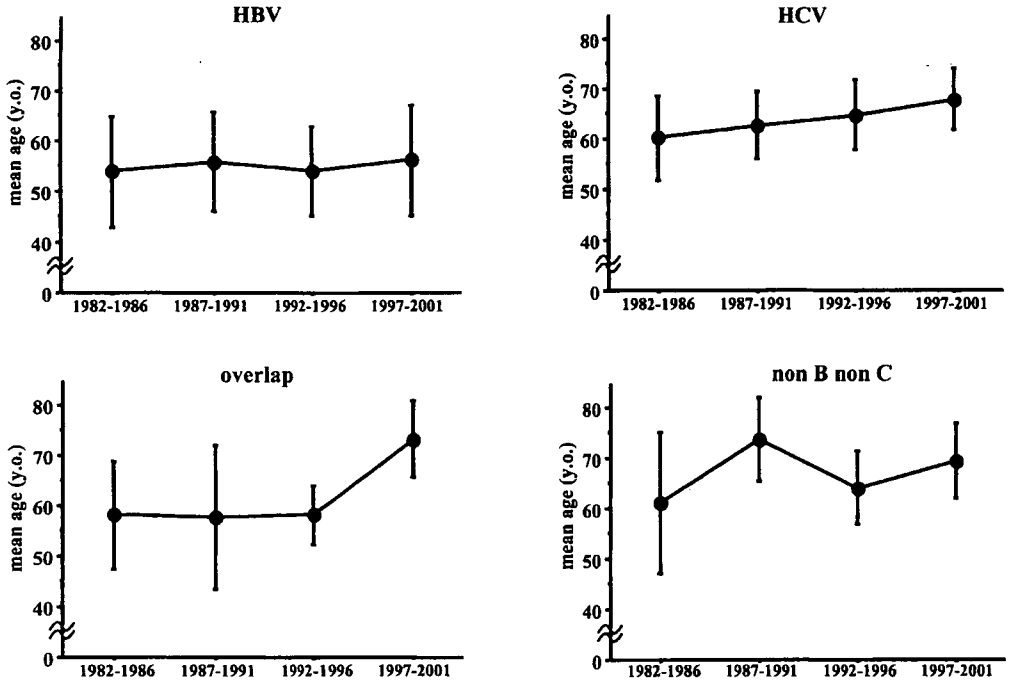

Figure 2. Sequential changes in the mean age of HCC patients categorized by etiology during the observation period. *P<0.05. The bars is standard deviation (SD).

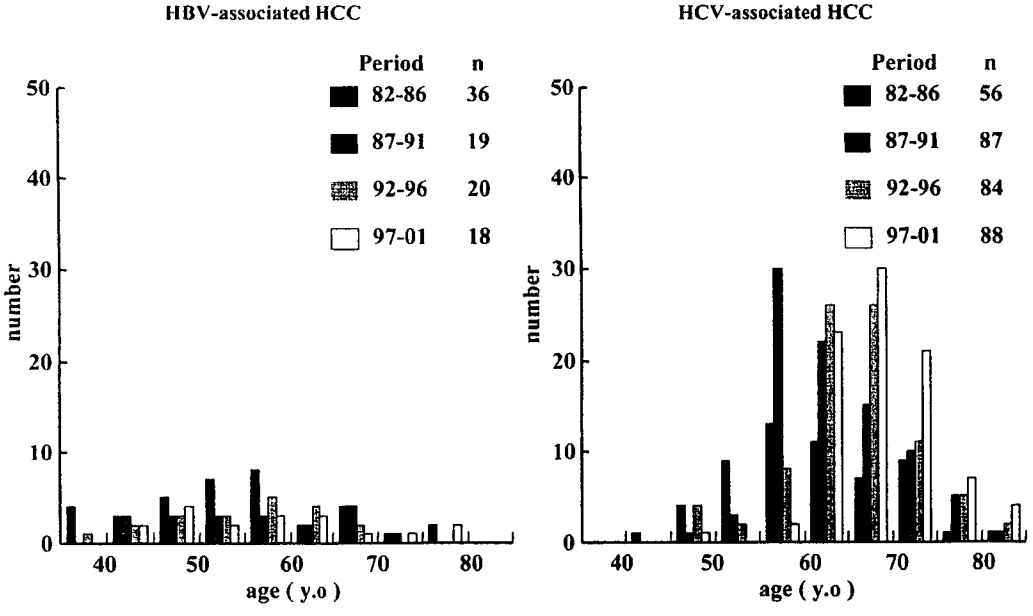

Figure 3. The age distribution of patients with HBV- and HCV-associated HCC during the four 5-year periods.

Table III shows the mean age and other characteristics at diagnosis of HCV-associated HCC in five-year intervals (1982-1986, 1987-1991, 1992-1996, and 1997-2001). In addition to mean age, the number of patients with Child-Pugh stage A showed a significant increase during the studied periods. Alcohol consumers significantly decreased during the periods.

In analysis of patients without alcohol consumption and Child-Pugh stage C in HCV-associated HCC, the mean ages in 1982-1986, 1987-1991, 1992-1996 and 1997-2001 were

Table I. The background of the 93 patients with HBV-associated HCC.

	No.	(%)	Mean age	(SD)	P-value
All	93	100	55	(10)	
Gender					
Male	71	76	53	(10)	
Female	22	24	60	(9)	0.0065[a]
Alcohol consumption					
Not excessive	84	90	55	(10)	
Excessive	9	10	52	(8)	NS[a]
IFN therapy					
(-)	91	98	55	(10)	
(+)	2	2	51	(3)	NS[a]
BMI					
<25	76	82	56	(10)	
≥25	17	18	54	(9)	NS[a]
Diabetes mellitus					
(-)	83	89	55	(11)	
(+)	10	11	58	(10)	NS[a]
Child-Pugh staging					
A	57	61	55	(1)	
B	28	30	56	(2)	
C	8	9	50	(3)	NS[b]
Tumor size					
<3 cm	38	41	56	(9)	
≥3 cm	55	59	54	(11)	NS[a]
Tumor no.					
Single	50	54	56	(10)	
Multiple	43	46	53	(10)	NS[a]

[a]Mann-Whitney U test. [b]ANOVA. SD, standard deviation; NS, not significant.

Table II. The background of the 315 patients with HBV-associated HCC.

	No.	(%)	Mean age	(SD)	P-value
All	315	100	64	(7)	
Gender					
Male	251	80	64	(7)	
Female	64	20	67	(7)	0.0032[a]
Alcohol consumption					
Not excessive	266	84	65	(7)	
Excessive	49	16	62	(7)	0.0107[a]
IFN therapy					
(-)	298	95	64	(8)	
(+)	17	5	66	(4)	NS[a]
BMI					
<25	255	81	63	(7)	
≥25	60	19	65	(8)	NS[a]
Diabetes mellitus					
(-)	229	73	65	(8)	
(+)	86	27	63	(7)	0.0173[a]
Child-Pugh staging					
A	207	66	65	(7)	
B	93	30	64	(8)	
C	15	4	60	(10)	0.0181[b]
Tumor size					
<3 cm	136	43	65	(7)	
≥3 cm	179	57	64	(8)	NS[a]
Tumor no.					
Single	165	52	65	(8)	
Multiple	150	48	64	(7)	NS[a]

[a]Mann-Whitney U test. [b]ANOVA. SD, standard deviation; NS, not significant.

62, 63, 64 and 68 years of age, respectively (1982-1986 vs. 1997-2001, p=0.0001) (Table IV).

Age-specific prevalence of HCV infection in the general population of studied area. The age-specific prevalence of HCVAb among the 13869 persons who visited Nagasaki prefecture Tarami Hospital for health screening from 1996 to 2002 is shown in Table V. Although the positive rate for HCVAb was 1.64% (277 of 13869) as a whole, it was higher in the group aged more than 60 years irrespective of gender.

Discussion

Our study was a single-center, hospital-based study designed to examine the sequential change in backgrounds among patients with HCC during the past 2 decades. More than 90% of our patients had chronic HBV or HCV infections. During the observation period, the number of HBV- and HCV-associated HCC cases decreased and increased in 1987-1991, respectively, and thereafter reached a plateau. These findings were consistent with previous reports from Japan (7,14). Additionally, the age-

Table III. The mean age and the other characteristics of HCV-associated HCC at diagnosis in 5-year intervals.

Period	1982-1986	1987-1991	1992-1996	1997-2001	Total	P-value
No.	56	87	84	88	315	
Age (years) (SD)	60 (8)	63 (7)	65 (8)	68 (6)	64 (7)	<0.0001
Gender						
Male	47	68	67	69	251	
Female	9	19	17	19	64	
Ratio	5.2	3.6	3.9	3.6	3.9	NS
Alcohol consumption						
Not excessive	42	68	75	81	266	
Excessive	14	19	9	7	49	
Ratio	3.0	3.6	8.3	11.6	5.4	0.0078
Diabetes mellitus						
(-)	39	64	64	62	229	
(+)	17	23	20	26	86	
Ratio	2.3	2.8	3.2	2.4	2.7	NS
Child-Pugh staging						
A	28	52	57	70	207	
B	25	32	22	14	93	
C	3	3	5	4	15	0.0160

SD, standard deviation; NS, not significant.

