INTRODUCTION

Hepatitis C virus (HCV) infection rarely resolves spontaneously once it becomes chronic [1]. Consequently, most patients in Japan with chronic HCV infection are likely to progress steadily to liver cirrhosis and hepatocellular carcinoma (HCC), which develops approximately 30 years after blood transfusion [2–4]. HCC is one of the most common malignancies, especially in Southeast Asia, and a major cause of death for patients with chronic HCV infection. In the early 1990s, interferon was introduced worldwide as a therapy for patients with chronic hepatitis C and was effective in inducing normalization of serum alanine aminotransferase (ALT) [5,6], eliminating HCV RNA [7,8], and improving liver histological findings [9–11] in patients with chronic hepatitis C.

To evaluate the effect of interferon therapy on the incidence of HCC and the risk of mortality for chronic hepatitis C patients, a randomized controlled trial is needed. However, a prospective randomized trial with untreated control patients is ethically impossible, because interferon therapy has already been established as a standard treatment for patients with chronic hepatitis C. Therefore, almost all chronic hepatitis C patients, except for cases with medical conditions such as depression, autoimmune disease and severe diabetes mellitus, have been treated with interferon in Japan. Recently, several investigators have reported this therapy as being effective for reducing the incidence of HCC among patients who showed normalization of ALT during and after interferon therapy, as well as among those in whom HCV was eradicated [12–17]. However, a reduced risk of HCC does not necessarily lead to improvement in survival. Indeed, little is known about the effects of interferon therapy on the mortality of patients with chronic hepatitis C. Several investigators [14, 18–23] have tried to evaluate the impact of interferon therapy on mortality. Four of these studies indicated that interferon therapy significantly reduced the mortality of compensated HCV-related cirrhotic patients [18,20] or of chronic hepatitis C patients including patients with compensated cirrhosis [21,23]. However, lack of analysis on response to interferon [18,20–23] or lack of information on disease-specific mortality [20,21] has made it difficult to evaluate the benefits of interferon for survival. Recently, Yoshida *et al.* [24] demonstrated that interferon therapy improved survival by preventing liver-related deaths of chronic hepatitis C patients showing a sustained virological response. However, whether a biochemical response to interferon therapy results in a reduced risk of mortality has not been investigated.

We conducted a multi-centre, large-scale, retrospective cohort study of patients with chronic hepatitis C, who had been enrolled at the end of 1997 at participating hospitals in order to analyse the effect of interferon therapy on the incidence of HCC. The aim of the present study was to examine the effect of interferon therapy on the mortality and causes of death among chronic hepatitis C patients.

PATIENTS AND METHODS

Patients

We recruited chronic hepatitis C patients from four previous studies which were conducted to assess the effect of interferon therapy on the incidence of HCC [12,14,15,17]. All patients meeting the following criteria were included in this study: (i) histological diagnosis of chronic hepatitis or cirrhosis; (ii) no history of clinical signs at entry into the study of complications of cirrhosis, i.e. ascites, jaundice, encephalopathy, or variceal bleeding; (iii) no evidence of HCC at entry into the study as assessed by ultrasonography and/or computed tomography; (iv) absence of serum hepatitis B surface antigen; (v) absence of co-existing liver diseases such as autoimmune hepatitis or primary biliary cirrhosis; (vi) absence of excessive alcohol consumption (>80 g/day); and (vii) absence of human immunodeficiency virus antibodies, as described previously [12,14,15,17]. A total of 3025 patients who met these criteria and whose initial sera tested positive for anti-HCV as determined by either first- or second-generation ELISA (Ortho Diagnostics, Tokyo, Japan) and HCV RNA were included in the study. The sera of patients who had been diagnosed as non-A, non-B hepatitis before anti-HCV testing became available (i.e. before 1989) had been frozen at −80 °C and were retrospectively assayed.

Of the 3025 chronic hepatitis C patients, 2762 had received interferon after 1987, when interferon became available in Japan. Interferon-treated patients received a 4–12-month course of interferon therapy, which was initiated within 1 month of liver biopsy. The remaining 263 patients did not undergo interferon therapy or any other antiviral therapy, including almost all patients with biopsy-proven chronic hepatitis who had refused interferon treatment due to adverse effects, lack of time for therapy, or their inability to undergo treatment as a consequence of depression, severe diabetes mellitus or other medical conditions.

Criteria for biochemical and virological responses to interferon therapy

The biochemical response during the follow-up up to 6 months after the completion of interferon therapy was defined according to previously described criteria with minor modifications [8,9]. In the sustained response group, ALT levels decreased to the normal range during therapy and remained within that range up to 24 weeks after therapy without any abnormal elevation. In the transient response group, ALT levels decreased to the normal range by the end of therapy, remained normal during therapy but returned to abnormal levels during the 24 weeks following interferon therapy. In the no-response group, ALT levels did not decrease to the normal range, or fluctuated during therapy and the subsequent 24 weeks. Both biochemical transient

and nonresponders were designated as nonsustained biochemical responders.

A sustained virological response was defined as HCV RNA negativity at more than 6 months after the cessation of interferon therapy. Patients showing positive HCV RNA at the same time were designated as nonsustained virological responders.

Histological evaluation

Liver biopsy was carried out before interferon therapy in all cases. Specimens were fixed in formaldehyde and embedded in paraffin. The sections were stained with haematoxylin-eosin and Azan-Mallory and analysed by two pathologists without any knowledge of the clinical and laboratory data. Histological findings were scored according to the classification of Desmet *et al.* [25].

Follow-up

The starting date of the follow-up for both the interferon-treated and untreated groups was defined as the date of liver biopsy. Biochemical examinations including α-fetoprotein and abdominal ultrasonography were carried out before interferon therapy and every 3–6 months thereafter at the outpatient clinic of the respective hospitals. The end of the follow-up was the date of death or the latest confirmation of survival. Follow-up data on the patients were obtained from the participating hospitals. Follow-up data that were not available from the hospitals were collected from the resident registry of the local municipal office. Death from liver-related disease was defined as death from HCC, liver failure determined by the presence of one or more of ascites, jaundice and hepatic encephalopathy, or variceal bleeding diagnosed on the basis of endoscopic findings of patients presenting with upper gastrointestinal haemorrhage.

Five untreated patients were observed for over 162 months, which corresponded to the longest period of observation of those treated with interferon. In these subjects, only the follow-up data up to 162 months were considered. Seventy-one patients whose follow-up period was shorter than 12 months were excluded from the study. The final numbers of study subjects were 2698 for the interferon-treated group and 256 for the untreated group.

Informed consent was obtained from each patient included in the study. The study protocol was in accordance with the Helsinki Declaration of 1975 (revised in 1983) and approved by the Ethical Committee of the Osaka University Graduate School of Medicine.

Statistical analysis

The chi-square test was used to compare the frequency of gender between the interferon-treated and untreated groups. The difference in age at liver biopsy and ALT between the two groups, expressed as median, was assessed for significance with the Student's *t*-test. The Wilcoxon rank-sum test was used to compare the distribution of age at liver biopsy and histological staging. Cumulative survival curves were determined with the Kaplan–Meier method, and the log-rank test was used to compare the cumulative survival rates.

The observed number of deaths was compared with the expected number, which was calculated by applying sex, 5-year age, 5-year calendar time, and cause-specific mortality rates for the general population in Japan, as prepared by the Statistics and Information Department, Japan Ministry of Health and Welfare [26]. The standardized mortality ratio (SMR) was expressed by dividing the observed number of deaths by the expected number of deaths. The standard error and the 95% CI of SMR were estimated by assuming Poisson's distribution, and differences in mortality between the study cohort and the general population were considered to be significant if the CI did not include unity.

Survival was also analysed by using Cox proportional hazards regression controlling for age (continuous variable), gender, stages of liver fibrosis (stage: 0/1/2/3/4) and time at liver biopsy (1991/1992). Risk ratios attributable to biochemical sustained, transient and no responses and to virological sustained and nonsustained responses were calculated in comparison with no treatment by using dummy variables.

Data analysis was performed with the SAS/PC statistical package (SAS Institute, Cary, NC, USA). All reported *P*-values were two-sided and $P < 0.05$ was considered to be significant.

RESULTS

Patient characteristics at entry

Of the 2698 patients treated with interferon, 901 (33.3%) had a sustained biochemical response, 701 (26.0%) a transient biochemical response and the remaining 1096 patients (40.6%) were classified as biochemical nonresponders. Serum HCV RNA remained negative at more than 6 months after cessation of interferon therapy in 738 (81.9%) of the sustained biochemical responders, designated as sustained virological responders, whereas serum HCV RNA remained positive in 133 (14.8%). Serum HCV RNA was not examined after the termination of interferon therapy in 30 sustained biochemical responders, who were excluded from the analysis according to virological responses to interferon. Positive HCV RNA after interferon therapy was detected in all of the biochemical transient and nonresponders.

