Table 1
The age-specific prevalence of confirmed anti-HCV positivity in 1993–1995 in Town C, Japan

Age (years)[a]	Male	Female	All
–29	2/119 (1.7%)	6/202 (3.2%)	8/321 (2.5%)
30–39	22/193 (11.4%)	29/442 (6.6%)	51/635 (8.0%)
40–49	52/403 (12.9%)	46/503 (9.1%)	98/906 (10.8%)
50–59	81/407 (19.9%)	130/607 (21.4%)	211/1014 (20.8%)
60–69	185/693 (26.7%)	277/919 (30.1%)	462/1612 (28.7%)
70–	122/385 (31.7%)	199/704 (28.3%)	321/1089 (29.5%)
Total	464/2200 (21.1%)	687/3377 (20.3%)	1151/5577 (20.6%)

[a] In 1995.

by RT-PCR in 1995. These 591 anti-HCV antibody-positive individuals who also tested positive for either HCVcAg or HCV RNA 6 months or more after the initial anti-HCV antibody testing were judged as having a persistent HCV infection.

3.2. Serum ALT and HCV core antigen in anti-HCV positive residents

Those individuals harboring persistent HCV infections were similar in both gender and age to previously infected individuals (Table 2). Although the mean titers of anti-HCV antibodies were greater in those individuals positive for HCVcAg in comparison to those negative for HCVcAg, but HCV RNA-positive, AST, ALT, and γ-GTP levels were similar between these two groups (Table 2). The frequency of HCV serotype I was significantly higher in the HCVcAg-positive individuals in comparison to the HCVcAg-negative, HCV RNA-positive individuals. The increased sensitivity of the HCVcAg FEIA in carriers infected with HCV serotype I may explain this observation [13]. The serum levels of both AST and ALT were significantly higher in individuals with persistent HCV infections than in those lacking any evidence of HCVcAg or HCV RNA. The serum levels of γ-GTP were significantly higher in HCVcAg-positive individuals than in those lacking detectable HCVcAg or HCV RNA. In chronically infected individuals, however, no correlation between HCVcAg concentrations and ALT levels could be observed (data not shown).

Of the 591 individuals diagnosed with persistent HCV infection in 1995, 511 had at least four available annual ALT measurements between 1993 and 2000. Sixty-three of these individuals reported having received interferon (IFN) treatment before 2002.

Of the 448 individuals who had not been treated with IFN, 162 (36.2%) had normal ALT levels in all tests (<35 IU/L) (Group N), while 286 (63.8%) had at least one abnormal ALT level (≥35 IU/L) during the examination period (Group A). There were no differences between these two groups in the HCVcAg levels determined in 1995 (data not shown).

3.3. Spontaneous elimination of HCV RNA

In 591 individuals who were judged as having a persistent HCV infection in 2000, serum samples in 2001 or 2002 were obtained from 302 individuals who had at least four available annual ALT measurements between 1993 and 2000 and had not been treated by IFN until 2002. These 302 serum samples were tested of HCV RNA using RT-PCR and a positive HCV RNA was detected in 282 of these 302 serum samples. Of the 20 individuals who were HCV RNA-negative, four individuals (case A, C, F and G in Fig. 1 and Table 3) were only positive for HCV RNA in 1995, 3 were confirmed positive for HCV RNA in any of the available stored samples until 2000 and 13 did not have detectable HCV RNA in any of the available stored samples spanning from 1995 to 2000. As a result, seven individuals (2.4%, 7/289) were determined to have spontaneously eliminated the HCV infection, based on RT-PCR (Fig. 1). For cases A, C, D, F, and G, there were insufficient serum quantities to re-test the 1995 samples. The titers of anti-HCV in the samples of cases F and G were very low in 1993 and became negative after 2001. The titers of anti-HCV in cases A–C also appeared to decrease over time (Fig. 1). In the seven individuals with apparent

Table 2
Characteristics of anti-HCV antibody-positive Town C residents, separated by HCV core antigen and HCV RNA status in 1995[a]

Characteristics	HCV core antigen ≥8 pg/mL (n=528)	HCV core antigen (−) and HCV-RNA (+) (n=63)	HCV core antigen (−) and HCV RNA (−) (n=245)	p value*
Gender, male female	209/319	26/37	90/155	0.69
Age	65.2 ± 10.4	66.5 ± 10.3	64.0 ± 10.9	0.19
Anti-HCV titer	9.0 ± 1.3[b,c]	6.6 ± 2.0[d]	5.0 ± 2.5	<0.001
HCV Serotype (I/II)[e]	303/115	30/22	NT[f]	0.03
AST	48.4 ± 41.2 (493)[c]	52.1 ± 35.1 (60)[d]	27.2 ± 149(231)	<0.001
ALT	42.9 ± 40.4 (493)[c]	44.8 ± 36.8 (60)[d]	25.0 ± 16.5 (231)	<0.001
γ-GTP	38.6 ± 51.5(493)[c]	41.4 ± 25.4 (60)	25.4 ± 293 (231)	<0.001

[a] Data shown as the means ± S.D. (number of individuals examined).
[b] p<0.001 vs. HCV core antigen (−) and HCV RNA (+) group, by Scheffe's test.
[c] p<0.001 vs. HCV core antigen (−) and HCV RNA (−) group, by Scheffe's test.
[d] p<0.01 vs. HCV core antigen (−) and HCV RNA (−) group, by Scheffe's test.
[e] Excluding individuals whose HCV serotype was undetermined.
[f] Not tested.
* Based on one-factor ANOVA, χ^2 test, or Fisher's exact test, as appropriate for the comparison across the groups.

