Original Paper

Hepatic Resection followed by IFN-α and 5-FU for Advanced Hepatocellular Carcinoma with Tumor Thrombus in the Major Portal Branch

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KEY WORDS:
HCC; IFN-α; 5-FU; Surgery; PVTT

ABBREVIATIONS:
Hepatocellular Carcinoma (HCC); Interferon (IFN); Alpha-Fetoprotein (AFP); Protein Induced by Vitamin K Antagonist or Absence (PIVKA-II); Portal Vein Tumor Thrombosis (PVTT); 5-Fluorouracil (5-FU); Transcatheter Arterial Embolization (TAE)

ABSTRACT

Background/Aims: The prognosis of hepatocellular carcinoma (HCC) invading the major branches of the portal vein (Vp3) is extremely poor. Recently, we reported the efficacy of combination therapy with subcutaneous interferon (IFN)-alpha and intra-arterial 5-FU for intractable HCC with Vp3. In this study, this therapy was applied for resectable advanced HCC (Vp3) as a postoperative adjuvant.

Methodology: Patients with HCC and tumor thrombi either in the major or first branch of portal vein were included (n=30). Fifteen consecutive patients with HCC and Vp3 were treated with at least 3 cycles of a combination therapy consisting of continuous arterial infusion of 5-FU (300mg/mm²/ day, 5 days/week, for the initial 2 weeks) and subcutaneous injection of IFN (5 MIU, 3 times/week, 4 weeks) as a postoperative adjuvant therapy following hepatic resection. Another 15 patients who underwent hepatic resection with no IFN/5-FU chemotherapy acted as controls.

Results: The results were as follows in the IFN/5-FU adjuvant treatment group; disease-free survival (n=11, 5-55 months), survival with recurrence (n=2, 9, 48 months), cancer death (n=1, 18 months), death from other causes but no recurrence (n=1, 22 months). The 1-year survival rate was 100% in patients treated with IFN/5-FU, and 41% in those without IFN/5-FU historical controls (n=15). There was a significant difference in disease-free and overall survival rates between the two groups (p=0.0033 and 0.0031).

Conclusions: Combination therapy with subcutaneous IFN and intra-arterial perfusion of 5-FU seems to be a promising postoperative adjuvant treatment modality for resectable HCC with Vp3.

INTRODUCTION

The prognosis of advanced hepatocellular carcinoma (HCC) remains poor, particularly in patients with tumor thrombi in the major branches of the portal vein [Vp3 (1)] (2-10). Even in resectable cases, the prognosis is extremely poor despite aggressive surgery, because of the very high incidence of recurrence in the residual liver (6,8,10-12). Therefore, a new strategy is required for these patients with advanced HCC and portal tumor thrombi.

We reported previously one patient with recurrent HCC and multiple lung, bone and liver metastases (13). The malignancy was uncontrollable by conventional therapies, but showed almost complete regression following administration of tegafur/uracil (UFT) and interferon (IFN)-α (13). The patient is still alive without relapse 5 years after the initiation of this treatment. This remarkable outcome prompted us to systematically investigate the beneficial effect of combination therapy using an anticancer drug and IFN-α in advanced HCCs. We also reported recently the efficacy of combination therapy of subcutaneously administered IFN-α and arterially infused 5-fluorouracil (5-FU) in 11 consecutive patients with unresectable HCC associated with Vp3 (14). Our results showed that this treatment regimen markedly decreased tumor size and levels of tumor markers with an encouraging response rate. It might therefore represent a promising regimen for advanced HCC with tumor thrombi in the portal vein. In the present study, we applied IFN/5-FU therapy for resectable advanced HCC (Vp3) as a postoperative adjuvant and examined its feasibility and efficacy.

METHODOLOGY

Patients

From 1986 to 2003, 538 patients with HCC were admitted and underwent curative hepatic resection

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in the Department of Surgery, Osaka University Hospital. Of these patients, 30 were included in this study based on the identification of a tumor thrombus either in the major or first branch of the portal vein (Vp3). Liver function tests and imaging techniques, including computed tomography (CT) with hepatic angiography and arterial portography, revealed that these cases were resectable and subsequently they underwent hepatectomy. Of the 30 patients, 15 recent consecutive patients, from 1998 to 2003, had an intra-arterial catheter inserted through the gastro-duodenal artery with an implanted drug delivery system (15) during the operation to facilitate postoperative adjuvant IFN/5-FU combined chemotherapy (*see below*) (14). They were treated with at least 3 cycles as a postoperative adjuvant. The demographics of these patients are shown in **Table 1**. The postoperative adjuvant was commenced as soon as possible postoperatively when the criteria for inclusion were satisfied. Fifteen previous patients, from 1987 to 2001, with the same tumor stage of advanced HCC and Vp3, underwent surgery with no combined IFN/5-FU postoperative adjuvant therapy. They were treated with appropriate local HCC therapy, and if there was recurrence postoperatively in the residual liver or other organ, no IFN/5-FU combined therapy was administered. The demographics of these patients are shown in **Table 2**. They were compared to 15 patients with postoperative adjuvant IFN/5-FU combined therapy after hepatic resection, in terms of features of HCC, hepatic function, surgery, clinical effects, disease-free and overall survival.