The demographic and clinical features of interferon-treated patients according to virological and biochemical responses to interferon and of untreated patients at the time of enrolment are summarized in Table 1. Untreated patients were significantly older than interferon-treated patients ($P = 0.04$), but frequency distribution of age at liver biopsy

Table 1 Characteristics of interferon-treated patients according to virological and biochemical responses to interferon and of untreated patients

	Interferon-treated							
	Virological response		Biochemical response					
	SVR (n = 738)	non-SVR (n = 1930)	SBR (n = 901)	TBR (n = 701)	BNR (n = 1096)	Total (n = 2698)	Untreated (n = 256)	P-value
Median age (range)	51 (20–72)	54 (20–76)	52 (20–73)	53 (20–75)	54 (20–76)	53 (20–76)	54 (21–72)	0.04
Age at biopsy (%)								
≤49	337 (45.7)	687 (35.6)	392 (43.5)	277 (39.5)	369 (33.7)	1038 (38.5)	75 (29.3)	0.12
50–59	240 (32.5)	759 (39.3)	303 (33.6)	280 (39.9)	428 (39.1)	1011 (37.5)	123 (48.9)	
≥60	161 (21.8)	484 (25.1)	206 (22.9)	144 (20.5)	299 (27.3)	649 (24.1)	58 (22.7)	
Sex (M/F)	507/231	1210/720	595/306	440/261	703/393	1738/960	157/99	0.32
Median ALT (U/L), SD	91	92	87	79	103	92	98	0.57
(range)	(7–1110)	(11–1195)	(7–1110)	(13–1195)	(13–828)	(7–1195)	(9–563)	
Stage of fibrosis (%)								
0	5 (0.7)	11 (0.6)	7 (0.8)	4 (0.6)	5 (0.9)	16 (0.6)	9 (3.5)	0.34
1	259 (35.1)	476 (24.7)	337 (37.4)	228 (32.5)	190 (17.3)	755 (28.0)	84 (32.8)	
2	263 (35.6)	614 (31.8)	297 (33.0)	238 (34.0)	349 (31.8)	884 (32.8)	40 (15.6)	
3	189 (25.6)	725 (37.6)	235 (26.1)	209 (29.8)	471 (43.0)	915 (33.9)	93 (36.3)	
4	22 (3.0)	104 (5.4)	25 (2.8)	22 (3.1)	81 (7.4)	128 (4.7)	30 (11.7)	

SVR, sustained virological responders; SBR, sustained biochemical responders; TBR, transient biochemical responders; BNR, biochemical nonresponders; ALT, alanine aminotransferase.

and the stages of liver fibrosis, gender and ALT did not differ significantly. In sustained biochemical responders, the ratio of male patients and median ALT levels were significantly higher for patients with HCV eradication than for those without it ($P < 0.001$, each), whereas median age and the frequency distribution of the stages of liver fibrosis were not significantly different between the two groups.

Follow-up data

The mean period of observation (total cases: 6.0 ± 2.2 years) of the interferon-treated and untreated patients was 5.8 and 8.0 years, respectively, with the former being significantly shorter than the latter ($P = 0.0001$) because interferon therapy was not introduced in Japan until 1987.

Table 2 Follow-up data for interferon-treated patients according to virological and biochemical responses to interferon and for untreated patients

	Interferon-treated						
	Virological response		Biochemical response				
	SVR (n = 738)	non-SVR (n = 1930)	SBR (n = 901)	TBR (n = 701)	BNR (n = 1096)	Total (n = 2698)	Untreated (n = 256)
Mean period of observation, year (SD)	5.7 (2.0)	5.8 (1.9)	5.6 (2.0)	5.7 (1.8)	5.9 (1.9)	5.8 (1.9)	8.0 (3.4)
No. of deaths	7	94	10	10	81	101	52
Liver-related deaths	1	68	1	5	63	69	42
Death from HCC	1	57	1	4	53	58	31
Death from other liver diseases	0	11	0	1	10	11	11
Liver-unrelated deaths	9	26	9	5	18	32	10

SVR, sustained virological responders; SBR, sustained biochemical responders; TBR, transient biochemical responders; BNR, biochemical nonresponders; HCC, hepatocellular carcinoma.

The sustained virological responders, nonsustained virological responders, sustained biochemical responders, transient biochemical responders and biochemical nonresponders were observed for a mean of 5.7, 5.8, 5.6, 5.7 and 5.9 years, respectively (Table 2).

We identified 153 deaths from all causes during the follow-up. The 153 patients who died consisted of 10 sustained biochemical responders (seven of whom were sustained virological responders and three of whom were sustained biochemical responders without HCV eradication), 10 transient biochemical responders, 81 biochemical nonresponders and 52 cases without interferon treatment. Death from all causes did not occur in 30 sustained biochemical responders whose serum HCV RNA was not examined after cessation of interferon therapy. Death from liver-related disease was identified in 111 (73%) of the 153 patients who died: only one death (10%) from liver-related disease (death from HCC) was found among sustained responders with HCV eradication, five (50%) among transient biochemical responders (death from HCC in four cases), 63 (78%) among biochemical nonresponders (death from HCC in 53 cases) and 42 (81%) among untreated patients (death from HCC in 31 cases) (Table 2).

Cumulative survival

The cumulative survival rates from all causes of death were found to be significantly higher for interferon-treated than for untreated patients ($P < 0.001$) (Fig. 1a) The respective 5-year survival rates of interferon-treated and untreated groups were 97.8 and 95.3%, and the 10-year survival rates 87.2 and 77.1%. The cumulative survival rates for sustained virological responders were significantly higher than for nonsustained virological responders ($P < 0.001$) (Fig. 1b), with 5-year survival rates of 99.5 and 97.1%, and 10-year survival rates of 97.8 and 81.9%, respectively. The cumulative survival rates for sustained biochemical responders were significantly higher than for nonsustained biochemical responders ($P < 0.001$). When nonsustained biochemical responders were divided into transient biochemical responders and biochemical nonresponders, the cumulative survival rates for the transient biochemical responders were significantly higher than for the biochemical nonresponders ($P < 0.001$) (Fig. 1c). The respective cumulative survival rates for sustained biochemical responders, transient biochemical responders and biochemical nonresponders were 99.2, 99.1 and 95.8% at the end of the fifth year and 97.8, 97.6 and 72.6% at the end of the 10th year. Among sustained biochemical responders, the cumulative survival rates for sustained virological responders and sustained biochemical responders without HCV eradication were 99.5 and 99.2% at the end of fifth year and 97.8 and 99.2% at the end of the 10th year, showing no statistical significance ($P = 0.18$).

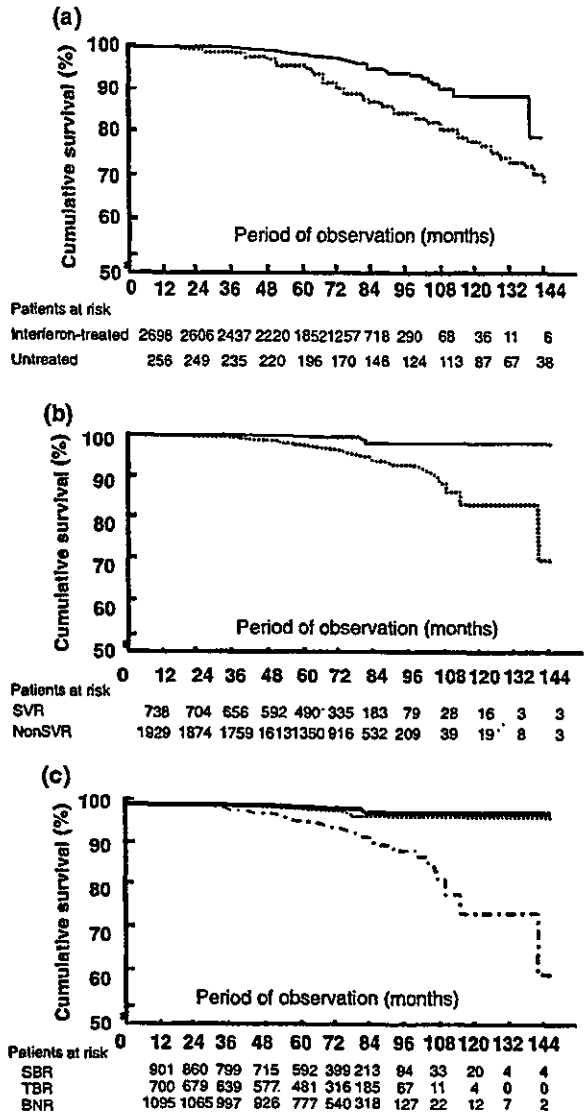

Fig. 1 Cumulative survival rates from all causes of death for patients with chronic hepatitis C. (a) For interferon-treated patients (solid line) and untreated patients (dotted line). (b) According to the virological response to interferon therapy: sustained virological responders (SVR) (solid line) and nonsustained virological responders (non-SVR) (dotted line). (c) In terms of the biochemical responses to interferon, sustained biochemical responders (SBR) (solid line), transient biochemical responders (TBR) (dotted line) and biochemical nonresponders (BNR) (dash-and-dot line).

