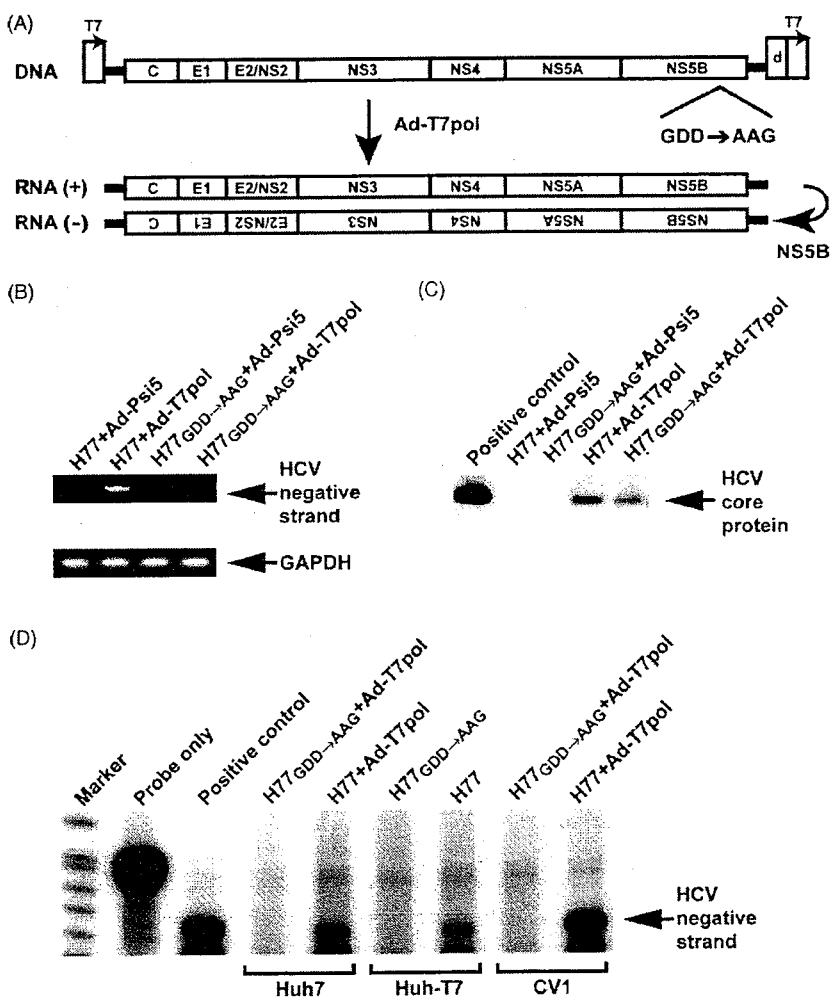

Fig. 2. (A) A control plasmid was prepared by mutating the active site motif from GDD to AAG in the NS5B RNA-dependent RNA polymerase sequence ($H77_{GDD \rightarrow AAG}$). (B) A qualitative strand-specific RT-PCR for negative-strand HCV RNA was performed as previously described (Lanford et al., 1995). (C) Western blotting analysis demonstrated that transfection/infection with H77 + Ad-T7-pol also resulted in HCV core protein production. (D) Ribonuclease protection assay demonstrated detectable negative-strand HCV RNA in CV-1, Huh7, and Huh-T7 cell lines on day 2.