Table IV. Mean age of HCV-associated HCC without excessive alcohol consumers and Child-Pugh stage C.

Year	1982-1986	1987-1991	1992-1996	1997-2001	Total
No.	40	66	71	77	254
Mean age (years)	62	63	64	68	65
SD	8	7	7	6	7

NS (1982-1986 vs 1987-1991)
NS (1987-1991 vs 1992-1996)
0.0338 (1992-1996 vs 1997-2001)
NS (1982-1986 vs 1992-1996)
NS (1987-1991 vs 1997-2001)
0.0012 (1982-1986 vs 1997-2001)
0.0001 (1982-1986 vs 1997-2001)

SD, standard deviation; NS, not significant.

specific prevalence of HCV infection in the general population of the studied area was also in agreement with Japanese epidemiological studies which showed a high prevalence of HCVAb in the population, 60 years of age and older (15-17).

In analysis of background features among HCC patients, HBV-associated HCC cases revealed no significant change, whereas the mean age of patients with HCV-associated HCC steadily increased from 60 to 68 years of age during the studied period. In patients with HCV-associated HCC, factors such as male gender, excessive alcohol consumption and diabetes mellitus, which are known to be risk factors for HCC, contributed to lowering the age of HCC occurrence. Furthermore, patients with Child-Pugh stage C were younger than those with stages A and B. When the mean age of HCV patients without alcohol consumption and Child-Pugh stage C, which may contribute to aging of HCV-associated HCC, was

Table V. Age-specific prevalence of HCV infection in Nagasaki prefecture, Japan.

Age	Male			Female			Total		
	No.	HCVAb(+)	(%)	No.	HCVAb(+)	(%)	No.	HCVAb(+)	(%)
0-19	0	0	0.00	0	0	0.00	0	0	0.00
20-29	7	0	0.00	7	0	0.00	7	0	0.00
30-39	594	3	0.51	303	4	1.32	897	7	0.78
40-49	2553	29	1.14	1051	9	0.86	3604	38	1.05
50-59	3188	38	1.16	1445	19	1.31	4633	56	1.21
60-69	2517	58	2.30	1309	37	2.83	3826	95	2.48
≥70	515	21	4.08	380	10	2.63	895	31	3.46
Total	9374	148	1.58	4495	79	1.76	13869	277	1.64

analyzed, a significant increase was also found during the studied period. Since the size and number of HCC cases was not associated with the mean age of HCV-associated HCC patients, it is unlikely that the delay in diagnosis of HCC accounted for aging of HCV-associated HCC cases. Indeed, it is possible that other factors contributed to the steady increase in the mean age of patients with HCV-associated HCC.

Japan with overall HCV prevalence rates similar to that of the United States (approximately 1-2%) but with higher incidence rates of hepatocellular carcinoma (8-10 times lower in the United States) is thought to have had earlier onset and peaks of the HCV epidemic than the United States (5,8,9). A recent study examined the constant evolutionary rate of HCV over time ('the molecular clock') in retrospectively collected serum samples of HCV carriers in Japan and the United States (18). The study concluded that HCV first appeared in Japan around 1882 and in the United States around 1910, whereas widespread dissemination occurred from 1940s to 1960s in Japan and from 1960s to 1980s in the United States. Risk factors for transmitting HCV were rampant during this period (e.g, injection drug use, needle sharing, and transfusion of unscreened blood and blood products) (19). It is speculated that these modes of transmission are responsible for differences in the age-specific prevalence of HCV infection in the general population. In the United States, the incidence of HCC continues to increase with the fastest rate among 40- to 50-year-old persons who have the highest rate of HCV infection (20,21). Thus, it is likely that the increasing mean age of patients with HCV-associated HCC in our study was associated with a shift toward an older-age group who had the highest rate of HCV infection.

It is known that 2-4 decades of chronic HCV infection is required to develop cirrhosis and subsequent HCC (22-25). The number of HCC cases has increased in Japan, because individuals infected with HCV during the past have grown old and have reached the cancer-bearing age. The prevalence of HCV infection in young Japanese persons is low and the incidence of HCVAb is very low because of preventative actions against HCV infection such as the screening of blood products for HCV and the use of sterile medical equipment (26). Additionally, we showed that the number of patients with HCV-associated HCC cases reached a plateau together with an increase in the mean age, although the present study was a single-center, hospital-based study. These findings indicate that a decrease in the prevalence of HCC in Japan, a country that is far advanced with regard to HCV-associated HCC, is expected in the near future. We believe that long-term experience in Japan helps to plan strategies against HCV-associated HCC and to cope with its long-term sequelae in many other countries worldwide.

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The Impact of Newer Treatment Modalities on Survival in Patients With Hepatocellular Carcinoma

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Background & Aims: Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide. However, although the therapeutic approaches for HCC have progressed rapidly, it remains unknown whether the current management of patients with HCC has reduced its mortality. We analyzed changes of survival rate in patients with HCC over a 20-year period. **Methods:** Between 1982 and 2001, 463 patients were diagnosed with HCC at our hospital. Subjects were enrolled in the current cohort according to the following inclusion criteria: HCC lesion measuring less than 3 cm in diameter, no evidence of extrahepatic metastasis, and no evidence of main portal vein infiltration/thrombosis. A total of 257 patients with HCC were recruited for this study, and categorized into 5-year intervals. **Results:** The survival rates improved significantly during the study period. When the patients were stratified according to Child-Pugh score, only patients with Child's B showed improved survival rates. Furthermore, patients with surgical resection or transarterial chemoembolization during the latter period had a better prognosis than those during the early period. **Conclusions:** Our findings suggest that the development of therapeutic interventions for HCC have led to improvements in the prognosis for HCC patients.

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide. Estimates of the incidence of cancer in 2000 indicate that primary liver cancer represented the fifth and eighth most common malignancy in men and women, respectively. The number of new cases is predicted to be 564,000, with 398,000 cases in men and 166,000 in women.[1] The geographic areas at highest risk are Eastern Asia, Middle Africa, and some countries of Western Africa.[1] Recently, a trend of increasing rates of HCC has been reported from several of the developed countries in North America and Europe.[2,3]

Although the age-adjusted rates of HCC incidence have increased between 1958 and 2000 in Japan,[4,5] the changes in the prognosis of HCC patients over this period are not understood fully. In the present study, we examined the changes in the survival rates of HCC patients over a 20-year period.

Patients and Methods

Patients

Between 1982 and 2001, 463 patients were diagnosed with HCC in the First Department of Internal Medicine at the Nagasaki University School of Medicine. Subjects were enrolled in the current cohort according to the following inclusion criteria: (1) HCC lesion measuring less than 3 cm in diameter, (2) no evidence of extrahepatic metastasis, and (3) no evidence of main portal vein infiltration/thrombosis. A total of 257 patients with HCC were recruited for this study. The diagnosis of HCC was based on α-fetoprotein (AFP) levels and imaging techniques, including ultrasonography, computed tomography, magnetic resonance imaging, hepatic angiography, and/or liver biopsy examination. The diagnostic criteria for HCC included confirmative liver biopsy examination or increased AFP levels (>20 ng/mL) and neovascularization in hepatic angiography and/or computed tomography. The cohorts of patients with HCC were divided into 5-year intervals (1982–1986, 1987–1991, 1992–1996, and 1997–2001). Table 1 indicates the diagnostic procedure(s) of the studied patients during each of the 5-year intervals.

Cause of Hepatocellular Carcinoma

Sera were stored at −80°C until they were used for the following assays. The diagnosis of chronic hepatitis C virus infection was based on the presence of anti-hepatitis C virus antibodies (microparticle enzyme immunoassay; Abbott Laboratories, Abbott Park, IL) and hepatitis C virus RNA, as detected by polymerase chain reaction. The diagnosis of chronic hepatitis B virus infection was based on the presence of hepatitis B surface antigen (enzyme-linked immunosorbent assay; Abbott Laboratories). Serum AFP level was measured by radioimmunoassay (Abbott Laboratories). The history of alcohol intake was noted from medical records. Habitual drinking was defined as an average daily consumption of an amount equivalent to 80 g of pure ethanol for a period of more than 10 years.

Treatment and Follow-Up Evaluation

All patients were assessed for surgical resection once they were diagnosed with HCC. These assessments were based on lobar involvement and liver functional status. The lobar involvement was evaluated by a combination of ultrasonography, computed tomography, magnetic resonance imaging, and hepatic angiography. Patients were considered not suitable for resection when they showed the following criteria: (1) bilobar involvement, (2) evidence of main portal vein infiltration/

Abbreviations used in this paper: AFP, α-fetoprotein; HCC, hepatocellular carcinoma; PEIT, percutaneous ethanol injection therapy; TACE, transarterial chemoembolization.

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Table 1. Diagnostic Procedure of the Studied Patients in 5-Year Intervals

	1982–1986	1987–1991	1992–1996	1997–2001
Diagnostic method (%)				
Histologic diagnosis	14 (37.8)	28 (45.2)	47 (66.2)	52 (59.8)
Hepatic angiography	23 (62.2)	34 (54.8)	17 (23.9)	30 (34.5)
Computed tomography only	0 (0)	0 (0)	7 (9.9)	5 (5.7)

thrombosis, (3) evidence of extrahepatic metastases, (4) Child's C cirrhosis, or (5) poor cardiac and respiratory performance status. If the patients were deemed not suitable for surgery or did not agree to undergo surgery, percutaneous ethanol injection therapy (PEIT) was the second choice of treatment offered to patients with HCCs less than 3 cm in diameter. The remaining patients without main portal vein thrombosis or extrahepatic metastasis were advised to undergo transarterial chemoembolization (TACE) irrespective of the size and number of tumors.