Standardized mortality ratio

Differences in mortality among interferon-treated and untreated patients from the general population were further assessed by calculating SMR, the ratio of the observed number of deaths to the expected number. Overall mortality

Table 3 Standardized mortality ratios (SMR) in patients with chronic hepatitis C according to virological and biochemical responses to interferon

	Overall deaths			Liver-related deaths			Liver-unrelated deaths		
	Observed	Expected	SMR (95% CI)	Observed	Expected	SMR (95% CI)	Observed	Expected	SMR (95% CI)
Untreated	52	19.2	2.7 (2.0–3.6)	42	1.9	22.2 (16.0–30.0)	10	17.3	0.6 (0.3–1.1)
Interferon-treated	101	112.7	0.9 (0.7–1.1)	69	12.6	5.5 (4.3–6.9)	32	100.0	0.3 (0.2–0.5)
Virological response									
Sustained (HCV RNA negative)	7	29.8	0.2 (0.1–0.5)	1	3.3	0.3 (0.0–1.7)	6	26.5	0.2 (0.1–0.5)
Nonsustained (HCV RNA positive)	94	82.2	1.1 (0.9–1.4)	68	9.2	7.4 (5.8–9.4)	26	73.0	0.4 (0.2–0.5)
Biochemical response									
Sustained response	10	36.5	0.3 (0.1–0.5)	1	4.0	0.3 (0.0–1.4)	9	32.5	0.3 (0.1–0.5)
Transient response	10	27.5	0.4 (0.2–0.7)	5	3.2	1.6 (0.5–3.7)	5	24.3	0.2 (0.1–0.5)
No response	81	48.8	1.7 (1.3–2.1)	63	5.4	11.6 (8.9–14.9)	18	43.3	0.4 (0.3–0.7)

Difference from the expected number of deaths was considered significant if 95% CI of SMR did not include unity.

for untreated patients (SMR: 2.7; 95% CI: 2.0–3.6) but not for the interferon-treated patients (SMR: 0.9; 95% CI: 0.7–1.1) was significantly higher than for the general population. Liver-related mortality was high for untreated patients (SMR: 22.2; 95% CI: 16.0–30.0) and also for interferon-treated patients, although to a lesser degree (SMR: 5.5; 95% CI: 4.3–6.9) (Table 3). For sustained virological responders overall mortality was low (SMR: 0.2; 95% CI: 0.1–0.5), and liver-related mortality (SMR: 0.3; 95% CI: 0.0–1.7) was equivalent to that for the general population. In contrast, liver-related mortality was high for nonsustained virological responders (SMR: 7.4; 95% CI: 5.8–9.4).

Sustained and transient biochemical responders showed a low overall mortality compared with that for the general population (SMR: 0.3; 95% CI: 0.1–0.5, and SMR: 0.4; 95% CI: 0.2–0.7, respectively), whereas overall mortality was high for biochemical nonresponders (SMR: 1.7; 95% CI: 1.3–2.1). Liver-related mortality was not high for sustained and transient biochemical responders (SMR: 0.3; 95% CI: 0.0–1.4, and SMR: 1.6; 95% CI: 0.5–3.7, respectively) compared with that for the general population, but it was high for biochemical nonresponders (SMR: 11.6; 95% CI: 8.9–14.9) (Table 3). Overall and liver-related mortality for sustained biochemical responders without HCV eradication was equivalent to that for the general population (SMR: 0.5; 95% CI: 0.1–1.5, and SMR: 0.0; 95% CI: 0.0–6.1, respectively).

Interferon-treated patients had a statistically lower risk of liver-unrelated death than the general population (SMR: 0.3; 95% CI: 0.2–0.5), whereas untreated patients did not (SMR: 0.6; 95% CI: 0.3–1.1).

Multivariate analysis

The effect of interferon on the risk of death was assessed by Cox proportional hazards regression controlling for age, gender, score of liver fibrosis and time at liver biopsy. Interferon therapy significantly reduced the risk of overall death to a ratio of only 0.47, in comparison with no treatment. When patients were classified according to virological responses to interferon, sustained virological responders showed reduced risks of overall death (risk ratio: 0.14; 95% CI: 0.056–0.352; $P < 0.001$) and liver-related death (risk ratio: 0.04; 95% CI: 0.005–0.301; $P = 0.002$) compared with untreated patients, whereas nonsustained virological responders did not. Similarly, sustained biochemical responders showed a lower risk of death from all causes (risk ratio: 0.16; 95% CI: 0.069–0.354; $P < 0.001$) and liver-related diseases (risk ratio: 0.03; 95% CI: 0.004–0.230; $P < 0.001$). Transient biochemical responders had a high, but still significantly reduced risk of overall death (risk ratio: 0.19; 95% CI: 0.083–0.445; $P < 0.001$) and liver-related death (risk ratio: 0.18; 95% CI: 0.063–0.532; $P = 0.002$), whereas the risk for nonresponders and untreated patients did not

Table 4 Risk of death in patients with chronic hepatitis C according to virological and biochemical responses to interferon

	All causes of deaths			Liver-related deaths		
	Risk ratio	95% CI	P-value	Risk ratio	95% CI	P-value
Untreated	1.00			1.00		
Interferon-treated	0.47	0.261–0.836	0.010	0.59	0.312–1.097	0.095
Virological response						
Sustained (HCV RNA negative)	0.14	0.056–0.352	<0.001	0.04	0.005–0.301	0.002
Nonsustained (HCV RNA positive)	0.59	0.327–1.057	0.08	0.76	0.402–1.417	0.380
Biochemical response						
Sustained response	0.16	0.069–0.354	<0.001	0.03	0.004–0.230	<0.001
Transient response	0.19	0.083–0.445	<0.001	0.18	0.063–0.532	0.002
No response	0.78	0.432–1.393	0.394	1.02	0.543–1.900	0.962

Adjusted for age, sex, score of liver fibrosis and period at liver biopsy.

change (Table 4). The risk of overall death for sustained biochemical responders without HCV eradication was lower than for untreated patients, although it did not reach a statistical significance (risk ratio: 0.31; 95% CI: 0.09–1.07; $P = 0.06$).

DISCUSSION

We previously demonstrated that interferon treatment could reduce the risk of HCC development in patients with chronic hepatitis C [12]. Following this, five retrospective studies [13–17] showed a similar effect of interferon on the risk of HCC, especially for virological and biochemical responders. These results suggest that interferon therapy for chronic hepatitis C can prevent the development of HCC, possibly leading to improvement in long-term survival. However, only a few previous studies have assessed the effects of interferon therapy on survival [18–24], and whether interferon therapy also reduces mortality from liver-related disease in patients with chronic HCV infection has not been thoroughly investigated. It is also still unclear what type of response to interferon results in the improvement of long-term survival.

To evaluate the effect of interferon therapy on the risk of mortality for chronic hepatitis C patients, a randomized controlled trial should be carried out. However, a prospective randomized trial with untreated control patients is ethically impossible, because interferon therapy has already been established as the standard modality for patients with chronic hepatitis C. Only two randomized controlled trials of a small number of HCV-related cirrhotic cases have evaluated the effect of interferon therapy on mortality [19,21], but with discrepant results. In contrast, large-scale prospective and retrospective cohort studies [23,24] indicate that interferon therapy for HCV-related cirrhosis or chronic hepatitis C improves long-term survival. In particular, Yoshida *et al.* [24] demonstrated in their recent retrospective cohort study that interferon therapy improved survival of chronic hepatitis C patients by preventing liver-related deaths. However, its beneficial effect was considered to be limited to patients with a sustained virological response.