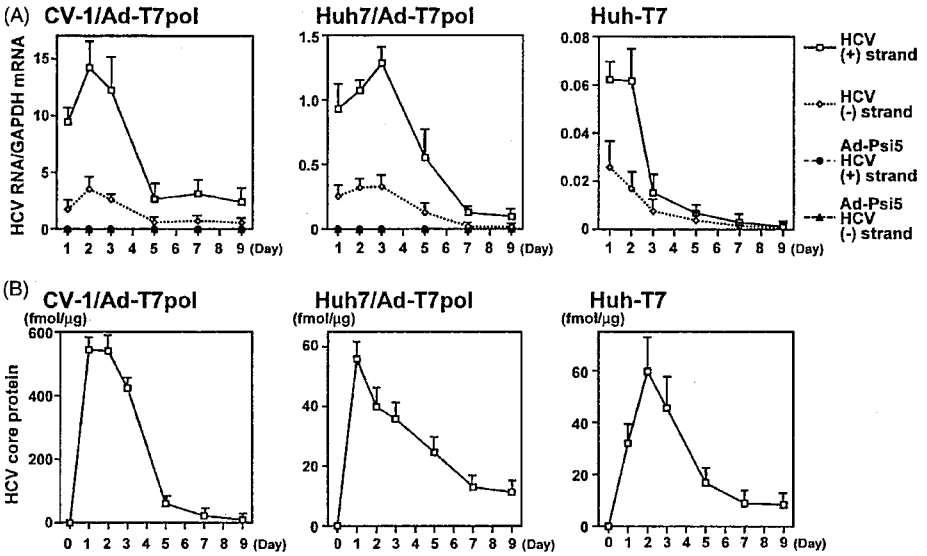

Fig. 3. (A) Real-time PCR of HCV positive- and negative-strand RNA was performed as described. Data are expressed as the strand-specific HCV copy number per molecule of GAPDH. Error bars indicate the mean ± standard error (S.E.) of four replicates. (B) A quantitative core ELISA measured HCV protein production in CV-1, Huh7, and Huh-T7 cell lines. Data are expressed as fmol (mean ± S.E.) of HCV core per μg of total protein for three replicates.

expression without inducing cell toxicity. Thus, the time course of strand-specific HCV RNA synthesis (Fig. 3A) and HCV protein production (Fig. 3B) was examined further in CV-1 and Huh7 cells (transfected with H77 and infected with Ad-T7pol), as well as Huh-T7 cells (transfected with H77). Both positive- and negative-strand HCV RNA were detectable for the entire 9-day experiment in each cell line. Positive-strand HCV RNA levels increased significantly after infection and continued to be expressed at high levels for 3 days and diminished thereafter. Negative-strand HCV RNA synthesis paralleled that of positive-strand throughout the time course; however, the quantity of negative-strand HCV RNA was approximately 10% of positive-strand levels. This is consistent with positive-/negative-strand ratios reported from infected human liver samples (Komurian-Pradel et al., 2004; Laskus et al., 1998). As expected, HCV RNA was not detected in CV-1 or Huh7 cells infected with the Ad-psi5 control vector.

Using an identical experimental approach, HCV core protein production was expressed strongly during days 1–3 in all cell lines examined, and diminished with similar kinetics as HCV RNA (Fig. 3B). Similar to HCV RNA, HCV core protein was detectable for the entire 9-day experiment in each cell line.

To circumvent potential perturbations in the cellular environment due to transfection/infection with viral vectors, experiments in a Huh7 cell line stably transfected with T7 polymerase (Huh-T7) were performed. After transfection of H77 into these cells, positive- and negative-strand HCV RNA were detected (Fig. 3A), as well as HCV core protein (Fig. 3B), throughout the entire 9-day experiment. Interestingly, HCV RNA levels were lower in Huh-T7 cells than in CV-1 and Huh7 cells, although core levels were not appreciably different between Huh7 and Huh-T7 cells.

3.4. IFN efficiently inhibits HCV expression

Utilizing these refined models of HCV replication, the inhibitory effects of IFN on HCV expression were examined. To determine the potential effects of IFN on cellular gene translation, the plasmid OS8 was transfected, and LacZ mRNA levels were measured in the presence of several doses of IFN. LacZ mRNA expression was slightly reduced; however, no significant toxicity in cells exposed to IFN was observed using trypan-blue staining (data not shown).

In CV-1 cells (Fig. 4A), HCV positive-strand RNA levels were significantly decreased in the presence of 1000 IU/mL IFN at day 2 (14.21 ± 3.95 versus 8.55 ± 0.61, $p < 0.05$). A trend toward reduced HCV RNA was also observed on day 3 (12.23 ± 5.43 versus 4.13 ± 0.74, $p < 0.10$). In Huh7 cells, a significant decrease of HCV positive-strand was observed on days 2 (1.07 ± 0.07 versus 0.63 ± 0.14, $p < 0.05$) and 3 (1.29 ± 0.13 versus 0.44 ± 0.15, $p < 0.05$). In Huh-T7 cells, a significant decrease was also observed on day 2 (0.06 ± 0.03 versus 0.03 ± 0.007, $p < 0.05$). For HCV negative-strand RNA (Fig. 4B), only day 3 IFN-treated CV-1 cells had significantly decreased levels compared to untreated cells (2.60 ± 0.41 versus 0.97 ± 0.31, $p < 0.05$).