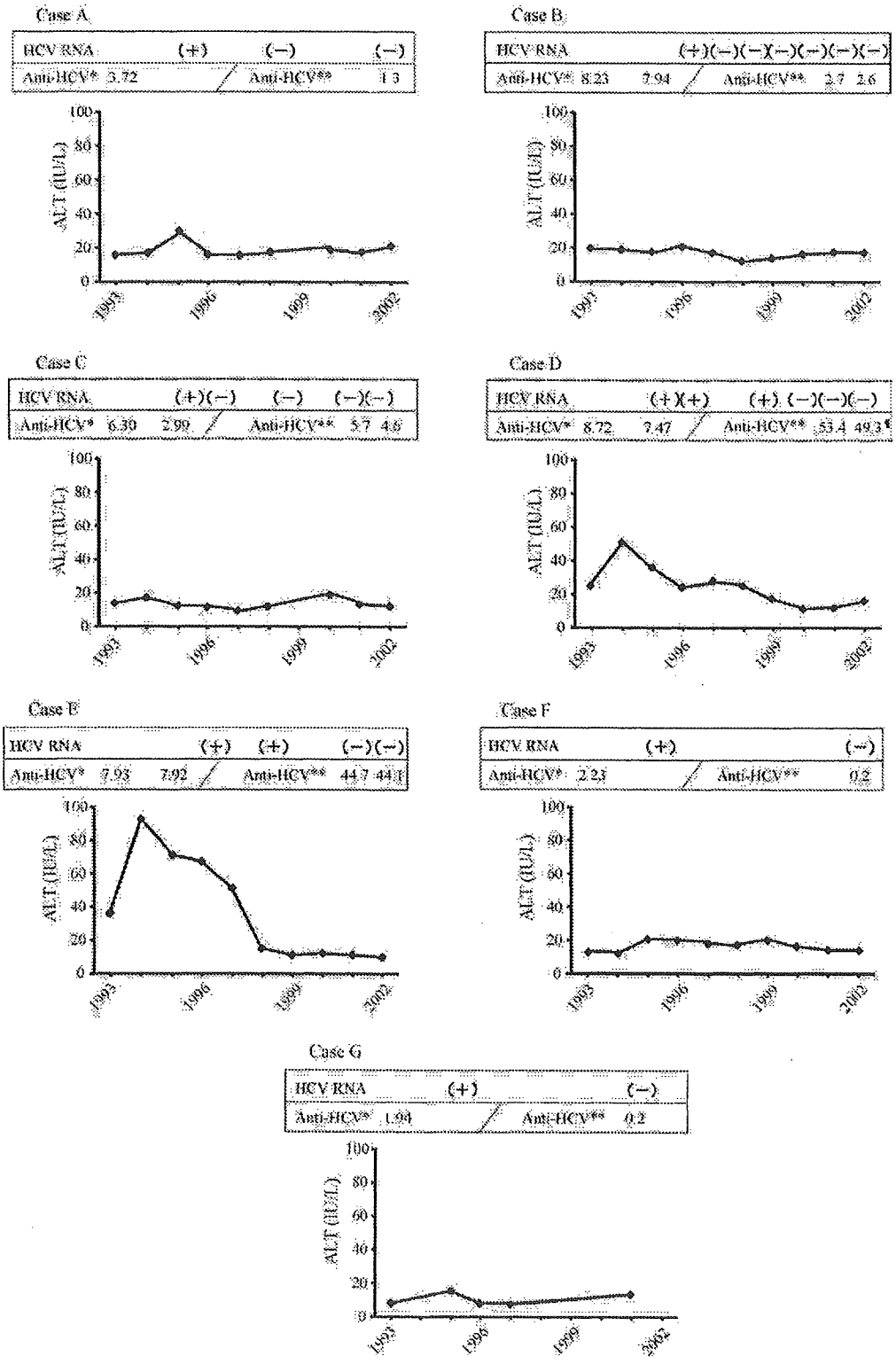

Fig. 1. Clinical course of individuals with spontaneous elimination of serum HCV RNA. Panels (A)–(G) represent the seven cases that were followed for 8 or 9 years. *Antibody against HCV (anti-HCV) were tested by a second generation enzyme immunoassay kit; titers higher than 1.0 were considered to be positive for anti-HCV antibody. **Since 2001, anti-HCV was evaluated by a third generation chemiluminescent enzyme immunoassay; samples with a signal/cut-off ratio of ≥1.0 were considered to be positive. ¶Data was from 2003.

Table 3
Characteristics of anti-HCV positive residents diagnosed with persistent HCV infection in 1995 whose serum HCV RNA were spontaneously eliminated between 1996 and 2002

Case	Age[a]/sex	HCV core antigen (pg/mL)	HCV serotype	ALT abnormality[b] (Group)	Platelets[c] ($\times 10^4/\mu L$)	Type IV collagen 7S[c] (ng/mL)
A	55/F	<8	ND[d]	N	25.5	4.7
B	60/F	33.4	I	N	25.0	3.9
C	64/F	<8	II	N	23.1	3.6
D	66/M	<8	II	A	25.3	3.9
E	68/M	28.2	II	A	17.1	3.3
F	73/M	<8	ND	N	20.6	4.5
G	76/M	<8	ND	N	20.7	3.8

[a] In 1995.
[b] Data were obtained between 1993 and 2000. N; persistently normal ALT levels, A; fluctuating or persistently abnormal ALT levels.
[c] Data were obtained in 2001, with the exception of case F for which data were obtained in 2002.
[d] Individuals whose HCV serotype was not determined.

Table 4
Comparison of demographic and virologic data of seven individuals whose serum HCV RNA were spontaneously eliminated to those of individuals remaining HCV RNA-positive

Characteristics	Elimination of HCV RNA ($n=7$)	Remained positive for HCV RNA ($n=282$)	p value*
Gender, male/female[a]	4/3	92/190	0.23
Age[a,b]	66.0 ± 7.2	65.3 ± 8.3	0.95
HCV core antigen (<20/>=20)[a]	5/2	33/249	<0.001
HCV Serotype (I/II)[a,c]	1/3	169/79	0.10
ALT[a,b]	28.9 ± 20.9	40.9 ± 38.7	0.16
ALT group (N/A)[d]	5/2	97/185	0.10

[a] Data were obtained in 1995.
[b] Data shown as the means ± S.D.
[c] Excluding individuals whose HCV serotype was undetermined or was not examined.
[d] N: persistently normal ALT levels, A: fluctuating or persistently abnormal ALT levels.
* Based on Mann–Whitney U test or Fisher's exact test as appropriate.

clearance of HCV RNA, five had HCVcAg levels below the assay's limit of detection; only HCV RNA could be detected by RT-PCR at initial testing in 1995 (Table 3). The incidence of low HCVcAg levels (below 20 pg/mL) [14] determined in 1995 was significantly higher in the individuals with spontaneous elimination of serum HCV RNA (5/7, 71.4%) than in those who remained HCV RNA-positive (33/282, 11.7%) ($P<0.001$) (Table 4). Five of the seven individuals (71.4%) also had persistently normal ALT levels (Group N), while two had at least one abnormal ALT level (Group A) in the measurements taken between 1993 and 2000 (Fig. 1, Table 3). In comparison to the individuals who remained HCV RNA-positive, those spontaneously eliminating HCV RNA were more likely to be male (57 versus 33%; $p=0.23$), be infected with HCV serotype II (75 versus 32%; $p=0.10$), and exhibit persistently normal ALT levels (71 versus 39%; $p=0.10$), although none of these trends were statistically significant (Table 4). In addition, platelet counts and type IV collagen 7S levels, which reflect the degree of liver fibrosis [15,16], were normal in all available measurements taken from these seven individuals in 2001 or 2002 (Table 3). The serum ALT levels were also normal during this period (Fig. 1).

4. Discussion

This study was performed in an area of Japan hyperendemic for HCV infection, where the prevalence of anti-HCV antibody positivity is 4- to 12-fold higher than that seen in the surrounding areas. The frequency of anti-HCV antibody positivity in this community is similar to that reported for other endemic areas of Japan [5]. In this population, as most residents displayed asymptomatic infections, with less than 5% reporting a history of acute hepatitis or jaundice (data not shown), the exact date of infection were typically unknown. Residents who tested positive for HCVcAg and/or HCV RNA more than 6 months after demonstrating anti-HCV seropositivity were considered to have a persistent HCV infection. The 70.5% prevalence of persistent infection in this population was similar to that previously reported [5,17]. The HCV serotype I was the most common seen in the study population, as reported for other Japanese populations [18,19], suggesting that Town C is similar to other HCV endemic areas of Japan.