As ours is a retrospective cohort study, it may be subject to several biases. The interferon-treated and untreated groups had different demographic characteristics, including age and gender. These factors were adjusted for multivariate regression analysis and considered when calculating SMR by applying the corresponding mortality for the general population. Severity of chronic liver disease was adjusted by using the stage of liver fibrosis for multivariate analysis. As the time of liver biopsy of untreated patients was earlier than for interferon-treated patients, mortality for untreated patients may be generally higher than for interferon-treated patients. To avoid this bias, we adjusted the time at liver biopsy for multivariate analysis, and 5-year time-specific mortality rates for the general population were prepared in the SMR analysis. Moreover, the number of untreated patients was small, because most Japanese chronic hepatitis C patients, except for cases with medical problems, have been treated with interferon. However, the relatively small number of untreated patients in comparison with the large number of interferon-treated patients is not likely to have resulted in a substantial overestimation of the effect of interferon therapy on survival as several of the biases already mentioned were controlled in the analyses.

When we compared the observed mortality with the expected mortality for the matched general population by calculating SMR, we were able to demonstrate that chronic hepatitis C patients had higher overall and liver-related mortality than the general population, and that the majority of deaths were liver-related. However, interferon-treated patients had a significantly lower risk of liver-unrelated mortality, whereas untreated patients did not. This may represent a selection bias in the use of interferon therapy, which included patients with no medical problems

except for having chronic liver diseases. However, our multivariate regression analysis clearly showed that interferon therapy reduced the risk of liver-related death in virological responders by 96% and in biochemical responders by 82–97%. These findings indicate that a significant reduction in the risk of death from all causes for patients treated with interferon, shown in the analysis of SMR, was not caused by a selection bias but is mainly attributable to the prevention of liver-related death by interferon therapy.

Our multivariate analysis made it clear that the risks of overall and liver-related deaths for chronic hepatitis C patients displaying a sustained virological response were 86 and 96% lower than for untreated patients. The risk reduction for sustained biochemical responders was almost equal to that for sustained virological responders. Similarly, the SMR analyses showed that liver-related mortality for these patients was equivalent to that for the general population. Thus, and as expected, when patients treated with interferon belong to the sustained virological or biochemical response group, they appear to have the highest long-term survival rate.

Of nonsustained virological responders, the risk of death from all causes and liver-related diseases for transient biochemical responders was significantly lower than for untreated patients, but higher than for sustained biochemical and virological responders. The same effects of interferon therapy on survival were observed in the SMR analyses. Although the follow-up period was not sufficiently long for a reliable and accurate examination of mortality, we would like to emphasize that the risk of death from all causes and liver-related diseases was significantly lower for chronic hepatitis C patients for whom interferon was effective in normalizing ALT than for patients who did not receive interferon, even when HCV was not eradicated. However, the risk of death from all causes and liver-related diseases was not reduced in biochemical nonresponders.

In conclusion, the findings reported here indicate that interferon therapy improves long-term survival in chronic hepatitis C patients showing a biochemical as well as a virological response by preventing liver-related deaths.

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特集

肝細胞癌の経皮的治療 (ラジオ波焼灼療法について)

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肝細胞癌の現状

我が国において肝臓病の患者さんは多く，その中でもB型肝炎ウイルスあるいはC型肝炎ウイルスによる慢性肝疾患が大部分を占めている。ウイルスの関連した慢性の肝障害は炎症の持続により慢性肝炎，肝硬変と進展しそして肝臓病の終末である肝細胞癌を発生することになる。肝硬変患者における肝細胞癌の発生頻度は高率であるが，慢性肝疾患のもっとも多い原因となるC型肝炎ウイルスによる肝硬変においては，年率5～7%に肝細胞癌を発生すると報告されている。また，肝硬変の死亡原因の約80%近くが肝細胞癌によるものである。このことは如何に肝細胞癌を的確に治療することが肝臓病の治療において最も重要であることを示している。

肝細胞癌の経皮的治療

肝細胞癌の治療には，外科的切除，血管造影下の肝動脈塞栓療法，化学療法など種々の治療法が試みられている。肝細胞癌は肝硬変を基礎に発症してくるため1個の癌結節を治療しても年率約20%の頻度で肝内に異所の再発を来たすとされている。宮崎大学医学部第二内科の成績でも，単発の肝細胞癌を治療しても肝内の異所再発は高率であった（図1）。このような肝細胞癌の再発様式を踏まえて肝細胞癌の治療はできうる限り患者さんに対する侵襲度を抑えた加療が第一選択となると考える。肝細胞癌に対する経皮的治療としては，従来超音波ガイド下エタノール注入療法（PEIT）が有効であり広くおこなわれていた（文献1）。我々の教室でも1999年以前は肝細胞癌の治療はPEITが主な治療法であった。PEITにおける肝細胞癌の治療は現在でも高

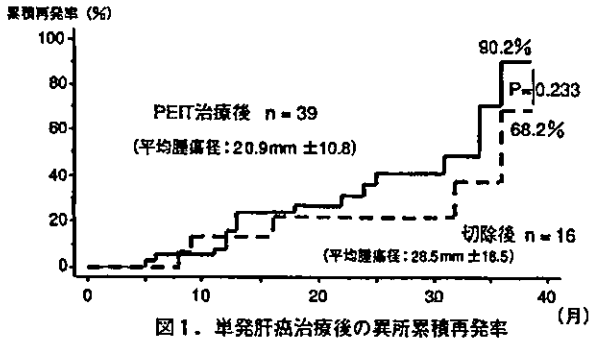

図1．単発肝癌治療後の異所累積再発率

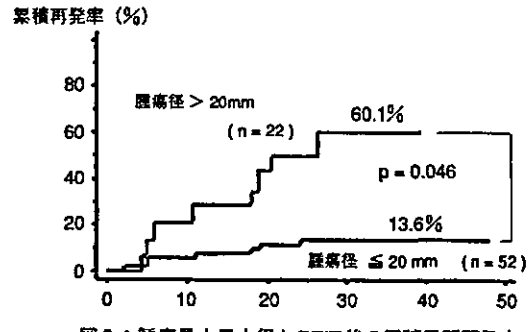

図2：腫瘍最大最大径とPEIT後の累積局所再発率

く評価されており，われわれの教室においても20㎜以下の肝細胞癌においては高い治療効果が得られていた。しかしながら，最大径20㎜以上の肝細胞癌に対しては，治療後3年の累積局所再発率は60.1%であり，腫瘍最大径20㎜が治療の限界であった（図2）。さらに，PEITにて腫瘍の完全壊死をはかるためには，腫瘍容積を超える量のエタノールを腫瘍内に充満させなければならない。通常1回の穿刺にて注入できるエタノール量は約2.0cc程度である。このため頻回の治療を必要とし，腫瘍径20㎜の肝細胞癌に対して，平均約6回の穿刺治療が必要であり，治療に3週間以上を費やしていたのが現状であった。また，1994年には本邦ではマイクロ波の誘電加熱による熱凝固を利用したマイクロ波凝固療法が，主に腹腔鏡下におこなわれるようになった（文献2）。しかしながら，経皮的におこなうには皮膚の熱傷など合併症の頻度が高く，また1回の穿刺凝固によって得られる有効な凝固範囲が狭いために，肝細胞癌を完全に壊死させるためには複数回の穿刺凝固が必要であり，煩雑なため肝細胞癌の経皮的治療の第一選択にはならなかった。

ラジオ波焼灼術は1996年にRossiらにより報告された治療法である（文献3）。1999年より日本にラジオ波焼灼療法が導入され，1999年2月より宮崎大学医学部第二内科でも導入され600例以上の治療をおこなってきた。これまでの治療成績をもとに，肝細胞癌のラジオ波焼灼療法に関して述べたいと思う。

ラジオ波焼灼装置

ラジオ波焼灼療法（RFA）とは，従来まで肝細胞癌の治療に応用されてきたマイクロ波凝固と同様の原理で高周波の誘電加熱作用を応用し，組織を凝固壊死させる治療法である。マイクロ波は2450MHzの周波数帯の電波を利用しているが，ラジオ波はこれより周波数の長い約450KHzの電波を使用している。電極先端より発生された電波が水分子である荷電体の運動を誘発し，そこに生じた分子間の摩擦熱により組織そのものを加熱する原理を利用している。RFA装置として本邦で使用される装置は3機種であるが，これら3つの機種に使用する電極が異なり，出力も異なる。また使用する電極の性状にて展開型の電極を使用する機種と，非展開型の電極を使用するタイプに大別される（図3）。我々