HCV core protein expression was approximately 10-fold higher in CV-1 cells compared to either Huh7 or Huh-T7 cells (Fig. 4C). IFN treatment of CV-1 cells did not appear to have a large effect on HCV core protein levels; however, HCV core levels were decreased in IFN-treated CV-1 cells compared to untreated cells at days 3 (423.51 ± 25.73 fmol/μg versus 190.92 ± 35.25 fmol/μg, $p < 0.05$) and 5 (60.24 ± 12.89 fmol/μg versus 34.15 ± 0.76 fmol/μg, $p < 0.05$). For Huh7 and Huh-T7 cells, HCV core expression was significantly reduced when treated with IFN compared to untreated cells at all time points ($p < 0.05$).

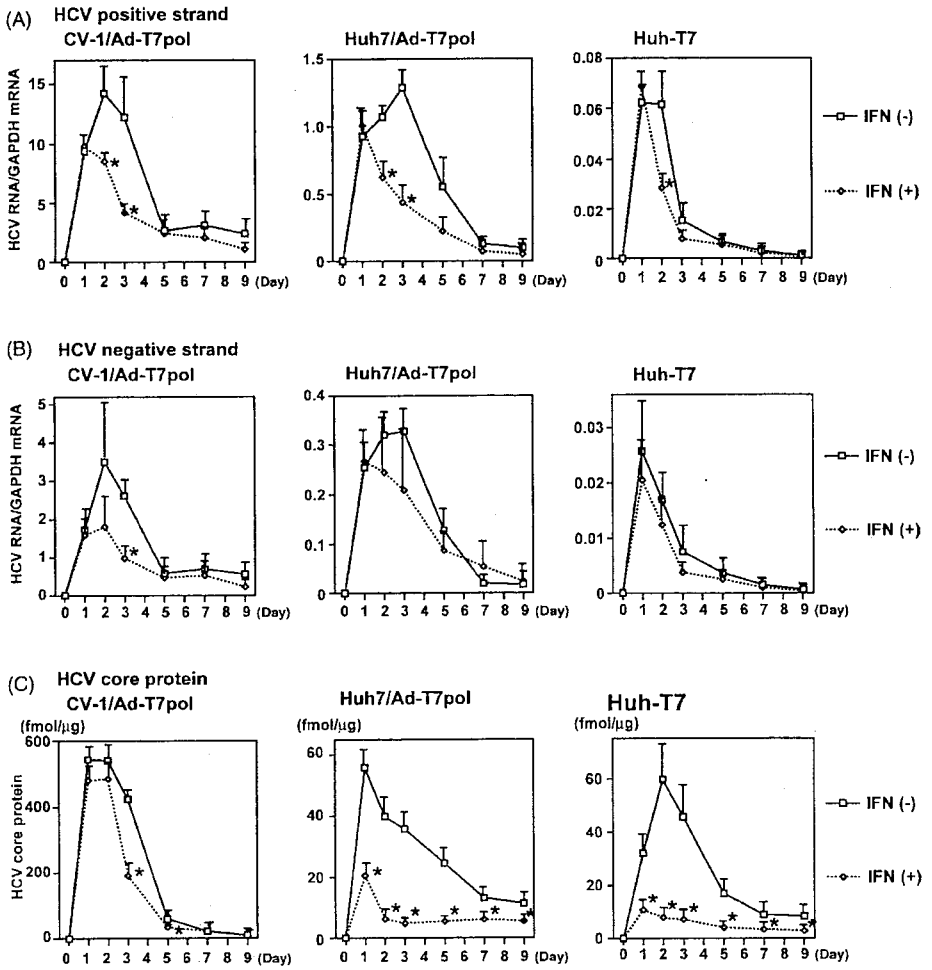

Fig. 4. (A) Real-time PCR of positive-strand HCV RNA was performed in the presence of 1000 IU/mL IFN. Error bar indicates mean ± S.E. for four replicates ($^{*}p < 0.05$; $^{**}p < 0.10$). (B) Real-time PCR of negative-strand HCV RNA was also performed in the presence of 1000 IU/mL IFN. (C) Quantitative core ELISA also demonstrated decreased HCV protein production in IFN-treated cells.