The TNM stage and grade of portal vein thrombus were classified according to the 3rd edition of the general rules for the clinical and pathological study of primary liver cancer by the Liver Cancer Study Group of Japan (1). The criteria for selection for intra-arterial combination treatment included; *1)* absence of extra-hepatic metastases, *2)* AST and ALT levels below 100 IU/L, *3)* a platelet count exceeding 80,000 /mm^3, *4)* successful implantation of intra-arterial catheter and drug delivery system, and *5)* a performance status (Eastern Cooperative Oncology Group, ECOG) (16) of level 0-1.

Treatment Regimen of IFN/5-FU Combined Chemotherapy and Follow-up after Surgery

After obtaining informed written consent, each patient was treated with subcutaneous administration of IFN-α (OIF, Otsuka Pharmaceutical Co., Tokyo) and an intra-arterial infusion of 5-FU (Kyowa Hakko Co., Tokyo). IFN-α (5x10^6 U, [5 MU]) was administered on days 1, 3, and 5 of every week (14). Continuous infusion chemotherapy (5-FU, 300mg/mm^3/day) through the proper hepatic artery was applied 5 days/week for 2 weeks via a catheter connected to a subcutaneously-implanted drug delivery system. All anti-cancer therapies were discontinued when adverse effects reached level 2 on the ECOG classification (16) (with the exception of platelet and leukocyte counts of less than 40,000 /mm^3 and 2,000 /mm^3, respectively, as these parameters were often low prior to treatment due to liver cirrhosis). In addition to serum chemistry, tumor markers such as alpha-fetoprotein (AFP) and PIVKA-II (Protein Induced by Vitamin K Antagonist or Absence) were measured at least once

TABLE 1. The Demographics of the IFN/5-FU Adjuvant Group (n = 15)

	Age	Sex	T	M	N	Vp	Stage	Operation	Alb	PT/HPT	ICGR-15	AFP	PIVKA-II	Virus
case 1	47	M	4	0	0	3	4A	left lobectomy	4.5	81/91	4	11400	7900	B
case 2	69	M	4	0	0	3	4A	extended anterior segmentectomy	3.7	86/85	21	768	14784	C
case 3	54	M	4	0	1	3	4A	right lobectomy	3.5	64/105	16	28	1847	B+C
case 4	47	M	4	0	0	3	4A	extended right lobectomy	3.4	74/67	26	27	2067	B+C
case 5	60	M	4	0	0	3	4A	extended posterior segmentectomy	3.9	71/69	16	<5	<40	B
case 6	80	M	4	0	0	3	4A	left lobectomy	4	74/66	26	19	1568	B+C
case 7	34	M	4	0	0	3	4A	extended left lobectomy	3.9	90/89	4	456	1153	B
case 8	66	M	4	0	0	3	4A	extended medial segmentectomy	3.3	75/87	15	5	298	C
case 9	54	M	4	0	0	3	4A	right lobectomy	4.5	77/62	14	8700	353617	B
case 10	54	M	4	0	0	3	4A	right lobectomy	3.7	65/85	21	32930	<40	B+C
case 11	69	M	4	0	0	3	4A	right lobectomy and pancreatoduodenectomy	4.1	90/93	17	7473	205	B+C
case 12	54	M	4	0	0	3	4A	left lobectomy	3.8	82/78	17	680	<40	C
case 13	56	F	4	0	0	3	4A	left lobectomy	3.6	71/63	19	13260	1039	C
case 14	62	M	4	0	0	3	4A	right lobectomy	3.6	63/73	18	23500	476	B+C
case 15	58	M	4	0	0	3	4A	right lobectomy	3.8	85/87	16	6500	1200	C

TNM stage and the grade of portal vein thrombus were classified according to the 3rd edition of the general rules of the clinical and pathological study of primary liver cancer by liver cancer study group of Japan.
Alb: serum albumin (g/dL); PT: Prothrombin time (%); HPT: Hepaplastin test (%);
ICGR-15: indocyanine green retention rate at 15 minutes (%);
AFP: alpha-fetoprotein (ng/mL) and PIVKA-II: Protein Induced by Vitamin K Absence (mAU/mL).