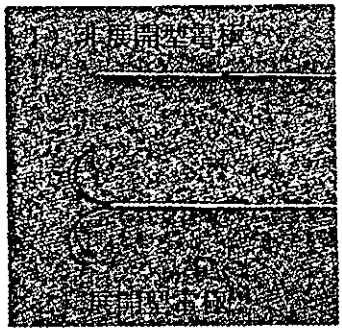

図3　RFAの穿刺電極

の施設では，1999年より2001年までは主に展開型の電極にてほぼ200例以上の治療をまた2001年より現在まではおもに非展開型の電極を用いて治療を行っている。展開型の電極穿刺針は針の中心部と展開した針の辺縁部より熱が発生するようになっているが，非展開型の針においては中心部のみより熱が発生するように設計されている，これらの針の性状により，出力装置の出力が50Wから最大150Wまでと異なり，その出力の差によって合併症の頻度も若干異なる。

穿刺電極は非展開型の電極また展開型の電極どちらも，有効な熱凝固範囲が最大30㎜に

及ぶように設計されているが，実際の焼灼範囲は肝硬変の組織の状態（繊維化の程度），腫瘍の形態（被膜の有無）などによって必ずしも均一ではない。我々の施設で行った結果では，展開型の電極（model30）による焼灼凝固範囲は治療後のCTでの計測により30.5×25.5㎜の楕円形を呈していた。また，焼灼範囲は周辺に熱伝導を妨げる大きな血管が存在するとcooling効果にて焼灼範囲が狭くなり抗腫瘍壊死効果の減弱が危惧されたが，われわれの成績では，比較的太い血管（門脈，肝静脈）の近傍に腫瘍が位置しても局所の再発率に優位差は生じなかった（文献4）。肝細胞癌の多くは被膜を有しているが，腫瘍の被膜内にて熱を発せさせると，結節内で熱は効率よく広がることが知られている（oven効果）。しかしながら，このoven効果のために，被膜外の組織に焼灼範囲が広がることはかえって困難となる。

RFAによる治療成績

1995年より我々の教室において治療をおこなった，1回の穿刺，焼灼において有効な焼灼範囲が得られたと判断した99結節の肝細胞癌において，そのRFAによる腫瘍効果をみた（文献4）。当初直径30㎜までの肝細胞癌を対象に治療をおこない，3年の局所無再発率がほぼ80%であり，RFAは肝細胞癌に対する有効な治療であると考えられた（図4）。しかしながら，約20%に局所の再発を認めたため，治療後の局所再発率に影響する種々の因子に関して検討した。その結果，腫瘍のサイズと腫瘍の存在位置が局所再発に最も影響する因子であることがわかった。腫瘍サイズが直径25㎜を超えた肝細胞癌では治療後の局所再発率が有為に高く，RFAによる1回の穿刺，焼灼では腫瘍最大径25㎜が適応の限界であると考えられた（図5）。これは，先ほどに述べたように腫瘍の壊死範囲の最短径が25㎜程度となることに関係しているように思われる。25㎜以上の被膜を有する肝細胞癌においては，oven効果にて凝固壊死範囲が直径25㎜以上に広がることは可能であるが，結節被膜を超えては壊死範囲が広がることはなく，被膜外の娘結節や，septumを有する腫瘍内の結節には壊死効果は及ばない可能性がある。腫瘍径が25㎜を超えるようになると，

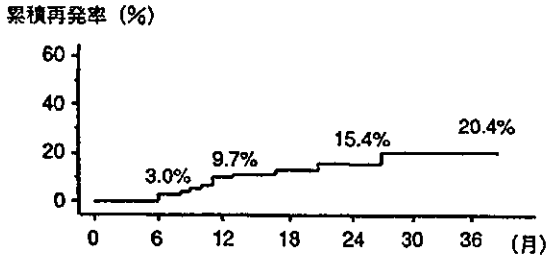

図4 RFA後の局所再発率

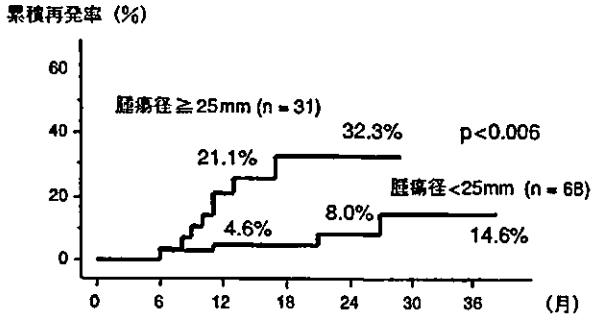

図5 RFA後の腫瘍最大径と局所再発率

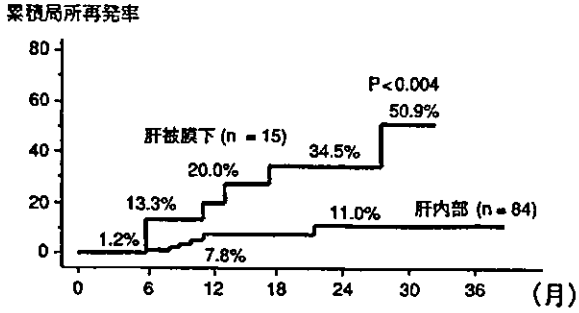

図6 腫瘍存在部位における局所再発率の差異

肝癌結節は効率に被膜外の腫瘍周辺に娘結節を持つようになる。これらの，娘結節にまで被膜を超えて熱凝固範囲を広げることが困難なため腫瘍最大径25㎜以上では1回の焼灼では局所再発率の上昇が見られることも1因と考えられる。また，腫瘍の存在位置においても局所再発率に差異が認められた（図6）。肝臓の辺縁，肝被膜下に存在する結節は，肝内部に存在する腫瘍に比べて局所再発率が有為に高かった。これは，肝辺縁，肝被膜下に存在する腫瘍に対しては穿刺針を腫瘍の中央部に穿刺することが困難であることがおおく，有効な壊死範囲を得ることが困難であることを示している。短期間の検討であるが我々の成績においては，現在，本邦にて使用されている展開型，非展開型の電極の差によって局所再発率の差はなかった。

今回のわれわれの検討において，1回の穿刺焼灼にて25㎜以上の腫瘍もしくは肝被膜下に存在する腫瘍の局所再発率は高かく，RFA単独治療の適応にはなりにくい。現在我々の施設においては，肝予備能の比較的保たれている症例においては，これらの肝細胞癌の治療は積極的に外科的切除もしくは侵襲をなるべく抑えた腹腔鏡下の凝固療法，あるいは小切開下の凝固療法などの治療を選択するようにしている。しかしながら，基礎の肝硬変の予備能が比較的悪く，また，複数箇所にすでに肝細胞癌が存在する症例においては外科的治療の適応も困難であることが多い。現在我々の施設では，最大径25㎜以上の肝細胞癌においては，肝機能が極端に低下していない限り（Child-Pugh分類Cを除く）先行して血管造影下に化学塞栓療法を施行した後にRFAを施行している。この治療法の利点は，リピオドールを集積させた肝細胞癌の周辺を全周性に覆うように凝固壊死領域を確保できることにある。このため結節被膜外に浸潤した肝細胞癌の娘結節の焼灼も可能となり，また，術後の画像的な効果判定が非常に確実に下せる。現在まで20結節以上の肝細胞癌にこの併用療法を応用しほぼ1年以上経過を観察しているが，これらの結節において，現在まで局所再発は認めていない。さらに肝被膜下に存在する肝細胞癌に対しては，経皮的にアプローチせずに，外科的に腹腔鏡下，胸腔鏡下にマイクロターゼ凝固療法，RFAを選択するようにしている。また，最近では熱凝固の進展を肝血流が妨げるため（cooling effect），肝血流遮断をおこなったうえで熱凝固の範囲の拡大をはかり，腫瘍径の大きな肝細胞癌にたいしてRFAをおこなう試みが報告されている（文献5）。

RFAの合併症

ラジオ波焼灼療法において重篤な合併症は少ない。しかしながら約40%以上の症例において術後2，3日続く38℃第の発熱を認めている。さらに，穿刺時の痛みに対して，鎮痛剤の追加投与が必要な症例は半数に達する。しかしながら，治療に伴う肝機能の悪化，肝不全への進行はほとんど経験していない（表1）。

表1 RFAによるおもな合併症

	展開型電極 (n = 160)	非展開型電極 (n = 123)
黄疸*	2 (1.8 %)	4 (3.3 %)
胸水貯留	2 (1.8 %)	1 (0.9 %)
胆道出血	2 (1.8 %)	0
腸管穿孔	1 (0.9 %)	0
肝梗塞	0	2 (1.8 %)
術中ショック	0	1 (0.9 %)