In the seven individuals who eliminated HCV RNA, the results of five individuals (cases A–C, F and G) were positive for HCV RNA only once, two of whom (cases F and

G) also were negative for HCV RNA only once. These data do not necessarily exclude the possibility of false positive or false negative HCV RNA results; however, the titers of anti-HCV in all five individuals were lower when the individuals were HCV RNA negative than when they were HCV RNA positive. Although the anti-HCV assays used were different before and after 2001, data from the individuals in the cohort who remained positive for HCV RNA did not show a similar decrease in titer values between the two assays (data not shown). Thus, the results of the HCV RNA and anti-HCV antibody testing, albeit not definitive, would seem to support the spontaneous elimination of HCV RNA in these individuals.

We demonstrated that serum HCV RNA was spontaneously eliminated in 7 of 20 individuals who were HCV RNA-negative in 2001/2002. The remaining 13 individuals who were HCVcAg-positive in 1995 were found to be HCV RNA-negative upon re-testing of all the available stored samples taken between 1995 and 2000. Although these 13 individuals may have undergone spontaneous HCV elimination, we could not confirm the elimination of HCV RNA using RT-PCR as the gold standard. Although the reason underlying this inconsistency is unclear, HCV RNA may have degraded in those blood samples that were stored at −30 °C until testing. As a result, we have conservatively estimated the frequency of HCV elimination in persistent infection at 2.4% (7/282).

Although HCV RNA detection was not performed every year, the presence of HCV RNA was assessed more than 6 years after the initial testing for of HCVcAg or HCV RNA in 1995; thus, the elimination rate was estimated to be approximately 0.4%/year, which is similar to that reported by Watanabe et al. [5]. In contrast, some investigators have reported higher elimination rates than that seen in this study [6–9]. Hattori et al. [7] demonstrated that 14% of pregnant female patients with chronic HCV infections lost positivity for serum HCV RNA without treatment during the follow-up (duration of average follow-up; 5.8 years) of parturition. Fujisawa et al. [6] showed that 8.3% of children with chronic hepatitis C exhibit spontaneous clearance of serum HCV RNA during follow-up (duration of infection; 5.5 years). Fukuizumi et al. [8] estimated a natural disappearance rate of serum HCV RNA positivity at 2.8% per year. These differences may be related to differences in the immune systems of pregnant and non-pregnant women or children and adults. In addition, there may be differences in the initial serum HCV RNA levels, the rate of HCV mutation, the duration of infection, or the HLA allelic frequencies. Further investigations of the factors influencing spontaneous HCV clearance will be necessary to address these discrepancies, which are ongoing in this cohort study.

The spontaneous elimination of HCV RNA has been observed to occur more frequently in females and in individuals with persistently normal ALT levels [5,20]. These observations, however, included individuals who had recovered from acute HCV infection. Although these individuals were positive for anti-HCV antibodies, positive for HCV RNA were not shown before the infection was cleared. In this study, we did not observe a relationship between female gender and the spontaneous elimination of HCV in individuals with persistent infections, which correlated well with the results of Fukuizumi et al. [8]. These results indicate that there is no significant association between gender and spontaneous HCV elimination in individuals with persistent infections.

Yokosuka et al. reported that 6 (2%) of 310 patients with type C chronic liver disease became negative for HCV RNA detection over a 3-year period; all 6 patients exhibited liver cancer with chronic active hepatitis or liver cirrhosis [9]. Individuals became seronegative for HCV RNA in the terminal stages of liver cancer, prompting the hypothesis that a reduction in the amount of the virus occurs with the loss of a suitable environment in which the virus can replicate. In this study, none of the individuals displaying a spontaneous loss of HCV infection had any evidence of hepatocellular carcinoma or liver cirrhosis (data not shown). There were significant differences in the HCVcAg levels measured initially in 1995 between those who eliminated the virus and those supporting persisting infections. Thus, an initial low HCV viral load may be an important factor in the clearance of HCV infection [5–8].

Fukuizumi et al. reported that spontaneous elimination was associated with low ALT levels [8]. In our study, Town C residents exhibiting spontaneous elimination of HCV RNA also tended to have low ALT levels in 1995 and persistently normal ALT levels during 1995 and 2000. However, this tendency was not statistically significant and Fukuizumi et al. did not investigate the association between spontaneous elimination of HCV RNA and sequential ALT levels [8]. The exact mechanism that serum HCV RNA was spontaneously eliminated is unclear. However, HCV elimination in HCV carriers with normal or only mildly elevated ALT levels likely differs from that in individuals with acute infection, in whom cellular immune responses induce severe liver enzyme elevation and these acute responses are critical for the clearance of HCV. The role for HCV elimination in individual with persistent infection requires further investigation.

In summary, we have provided evidence for the spontaneous elimination of HCV RNA. This elimination occurred only rarely and was associated with low initial HCV viral loads, but was not associated with age, gender, or ALT levels. In addition, we could not observe an association between HCV viral load and ALT levels.

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Timing of interferon therapy and sources of infection in patients with acute hepatitis C

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Abstract

Background/Aims: Controversy over the selection of patients and optimum therapeutic method for acute hepatitis C has continued. The aims of this study were to investigate the source of infection, and to evaluate the timing of interferon (IFN) therapy in patients with acute hepatitis C in Japan.

Methods: The records of 102 patients from 12 facilities in Japan who developed acute hepatitis C after 1990 were investigated. In the patients treated with IFN, we performed multivariate analysis to investigate factors related to sustained virological response (SVR).

Results: Medical procedure was the most common source of infection, accounting for 32.4% in the 102 patients (33/102). Of 81 patients treated with IFN, 71 patients were followed after IFN therapy, and 57/71 (80.3%) had SVR. The SVR rate was significantly higher in patients treated with IFN within 24 weeks from onset of symptoms than the SVR rate in those treated after 25 weeks ($P = 0.0016$). Multivariate analysis revealed that only the duration between onset of symptoms and initiation of IFN therapy (within 24 weeks) was related to SVR.

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Abbreviations: HCV, hepatitis C virus; IFN, interferon; ALT, alanine aminotransferase; SVR, sustained virological response; Peg-IFN, pegylated interferon

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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.

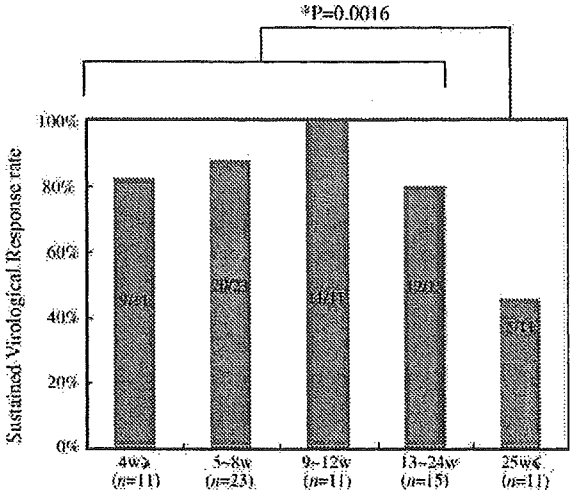

Fig. 1. Sustained virological response rate according to duration between onset of symptoms and initiation of IFN therapy. The groups treated with IFN 0–24 weeks after onset of symptoms and treated after 25 weeks were compared. Comparison by the Chi-square test. (*) Statistically significant; w, week.