4. Discussion

Because of the cytotoxic nature of vaccinia virus, long-term evaluation of HCV RNA synthesis and protein production, as well as characterization of the inhibitory effects of antiviral agents, such as IFN and RBV, was not possible using our previous replication model. By using adenovirus-derived T7 vectors, vaccinia-induced cytotoxicity was removed, allowing sustained detection of HCV replication and protein production for 9 days in multiple cell types. The refined binary HCV replication system efficiently synthesized HCV negative-strand RNA, an important indicator of ongoing, active viral replication, in an NS5B-dependent manner, as no negative-strand HCV RNA was detected upon transfection of an NS5B mutant. Using Huh7 cell lines stably expressing T7 polymerase (Huh-T7), dependence of the replication models on any viral vectors was removed. Quantities of HCV RNA synthesis and protein production in Huh-T7 cells were not as robust as in CV-1 or Huh7 cells transfected with Ad-T7. However, sustained HCV replication in Huh-T7 cells, with no obvious signs of cytotoxicity, suggests that this viral vector-independent replication model will be useful for future studies of virus–host interactions and the development of antiviral agents with activity against HCV genotype 1.

These binary systems offer several advantages over currently available HCV replication systems. First, these replication models do not require continuous antibiotic selection as do current replicon systems (Blight et al., 2000; Frese et al., 2001; Guo et al., 2001; Lohmann et al., 1999). Second, the requirement of highly adaptive viral mutations for efficient replicon activities that are not necessarily viable in vivo (Bukh et al., 2002) may limit the interpretability of certain findings obtained from replicon systems. Because the refined replication models do not require continuous selection and do not possess highly 'adaptive' viral mutations, they are more authentic for characterization of antiviral agents, virus–host interactions, and viral fitness. Third, these replication systems can be used to study HCV replication in a variety of hepatocyte- and non-hepatocyte-derived cell types; in contrast, replicon systems only replicate efficiently in Huh7 cells. Most importantly, these replication systems use a full-length infectious genotype 1a cDNA construct that yields an

Table 1
Several similarities and differences between the vaccinia and adenovirus systems exist

	Vaccinia	Ad-T7
T7 polymerase delivery	Vaccinia virus	Adenovirus
Cytotoxicity	Yes	No
HCV replication	(+) and (−) strand synthesis; high levels	(+) and (−) strand synthesis; low but effective levels
Duration of replication	24 h	9 days
HCV protein production	Yes	Yes
Quasispecies generation	Yes	Yes
IFN inhibits replication	Yes	Yes

authentic dual-function template in vivo that is both translated and transcribed. Moreover, transfected cells in our replication systems are able to express all HCV structure and non-structural proteins (Lin et al., 2005). Thus, they are more likely to carry out authentic HCV RNA replication than replicon systems based on sub-genomic constructs.

Several significant differences exist between the vaccinia and adeno-T7 replication systems (Table 1). Both are capable of positive- and negative-strand HCV RNA synthesis, protein production, and quasispecies generation without the need for cell culture adaptive mutations (Chung et al., 2001; Contreras et al., 2002; Blackard and Hiasa et al., unpublished data). HCV replication is inhibited significantly by IFN in both systems. However, the vaccinia-based system replicates at much higher levels than the adeno-T7-based system; yet, HCV RNA synthesis occurs for at least 9 days in the former due to the lack of vector-induced cytotoxicity. Nonetheless, the decrease of HCV RNA synthesis and protein production after 3 days suggests an inhibitory effect exerted by key host cells proteins, such as protein kinase R (PKR), since adenoviruses do not inhibit PKR function as does vaccinia virus (Chang et al., 1992; Watson et al., 1991). Further examination of host antiviral pathways that limit robust long-term viral replication in culture is necessary.

We used these refined replication systems to explore the kinetics of HCV RNA synthesis and protein production in the presence of IFN. When 1000 IU/mL of IFN was added to the culture medium of HCV-expressing cells, there was no difference in HCV positive- or negative-strand quantity compared to untreated cells at day 1. Despite this lack of short-term antiviral activity, HCV RNA was significantly decreased in IFN-treated cells at days 2 and 3, suggesting that the full effects of IFN may require at least 24 h.

This cell-based HCV replication system has already been used to examine the interaction between HCV protein expression and host type I IFN signaling components in the Jak-STAT kinase pathway (Lin et al., 2005). Recently, in vitro systems that support infectious HCV production have been reported. However, these systems are based on HCV genotype 2a (Wakita et al., 2005; Zhong et al., 2005) and do not support replication in cells other than the highly permissive Huh-7 cell line and its derivatives. Importantly, the replication systems described here are based on genotype 1a isolate and replicate in several hepatocyte- and non-hepatocyte-derived cell lines. Thus, these refined replication models provide the opportunity to explore HCV molecular biology and the interactions between antiviral agents and specific HCV and/or host proteins that are relevant to genotype 1 infection.

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