＊ 黄疸 ： 総ビリルビン値 4.0mg/dl以上)

また，穿刺に伴う腹腔内出血はこれまでの約600例の経験において2例に認めている。いずれの症例も保存的に止血したが，これら2症例は肝被膜下に存在した症例であり，治療後の熱による止血操作が十分におこなえなかった症例である。本来，RFAによる治療は穿刺針を抜去の際に穿刺ルートを熱凝固しながら抜去できるために，出血の可能性は低く，軽微であれば，腹水の貯留した症例においても安全に施行できる治療法である。さらに予想される重篤な合併症は周辺臓器への熱の波及に伴う臓器損傷である。肝周辺とくに腹部の手術の既往のある患者さんにおいては，肝被膜に癒着した臓器に熱凝固が及び臓器損傷をきたす可能性がある。また，非展開型の穿刺電極は出力数も大きいため，肝被膜外に突出した肝細胞癌に物理的な圧力がかかり，癌結節を破裂させ，肝被膜外へ組織を散布させてしまう可能性が高い。2001年Llovetらは30㎜以上の肝細胞癌にたいし，行ったRFA治療にて32結節中4結節の治療において腹膜播種をきたしたと報告したが（文献6），この論文の対象になった結節は最大径が30㎜以上と比較的大きな腫瘍であり，ほとんどの結節が肝被膜下に存在している。これらの結節は，先ほど述べたようにわれわれの施設においてはRFAの単独治療では適応しないと考えられる結節であるため，我々の経験では，腹膜播種の頻度はこれらの報告より極端に低い。しかしながら，我々の教室においても約600結節の治療中2結節に腹膜播種をきたした。これらの結節は腫瘍径約20㎜であるが，2結節ともに肝被膜下に存在していた。さらに，使用した電極針が非展開型（cool-tip type）を使用しており，被膜下に存在した結節の治療経過中に物理的な力が働き，腹腔内播種をきたしたのではないかと考えられる。

ま と め

ラジオ波焼灼療法は，1回の治療にて径25㎜までの腫瘍を凝固できる治療法である。これまで，小肝細胞癌の治療にも約1ヶ月の入院期間が必要であったが，現在ではクリニカルパスの導入とともに，約1週間程度の入院にて治療が完結できるようになり，患者さんのQOLも大きく改善している。RFAは短期間に肝細胞癌を有効に治療できる手段として今後，内科的経皮的治療法の第一選択となると考えられる。しかしながら，その治療効果はオールマイティではなく，その短所と長所，あるいは使用する機器の違いによる合併症の差などを十分理解したうえで導入することが必要である。

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HCV core antigen as an alternative to NAT to detect HCV viremia

A recent report in TRANSFUSION by Laperche and coworkers[1] adds to growing evidence that the detection of HCV core antigen is a useful alternative to the detection of HCV RNA. We compared HCV core antigen with NAT for detection of HCV RNA in a Japanese population with a high prevalence of HCV infection

The adult residents of a village in Miyazaki Prefecture, Japan, were followed from 1984 to 2001 as part of the prospective population-based Miyazaki Cohort Study.[2] This community has high prevalences of both anti-HTLV-I (approx., 27%) and anti-HCV (approx., 23%). Serum samples and demographic information were collected at annual government-sponsored health examinations. A cross-sectional study was conducted using sera samples from 90 anti-HCV+ village residents. HCV core antigen was detected by fluorescent EIA[3] (Immunocheck F-HCV Ag Core, International Reagents Co., Kobe, Japan); levels of at least 15 pg per mL were considered positive according to the manufacturer's instructions. HCV RNA was detected by NAT using RT-PCR (Amplicor HCV test v. 1.0, Roche Diagnostic Systems, Inc., Branchburg, NJ). The NAT assay was modified so that the volume of sample amplified was 25 µL instead of 5 µL.

Among the 90 anti-HCV+ village residents, HCV core antigen was detected in 68 (76%) and HCV RNA was detected in 76 (84%). Age, sex, anti-HTLV-I status, or ALT level did not influence the detection of HCV core antigen or HCV RNA. Considering the HCV RNA test as the reference assay, HCV core antigen was detectable in 65 of 76 (85.5%) residents who were positive for the presence of HCV RNA (Table 1). Considering the HCV core antigen test as the reference assay, HCV RNA was detectable in 65 of 68 (95.6%) residents who were positive for the presence of HCV core antigen. The specificity of the assays relative to each other is shown in Table 1.

TABLE 1. Comparison of HCV core antigen and HCV RNA in 90 subjects with anti-HCV

	HCV RNA		
HCV core antigen	Positive*	Negative*	Total
Positive†	65 (85.5)	3 (21.4)	68
Negative	11 (14.5)	11 (78.6)	22
Total	76	14	90

* Data are reported as number (%).
† Positive defined as ≥15 pg/mL.

Using HCV RNA as the "gold standard," the sensitivity of the HCV core antigen test (85.5%) is in agreement with a previous report of 90.3 percent sensitivity in a sample of anti-HCV+ blood donors and hospital patients, with similar HCV core antigen and HCV RNA assays.[4] A new version of the HCV core antigen assay has been developed that is nearly as sensitive as the HCV RNA assay used in this study;[5] the use of the older assay is a limitation of this study.

Three NAT-negative individuals in the present study had detectable HCV core antigen (range, 109-189 pg/mL). Because the levels of HCV core antigen were well above the cutoff value for a positive test, false-positive results seem unlikely. Nevertheless, given the high sensitivity of the modified HCV RNA assay used, which can detect as few as 100 IU of HCV RNA per mL of serum, false-negative HCV RNA results also seem unlikely. Sequence variation in the amplification target for the RT-PCR assay may account for the observed discrepancy in assay results.

The HCV core antigen assay is less expensive and easier to perform than the assay for HCV RNA. This study suggests that the HCV core antigen assay may be a useful method for identifying active HCV infection in situations where NAT is not available.

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Enhanced expression of growth factors and imbalance between hepatocyte proliferation and apoptosis in the livers of rats fed a choline-deficient, L-amino acid-defined diet

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Abstract

In a rat model of hepatocarcinogenesis induced by a choline-deficient, L-amino acid-defined (CDAA) diet, hepatocellular carcinoma (HCC) occurs in conjunction with fatty liver, hepatocyte injury and regeneration, fibrosis and cirrhosis. This is similar to human HCC development with cirrhosis. The aim of this study is to clarify sequential changes in the expression of growth and growth inhibitory factors, and hepatocyte proliferation and apoptosis during development of preneoplastic nodules in rats fed a CDAA diet. The expression of hepatocyte growth factor was stimulated at about the same time as CDAA diet-induced liver injury within 1 week. Hepatocyte growth factor reached a maximum level of expression from 4 to 8 weeks. Transforming growth factor (TGF)-α expression increased from 4 to 40 weeks. Although TGF-β, a growth inhibitory factor for hepatocytes, was also expressed with a peak from 4 to 8 weeks followed by a gradual decrease until 48 weeks, expression of cyclin D1 and hepatocyte proliferation continued to be stimulated throughout the experimental period. Additionally, the number of apoptotic hepatocytes was markedly reduced after peaking at 8 weeks. These results suggest that some hepatocytes in the livers of rats fed a CDAA diet may escape from TGF-β-induced growth inhibition and apoptosis, leading to development of preneoplastic nodules.

Keywords: Hepatocarcinogenesis; Hepatocyte growth factor; Transforming growth factor-α; Heparin binding-epidermal growth factor-like growth factor; Transforming growth factor-β; Cell proliferation; Apoptosis

1. Introduction

The development of hepatocellular carcinoma (HCC) is a lengthy process, and is strongly associated with liver cirrhosis or fibrosis. These preconditions are most often a consequence of chronic viral hepatitis due to hepatitis B virus or hepatitis C virus infection. Although viral proteins have been reported to play a role in hepatocarcinogenesis [1,2], this is not sufficient to explain all mechanisms of HCC development, indicating the involvement of endogenous factors, such as prolonged exposure to growth factors due to repeated hepatocyte injury. Recently, Nakae et al. have reported a rat model of hepatocarcinogenesis induced by a choline-deficient, L-amino acid-defined (CDAA) diet. This system is an appropriate in vivo experimental model, in which HCCs develop without exposure to exogenous carcinogen [3]. In this rat model, HCCs occur in conjunction with fatty liver, hepatocyte death and subsequent regeneration, fibrosis, and eventual cirrhosis. This is similar to the histopathological sequence of human HCC development with cirrhosis [4]. The repeating cycles of hepatocyte injury and regeneration [5], inhibition of apoptosis [6], oxidative stress [7], hypomethylation of DNA and RNA [8], and chronic activation of protein kinase C [9] are known to be involved in hepatocarcinogenesis induced by choline-deficiency.