After initial treatment, the studied patients underwent measurement of AFP levels and liver function biochemistry every 1–3 months of the follow-up period, and ultrasonography was performed every 3–6 months. Patients in whom recurrence of HCC was suspected were evaluated further by computed tomography and/or magnetic resonance imaging. The assessment of treatment for recurrent HCC was based on lobar involvement and liver functional status in the same manner as that for the initial treatment. Radiofrequency ablation or liver transplantation for HCC was started at our institution in 2002. Therefore, none of the patients were treated by radiofrequency ablation or transplantation between 1982 and 2001. Furthermore, none of the subjects in our study received either of these treatments for recurrent HCC during the follow-up period.

The closing date of the current study was April 2005 or the time of a patient's death. If a patient had not been monitored in our hospital for more than 1 year, the patient was considered lost to follow-up evaluation.

Statistical Analysis

The time of survival was measured from the time of HCC diagnosis to the time of death, or until the time of writing. The data were analyzed by the Mann–Whitney test for continuous ordinal data, χ^2 test with Yates' correction and the Fisher exact test for the association between 2 qualitative variables, and Kaplan–Meier survival analysis. Parametric comparisons were assessed by analysis of variance. The significance of individual differences was evaluated by use of the Scheffe's test. The standard error was calculated based on the binomial model for the response proportion. P values of less than .05 were considered statistically significant.

Results

Clinical Features of the Studied Patients

A total of 257 patients were enrolled in this study, and were followed up for a mean of 3.64 years (range, 1.41–22.38 y). Patient characteristics at the time of HCC diagnosis are presented in Table 2. There were 187 men (72.8%) and 70 women (27.2%) who were HCC patients (mean age, 63 y). The proportion of patients diagnosed with hepatitis B virus-associated HCC was 17.9% (46 of 257), whereas 72.3% (186 of 257) showed hepatitis C virus-associated HCC, and an additional 3.9% (10 of 257) showed HCC associated with both viruses. Three of the remaining 15 patients had a history of significant alcohol intake and 12 had no known cause. Child-Pugh grade A was recorded in 63.8% (164 of 257), grade B was noted in 30.7% (79 of 257), and grade C was noted in 5.5% (14 of 257) of the patients. Solitary HCC was detected in 61.1% (157 of 257) of all cases. The AFP values were normal (<20 ng/mL) in 36.6% (94 of 257), 21–200 ng/mL in 37.0% (95 of 257), and more than 201 ng/mL in 26.4% (68 of 257). Of the studied patients, 11.3% (29 of 257) underwent surgical resection, 43.6% (112 of 257) received PEIT, 34.6% (89 of 257) underwent TACE, and 10.5% (27 of 257) received palliative care only.

Table 3 presents the characteristics at diagnosis of HCC in 5-year intervals (1982–1986, 1987–1991, 1992–1996, and 1997–2001). The mean age at the time of HCC diagnosis increased steadily and the number of patients with Child-Pugh grade A or AFP value of less than 20 ng/mL showed a significant increase during the studied periods. There were no significant differences in the cause of liver disease between the 5-year

Table 2. Background Features of the Studied 257 Patients at Baseline

	Number	%
Patients	257	100
Average age at diagnosis (SD)	63 (9)	
Sex		
Male	187	72.8
Female	70	27.2
Cause of liver disease		
HBV	46	17.9
HCV	186	72.3
HBV + HCV	10	3.9
Alcohol	3	1.2
Unknown	12	4.7
Child–Pugh staging		
A	164	63.8
B	79	30.7
C	14	5.5
Tumor lesions		
Solitary	157	61.1
Not solitary	100	38.9
AFP level, *ng/mL*		
≤20	94	36.6
20–200	95	37.0
>200	68	26.4
Therapy		
Surgical resection	29	11.3
PEIT	112	43.6
TACE	89	34.6
Only palliative care	27	10.5

HBV, hepatitis B virus; HCV, hepatitis C virus.

Table 3. Characteristics of the Studied Patients in 5-Year Intervals

	1982–1986	1987–1991	1992–1996	1997–2001	*P* value
Number of patients	37	62	71	87	
Average age (SD)	58.5 (10.3)	61.8 (8.5)	62.9 (7.0)	66.6 (8.0)	<.0001
Male/female	25/12	44/18	51/20	67/20	.7026
Child–Pugh staging (%)					
A	15 (40.5)	37 (59.7)	47 (66.2)	65 (74.7)	
B	20 (54.1)	22 (35.5)	21 (29.6)	16 (18.4)	
C	2 (5.4)	3 (4.8)	3 (4.2)	6 (6.9)	.0387
Number of tumor lesions (%)					
Solitary	24 (64.9)	39 (62.9)	42 (59.2)	52 (59.8)	
Not solitary	13 (35.1)	23 (37.1)	29 (40.8)	35 (40.2)	.9239
AFP level, *ng/mL* (%)					
≤20	4 (10.8)	23 (37.1)	23 (32.4)	44 (50.6)	
21–200	14 (37.8)	23 (37.1)	27 (38.0)	31 (35.6)	
>200	19 (51.4)	16 (25.8)	21 (29.6)	12 (13.8)	<.0001

intervals (data not shown). As shown in Table 4, surgical resection for HCC has decreased drastically since 1989 when PEIT was introduced into our hospital. Patients who underwent liver transplantation or radiofrequency ablation were not included in this study because these therapies were only introduced into our hospital in 2002. When the patients were categorized according to the Japan integrated staging score,[6,7] which combines the Child-Pugh classification and TNM staging, there were no significant changes between the 5-year intervals (Table 5).

Independent Predictors of Survival Rate

Table 6 indicates the results of univariate and multivariate analyses using the Cox proportional hazards model. According to univariate analysis, 6 of 9 factors (Child-Pugh grade B or C, not solitary HCC, AFP level >200, treatment by PEIT, TACE, and study period between 1982 and 1991) significantly affected the survival rate in the patients with HCC. When multivariate analyses were performed on the 5 significant variables (Child-Pugh score, number of tumors, AFP level, treatment by TACE, study period), 4 of the factors (Child-Pugh grade B or C, not solitary tumor, AFP level >200, and study period between 1982 and 1991) were found to be independent prognostic indicators.

Change of Survival Rate in 5-Year Intervals

Figure 1 indicates the cumulative survival rates for cohorts of HCC patients during each of the 5-year intervals between 1982 and 2001, and shows improved prognosis during the studied periods (1982–1986 vs 1997–2001, $P < .0001$; 1982–

Table 4. Therapeutic Procedure of the Studied Patients in 5-Year Intervals

	1982–1986	1987–1991	1992–1996	1997–2001
Therapy (%)				
Surgical resection (%)	14 (37.8)	13 (20.9)	0	2 (2.3)
PEIT (%)	0	15 (24.2)	47 (66.2)	50 (57.5)
TACE (%)	17 (45.9)	25 (40.2)	17 (23.9)	30 (34.5)
Only palliative care (%)	6 (16.3)	9 (14.5)	7 (9.9)	5 (5.7)

Table 5. Categorization According to JIS Score of the Studied Patients in 5-Year Intervals

	1982–1986	1987–1991	1992–1996	1997–2001	Total
Number of patients	37	62	71	87	257
JIS score (%)					
0	9 (24)	16 (26)	22 (31)	27 (31)	74 (29)
1	11 (30)	22 (35)	22 (31)	31 (36)	86 (33)
2	12 (32)	14 (23)	15 (21)	21 (24)	62 (24)
3	4 (11)	8 (13)	5 (7)	5 (6)	22 (9)
4	1 (3)	2 (3)	7 (10)	3 (3)	13 (5)
5	0	0	0	0	0
	NS	NS	NS		
	NS	NS			
	NS				

JIS, Japanese integrated staging score (Scheffe's test).

Table 6. Univariate and Multivariate Analyses of Prognostic Factors for the Studied Patients

Variable	Univariate analysis		Multivariate analysis	
	P	Relative risk (95% CI)	*P*	Relative risk (95% CI)
Age, y: ≥63 vs <63	.6055	1.09 (.79–1.48)		
Sex: male vs female	.3484	1.18 (.83–1.67)		
Child–Pugh score: B or C vs A	<.0001	3.01 (2.18–4.15)	<.0001	2.58 (1.83–3.62)
Number of tumors: not solitary vs solitary	.0009	1.70 (1.24–2.32)	.0025	1.64 (1.91–2.27)
AFP level, ng/mL: >200 vs ≤200	<.0001	2.02 (1.46–2.80)	.0032	1.67 (1.19–2.34)
Therapy				
Surgical resection vs other therapy	.1729	.72 (.44–1.56)		
PEIT vs other therapy	.0028	.62 (.45–0.85)		
TACE vs other therapy	<.0001	1.91 (1.40–2.59)	.0691	1.65 (.96–2.83)
Period: 1982–1991 vs 1992–2001	.0005	1.72 (1.27–2.34)	.0090	1.63 (1.13–2.35)

CI, confidence interval.