TABLE 2 The Demographics of the Non-IFN/5-FU Adjuvant Group (n=15)

	Age	Sex	T	M	N	Vp	Stage	Operation	Alb	PT/HPT	ICGR-15	AFP	PIVKA-II	Virus
case 16	72	M	4	0	1	3	4A	right lobectomy	4.3	ND/89	15	10876	ND	nonAnonB
case 17	56	M	4	0	0	3	4A	right lobectomy	3.2	ND/74	23	377	ND	nonAnonB
case 18	42	M	4	0	0	3	4A	right lobectomy	4.1	ND/82	14	67	ND	B
case 19	65	M	4	0	0	3	4A	extended left lobectomy	4.0	ND/133	6	5	ND	B
case 20	58	M	4	0	0	3	4A	right lobectomy	3.7	ND/72	16	227	ND	nonAnonB
case 21	61	M	4	0	0	3	4A	right lobectomy	3.7	ND/75	-	10256	ND	nonAnonB
case 22	62	M	4	0	0	3	4A	lateral segmentectomy	3.8	ND/73	19	105360	ND	ND
case 23	34	M	4	0	0	3	4A	right lobectomy	2.9	61/59	6	10332	10240	B
case 24	56	M	4	0	0	3	4A	left lobectomy	3.1	76/52	11	75	1450	B
case 25	48	M	4	0	0	3	4A	right lobectomy	2.8	58/57	14	9	62.5	B
case 26	54	M	4	0	1	3	4A	extended right lobectomy	3.0	85/93	-	1500	21300	-
case 27	58	M	4	0	0	3	4A	right lobectomy	4.2	84/72	19	2208	62.5	B
case 28	63	M	4	0	0	3	4A	extended posterior segmentectomy	3.4	72/84	15	21	62.5	-
case 29	69	F	4	0	0	3	4A	right lobectomy	3.7	97/97	7	2900	571	C
case 30	67	M	4	0	0	3	4A	extended posterior segmentectomy	2.9	67/60	35	4733	18625	C

TNM stage and the grade of portal vein thrombus were classified according to the 3rd edition of the general rules of the clinical and pathological study of primary liver cancer by liver cancer study group of Japan.
Alb: serum Albumin (g/dL); PT: Prothrombin time (%); HPT: Hepaplastin test (%);
ICGR-15: indocyanine green retention rate at 15 minutes (%);
AFP: alpha-fetoprotein (ng/mL) and PIVKA-II: Protein Induced by Vitamin K Absence (mAU/mL). ND: not done.

every month. An abdominal CT scan or dynamic magnetic resonance imaging (MRI) was also performed before and after treatment, at least once every 3 months. The objective response was classified according to the ECOG criteria (16).

Statistical Analysis

Survival curves were constructed using the Kaplan-Meier method (17). Survival curves were compared using the log-rank test. The features of HCC, biochemistry, ICGR-15, and virus status were compared using the Mann-Whitney test. The level of tumor markers (AFP, and PIVKA-II) was compared by the Wilcoxon matched-pair test. Significance was interpreted as $p<0.05$.

RESULTS

Features of Preoperative Hepatic Function, Hepatocellular Carcinoma, and Surgery

The features of preoperative hepatic function are shown in **Table 3**. There was no significant difference between the IFN/5-FU adjuvant and non-IFN/5-FU adjuvant groups in the preoperative hepatic function; serum albumin (g/dL), prothrombin time (PT, %), hepaplastin test (HPT, %), indocyanine green retention rate at 15 minutes (ICGR-15, %). No difference was also demonstrated in terms of tumor stage, surgical procedure, including AFP (ng/mL) and PIVKA-II (mAU/mL) (**Tables 1 and 2**).

Clinical Effects, Disease-free and Overall Survival

All 30 patients in this study were discharged without major complications. The results of the IFN/5-FU adjuvant treatment group were as follows; disease-free survival (n=11) (5-55 months), survival with recurrence (n=2) (13, 48 months), cancer death (n=1) (18 months), death from other causes with no recurrent cancer lesion in the residual liver (n=1) (22 months). The summary of these results in each case is shown in **Table 4**. With respect to Cases 2 and 8, IFN/5-FU combined therapy could not be continued over 4 cycles, due to technical difficulties with the catheter. After stopping the treatment, recurrent lesions appeared in the residual liver in these two patients, at 18 and 8 months after surgery, respectively. They were treated again after insertion of the arterial catheter with intervention (IVR). The lesion was well-controlled over 30 months in Case 2. In Case 8, the recurrent lesion completely disappeared after re-treatment with IFN/5-FU combined chemotherapy (complete response, CR), however, the patient died suddenly due to cardiac failure secondary to ischemic heart disease.