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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The peroxisome proliferator-activated receptor-γ agonist, pioglitazone, inhibits fat accumulation and fibrosis in the livers of rats fed a choline-deficient, L-amino acid-defined diet

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Abstract

Administration of a choline-deficient, L-amino acid-defined (CDAA) diet to rats causes steatohepatitis, hepatic fibrosis, and hepatocellular carcinoma, a pathology similar to that observed in non-alcoholic steatohepatitis (NASH). The aim of this study was to evaluate if a peroxisome proliferator-activated receptor (PPAR)-γ agonist, pioglitazone (PGZ), could ameliorate CDAA diet-induced fatty liver and cirrhosis. Rats were fed a CDAA diet for 1 week and were given the CDAA diet for an additional week with or without PGZ (2-week model). Also, after administration of the CDAA diet for 12 weeks, rats were administered the CDAA diet for an additional 4 weeks with or without PGZ (16-week model). The CDAA diet, administered for either one or 12 weeks, induced fatty liver or cirrhosis with up-regulation of hepatic PPAR-γ expression, respectively. In the 2-week model, rats treated with PGZ for 1 week demonstrated significantly lower hepatic triglyceride content and serum levels of tumor necrosis factor-α. In the 16-week model, treatment for 4 weeks with PGZ ameliorated hepatic fibrosis with a decrease in the expression of procollagen, α-smooth muscle actin, and transforming growth factor-β1 in comparison to rats without PGZ. These results suggest that PPAR-γ agonist is a potential therapeutic modality to treat NASH.

Keywords: CDAA; NASH; PPAR-γ agonist; Fatty liver; Hepatic fibrosis

1. Introduction

Non-alcoholic steatohepatitis (NASH) is caused by the accumulation of excess fat in the liver. This disease may progress to cirrhosis, occasionally causing hepatocellular carcinoma [1,2], NASH is also closely associated with type 2 diabetes mellitus and obesity; insulin resistance has been implicated as a possible factor in NASH development [3–5]. The mechanism of this disease is still obscure, however, in part due to the lack of an appropriate experimental model. The choline-deficient, L-amino acid-defined (CDAA) diet, a semi-synthetic diet containing no choline and limited methionine in the absence of any known carcinogens, may prove such a model. Administration of the CDAA diet to rats induces hepatocellular carcinoma (HCC) in conjunction with fatty liver, hepatocyte injury and regeneration, fibrosis, and cirrhosis [6,7]. This phenotype is similar to the development of human HCC with cirrhosis, especially that observed in NASH.

Peroxisome proliferator-activated receptor-γ (PPAR-γ), a member of the nuclear receptor superfamily, was originally defined as an essential factor in adipogenesis [8]. The PPAR subfamily consists of three isotypes, PPAR-α, PPAR-γ, and PPAR-β/δ; these three PPAR isotypes exhibit distinct

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patterns of tissue distribution [9]. PPAR-γ agonists are widely used in patients with insulin resistance and diabetes. PPAR-γ agonists have also been reported to inhibit cell proliferation and collagen expression in primary hepatic stellate cells (HSC) both in vitro and in vivo [10,11]. These results suggest a potential therapeutic value for PPAR-γ agonists in treating NASH. It is unclear, however, if the dosages of PPAR-γ agonist used for the treatment for patients with type 2 diabetes mellitus will be effective for NASH therapy. Even if effective for the early prevention of liver disease, it remains unknown whether PPAR-γ agonist will be effective if given after the disease has progressed to fatty liver or liver cirrhosis.

This study examined the effects of treating CDAA-fed rats with the PPAR-γ agonist, pioglitazone (PGZ; 1.0 mg/(kg day)), an agent widely used to treat patients with type 2 diabetes mellitus, after the development of fatty liver and liver cirrhosis. We have demonstrated that PGZ administration suppresses fat accumulation in the liver and hepatic fibrosis in these rats, suggesting that hepatic PPAR-γ may be a useful therapeutic target in humans suffering from NASH.

2. Materials and methods

2.1. Animals

Six-week-old male Fischer 344 rats were purchased from Kyushu Experimental Animal Supply (Kumamoto, Japan). The animals were maintained at constant room temperature (25 °C) and provided free access to water and food throughout the study. After a 1-week acclimation period on a standard diet, rats were switched to a CDAA diet (Dyets Inc., Bethlehem, PA) and were sacrificed at 1, 2, 12, and 16 weeks after beginning the CDAA diet. Blood was obtained by cardiac puncture. The liver was immediately excised and the wet weight of the liver was determined. Samples were either immediately subjected to histological analysis or frozen in liquid nitrogen and stored at −80 °C until analysis. All animal procedures were performed according to approved protocols in accordance with the ethical committee of University of Miyazaki.

2.2. Compounds and animal treatment

The PPAR-γ agonist PGZ was kindly provided by Takeda Pharmaceutical Co. (Tokyo, Japan). All rats were continuously fed a CDAA diet with or without PGZ until the end of the experiment. When indicated, PGZ was given by gavage once a day at a dose of 1 mg/(kg day), a dosage comparable to that used to treat humans. Rats were randomly divided into four groups. Groups 1 and 3, administered the CDAA diet for 2 or 16 weeks, respectively, served as control groups. One or 12 weeks after beginning administration of the CDAA diet, the experimental animals in groups 2 and 4 received PGZ for an additional one or 4 weeks with the CDAA diet, respectively. Thus, groups 1 and 2 were 2-week model, and group 3 and 4 were also 16-week model.

2.3. Serum markers

Serum levels of alanine aminotransferase (ALT), glucose, insulin, and hyaluronic acid were determined in the experiments. Serum levels of tumor necrosis factor (TNF)-α were measured using a rat cytokine ELISA kit (Biosource, Camarillo, CA).

2.4. Reverse transcription-polymerase chain reaction

Total RNA was extracted from liver tissue using the acid guanidinium thiocyanate:phenol:chloroform method. One microgram of RNA in a 20-μl mixture was reverse transcribed with Molony murine leukemia virus reverse transcriptase MMLV (TaKaRa, Tokyo, Japan) at 42 °C for 60 min in the presence of random hexamers. The following primers were used: 5′-ATCAGCTCTGTGGACCTCTCTGTG-3′ (sense) and 5′-AGCTTCAATCGGATGGTTCTTCGG-3′ (antisense) for rat PPAR-γ (product size 380 base pairs [bp]), 5′-AACAGGACTTCTTCGGAACCAC-3′ (sense) and 5′-CATTTGCACCACTTGTGGCTTC-3′ (antisense) for rat procollagen α2(I) (product size 422 bp) [12], and 5′-ACTCTACCCACGGCAAGTTCA-3′ (sense) and 5′-GGCAGTGATGGCATGGACT-3′ (antisense) for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (product size 483 bp). Five microliters of the reverse-transcribed mixture was amplified by polymerase chain reaction (PCR) in a 25-μl volume (TaKaRa). PCR reactions were initially denatured at 94 °C for 2 min and cycled at 94 °C for 30 s, 53 °C (PPAR-γ), 56 °C (type I procollagen α2), or 59 °C (GAPDH) for 30 s, and 72 °C for 30 s. Thirty-two cycles were performed to amplify PPAR-γ and procollagen α2(I), while 30 cycles were performed to amplify GAPDH using a GeneAmp PCR system 9700 (PE Applied Biosystems, Foster City, CA). Type I procollagen α2 PCR products were examined by 1.2% agarose gel electrophoresis and visualized with ethidium bromide. Densitometric analysis examined PCR product semi-quantities by measuring the absorbance with a Bio-1D apparatus (M & S Instruments Trading Inc., Tokyo, Japan). The magnitudes of gene expression were calculated as relative intensity against GAPDH mRNA levels. The mean relative intensity of type I procollagen α2 in group 3 was normalized to a value of 1.