Several growth factors play an important role in liver regeneration. Once hepatic injury occurs, liver regeneration is stimulated by hepatocyte growth factor (HGF), transforming growth factor (TGF)-α, and heparin binding-epidermal

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growth factor-like growth factor (HB-EGF). These growth factors induce hepatocyte proliferation mainly through the mitogen-activated protein kinase pathway and G1 cyclins [10]. On the other hand, TGF-β1 has been shown to be involved in the development of cirrhosis [11–14], and to inhibit proliferation of hepatocytes [15–18]. TGF-β1 suppresses the growth factor-induced signals which stimulate cyclin D1 and cyclin E, thereby resulting in the inhibition of DNA synthesis [10]. These growth and growth-inhibitory factors act in concert to regulate proliferation of hepatocytes precisely during liver regeneration in vivo, and the impaired cooperation of growth factors and the escape by some initiated cells from growth inhibitory stimuli are hypothesized to be contributing factors in HCC development.

In this study, we clarify the relationship between the expression of growth factors and hepatocyte proliferation following CDAA diet-induced hepatic injury. We have investigated the sequential changes in blood and serum markers, expression of growth and growth-inhibitory factors, and proliferation and apoptosis of hepatocytes during the development of cirrhosis and preneoplastic nodules.

2. Materials and methods

2.1. Animals

Six-week-old male Fischer 344 rats were obtained from Kyushu Experimental Animal Supply (Kumamoto, Japan). The animals were maintained under constant room temperature (25 °C) and provided free access to water and the indicated diet throughout the study. The protocol for these animal studies was approved by the ethical committee of Miyazaki Medical College (Miyazaki, Japan).

After a 1-week acclimation period on a standard diet, the rats were switched to a CDAA diet (Dyets Inc., Bethlehem, PA), and sacrificed at pre-determined intervals over the following 48 weeks. Blood was obtained from the bifurcation of the abdominal aorta. The number of platelets, serum alanine amintransferase (ALT), and albumin were determined. The liver and spleen were immediately excised, and the wet weight of these organs was determined. Samples were subjected to histological analysis or frozen in liquid nitrogen and stored at −80 °C until analysis.

2.2. Northern blot analysis

Total RNA was extracted from liver tissues of rats fed a CDAA diet by the acid guanidinium thiocyanate:phenol: chloroform method. Growth factor mRNAs were detected using the following PCR-amplified cDNA probes: the 2274-bp fragment corresponding to bases 20–2306 of rat hepatocyte growth factor/scattering factor (HGF/SF) cDNA [19], the 240-bp fragment corresponding to bases 57–297 of rat transforming growth factor (TGF)-α [20], the 801-bp fragment corresponding to bases 690–1498 of rat heparin binding-epidermal growth factor like growth factor (HB-EGF) [21] and the 438-bp fragment corresponding to bases 518–956 of rat TGF-β1 [22]. Cyclin D1 transcript was detected using a 427-bp rat cyclin D1 cDNA [23].

2.3. Histopathological and immunohistochemical analysis

Two 5 mm thick slices from the two major liver lobes (left lateral and median lateral lobes) were fixed in 10% formalin and embedded in paraffin. Four or five serial 4 μm sections were prepared from each fixed liver slice. The first was stained with hematoxylin and eosin (HE) or azan for histological examination. The remaining three sections were subjected to immunohistochemical analysis. After boiling in distilled water for 10 min, slides were incubated with a rabbit polyclonal antibody against the placental form of rat liver glutathione *S*-transferase (GST-P) (Medical and Biological Laboratories, Nagoya, Japan), an anti-proliferating cell nuclear antigen (PCNA) monoclonal mouse antibody (Dako Japan, Kyoto, Japan) or a rabbit polyclonal antibody against single-stranded DNA (Dako Japan) [23]. Goat anti-mouse or anti-rabbit IgG was then applied followed by an avidin–biotin–peroxidase complex and chromatin 3′,3′-diaminobenzidine. The PCNA labeling indices were calculated as percentage of labeled hepatocytes counted by light microscope. The number of apoptotic cells, which were stained with the anti-single-stranded DNA antibody, was analyzed.

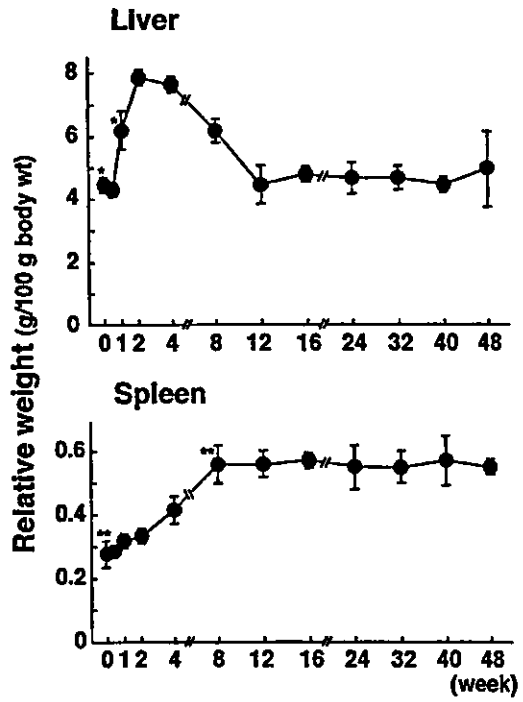

Fig. 1. Changes in the relative liver and spleen weights during CDAA diet administration. The ratios of liver and spleen weight to body weight (g/100 g body weight) in rats fed a CDAA diet were determined at the indicated time points (n = 5 or 10). Administration of a CDAA diet-induced a transient increase in liver weight from 2 to 4 weeks, and gradual enlargement of the spleen until 8 weeks ($^*P = 0.001$, $^{**}P < 0.001$).

2.4. Statistical analysis

Unless otherwise specified, data are expressed as mean ± S.D. Statistical parameters were ascertained using Statview J-4.5 software (Abacus Concepts Inc., Berkeley, CA). Differences between means were assessed by the unpaired Student's *t*-test. The significance level was set at $P < 0.05$.

3. Results

3.1. Sequential changes in the weight of the liver and spleen during CDAA diet-induced cirrhosis development

The weight of the liver and spleen was measured periodically during CDAA diet administration (Fig. 1). The relative liver weight increased with statistical significance at 1 week ($P = 0.001$), and reached a peak weight from 4 to 8 weeks. After this, the liver weight gradually decreased until after 12 weeks the liver was the same weight as before CDAA diet feeding. Histologically, fat deposition was observed in zones 2 and 3 of hepatocyte lobuli 3 days after the start of the CDAA diet, and quickly expanded, resulting in diffuse fatty liver at 1 week (Fig. 2A and B). Collagen fibers began to extend at 4 weeks, followed by development of cirrhosis (Fig. 2C, E and F). GST-P positive nodules were observed in some rats fed a CDAA diet for 4 weeks (Fig. 2D). Administration of the CDAA diet for 8 weeks resulted in a gradual increase in the relative spleen weight ($P < 0.001$) (Fig. 1). Persistent enlargement of the spleen was observed in rats fed a CDAA diet from 8 to 48 weeks. These results indicate that an increase in the liver and spleen