In the other group that received no adjuvant IFN/5-FU therapy, almost all patients (11 of 15) died of recurrent cancer within 2 years. All patients developed recurrences in the residual liver, 2 also had lung metastasis, and one had lung and lymph node metastasis. Recurrence was identified within 1 year of hepatic resection in 12 of the 15 patients, and 11 died within 2 years. These clinical results for the control group are summarized in **Table 5**.

With respect to survival, the overall survival rates at 1 and 3 years were 100% and 74%, respectively, in patients on IFN/5-FU combination therapy (n=10), and 41% and 22%, respectively, in the historical controls with no IFN adjuvant (n=15). In addition, the

TABLE 3 Feature of Hepatic Function

	IFN/5-FU postoperative adjuvant (n=15)	Non-IFN/5-FU postoperative adjuvant (n=15)	
	mean ± SD (range)	mean ± SD (range)	p value
Albumin (g/dL)	3.8±0.4 (3.4-4.5)	3.5±0.5 (2.8-4.3)	NS
Prothrombin time (%)	75.7±8.3 (65-90)	75.0±13.2 (61-97)	NS
Hepaplastin test (%)	80.6±13.9 (62-105)	78.1±20.2 (52-133)	NS
ICGR-15 (%)	16.3±7.8 (4-26)	15.4±7.8 (6-35)	NS

TABLE 4 The Prognosis and Pathological Findings of the IFN/5-FU Adjuvant Group (n=15)

	Recurrence		Period		Prognosis		Histology	
	Recurrence	Recurrent site	Disease-free period	Survival period	prognosis	Cause of death	Cancer	Non-cancer
case 1	-	-	55	55	alive	-	EdIII(por)	B'-
case 2	+	liver	18	48	alive	-	EdII(mod)	CAH+
case 3	-	-	30	30	alive	-	EdII(mod)	B-
case 4	-	-	24	24	alive	-	EdIII(por)	CAH+
case 5	-	-	24	24	alive	-	EdIII(por)	B'-
case 6	-	-	22	22	alive	-	EdIII(por)	B'-
case 7	+	liver, lung	7	18	died	cancer	EdIII(por)	B'-
case 8	+	liver	8	22	died	cardiac failure*	EdII(mod)	B'-
case 9	-	-	13	13	alive	-	EdIII(por)	B'-
case 10	+	lymph node	6	13	alive	-	EdIII(por)	B-
case 11	-	-	12	12	alive	-	EdIII(por)	B'-
case 12	-	-	12	12	alive	-	EdII(mod)	B'-
case 13	-	-	10	10	alive	-	EdIII(por)	B-
case 14	-	-	6	6	alive	-	EdII(mod)	B'-
case 15	-	-	5	5	alive	-	EdII(mod)	CAH+

* Although the recurrent lesion had completely disappeared after the re-treatment with IFN/5-FU combined chemotherapy (CR), he died suddenly due to cardiac failure of ischemic disease.

1- and 3-year disease-free survival rates were 72% and 60%, respectively, in patients on IFN/5-FU combination therapy (n=10), and 39% and 20%, respectively, in historical controls with no IFN adjuvant (n=15). There was a significant difference in disease-free and overall survival between these two groups (overall; $p=0.0031$, disease-free; $p=0.0033$). The overall survival curves are shown in **Figure 1**.

A Representative Case Successfully Treated with IFN/5 FU Adjuvant Therapy and Hepatic Resection

A representative patient who was successfully treated is described below.

Case 1

A 47-year-old man with hepatitis B virus infection, advanced HCC (massive type in left lobe) and portal thrombus in the left branch of the major trunk (**Figure 2**) underwent left lobectomy in October 1998. Tumor markers were highly elevated before surgery (AFP: 11,400ng/mL, PIVKA-II: 7,900mAU/ mL). Histopathological examination of the resected tissue showed poorly-differentiated HCC with Vp3 and metastasis to the gallbladder (**Figure 2**). For the prevention of recurrent tumor development, a combination of IFN/5-FU therapy (4 cycles) was administered over 7 months. No recurrence of the tumor occurred after cessation of treatment and no tumor progression has been observed for 55 months after surgery.

Adverse Effects

No leukopenia, thrombocytopenia, or myelosuppression was observed in the 15 patients of the IFN/5-FU group. Other adverse effects were, in general, clinically manageable. Fever was commonly observed but was easily controlled by non-steroidal anti-inflammatory drugs prior to IFN injection. No depression due to IFN administration was observed in any of the 15 patients.