2.5. Histopathological and immunohistochemical analysis

Liver samples were fixed in 10% formalin and embedded in paraffin. For histological examination, 5-μm slices were stained with hematoxylin and eosin (HE) or Azan dye. Immunohistochemical analysis of α-smooth muscle actin (ASMA) (Dako Japan, Kyoto, Japan) was performed with paraffin-embedded sections as previously described

[13]. Rabbit anti-rat IgG (Novocastra, Burlingame, CA) was applied to samples, followed by an avidin–biotin-peroxidase complex and chromatin 3′,3′-diaminobenzidine for detection of bound antibody.

2.6. Western blot analysis

Liver tissues were solubilized using T-PER tissue protein extraction reagent (Piece Biotechnology, IL). Sample proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and blotted onto polyvinylidine difluoride filters. After blocking with 1% bovine serum albumin, filters were incubated with anti-PPAR-γ polyclonal antibody (Santa Cruz Biotechnology, CA) or anti-ASMA monoclonal antibody (Dako Japan). Bound antibody was detected using horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies (Amersham Biosciences, Buckinghamshire, UK), respectively. Proteins were then visualized with ECL Western blotting detection kit (Amersham).

2.7. Hepatic triglyceride content and TGF-β1 expression

Hepatic triglyceride content was determined as previously described [14]. Triglyceride content is expressed as mg/g of the wet liver. TGF-β1 expression in the liver was measured using a Quantikine assay kit (R & D systems, Minneapolis, MN). After the preparation of liver lysates, samples were equalized for protein concentration as previously described prior to measuring TGF-β1 levels [13]. TGF-β1 protein levels measured the active form, detected by the addition of HCl [15]. The resulting values are expressed as ng/g of the wet liver.

2.8. Statistical analysis

Results are presented as means ± S.D. Statistical parameters were ascertained using StatView J-4.5 software (Abacus Concepts, Inc., Berkeley, CA). Differences between means were assessed by the Mann–Whitney *U*-test. The significance level was set at $P < 0.05$.

3. Results

3.1. PPAR-γ expression in the liver during CDAA diet administration

We have previously reported that diffuse fatty liver was observed in the animals 1 week after beginning of the CDAA diet [16]. The development of cirrhosis was also recognized at 12 weeks [16]. As PPAR family members are implicated in adipogenesis, we examined PPAR-γ expression during stages of the CDAA diet in which fat accumulation and liver fibrosis were induced. Although no hepatic expression of PPAR-γ could be observed in normal rats, PPAR-γ expression was observed after one and 12 weeks of CDAA diet administration (Fig. 1). These findings indicate that increased expression of PPAR-γ was observed in conjunction with the development of fatty liver and liver cirrhosis. Thus, PPAR-γ is a potential therapeutic target for treatment of fatty liver and hepatic fibrosis in this animal model.

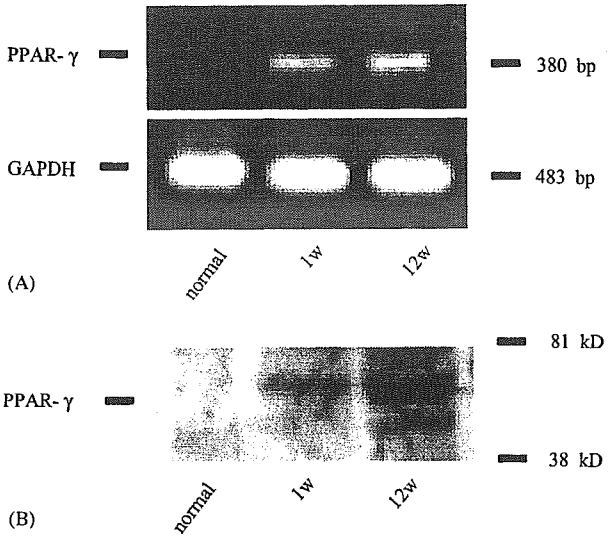


Fig. 1. Hepatic PPAR-γ expression in rats fed a CDAA diet. PPAR-γ expression in the livers of four rats at each time point was analyzed by reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis. No hepatic expression of PPAR-γ mRNA was observed in normal rats. Administration of a CDAA diet induced the up-regulation of PPAR-γ expression in the liver at weeks 1 and 12. Representative results of RT-PCR (A) and Western blot analysis of PPAR-γ protein expression (B) are shown.

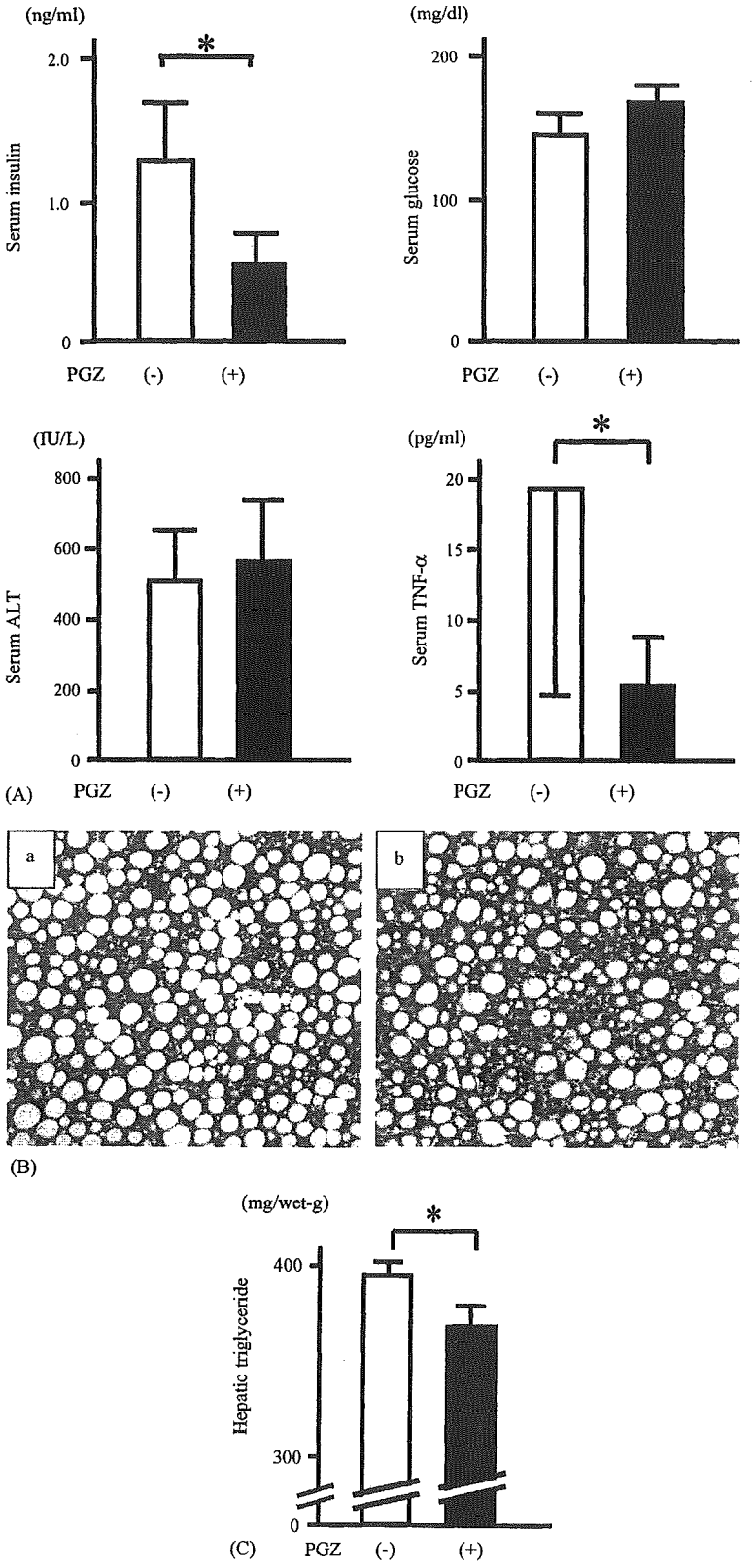
3.2. Serological and histological analysis during fatty liver development (2-week model)