1986 vs 1987–1991, $P = .0494$; 1987–1991 vs 1997–2001, $P = .0263$).

By stratification according to the Child–Pugh score at baseline, the survival rate of patients with Child's B increased significantly during the studied periods (1982–1986 vs 1997–2001, $P < .0001$; 1982–1986 vs 1992–1996, $P = .0029$; 1987–1991 vs 1997–2001, $P = .0429$; 1987–1991 vs 1997–2001, $P = .0001$) (Figure 2). However, the survival rates of patients with Child's A or Child's C were not significantly different throughout the studied periods.

Furthermore, when the patients were categorized according to the initial treatment, the survival rates of patients undergoing surgical resection (1982–1986 vs 1987–1991, $P = .0487$) or TACE (1982–1986 vs 1997–2001, $P < .0001$; 1987–1991 vs 1997–2001, $P = .0040$) showed a significant increase during the studied periods (Figure 3). However, there was no significant difference in the survival rate of patients undergoing PEIT.

Discussion

The prognosis for patients with HCC remains poor because recurrence of HCC is common and most patients with HCC also show underlying cirrhosis. Furthermore, these patients do not tolerate cytotoxic therapy or extensive resection. Possible curative therapies, including surgical resection, liver transplantation, or percutaneous treatments, benefit only a small proportion of patients and these therapeutic approaches can improve life expectancy.[8–13] In Japan and other countries, surveillance programs have led to an increase in the application of curative therapies.[14–16] In addition, several therapies have been proposed for patients who will not benefit from a radical approach. Of these approaches, only TACE has been shown to improve survival in properly selected candidates.[17,18] However, to date, no consensus agreement has been achieved on a common treatment strategy for patients with HCC worldwide.[19–21]

Tumor size can be crucial in the selection of therapeutic options and the prognosis of HCC patients. Although surgical resection and TACE are considered effective treatments for HCC irrespective of tumor size, PEIT has been used only as a potentially curative treatment for HCCs less than 3 cm in diameter in Japan.[22,23] This is likely because the total amount of ethanol injected is less than 10 mL, and it often is difficult to penetrate through all parts of the tumor nodules when the HCC is larger than 3 cm in diameter. Furthermore, in our institution PEIT was selected for patients with HCCs less than 3 cm in diameter throughout the studied periods. Therefore, to select for patients who were able to receive effective therapeutic options, including PEIT, we added HCC lesions measuring less than 3 cm in diameter to the inclusion criteria for the enroll-

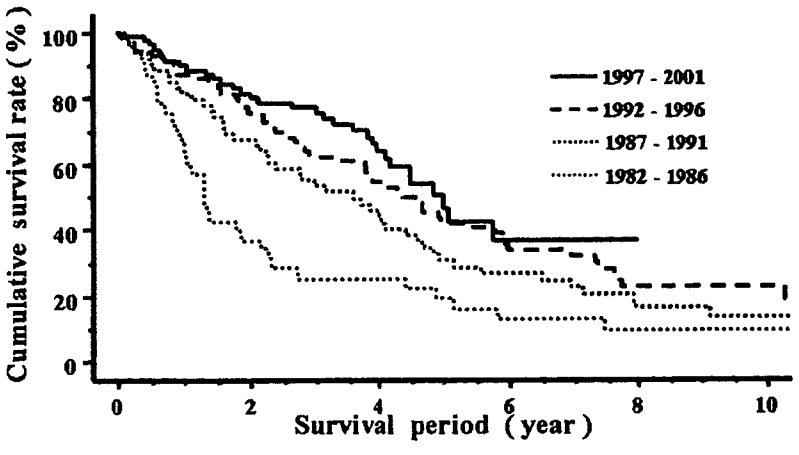

Figure 1. The cumulative survival rates in patients with HCC from 1982 to 1986 (· · ·), 1987 to 1991 (●●●), 1992 to 1996 (—), and 1997 to 2001 (—) are shown separately. For 1982–1986 vs 1997–2001, $P < .0001$; 1982–1986 vs 1987–1991, $P = .0494$; 1987–1991 vs 1997–2001, $P = .0263$.

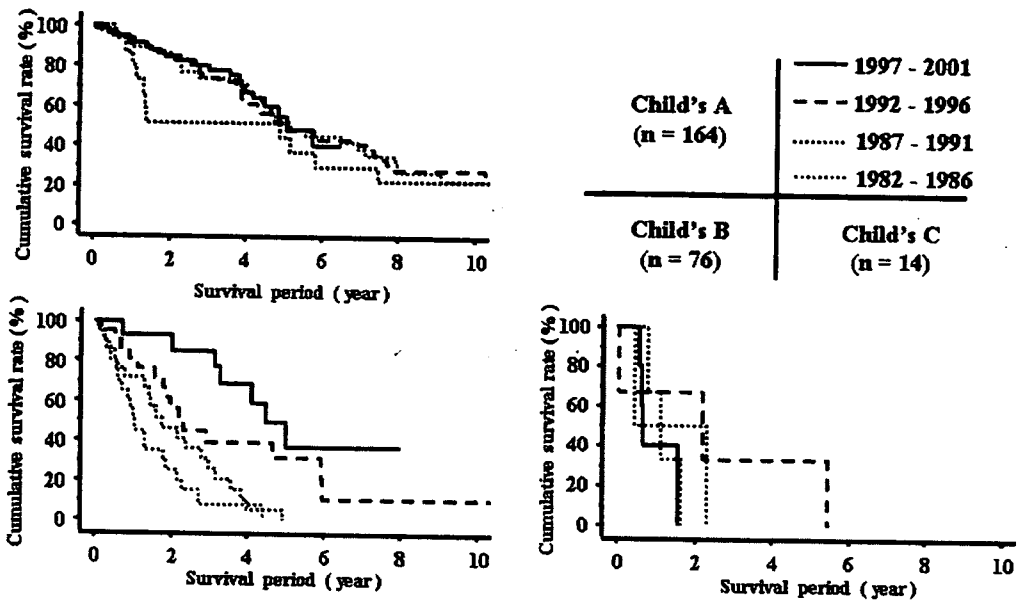

Figure 2. The cumulative survival rates in patients with HCC from 1982 to 1986 (· · ·), 1987 to 1991 (●●●), 1992 to 1996 (—), and 1997 to 2001 (—) according to Child-Pugh staging are shown separately. Child-Pugh A (n = 164), P = NS. Child-Pugh B (n = 76), 1982–1986 vs 1997–2001, $P < .0001$; 1982–1986 vs 1992–1996, $P = .0029$; 1987–1991 vs 1997–2001, $P = .0001$; 1987–1991 vs 1997–2001, $P = .0429$. Child-Pugh C (n = 14), P = NS.

ment of subjects in the current cohort. Although there were significant differences in several factors between the 5-year intervals, the Japan integrated staging score, which has been proposed as a useful prognostic staging system for HCC, showed no significant change.[7] In statistical analyses, the study period was found to be an independent prognostic indicator and the cumulative survival rates showed a significant increase during the studied periods. The survival of our subjects was consistent with previous reports.[3,24] Furthermore, categorized analyses indicated that patients with Child-Pugh grade B, surgical resection, or TACE showed improved prognosis during the studied periods.

It is extremely difficult to determine the optimal treatment choice for HCC patients with Child-Pugh grade B because

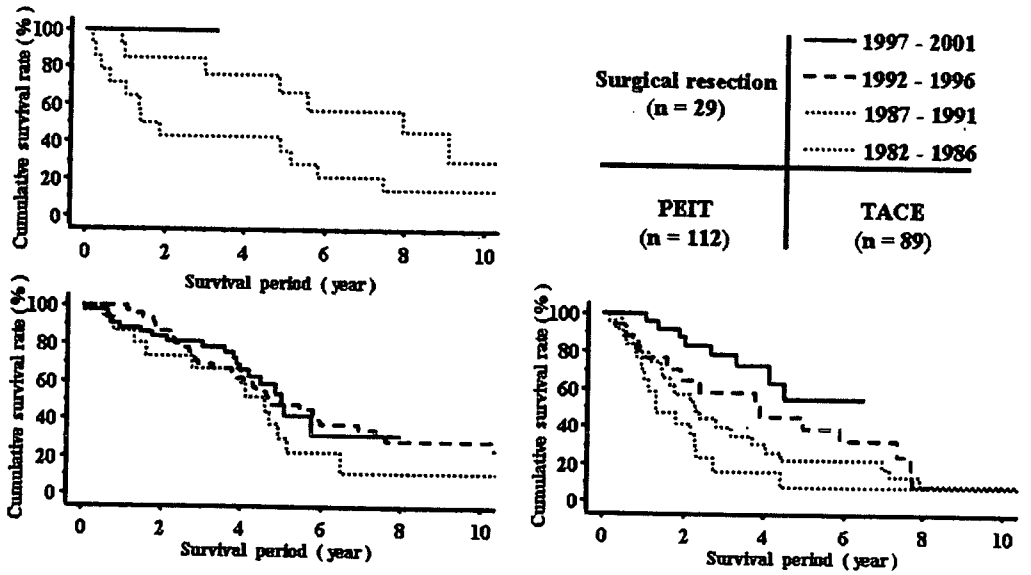

Figure 3. The cumulative survival rates in patients with HCC from 1982 to 1986 (· · ·), 1987 to 1991 (●●●), 1992 to 1996 (—), and 1997 to 2001 (—) according to their respective treatments are shown separately. Surgical resection (n = 29), 1982–1986 vs 1987–1991, $P = .0487$. PEIT (n = 112), P = NS. TACE (n = 89), 1982–1986 vs 1997–2001, $P < .0001$; 1987–1991 vs 1997–2001, $P = .0040$.