DISCUSSION

In the present study, a combination therapy of subcutaneous administration of IFN-α and arterial infusion chemotherapy with 5-FU was applied as a postoperative adjuvant to 15 consecutive patients with resectable HCC associated with Vp3, following hepatic resection. Our results showed that this treatment regimen markedly decreased the incidence of recurrence in the residual liver and significantly prolonged the disease-free and overall survival periods compared with historical controls, as shown in **Figures 1 and 2**. Unexpectedly, the 1-year overall survival rate was 100% in the IFN/5-FU treatment group. These results showed that combination therapy with subcuta-

TABLE 5. The Prognosis and Pathological Findings of the Non-IFN/5-FU Adjuvant Group (n=15)

	Recurrence		Period		Prognosis		Histology	
	Recurrence	Recurrent site	Disease-free period	Survival period	prognosis	Cause of death	Cancer	Non-cancer
case 16	+	liver	50	58	died	cancer	EdIII(por)	CAH
case 17	+	liver, lung	1	3	died	cancer	EdIII(por)	B'-
case 18	+	liver	1	5	died	cancer	EdIII(por)	B'-
case 19	+	liver	12	18	died	cancer	EdIII(por)	unknown
case 20	+	liver	5	5	died	cancer	EdII(mod)	BA'+
case 21	+	liver	42	63	died	cancer	EdIII(por)	B+
case 22	+	liver	4	33	died	unknown	EdIII(por)	B+
case 23	+	liver	5	10	died	cancer	EdIII(por)	CIH-
case 24	+	liver	3	5	died	cancer	EdIII(por)	glissonitis,se
case 25	+	liver	2	4	died	cancer	EdIV (undifferentiated)	glissonitis
case 26	+	liver	1	3	died	cancer	EdIV (undifferentiated)	chr.glissoniti
case 27	+	liver	31	58	died	cancer	EdIII(por)	B-
case 28	+	liver, lymph node	6	6	died	cancer	EdIII(por)	chr.glissoniti
case 29	+	liver, lung	3	8	died	cancer	EdIII(por)	CIH
case 30	+	liver	4	7	died	cancer	EdIII(por)	B-

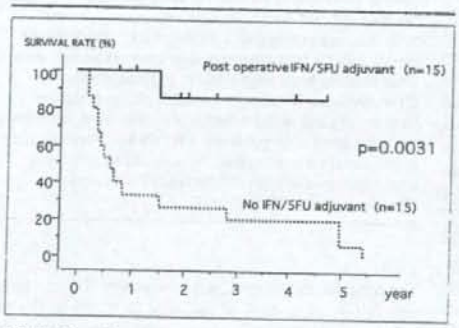

FIGURE 1 Overall survival rates of patients grouped according to whether they received IFN/5-FU combined chemotherapy or not as a postoperative adjuvant following hepatic resection. There was a statistically significant difference in survival ($p=0.0031$).

neous IFN and intra-arterial 5-FU may be therefore a promising treatment modality for resectable HCC with Vp3, as a postoperative adjuvant.

Development of tumor thrombi in a major branch or main trunk of the portal vein is a frequent terminal feature of HCC, either with primary or recurrent tumors. The prognosis of such patients is extremely poor and survival is generally limited to a few months after diagnosis (2-10). For these advanced HCCs, conventional therapies like percutaneous ethanol injection, microwave coagulation therapy, and transcatheter arterial embolization (TAE) are generally not indicated due to lack of efficacy and associated complications (6,8,18). Arterial infusion chemotherapy has also been attempted, but its effectiveness remains unsatisfactory (5,19,20). Even in resectable cases, the prognosis is extremely poor despite aggressive surgery, because of the very high incidence of recurrence in residual liver (7,9,10-12). In addition, the recurrent lesions are very severe and massive in almost all cases. Based on this point of view, in the absence of effective pre- and/or postoperative adjuvant therapy, hepatic resection should not be offered in such cases; no treatment except hepatic resection would be anticipated to improve AFP status or long-term survival. To date, several reports have mentioned the feasibility of hepatic resection for patients with portal vein tumor thrombosis (PVTT), however the outcome of this treatment is in general unsatisfactory. A recent study (21) reported very low rates of disease-free and overall survival for HCC with PVTT. In that report, disease-free survival rates at 1, 3, and 5 years were 15.0, 5.0 and 5.0%, respectively; overall survival rates at 1, 3 and 5 years were 30.0, 13.0 and 13.0%, respectively. Several other previous reports (7,9,10-12,22) were similar. Compared with these previous reports, our clinical outcome using IFN/5-FU combined therapy as a postoperative adjuvant was excellent and highly satisfactory, in terms of disease-free and overall survival rates.

The anti-tumor effects of systemic IFN-α therapy in HCC remain controversial. Several studies with IFN-α alone demonstrated only a minimal clinical effect (23-26). In a randomized controlled study, Lai *et al.* (27) demonstrated the beneficial effect of IFN-α with a 31% response rate in patients with inoperable HCC. In other studies, systemic combination therapy with IFN-α and doxorubicin was also found to be ineffective with a response rate ranging from 3 to 17% (28-30). The response rate in combination therapy with IFN-α and multiple anticancer agents including 5-FU and doxorubicin was 26% (31), and appeared better than other combination therapies with doxorubicin only.