We examined the effects of PGZ administration on various serum markers. Serum insulin levels were significantly reduced in the PGZ-treated rats (Fig. 2A), whereas serum levels of glucose and ALT were unaffected by this treatment. PGZ treatment also lowered serum levels of TNF-α. We examined the effect of PGZ treatment on fat accumulation in the liver. HE staining demonstrated that administration of the CDAA diet to animals for 2 weeks resulted in marked fat accumulation (Fig. 2B-a, group 1). Treatment with PGZ (group 2) slightly improved this fat accumulation (Fig. 2B-b), in comparison with the extent observed in group 1.

PGZ also significantly decreased triglyceride content in the liver (Fig. 2C), indicating that PGZ resolves the fatty changes in liver tissues induced by the CDAA diet.

3.3. Histological and biochemical analysis during development of liver cirrhosis (16-week model)

In group 3 animals, livers were enlarged, yellowish, and multinodular (Fig. 3A-a). Marked liver fibrosis was observed


(ng/ml)
Serum insulin
2.0
1.0
0
*
PGZ (-) (+)
(mg/dl)
Serum glucose
200
100
0
PGZ (-) (+)
(IU/L)
Serum ALT
800
600
400
200
0
PGZ (-) (+)
(A)
(pg/ml)
Serum TNF-α
20
15
10
5
0
*
PGZ (-) (+)
a
b
(B)
(mg/wet-g)
Hepatic triglyceride
400
300
0
*
(C) PGZ (-) (+)

after a 16-week administration of a CDAA diet (Fig. 3A-c). In group 4, a 4-week PGZ treatment during weeks 12–16 significantly decreased liver fibrosis (Fig. 3A-b and A-d). PGZ treatment also decreased type I procollagen α2 mRNA expression (Fig. 3B) and hepatic TGF-β1 concentration (Fig. 3C). Although serum levels of ALT and glucose were indistinguishable between groups 3 and 4, serum concentrations of insulin and hyaluronic acid were significantly reduced in comparison to control (non-PGZ-treated) rats given a CDAA diet (Fig. 3D). These results suggest that PGZ resolved hepatic fibrosis.

3.4. ASMA expression in the liver of rats fed a CDAA diet and the effects of PGZ treatment (16-week model)

We examined the effects of PGZ on hepatic stellate cell (HSC) activation during the development of fibrosis using immunohistochemical analysis of ASMA expression. Although the number of activated HSCs expressing ASMA increased during administration of the CDAA diet, PGZ treatment from weeks 12 to 16 dramatically reduced the number of ASMA-positive cells in the liver (Fig. 4A). In addition, the expression of ASMA protein decreased in the liver at week 16 (Fig. 4B).

These results suggest that treatment with PGZ ameliorates CDAA diet-induced liver fibrosis via suppression of HSC activation and proliferation.

4. Discussion

The pathological changes of the livers of rats fed the CDAA diet, characterized by diffuse fatty infiltration and inflammation followed by development of cirrhosis and HCC, are similar to those changes seen in patients with NASH. Additionally, increases in hepatic mRNA expression and serum level of TNF-α were observed in these rats compared with normal rats (data not shown). TNF-α is known to play an important role in the development of hepatic steatosis; patients with NASH exhibit significant increases in serum TNF-α [17]. Therefore, although the pathogenic mechanisms underlying NASH are not well understood, a rat animal model fed a CDAA diet is considered to be an appropriate model to investigate the pathogenesis of NASH and examine potential therapeutic agents.

In this study, we demonstrated that PPAR-γ expression was upregulated in the livers of rats fed a CDAA diet. The expression of PPAR-γ is closely associated with the development of fatty liver and hepatic steatosis [18–21]. Schadinger et al. [22] recently reported that PPAR-γ2 induces steatosis in hepatocytes via pathways regulating de novo lipid synthesis. Nakae et al. [6] have reported that oxidative DNA damage induced by the CDAA diet was significantly greater than that resulting from a choline-deficient diet. As oxidative signals induce and activate PPAR-γ [23], the induction of oxidative stress in the livers of rats fed the CDAA diet likely stimulates hepatocytes expression of PPAR-γ. In agreement with previous studies demonstrating a role for oxidative stress in human NASH [24,25], the expression of PPAR-γ may be associated with NASH development. Therefore, rats fed a CDAA diet are a suitable experimental model for NASH. In addition, PPAR-γ expression may be a potential therapeutic target for the treatment of human NASH.

As NASH is associated with insulin resistance, preliminary data using PPAR-γ agonists to improve this condition in patients with NASH have suggested a potential reversal of the adverse effects of NASH on the liver through selective modulation of PPAR-γ activity [26–28]. These reports demonstrated that hepatic fat content was decreased and hepatic fibrosis was significantly improved in patients with NASH treated with PPAR-γ agonists (PGZ or rogiglitazone) for 48 weeks. In addition, serum insulin levels, but not serum glucose levels, were reduced by PGZ administration in humans and rats [27,29]. The presence of insulin resistance in our experimental model appears to be negative; serum levels of glucose and insulin did not differ between rats fed a normal diet and those fed a CDAA diet for 1 week, respectively (data not shown). Serum insulin levels, however, were significantly reduced in PGZ-treated rats, despite unaltered serum glucose levels (Fig. 2A), indicating that insulin sensitivity is increased in these animals. These results also suggest that PGZ modulates PPAR-γ activity in human NASH patients in a manner similar to that seen in our experimental model.