those patients progress to hepatic failure as a result of invasive therapy more often than those with grade A. Accordingly, our results may indicate that the choice of therapy for HCC patients during the latter period was more appropriate than that during the early period; this improvement in therapeutic choice occurred concurrently with advances in radiologic assessment. In addition, the improved prognosis in HCC patients with surgical resection or TACE may be associated with advances in surgical procedure and instrumentation. In fact, the surgical mortality rate for hepatectomy has decreased from the 10%–20% that was observed in the 1980s to less than 5% today.[8] However, the survival rates of the cohorts categorized according to the initial treatment do not necessarily reflect the efficacy of each treatment because patients with recurrent HCC commonly undergo additional therapies that are different from the initial treatment. On the other hand, the mortality from variceal hemorrhage in cirrhotic patients has decreased because of advanced management, such as variceal ligation and pharmacologic treatment. Furthermore, long-term nutritional supplementation with oral branched-chain amino acids has been useful in the prevention of progressive hepatic failure.[25–28] These supportive treatments may have contributed to improvement of the survival rate of our subjects.

Although our data showed an improvement in the prognosis of HCC patients over the past 20 years, HCC remains a fatal disease. Recently, radiofrequency ablation and liver transplantation have been added to the therapeutic options for HCC and new approaches also have been suggested, including proton beam irradiation, intrahepatic infusion of yttrium-90 microspheres, and immunotherapy.[10,29–33] In addition to the evaluation of these newer therapeutic modalities, advances in the prevention and early diagnosis of HCC also are needed to achieve further improvement of the prognosis of this disease.

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Different mechanisms for anti-tumor effects of low- and high-dose cyclophosphamide

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Abstract. It is known that, besides its direct cytotoxic effect as an alkylating chemotherapeutic agent, cyclophosphamide also has immuno-modulatory effects, such as depletion of $CD4^+CD25^+$ regulatory T cells. However, its optimal concentration has not yet been fully elucidated. Therefore, we first compared the effects of different doses of cyclophosphamide on T cell subsets including $CD4^+CD25^+$ T cells in mice. Cyclophosphamide (20 mg/kg) decreased the numbers of splenocytes, $CD4^+$ and $CD8^+$ T cells by ~50%, while a decline in $CD4^+CD25^+$ T cell number was more profound, leading to the remarkably lower ratios of $CD4^+CD25^+$ T cells to $CD4^+$ T cells. In contrast, 200 mg/kg cyclophosphamide severely decreased the numbers of all the T cell subsets by >90% although the decreased ratios of $CD4^+CD25^+$ T cells to $CD4^+$ T cells were still observed. Next, low-dose cyclophosphamide significantly inhibited *in vivo* growth of murine hepatoma MH129 tumor in immuno-competent but not immuno-deficient mice. This anti-tumor effect was abolished by $CD4^+CD25^+$ T cell repletion. In contrast, high-dose cyclophosphamide exhibited similar anti-tumor effects in both mice. In addition, contrary to antibody-mediated $CD4^+CD25^+$ T cell depletion, administration of low-dose cyclophosphamide after tumor inoculation was more efficacious than the prior administration. Our data show that low-dose cyclophosphamide selectively depletes $CD4^+CD25^+$ T cells, leading to enhanced anti-tumor effects against pre-existing tumors, while the anti-tumor effect of high-dose cyclophosphamide is solely attributed to its direct cytotoxicity. These findings appear to be highly crucial in a clinical setting of combined chemotherapy and immunotherapy for cancer treatment.

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Key words: cyclophosphamide, $CD4^+CD25^+$ naturally occurring regulatory T cells, tumor immunity, MH129 hepatoma cells, effector T cells

Introduction

Chemotherapy and immunotherapy are generally regarded as unrelated or even mutually exclusive in cancer treatment, because chemotherapy kills not only target cancer cells but also immune cells, inducing systemic immune suppression and dampening the therapeutic efficacy of immunotherapy. In this regard, however, cyclophosphamide appears an exceptional chemotherapeutic agent (1,2). Besides its direct cytotoxic effect as an alkylating agent, cyclophosphamide is reported to modulate the immune system in hosts (1,2). Examples for this include (i) enhancement of dendritic cell-based anti-tumor immunity by increased tumor antigens released from tumor cells dying of cyclophosphamide-induced apoptosis (3), (ii) increased type-I interferon production and evolution of $CD44^{hi}$ memory T cell response by cyclophosphamide (4), (iii) induction of homeostatic T cell proliferation by cyclophosphamide-mediated lymphopenia that enhances some cancer vaccines (5,6), and (iv) down-regulation of T-cell derived IL-10 and TGF-β productions by cyclophosphamide (7). More importantly, recent studies show selective suppression by cyclophosphamide of $CD4^+CD25^+$ naturally occurring regulatory T cells (8-11), which are widely believed to play a key role in immune tolerance (12). Although it is widely believed that 'low-dose' cyclophosphamide augments the immune response, the optimal concentration has not yet been fully elucidated. Thus, the amounts of cyclophosphamide used vary from 10 to 300 mg/kg in studies on immuno-potentiation of low-dose cyclophosphamide (2) and from 30 to 200 mg/kg in those on cyclophosphamide-mediated $CD4^+CD25^+$ T cell suppression (8-11). In this article, therefore, we compared the effects of different doses of cyclophosphamide on T cell subsets including $CD4^+CD25^+$ T cells and also on tumor immunity in mice. Our results clearly demonstrate that low-dose (20 mg/kg), but not high-dose (200 mg/kg), cyclophosphamide selectively suppresses the number of $CD4^+CD25^+$ T cells but spares those of conventional $CD4^+$ and $CD8^+$ T cells in spleen, and efficiently inhibits, through $CD4^+CD25^+$ T cell

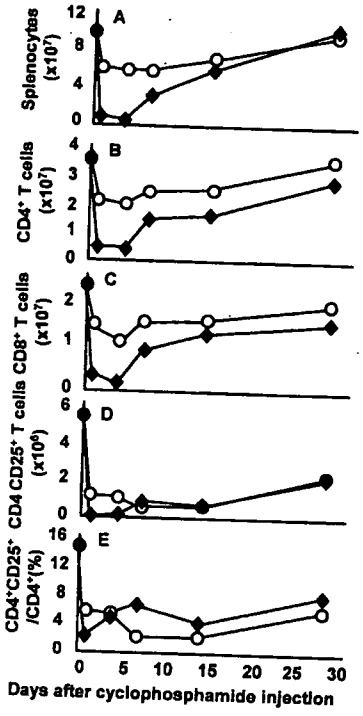

Figure 1. Alterations in T cell subsets following intraperitoneal injection of low- or high-dose cyclophosphamide. After 1,4,7,14 and 28 days after intraperitoneal injection of 20 or 200 mg/kg cyclophosphamide, numbers of splenocytes, $CD4^+$, $CD8^+$ and $CD4^+CD25^+$ T cells were determined as described in the Materials and methods. The data are means of two mice in each group. ○ and ●, 20 and 200 mg/kg cyclophosphamide, respectively. The same experiments were repeated at least twice with similar results.

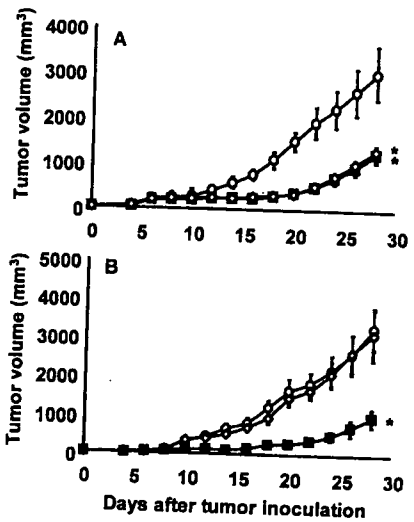

Figure 2. Anti-tumor effects of low- and high-dose cyclophosphamide in MH129 tumor-bearing C3H/HeN and nude mice. Mice (A, C3H/HeN; B, BALB/c nu/nu) were inoculated with $5x10^5$ MH129 cells on day 0. Groups of mice were also treated with 20 or 200 mg/kg cyclophosphamide on day 7, and injected with $4x10^6$ $CD4^+CD25^+$ T cells on day 8. Tumor sizes were monitored for 4 weeks. ○, control mice; ◇, mice treated with 20 mg/kg cyclophosphamide; ■, mice treated with 200 mg/kg cyclophosphamide; ▲, mice treated with 20 mg/kg cyclophosphamide and $4x10^6$ $CD4^+CD25^+$ T cells. The data are means ± SEM (n=8). *$p<0.05$, versus control mice (t-test). The same experiments were repeated at least twice with similar results.

depletion, *in vivo* growth of pre-existing murine hepatoma MH129 tumor. These findings appear to be highly crucial in a clinical setting of combined chemotherapy and immunotherapy for cancer treatment.