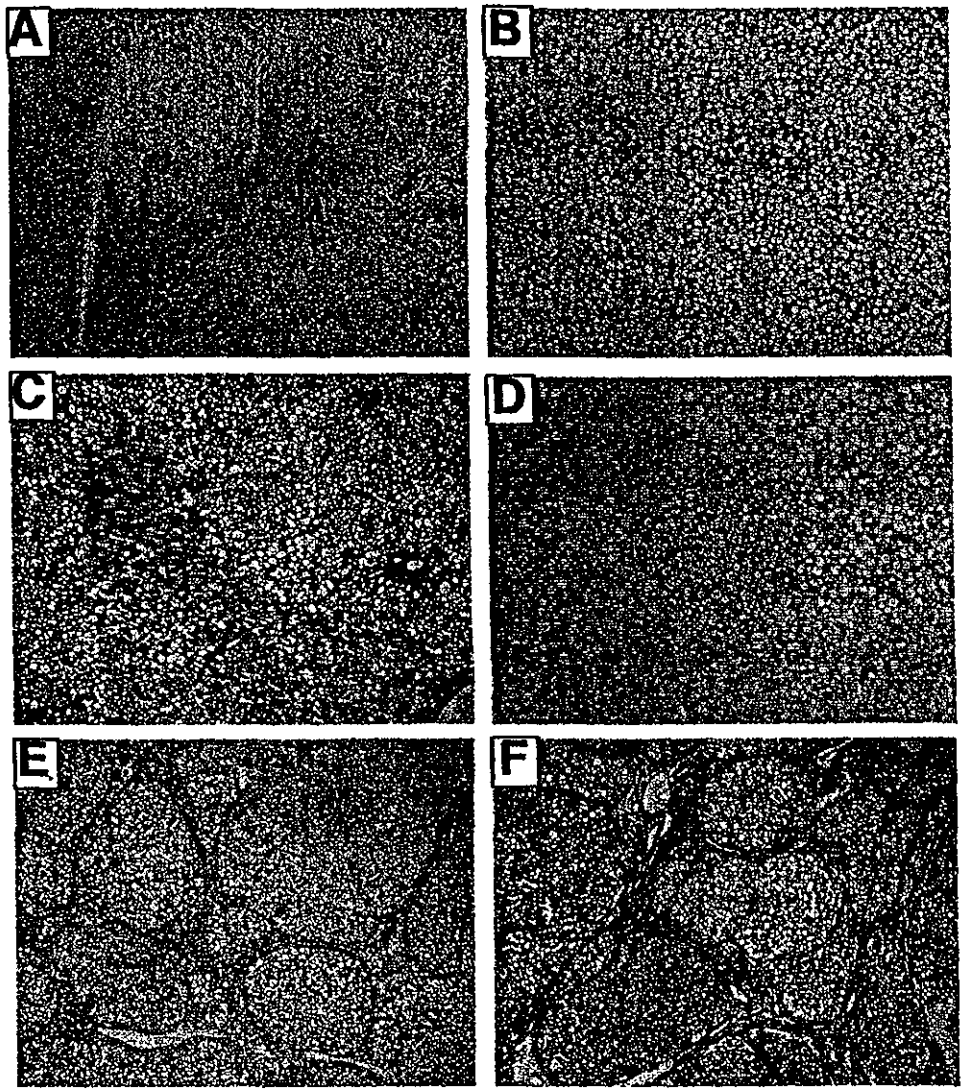

Fig. 2. Sequential histological changes in the livers of rats fed a CDAA diet. Liver tissue was obtained from rats fed a CDAA diet for 3 days (A), 1 weeks (B), 4 weeks (C and D), 12 weeks (E), and 24 weeks (F), and stained with HE (A, B, E and F), azan (C) or anti-GST-P antibody (D), as described in Section 2. Fat deposition was observed in zones 2 and 3 of hepatocyte lobuli on day 3, and then expanded. Collagen fibers began to extend starting at 4 weeks, followed by development of cirrhosis. GST-P-positive nodules were observed in a few rats fed a CDAA diet for 4 weeks [magnifications 40× (A–C, E and F) and 100× (D)].

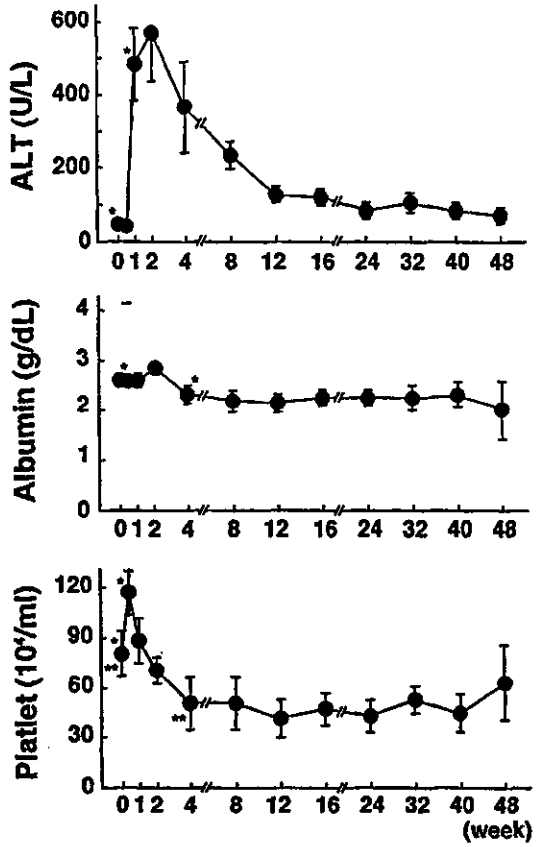

Fig. 3. Changes in serum levels of ALT and albumin and the number of platelet in rats fed a CDAA diet. A transient increase in serum ALT was observed from 2 to 4 weeks of administration of a CDAA diet ($n = 5$ or 10) ($^*P < 0.001$). The level of serum albumin was reduced 8 weeks after feeding the CDAA diet ($n = 5$ or 10) ($^*P < 0.001$). The number of platelets continued to show a decrease from 4 to 48 weeks following a transient increase on day 3 ($n = 5$ or 10) ($^*P = 0.005$, $^{**}P < 0.001$).

weights are associated with development of fatty liver and cirrhosis.

3.2. Hepatocyte injury was induced in the early weeks of CDAA diet administration, followed by development of cirrhosis

To evaluate the liver damage during CDAA diet feeding, we examined sequential changes in serum ALT, a sensitive indicator of hepatocyte injury (Fig. 3). Rats fed a CDAA diet for 1 week showed a marked increase in serum ALT levels ($P < 0.001$), although this effect was not observed after 3-day administration of the CDAA diet. The serum ALT levels reached a peak at the 2nd week, and then gradually decreased during the following 10 weeks. A mild elevation of serum ALT levels continued from 12 weeks throughout the experimental period. Although serum albumin, a marker for biosynthetic function of the liver, showed a slight transient increase in rats fed a CDAA diet for 2 weeks, serum albumin then decreased with statistical significance to lower levels than were present at the start of administration of the CDAA diet ($P < 0.001$) (Fig. 3). These decreased levels continued from 4 to 48 weeks. In addition, platelets, which are reduced in parallel with development of cirrhosis, increased to a maximum level after 3 days of a CDAA diet administration ($P = 0.005$), and then rapidly decreased within 4 weeks (Fig. 3). The numbers of platelets from 4 to 40 weeks was reduced to 50–60% of the levels observed in rats before CDAA diet feeding ($P < 0.001$). These results indicate that CDAA diet-induced hepatic injury occurs mainly in the early phase of diet administration. This damage is followed by the development of cirrhosis, resulting in a decrease in serum albumin and platelets.

3.3. Increased expression of growth factors and cyclin D1 in the livers of rats fed a CDAA diet

To examine growth stimuli following CDAA diet-induced liver injury, we evaluated the expression of growth factors and cyclin D1, a growth factor-dependent G1 cyclin (Fig. 4). Low-level expression of HGF mRNA was observed in normal liver tissue. However, expression of HGF was stimulated in rats fed a CDAA diet for 3 days. The level of HGF expression increased to a maximum level after 4 weeks of treatment, and then decreased until week 12. In contrast, expression of TGF-α mRNA, which was also detected in normal liver tissues, gradually increased beginning at 2 weeks, and reached a peak from 24 to 40 weeks. HB-EGF transcripts were barely detectable in normal liver tissues. However, low-level expression of HB-EGF was detected after 1 week of CDAA diet feeding, and moderately increased from 4 to 8 weeks, followed by low expression. In parallel with expression of these growth factors, cyclin D1 expression was significantly stimulated 1 week after CDAA diet administration, and enhanced expression of cyclin D1 continued throughout the experimental period.

Expression of TGF-β1, which plays an important role in hepatic fibrosis [11–14] and is an inhibitory growth factor for hepatocytes [15–17], also increased in rat livers treated with a CDAA diet for 1 week. Expression of this mRNA reached a maximum level from 4 to 8 weeks of treatment, followed by a gradual decrease until 48 weeks. Collagen fibers begin to extend after 4 weeks of CDAA diet feeding (Fig. 2C), resulting in the development of cirrhosis until 30 weeks [24,25]. Therefore, the sequential changes in TGF-β1 expression correlate closely with this development of cirrhosis.

3.4. Accelerated proliferation and decreased apoptosis of hepatocytes during administration of a CDAA diet

Next we examined the proliferation of hepatocytes in GST-P-positive nodules, which were considered to be precancerous lesions, and the surrounding GST-P-negative areas of livers from rats fed the CDAA diet (Fig. 5A). The number of hepatocytes staining positive for PCNA, an S-phase marker, in GST-P-negative areas increased with sta-