Using intra-arterial infusion chemotherapy and systemic IFN-α administration, Urabe *et al.* (32) reported a response rate of 47% in patients with Vp3. More recently, Chung *et al.* (33) reported a partial response in 33% (6/18) of patients with major portal vein thrombosis or distant metastases, who received systemic combination therapy with IFN-α and cisplatin (CDDP). We also reported the anti-tumor effect of IFN/5-FU combined chemotherapy; complete or partial response was observed in 63% (5/8) of patients (14). It is difficult to accurately compare the effectiveness of various therapeutic regimens among different studies because of patient selection bias in liver function or extent of tumor progression and differences in the evaluation methods of the clinical effect. Although the limitations in comparing the clinical response between studies cannot be neglected, the marked effect and acceptable toxicity of our therapy in HCC patients with extremely poor prognosis suggests that combination therapy of IFN-α and 5-FU may be a promising treatment regimen.

This incredible clinical effect of IFN/5-FU combined chemotherapy after surgery could not be induced by 5-FU chemotherapy alone as a postoperative adjuvant. Cases 24 and 25 were treated with intra-arterial perfusion of 5-FU alone. Both patients refused the IFN/5-FU combined chemotherapy because of their experience of depression induced by IFN-α for treatment of HCV infection. These two patients developed tumor recurrence in residual liver at 3 and 4 months postoperatively, and died of cancer 8 and 7 months postoperatively, respectively, despite 5-FU intra-arterial perfusion chemotherapy without IFN as a postoperative adjuvant.

With respect to postoperative adjuvant therapy for HCC, a recent review mentioned systemic chemotherapy, hepatic-artery chemotherapy or TAE, as well as a combination of these therapies did not improve overall or disease-free survival after potentially curative surgery for localized HCC (34). This report referred to pre- and/or post-adjuvant therapy only for resectable low stage HCCs, and not for advanced cases, with common prevention of recurrence in the liver. To date, there are few reports about adjuvant therapy for advanced HCC with PVTT. Radiotherapy was effective for reduction of the size of tumor thrombus, especially combined with TAE in recent reports of the treatment of PVTT (35-37). However, no prolongation of survival was expected and it may be considered as a preoperative adjuvant therapy. Another report suggested that for long-term survival in advanced HCC with tumor thrombus, preoperative TAE is necessary and the ICGR-15 (%) should be better than 20% (38). The survival rates at 1 and 3 years in that study were 82% and 42%, respectively. Their results compared favorably with the previous reports, and our data were better than their results in terms of overall survival at 1 and 3 years (100% and 74%, respectively, in the present study). With respect to liver function, a low ICGR-15 was the one of the conditions for long-term survival after surgery. In our report, the ICGR-15 in 40% of patients (4 of 10) in the post-adjuvant IFN/5-FU group was worse than 20%. Therefore, our present study is the first to describe a promising strategy for advanced HCC with PVTT, with 1-year survival rate of 100%.

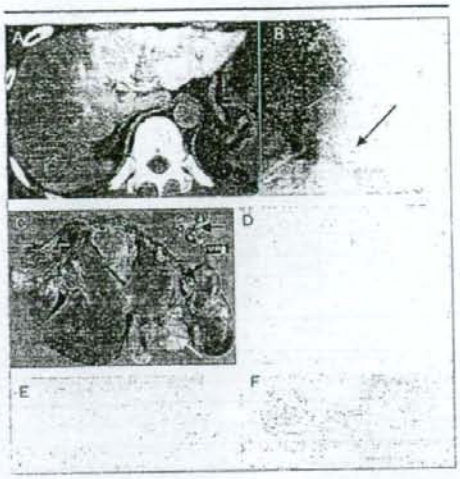

FIGURE 2 Case 1. **(A)** CT arteriography (CT-A) showing the main tumor of the left lobe of the liver. The tumor is massive with tumor thrombus of the left branch. **(B)** Arterial portography demonstrates tumor thrombus extending up to the main trunk of the portal vein (arrow), from the main tumor occupying the whole left lobe. Arrow; tumor thrombus in the major branches of the portal vein. **(C)** Morphological findings of the resected left lobe of the liver. Black arrow; portal venous thrombus of the main trunk. White arrow; metastatic lesion of the gallbladder. **(D)** Pathological findings of the main tumor of the resected liver. The postoperative histological examination revealed poorly-differentiated HCC with Vp3 **(E)** and metastasis to gallbladder **(F)**.