Materials and methods

Cell lines and mice used. MH129 cells, a mouse hepatoma cell line (13), were maintained in RPMI-1640 medium with 10% fetal calf serum and antibiotics. Six-week-old female C3H/HeN and BALB/c nu/nu mice were purchased from Charles River Japan (Tokyo, Japan) and kept in a specific pathogen-free facility. All experiments were conducted in accordance with the principles and procedures outlined in the Guideline for the Care and Use of Laboratory Animals of Nagasaki University.

Studies on the effect of cyclophosphamide on T cell subsets in the spleen. C3H/HeN mice were intraperitoneally injected with cyclophosphamide (20 or 200 mg/kg; Sigma, St. Louis, MI), and numbers of splenocytes, $CD4^+$, $CD8^+$ and $CD4^+CD25^+$ T cells were monitored for up to 4 weeks. The numbers of splenocytes were counted using a hemocytometer after lysis of red blood cells with ammonium chloride. The numbers of $CD4^+$, $CD8^+$ and $CD4^+CD25^+$ T cells were determined with FITC-conjugated anti-mouse CD4 (H129.19), PE-conjugated anti-CD8 (53-6.7) and PE-conjugated anti-CD25 (7D4) (PharMingen, San Diego, CA), respectively, on a FACScan flow cytometre using CellQuest software (BD Biosciences, Mountain View, CA).

Studies on the effect of low-dose cyclophosphamide on in vivo MH129 tumor growth. MH129 cells ($5x10^5$ cells/mouse) were subcutaneously injected into the flanks of mice. Tumor sizes were determined from caliper measurement using the standard formula (length x width^2/2). Groups of mice were treated by either intraperitoneal injection of cyclophosphamide (20 or 200 mg/kg) (Sigma) or 500 μg/mouse anti-CD25 antibody before or after tumor cell inoculation. A group of mice were also intraperitoneally injected with $4x10^6$ cells purified $CD4^+CD25^+$ T cells at the indicated time point. Anti-CD25 monoclonal antibody was purified from ascites of mice intraperitoneally injected with hybridoma PC61 using HiTrap™ protein G HP column (Amersham, Piscataway, NJ). $CD4^+CD25^+$ T cells (>90% pure) were isolated from splenocytes of naïve mice using a SpinSep Murine $CD4^+$ T-Cell kit (Veritas, Tokyo, Japan) and MACS CD25 MicroBead kit (Miltenyi Biotech, Bergisch Gladbach, Germany). Confirmation of $CD25^+$ cell depletion by PC61 was determined by staining splenocytes 4 days after antibody treatment with anti-CD25 antibody that recognize a different epitope of CD25 (7D4).

Histology. Tumor histology was examined on formalin-fixed tissue sections stained with hematoxylin and eosin (H&E).

Results

Low-, but not high-dose cyclophosphamide selectively depletes $CD4^+CD25^+$ T cells. We first examined the effects of low- and high-dose cyclophosphamide on the kinetics of T

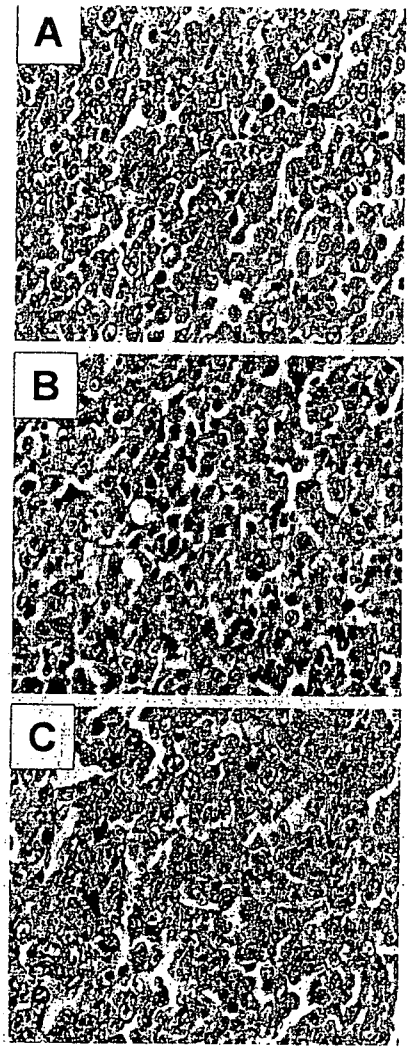

Figure 3. H&E staining of MH129 tumors from control mice (A) and mice treated with 20 or 200 mg/kg cyclophosphamide (B and C). Tumors were removed on day 22 from mice. Magnification of x400.

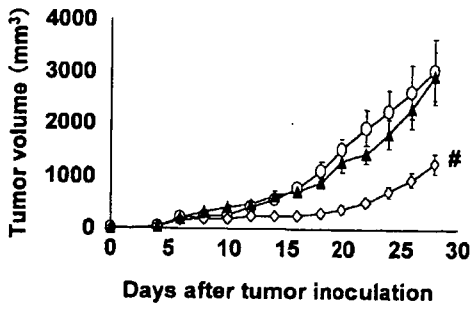

Figure 4. Anti-tumor effects of low-dose cyclophosphamide with/without repletion of CD4+CD25+ T cells in MH129 tumor-bearing C3H/HeN mice. Mice were inoculated with $5x10^5$ MH129 cells on day 0. Groups of mice were also treated with 20 mg/kg cyclophosphamide on day 7, and injected with $4x10^6$ CD4+CD25+ T cells on day 8. Tumor sizes were monitored for 4 weeks. ○, control mice; ◇, mice treated with 20 mg/kg cyclophosphamide; ▲, mice treated with 20 mg/kg cyclophosphamide and $4x10^6$ CD4+CD25+ T cells. The data are means ± SEM (n=8). *p<0.05, versus control mice (t-test). The same experiments were repeated at least twice with similar results.

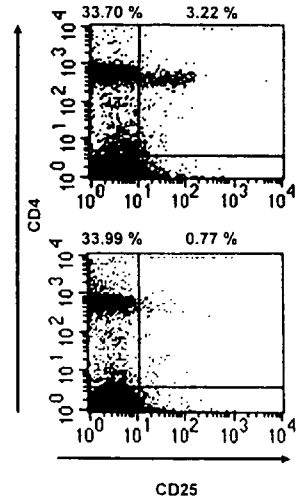

Figure 5. Flow cytometric analysis of CD4 and CD25 expression on splenocytes in mice untreated (A) or treated with 0.5 mg anti-CD25 antibody (B). Four days after antibody treatment, splenocytes were analyzed for CD4 and CD25 expressions by FACScan as described in Materials and methods.

lymphocyte subsets in the spleens of C3H/HeN mice. Flow cytometric analysis of splenocytes was performed 1, 4, 7, 14 and 28 days after intraperitoneally injecting 20 or 200 mg/kg cyclophosphamide (day 0). Fig. 1 shows that 20 mg/kg cyclophosphamide decreased the numbers of splenocytes, CD4+ and CD8+ T cells by ~50% from day 1. The decrease peaked on day 4 and continued for at least 2 weeks. However, a decline in CD4+CD25+ T cell number was more profound (~85% decrease) and recovered more slowly than CD4+ cells, thus leading to the remarkably lower ratios of CD4+CD25+ T cells to CD4+ T cells throughout the experimental period. In contrast, 200 mg/kg cyclophosphamide severely decreased the numbers of all the T cell subsets examined by >90% although the decreased ratios of CD4+CD25+ T cells to CD4+ T cells were still observed. These data clearly indicate the selective suppression of CD4+CD25+ regulatory T cells by low-dose, but not high-dose, cyclophosphamide.

Anti-tumor effect of low-dose cyclophosphamide is immune-mediated, while that of high-dose cyclophosphamide is attributed solely to direct cytotoxic effect. To see how critically the aforementioned phenomenon plays a role in tumor immunity, anti-tumor effects of low- and high-dose cyclophosphamide were compared in an *in vivo* tumor model with a mouse hepatoma cell line, MH129, and syngeneic immunocompetent C3H/HeN and immuno-incompetent nude mice. Surprisingly, both 20 and 200 mg/kg cyclophosphamide injected 7 days after tumor cell inoculation significantly suppressed the growth of MH129 tumors in C3H/HeN mice (Fig. 2A). However, the suppressive effect of low-dose cyclophosphamide was no longer observed in nude mice (Fig. 2B), indicating that the anti-tumor effect of low-dose cyclophosphamide observed in immuno-competent mice appears to be attributed to anti-tumor immunity. In contrast, high-dose cyclophosphamide was equally effective in both mice, indicating that the effect is solely due to direct cytotoxicity.