While PPAR-γ agonists have improved ALT levels and steatosis in NASH patients [27–29], these agonists have also been reported to induce liver injury [30,31]. In this report, however, it was not observed any alterations in serum ALT levels which result was similar to other report [32]. Previously, we demonstrated an increased apoptosis of hepatocytes in rats fed a CDAA diet, which did not correlate with changes in serum ALT levels [16]. Hepatocyte apoptosis is also significantly increased in patients with NASH [33,34]. In addition, PPAR-γ agonists inhibit the production of monocyte inflammatory cytokine, which may contribute to tissue damage [35]. In this study, serum levels of TNF-α were significantly reduced by PGZ administration. As TNF-α contributes to the

Fig. 2. Serum markers, histopathological findings and hepatic triglyceride levels during fatty liver development after a 2-week administration of the CDAA diet. (A) Treatment with PGZ significantly reduced serum levels of insulin, while serum glucose and ALT levels were unaffected by treatment (with or without a 1-week treatment with PGZ; $n = 4$). Serum TNF-α expression was also significantly reduced by a 1-week treatment with PGZ ($n = 8$) compared with untreated CDAA-fed controls ($n = 8$) ($^*P < 0.05$). (B) Representative photomicrographs of liver from treated animals are shown. Two-week administration of the CDAA diet resulted in a marked fat accumulation (a). A 1-week administration of PGZ slightly reversed this fat accumulation (b) (HE staining, original magnification ×100). (C) Hepatic triglyceride levels were significantly reduced by a 1-week treatment with PGZ beginning at week 2 ($n = 4$) in comparison with untreated CDAA-fed controls ($n = 4$) ($^*P < 0.05$).

induction of hepatocyte apoptosis, PGZ treatment may reduce hepatocyte apoptosis without altering serum ALT levels especially in this model. Furthermore, the differences between rat and human, variations in the amount of specific cofactors, or the presence of different isoforms of PPAR-γ [23,36] may be due to different effect of PGZ on serum ALT levels.

PGZ administration reduced hepatic triglyceride levels in rats fed a CDAA diet. Although the mechanisms by which PPAR-γ agonists reduce hepatic triglyceride content have not been clarified, PPAR-γ regulates the expression of genes involved in adipocyte differentiation and lipid storage. PPAR-γ agonists reduced the total lipid content of the livers of ob/ob mice or alcohol administered rat [37]. PPAR-γ agonists up-regulate the expression of lipid-related genes, including fatty acid transporters and uncoupling protein (UCP)-2 [38], and down-regulate the expression of leptin. In addition, non-parenchymal cells, such as Kupffer cells, may be a potential pharmaceutical target of PGZ in the early stages of hepatocarcinogenesis in rats fed a CDAA diet [39]. Prostaglandin E2 derived from activated Kupffer cells results in triglyceride accumulation in the liver; PPAR-γ agonists suppress Kupffer cell activation [40,41]. Increases in the hepatic mRNA

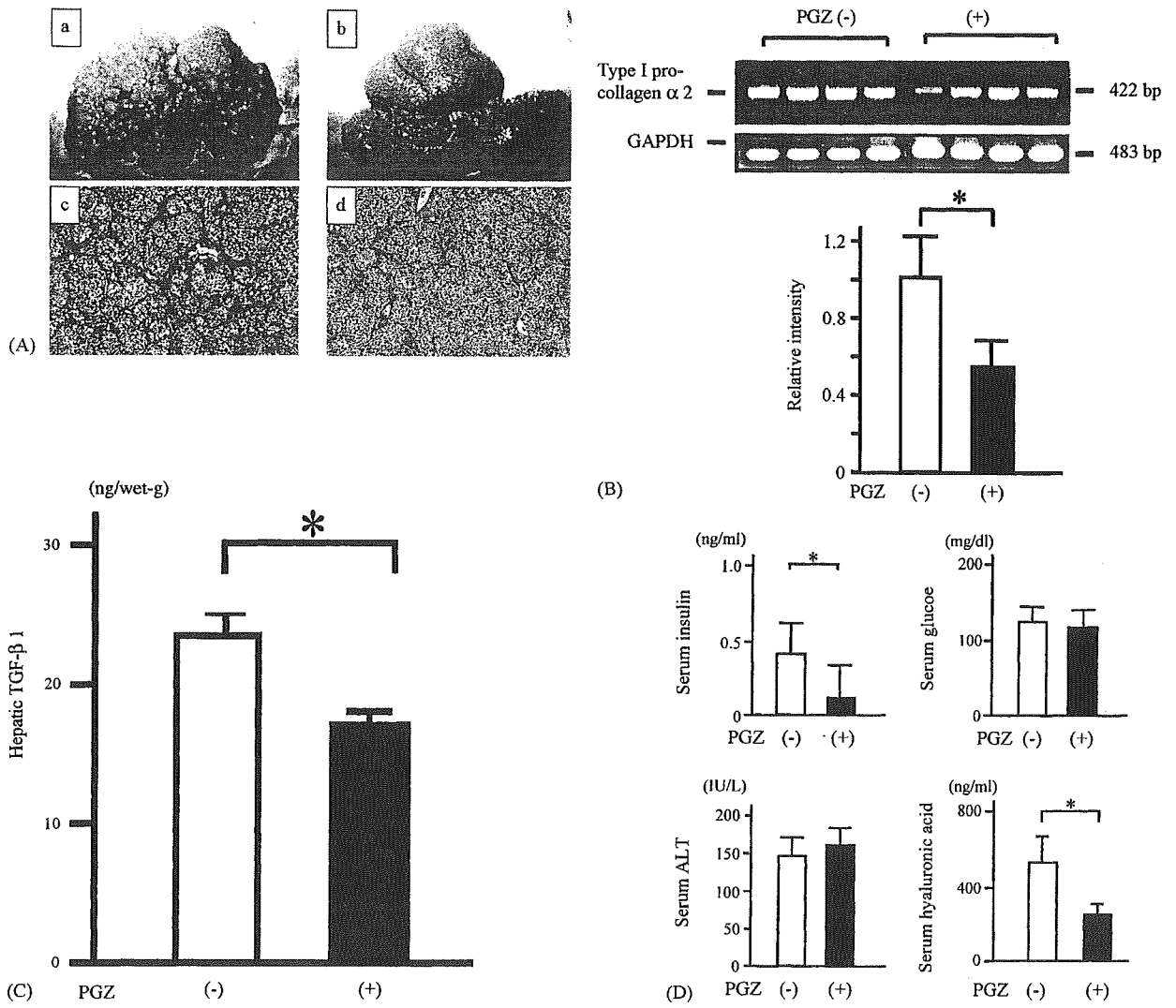


Fig. 3. Histopathological findings, serum markers, type I procollagen α2 mRNA and hepatic TGF-β1 protein levels during the development of liver cirrhosis. (A) Liver tissues were obtained from rats fed a CDAA diet for 16 weeks with (b and d) or without (a and c) PGZ treatment during the final 4 weeks. CDAA diet administration resulted in the development of multinodular liver (a) and liver fibrosis (c). PGZ treatment from weeks 12 to 16 decreased liver surface nodules (b) and fibrosis (d). (a and c) Representative macroscopic appearance of livers. (c and d) Representative photomicrographs of liver sections (Azan staining, original magnification ×40). (B and C) PGZ treatment during weeks 12–16 decreased type I procollagen α2 mRNA expression (B) and hepatic TGF-β1 protein content (C) in the liver at week 16 (with or without PGZ; $n=4$) ($^*P<0.05$). (D) PGZ treatment during weeks 12–16 also significantly decreased serum concentrations of insulin and hyaluronic acid at week 16 (with or without 4 weeks of PGZ treatment; $n=4$) ($^*P<0.05$). Neither serum glucose nor ALT levels differed between these two groups.