Myelosuppressive adverse effects are particularly important in patients with HCC. This is not only because thrombocytopenia and/or leukopenia are often present before the initiation of anticancer therapy, but also because treatment often has to be discontinued due to these side-effects. Another advantage of this combination therapy is the markedly low incidence of myelosuppressive side-effects; no patient developed leukopenia in this study (data not shown).

Other side-effects were also well controlled by conventional treatment. These relatively mild side-effects allowed continuation of treatment, and may enhance the marked clinical effect because treatment was never interrupted due to adverse effects. In addition, the QOL of patients in this study was excellent, because IFN/5-FU adjuvant therapy was performed at outpatient clinics. No hospital admission was necessary to receive IFN injection combined with intra-arterial perfusion chemotherapy. The patients could maintain their social life while on IFN/5-FU adjuvant

therapy. Moreover, they had no symptoms related to liver dysfunction.

In conclusion, our present study indicated that combination chemotherapy with subcutaneous IFN-α and intra-arterial 5-FU is a promising strategy for resectable HCC with tumor thrombus in major branches of the portal vein, as a postoperative adjuvant therapy following surgery. To obtain conclusive evidence of the effect of this treatment, a large phase II trial and further investigation are essential.

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FOOTNOTE

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CASE REPORT

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Successful treatment of multiple hepatocellular carcinoma with tumor thrombi in the major portal branches by intraarterial 5-fluorouracil perfusion chemotherapy combined with subcutaneous interferon-alpha and hepatectomy

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Abstract We experienced a patient who received successful treatment for multiple hepatocellular carcinoma (HCC) nodules, with tumor thrombi in the major portal branches, with intraarterial 5-fluorouracil perfusion chemotherapy combined with subcutaneous interferon-alpha administration. The patient was a 50-year-old man with hepatitis C virus and HCC. The tumors consisted of a 5-cm main nodule in the right lobe (segment 8) and multiple intrahepatic metastases. The tumor also involved portal vein thrombosis throughout the right portal branch. After two cycles of interferon-alpha/5-fluorouracil combination chemotherapy, tumor markers demonstrated a decreasing tendency. Nine months after the initiation of this therapy, the tumors were limited to the right lobe and were surgically removed by S8 subsegmentectomy, S5 partial hepatectomy, and portal thrombectomy. The serum levels of both alpha-fetoprotein and protein induced by vitamin K absence II fell to normal levels after hepatic resection. Fifty-eight months after the first treatment, he is alive with several recurrent nodules in the liver. In conclusion, the interferon-alpha/5-fluorouracil combination therapy is a useful treatment for HCC in patients who have multiple intrahepatic metastases and portal vein thrombosis. In addition to this therapy, combined modality therapy including, for example, surgical resection, can sometimes have a dramatic therapeutic effect, shown by tumor markers reverting to normal levels.

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Key words Hepatocellular carcinoma · Portal vein tumor thrombi · Interferon · Chemotherapy · Hepatectomy

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide.[1] The prognosis of HCC remains unsatisfactory in spite of newly developed therapeutic modalities, such as radiofrequency ablation and microwave coagulation therapy.[2,3] Especially, the prognosis of HCCs with macroscopic tumor thrombi in the major branch of the portal vein (Vp3–4) is extremely poor.[4] Most HCC patients with Vp3–4 tumors develop recurrences, and half of them die within 1 year after surgery even if curative resection is performed.[5] The prognosis of unresectable patients with Vp3–4 is much worse, and most patients die within several months.[6–8] Therefore, the development of new antitumor therapy for HCC patients is urgent and mandatory.

Recently, we reported that intraarterial 5-fluorouracil (5-FU) perfusion chemotherapy combined with subcutaneous interferon-alpha (IFN-α) administration showed an excellent clinical response in patients with advanced HCC with macroscopic tumor thrombi in the major branch of the portal vein.[9–11] However, the clinical response rate of this combination theraphy is about 50%.[10] In addition, complete remission was not always achieved, even in patients for whom the treatment was considered effective (i.e., CT showed all enhanced regions in the liver disappeared). In this report, we describe our experience of a patient with HCC associated with liver cirrhosis due to hepatitis C, who, when first admitted was assessed as unresectable because of multiple intrahepatic metastases and major portal vein thrombosis. He was given subcutaneous injections of IFN-α and intraarterial infusions of 5-FU, and subsequently hepatic resection and thrombectomy were performed. This led

to the dramatic eradication of multiple tumor nodules and portal vein thrombosis, and the reduction to normal range of serum levels of both alpha-fetoprotein (AFP) and protein induced by vitamin K absence II (PIVKA-II).