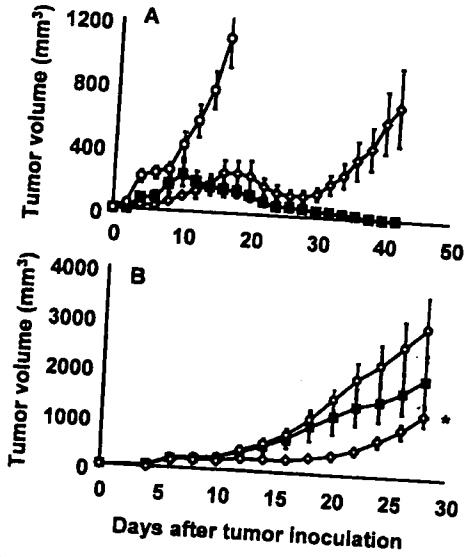

Figure 6. Comparison of anti-tumor effects of low-dose cyclophosphamide and anti-CD25 antibody administered before and after tumor cell inoculation in C3H/HeN mice. C3H/HeN mice were inoculated with $5x10^5$ MH129 cells on day 0. Groups of mice were also treated with 500 μg/mouse anti-CD25 antibody (A) or 20 mg/kg cyclophosphamide (B) on day -4 or +4. Tumor sizes were then monitored for 4 or 7 weeks. ○, control mice; ◇, mice treated with cyclophosphamide or anti-CD25 antibody after tumor cell inoculation; ■, mice treated with cyclophosphamide or anti-CD25 antibody before tumor cells inoculation. The data are means ± SEM (n=8). *$p<0.05$, versus control mice. The same experiments were repeated at least twice with similar results.

In histological examinations (Fig. 3), tumors from mice treated with low-dose cyclophosphamide showed higher intratumoral lymphocyte infiltrations as compared to those in control and high-dose cyclophosphamide-treated mice, data compatible with immune and non-immune mediated anti-tumor effects of low- and high-dose cyclophosphamide, respectively.

Anti-tumor effect of low-dose cyclophosphamide is mediated by depletion of CD4+CD25+ T cells. The next study was performed to evaluate whether anti-tumor immunity induced by low-dose cyclophosphamide resulted from the selective CD4+CD25+ T cell depletion. For this purpose, following MH129 inoculation on day 0 and low-dose cyclophosphamide injection on day 7, CD4+CD25+ T cells ($4x10^6$ cells/mouse) purified from naïve mice were injected on day 8. Fig. 4 shows that repletion of CD4+CD25+ T cells completely abolished the anti-tumor effect of low-dose cyclophosphamide. These data clearly implicate the anti-tumor effect of low-dose cyclophosphamide in its selective depletion of CD4+CD25+ T cells.

Timing of administration to obtain the optimal anti-tumor immunity is different between low-dose cyclophosphamide and anti-CD25 antibody. Because monoclonal anti-CD25 antibody (PC61) has been widely used to deplete CD4+CD25+ T cells in previous studies (14-16), the consequence of antibody-mediated CD4+CD25+ T cell depletion was compared to that of cyclophosphamide-mediated CD4+CD25+ T cell depletion in the MH129 tumor model. In our preliminary dose-escalating experiment, intraperitoneal injection of 0.5 mg anti-CD25 antibody (PC61) maximally depleted CD4+CD25+ T cells (Fig. 5). Fig. 6 shows that four day-prior injection of anti-CD25 antibody completely eradicated MH129 tumors but, 4 days later, the injection only transiently inhibited tumor growth, which is consistent with data previously reported with several cell lines including Meth A, MOPC-70A and RL Male1 cells (15,16). However, the results were opposite in the case of cyclophosphamide; injection of cyclophosphamide on day -4 of tumor cell inoculation was less effective than that on day +4.

Discussion

Although cyclophosphamide-induced suppression of CD4+CD25+ regulatory T cells has previously been demonstrated, attention has not been focused on the concentrations of cyclophosphamide used. Thus, the amounts used varied from 30 to 200 mg/kg in mice and rats (8-11). Furthermore, an immuno-enhancing effect has also been described with doses of cyclophosphamide ranging from 10 to 300 mg/kg (2). Therefore, we first compared the outcomes of high-dose (200 mg/kg) and low-dose (20 mg/kg) cyclophosphamide administration on T cell subsets in mice. Our data clearly demonstrate that, although both high- and low-dose cyclophosphamide markedly suppressed the number of CD4+CD25+ T cells and the ratios of CD4+CD25+ T cells to CD4+ T cells, their effects on the numbers of CD4+ T and CD8+ T cells were quite different. Thus, decreases in absolute numbers of CD4+ T and CD8+ T cells by high-dose cyclophosphamide were much more evident than those by low-dose cyclophosphamide (>90% versus ~50% decreases). These observations indicate that CD4+CD25+ regulatory T cells are selectively suppressed, but conventional, effector T cells appear to be spared by low-dose cyclophosphamide. In contrast, high-dose cyclophosphamide induces quantitatively substantial decreases in all T cell subsets, presumably eradicating both regulatory and effector T cells.

Regarding the mechanism(s) for this selective depletion of CD4+CD25+ T cells with cyclophosphamide, Lutsiak *et al* (10) showed the increased sensitivity of CD4+CD25+ T cells to apoptosis in 100 mg/kg cyclophosphamide-treated mice, leading to the impaired homeostatic proliferation of this T cell subpopulation. Ercolini *et al* (11) also demonstrated selective depletion of the cycling population of CD4+CD25+ T cells with 100 mg/kg cyclophosphamide. In addition, Ikezawa *et al* (9) and Lutsiak *et al* (10) found that cyclophosphamide suppressed not only number but also functional property of CD4+CD25+ T cells.

The long-lasting depletion of CD4+CD25+ T cells by low-dose cyclophosphamide enabled us to observe the effect of cyclophosphamide on tumor immunity. Strong growth inhibition of MH129 tumors by low-dose cyclophosphamide in the syngeneic immuno-competent but not immuno-incompetent nude mice, extensive intratumoral lymphocyte infiltration and disappearance of this anti-tumor effect by CD4+CD25+ T cell repletion collectively indicate that the anti-tumor effect of low-dose cyclophosphamide is mediated by its immuno-enhancing potential induced by CD4+CD25+ T cell depletion,

rather than its direct cytotoxic effect as an alkylating agent. In contrast, the similar anti-tumor effects of high-dose cyclophosphamide in mice and sparse intratumoral lymphocyte infiltration indicates that the anti-tumor effect of high-dose cyclophosphamide is solely comprised of the direct cytotoxic effect. Thus, we definitely demonstrate the difference in the effects of low- and high-doses of cyclophosphamide on anti-tumor immunity. Thus, low-dose cyclophosphamide enhances anti-tumor immunity by selectively depleting $CD4^+CD25^+$ regulatory T cells, while high-dose cyclophosphamide does not because of elimination of both effector and regulatory T cells. Our data suggest that 50% decreases in conventional, effector $CD4^+$ T and $CD8^+$ T cell numbers by low-dose cyclophosphamide are unlikely to affect overall immune reaction, but the >90% decreases seem to be critical.

Although the timing of cyclophosphamide injection to obtain the optimal result is controversial in other literatures (17,18), administration of cyclophosphamide after tumor inoculation is more efficacious than prior injection in this study. These data are similar to those in the recent report obtained with the agonistic anti-glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR) antibody (DTA-1) (19). In contrast, the anti-tumor effect of anti-CD25 antibody-mediated $CD4^+CD25^+$ T cell depletion is higher when antibody is given before tumor cell inoculation than after tumor cell injection in the present and previous studies (15,16). This is at least partly because CD25 is also expressed on activated T cells (14). Thus, anti-CD25 antibody can eliminate not only regulatory cells but also activated effector cells (both $CD4^+CD25^+$) when administered after tumor cell inoculation. On the contrary, low-dose cyclophosphamide does not seem to affect activated $CD4^+CD25^+$ effector T cells. Anti-GITR antibody stimulates the activated $CD4^+CD25^+$ effector T cells (20). In this regard, use of cyclophosphamide or agonistic anti-GITR antibody appears to be more practical in a clinical setting.

Since recent studies show an increase in $CD4^+CD25^+$ T cells in patients with various cancers (21-23), suppression of number and/or function of $CD4^+CD25^+$ T cells may be critical for successful anti-cancer immunotherapy. It may be worthy noting here that selective suppression of $CD4^+CD25^+$ T cells has also been recently described in methotrexate (8) and fludarabine (24).

It should be emphasized here that the low-dose cyclophosphamide we used in this study (20 mg/kg) approximately corresponds to a dose of cyclophosphamide commonly used in cancer chemotherapy in humans (1000 mg/m^2) (25). Furthermore, 200-300 mg/m^2 cyclophosphamide has widely been used as an immuno-potentiating agent in cancer immunotherapy (2). Therefore, it may be particularly important to scrutinize the effect of lower-dose cyclophosphamide (20 mg/kg or less) on immune function including number and function of $CD4^+CD25^+$ regulatory T cells in humans.

In conclusion, we here report that low-dose (20 mg/kg) cyclophosphamide selectively depletes $CD4^+CD25^+$ T cells, thereby enhancing anti-tumor immunity. These findings appear to be highly critical in terms of combined chemotherapy and immunotherapy for cancer treatment in humans. Further studies on elucidating the molecular mechanisms for cyclophosphamide-mediated suppression of regulatory T cells will help us better understand regulatory T cell physiology and develop novel strategies for cancer treatment.