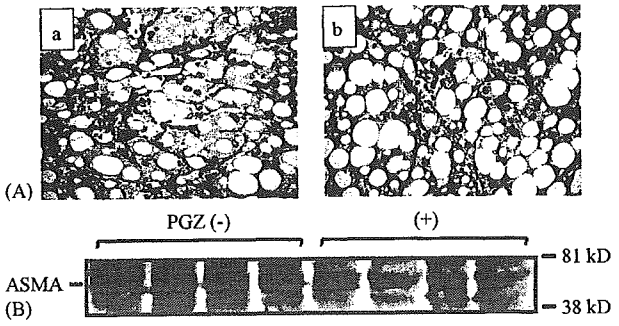


Fig. 4. The expression of α-smooth muscle actin (ASMA). (A and B) Immunohistochemical (A) and Western blot analysis (B) of ASMA examined the effects of PGZ treatment on fibrosis development. Liver tissues were obtained from rats fed a CDAA diet for 16 weeks with (b) or without (a) PGZ treatment during weeks 12–16 ($n = 4$). (A) Representative photomicrographs of liver sections are shown (original magnification ×100). PGZ treatment reduced the number of ASMA-positive cells (b). (B) PGZ treatment also decreased the overall expression of ASMA protein in the liver at week 16.

expression of TNF-α in CDAA treated-rats in comparison to the levels observed in normal rats (data not shown) might also be associated with Kupffer cell activation. As adipocyte differentiation, lipid storage, apoptosis and Kupffer cell activation may contribute to the development of fatty liver and hepatocyte injury and the ability to influence these processes with agonists appears to be a therapeutic approach.

Kawaguchi et al. [32] recently reported that PGZ improved hepatic steatosis and prevented liver fibrosis in rats fed a CDAA diet. As the PGZ was mixed into CDAA chow and was administered from the beginning of CDAA feeding, the daily dose of PGZ could not be evaluated. In this study, we administered 1.0 mg/(kg day) of PGZ, an identical dose as that used for the treatment for patients with type 2 diabetes mellitus (approximately 0.5–1.0 mg/(kg day)). Using a rat model of NASH, this study demonstrated that clinical doses of PGZ effectively reduced steatosis and liver fibrosis, hepatic levels of TGF-β1, and the expression of type I procollagen α2 mRNA. In addition, serum hyaluronic acid levels, which reflect the degree of hepatic fibrosis in patients with chronic liver disease such as NASH [17], were also reduced in this model following PGZ treatment. These data document that clinical use of PGZ for NASH is both practical and effective.

We have also demonstrated that PGZ induced an antifibrotic effect in a rat model of liver fibrosis induced by administration of the CDAA diet. Oral administration of PPAR-γ agonists has been reported to reduce extracellular matrix deposition and HSC activation in both toxic and cholestatic models of liver fibrosis in vitro and in vivo [11,42]. PPAR-γ agonists inhibit the proliferation of HSCs; increased levels of PPAR-γ inhibit TGF-β1-induced collagen synthesis [11]. TGF-β plays an important role in liver fibrogenesis and activates HSC autocrine mechanisms [43]. While the expression of TGF-β1 is stimulated during liver fibrogenesis in rats fed a CDAA diet [16], TGF-β1 expression was suppressed by PGZ treatment in this study. Furthermore, PGZ treatment decreased the expression of ASMA, as assessed by immunohistochemical analysis and Western blot analysis. Although PGZ administration did not significantly affect PPAR-γ expression in liver tissues at 16 weeks of CDAA diet (data not shown), a decrease in hepatic expression of TGF-β1 and ASMA is likely due to the inhibition of HSC proliferation and activation by PGZ.

In this study, we demonstrate that PPAR-γ expression is stimulated in the livers of rats fed a CDAA diet. Administration of a clinical dose of a PPAR-γ agonist, PGZ, effectively reduced fatty accumulation in the liver tissues and inhibited the development of cirrhosis. As rats fed a CDAA diet are an appropriate experimental model for the liver phenotype exemplified in NASH, PGZ may be a useful therapy for patients with NASH. Further investigation will be needed to clarify the role of PPAR-γ in NASH development and to evaluate the effect of other PPAR-γ agonists on hepatic steatosis and fibrosis.

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Short communication

Development of a rapid semi-quantitative immunochromatographic assay for serum hepatocyte growth factor and its usefulness in acute liver failure

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Abstract

Measurement of serum human hepatocyte growth factor (HGF) by enzyme-linked immunosorbent assay (ELISA) is useful for the early diagnosis and prediction of prognosis of patients with acute liver failure (ALF). This ELISA methodology, however, is neither rapid nor convenient for use at the bedside. In this study, we have developed a rapid semi-quantitative immunochromatographic (IC) assay and evaluated its usefulness in assessing patients with acute hepatic injury. Only 100 μl of serum is required; the assay can be easily completed in 20 min. The values obtained using this novel assay correlated well with the values obtained using the standard ELISA protocol. In addition, the values obtained in the IC assay correlated with clinical course; increased serum HGF levels were associated with an increased frequency of ALF and death. These results indicate that this rapid semi-quantitative IC assay for HGF is useful for the early diagnosis of ALF and prediction of clinical outcome in acute hepatic injury.

Keywords: HGF; Rapid semi-quantitative assay; Acute liver failure; Immunochromatography

1. Introduction

Acute liver failure (ALF) is a rare clinical syndrome characterized by the abrupt onset of jaundice, coagulopathy, and encephalopathy in previously normal individuals [1,2]. Approximately 1% of patients with acute hepatitis develop ALF, including approximately 700 patients a year in Japan. Although living donor liver transplantation has contributed greatly to improved survival of patients with ALF [3], the prognosis of those who are unable to receive a liver transplantation is still poor [4–6]. Therefore, early diagnosis of ALF, facilitating the beginning of intensive care, is critical to improve the prognosis of patients [6].

Hepatocyte growth factor (HGF) was originally purified from patients with ALF [7,8]. We have previously established

Abbreviations: AH, acute hepatitis; ALF, acute liver failure; AOC, acute liver failure on chronic liver disease; CH, chronic hepatitis; ELISA, enzyme-linked immunosorbent assay; HBV, hepatitis B virus; HGF, hepatocyte growth factor; IC, immunochromatographic; PT, prothrombin time

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