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The up-regulation of type I interferon receptor gene plays a key role in hepatocellular carcinoma cells in the synergistic antiproliferative effect by 5-fluorouracil and interferon-α

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Abstract. Combination therapy with interferon (IFN)-α and 5-fluorouracil (5-FU) has been reported to show an improved therapeutic efficacy in patients with advanced hepatocellular carcinoma (HCC) but the mechanism behind this has not been completely elucidated. We examined the molecular events underlying the antiproliferative effects of IFN-α and 5-FU in combination using six human HCC cell lines. When the antiproliferative effects of administering IFN-α and 5-FU together were analyzed using isobolograms, we found that the cell lines could be divided into two groups: the S-group containing three cell lines, which showed synergistic effects, and the A-group, containing the remaining three cell lines, which showed additive effects. Real-time RT-PCR and Western blot analyses revealed that the expression levels of type I IFN receptor subunits, IFNAR1 and IFNAR2, were specifically up-regulated by 5-FU in all three cell lines of the S-group with the exception of IFNAR2 in one cell line, but not in those of the A-group. IFN-α modulated the protein expression levels of six enzymes regulating sensitivity to 5-FU, but none of them were down- or up-regulated in the same way in all members of the S- or A-group. In conclusion, the 5-FU-induced modulation of IFN receptor expression could play a pivotal role in the therapeutic efficacy of IFN-α combined with 5-FU. Measuring the expression levels of IFN receptors, and their ability to be up-regulated, may be a promising method for selecting HCC patients for this type of combination therapy.

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Abbreviations: IFN-α, interferon α; 5-FU, 5-fluorouracil; HCC, hepatocellular carcinoma; IFNAR1, type I IFN receptor subunit 1; IFNAR2, type I IFN receptor subunit 2; FdUMP, 5-fluoro-2'deoxyuridine-5'-monophosphate; TS, thymidylate synthase; TP, thymidine phosphorylase; DPD, dihydropyrimidine dehydrogenase; OPRT, orotate phosphoribosyl transferase; UP, uridine phosphorylase; TK, thymidine kinase; RT-PCR, reverse transcription-polymerase chain reaction

Key words: interferon-α, 5-fluorouracil, combination therapy, synergism, interferon receptor, hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC), the most common primary liver cancer, is one of the most frequent and aggressive malignant tumors, and its incidence is increasing. The surgical resection of hepatic lesions is the most effective treatment for patients with HCC, and local therapeutic approaches, such as transcatheter arterial embolization (1), percutaneous transhepatic ethanol injection (2), microwave coagulation (3), and radiofrequency ablation (4) have also been reported to be effective. However, these therapies are still not effective for patients with advanced HCC, who are often not suitable for surgery and whose 5-year survival rate is extremely low (5). One chemotherapeutic strategy is combined chemotherapy with 5-fluorouracil (5-FU) and interferon (IFN)-α. Monden and colleagues have reported a beneficial therapeutic effect in a patient with recurrent HCC and multiple lung and bone metastases (6-8). However, this combined treatment was accompanied by increased toxicity, as manifested by an elevated incidence of mucositis and neurological and hematological side effects (9). It is therefore important to understand

the exact mechanism of this combination therapy so that patients likely to respond can be selected and unnecessary side effects can be avoided.

5-FU has two major antitumor mechanisms: one involves its active metabolite 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP), inhibiting the activity of thymidylate synthase (TS) and consequently DNA synthesis; the other is related to the incorporation of 5-FU metabolite into RNA and DNA, thereby disrupting normal RNA processing and function. The sensitivity of cancer cells to 5-FU is often influenced by the enzymes affecting 5-FU metabolism, including dihydropyrimidine dehydrogenase (DPD), orotate phosphoribosyl transferase (OPRT), thymidine phosphorylase (TP), uridine phosphorylase (UP), and thymidine kinase (TK). In contrast, IFNs are divided into type I and type II. The human type I IFN family is composed of at least 14 structurally related IFN-α subtypes and single IFN-ß and IFN-ω subtypes. Type I IFNs have various biological activities, including antiviral, anti-proliferative, immunomodulatory (10-12), and anti-angiogenic effects (13,14), mediated by the type I IFN receptor. This receptor is composed of two functional transmembrane subunits, type I IFN receptor subunit 1 (IFNAR1) and subunit 2 (IFNAR2), cooperating to form a high-affinity receptor for all type I IFNs (15,16). IFNAR2 is the major binding subunit and IFNAR1 is necessary for the tight binding. Of these receptor molecules, the expression levels of the type I IFN receptor were closely correlated with the response rates to IFN treatment in patients with chronic hepatitis C (17), and overexpression of the IFNAR2 markedly increased the anti-proliferative activity of IFNs and their capacity to induce apoptosis (18), suggesting that the type I IFN receptor is a key molecule for the antitumor activity of IFN-α.

Concerning possible mechanisms behind IFN-α and 5-FU showing improved therapeutic efficacy, increased FdUMP concentrations, decreased protein level of TS, an increase in TS inhibition rate and TP activity, and an alteration in 5-FU pharmacokinetics by combined IFN-α have been reported (19-24). Eguchi *et al* have reported that augmentation of the antitumor effect of 5-FU by IFN-α might in part be attributable to the up-regulation of $p27^{Kip1}$ blocking cell cycle progression (25). However, none of these theories provide a consistent mechanism for the exact rationale of this combination therapy. Furthermore, almost all studies have assumed that IFN-α plays a role in modulating the antitumor activity of 5-FU.

In this study, we provide evidence that the modulation of IFN receptor expression by 5-FU is specifically associated with the improved efficacy rather than the cellular modulation of the enzymes that regulate the sensitivity to 5-FU by IFN-α.

Materials and methods

Drugs. Natural human IFN-α was purchased from Otsuka Pharmaceutical Co., Ltd. (OIF, Tokyo, Japan) and 5-FU was purchased from Kyowa Hakko Kogyo Co., Ltd. (5-FU Injection 250 Kyowa, Tokyo, Japan).

Cell lines. HCC cell lines, KIM-1, KYN-1, KYN-2, KYN-3, HAK-1A and HAK-1B (26-30), were grown in Dulbecco's modified Eagle's medium (Nissui Seiyaku Co., Tokyo, Japan) with 10% fetal bovine serum (FBS) (FETALCLONE III, Hyclone, UT, USA) in a humidified atmosphere of 5% CO_2 at 37°C.

Antiproliferation test. Cells were seeded into 96-well plates at 1,000 cells/0.1 ml/well and incubated overnight. On the following day, 100 µl aliquot containing IFN-α and 5-FU was added and cultured for a further 5 days. In study of schedule-dependent synergy, HCC cells were seeded into 12-well plates at 5,000 cells/1 ml/well. On the following day, 1-ml aliquot containing drugs was added. After a further 3 days, culture medium was exchanged for fresh medium including another drug and cultured for a further 3 days. The number of viable cells was estimated by activity of cellular dehyrogenases using WST-8 reagent (Cell Counting Kit-8, DOJINDO, Kumamoto, Japan) (31).

Isobologram analysis. To analyze the mode of interaction between 5-FU and IFN-α, the combined doses that reduced cell growth by 50% were plotted as isobolograms, according to the method of Steel and Peckham (32). The envelope of additivity surrounded by mode I (heteroaddition), IIa and IIb (isoaddition) curves, was constructed based on the dose-response curves of IFN-α and 5-FU alone. Thus, when the data points for the combined drugs fell within this envelope, the combined effect was judged to be additive. When the points fell in the area under the envelope of additivity, the combined effect was judged as synergistic, because in this case, 50% inhibition was produced by a lower concentration than predicted on an additive basis.

cDNA preparation and quantitative real-time RT-PCR. Total RNA was extracted using Isogen (Nippon Gene Co., Ltd., Tokyo, Japan) and reverse transcribed using a reverse transcription system (Promega Corp., Madison, WI) according to the manufacturer's instructions. RT-PCR was performed with an ABI PRISM 7300 (PE Applied Biosystems, Foster City, CA). The sequences of the primers and probes are shown in Table I, and those for IFNAR1, IFNAR2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Applied Biosystems.

Western blotting. HCC cells were cultured with various concentrations of 5-FU or IFN-α. Total protein was extracted using protein extraction reagent (M-PER™, Pierce, Rockford, IL) supplemented with protease inhibitors (Halt™ protease inhibitor cocktail kit, Pierce). Cell lysates were subjected to SDS-PAGE and transferred to Immobilon-P transfer membranes (Millipore, Bedford, MA). After blocking, membranes were probed with anti-TS, -OPRT and -TP monoclonal and anti-DPD polyclonal antibodies (a gift from TAIHO Pharmaceutical Co., Ltd., Tokyo, Japan), monoclonal antibody against TK (abcam, Cambridge, UK), polyclonal antibodies against IFNAR1, IFNAR2 and UP (Santa Cruz Biotechnology, Santa Cruz, CA). The proteins were visualized using HRP-conjugated antibodies followed by enhanced chemiluminescence (Pierce). The intensity of luminescence was quantified using an image analysis system (LAS-1000, Fuji Film, Tokyo, Japan).

Silencing IFNAR1 and IFNAR2 genes. Stealth™ RNAis (Invitrogen™ Life Technologies, San Diego, CA) were used