Case report

The patient was a 50-year-old man with a history of blood transfusion at 33 years for treatment of hemorrhage due to a traffic accident. Abnormal liver function was identified for the first time at 38 years of age, and chronic hepatitis due to infection with hepatitis C virus was diagnosed. At age 48 years, multiple liver tumors were found by ultrasound examination. The tumors were diagnosed as HCC by computed tomography (CT) and magnetic resonance imaging (MRI) and consisted of a main nodule 5 cm in diameter in the right lobe (segment 8) and multiple intrahepatic metastases around the main nodule and in segment 3. In addition, the tumor involved portal vein thrombosis throughout the right portal branch.

Clinical tests on admission indicated abnormal liver function. The levels of aspartate aminotransferase (AST; 63 IU/l), alanine aminotransferase (ALT; 97 IU/l), and total bilirubin (T-Bil; 1.8 mg/dl) were abnormally high. Prothrombin time (PT) was 63% and the indocyanine green test rate at 15 min (ICGR-15) was 27%. Elevation of the tumor markers AFP, at 43 ng/ml, and PIVKA-II, at 8127 mAU/ml, revealed advanced HCC. After hospitalization, the background liver cirrhosis was defined as B according to the Child-Pugh classification. Because there were multiple nodules in the whole liver and thrombosis in the major portal branch, hepatectomy and embolization therapy were judged to be contraindicated. After the permission of the ethics committee at Osaka University Hospital and written informed consent were obtained, the patient was treated with subcutaneous administration of IFN-α (OIF; Otsuka Pharmaceutical, Tokyo, Japan) and intraarterial infusion of 5-FU (Kyowa Hakko, Tokyo, Japan). The regimen was as follows: IFN-α (5×10^6 U) was administered on days 1, 3, and 5 of each week for 4 weeks, and continuous infusion chemotherapy (5-FU, 300 mg/m^2 per day) through the proper hepatic artery was performed every 2 weeks for 4 weeks via a catheter connected to a subcutaneously implanted drug delivery system.[9]

After two cycles of this IFN-α/5-FU combination chemotherapy, tumor markers demonstrated a decreasing tendency. The level of PIVKA-II fell to 361 mAU/ml. The AFP level demonstrated no marked elevation (Fig. 1). After three cycles of IFN-α/5-FU combination chemotherapy, the main nodule had decreased in size and the tumor thrombus in the first branch of the portal vein and some of satellite lesions were disappeared (Fig. 2a–d). Nine months after the initiation of this therapy, the tumors were limited to the right lobe and the tumor thrombus had disappeared. Therefore, we could avoid right lobectomy, which would have been an extensive resection in this patient, and we surgically removed the tumor by S8 subsegmentectomy with S5 partial hepatectomy, and portal thrombectomy. The macroscopic finding of portal vein tumor thrombus was necrotic (Fig. 3a). Histological examination revealed that there were no intrahepatic metastatic lesions in the resected specimen. Moreover, the portal vein thrombosis had become organized (Fig. 3a) and showed no evidence of malignant cells (Fig. 3c). Serum levels of both AFP and PIVKA-II fell to normal after the hepatic resection. Furthermore, one cycle of IFN-α/5-FU combination chemotherapy was performed as postoperative adjuvant therapy, and the serum levels of these markers were maintained at normal levels, and no evidence of recurrence was shown by computed tomography for 8 months after the hepatic resection.

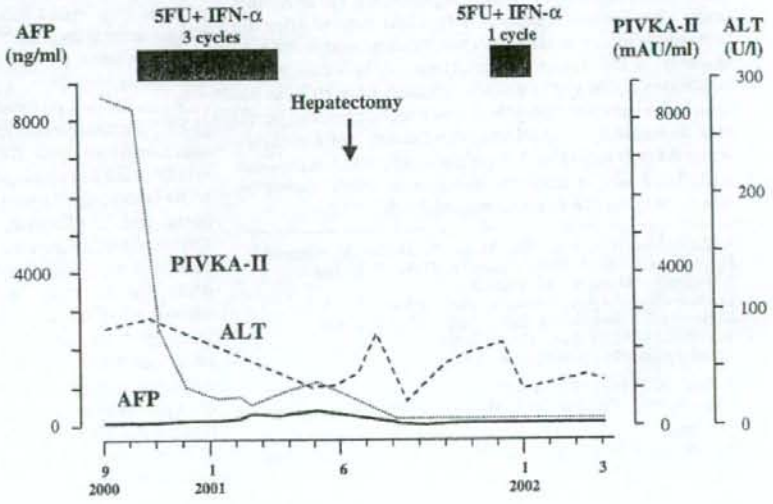

Fig. 1. Clinical course of the patient. Serum alpha-fetoprotein (*AFP*), protein induced by vitamin K absence II (*PIVKA-II*), and alanine aminotransferase (*ALT*) levels are indicated by the *solid line*, *dotted line*, and *dashed* line, respectively. *5-FU*, 5-fluorouracil; *IFN-α* interferon-alpha

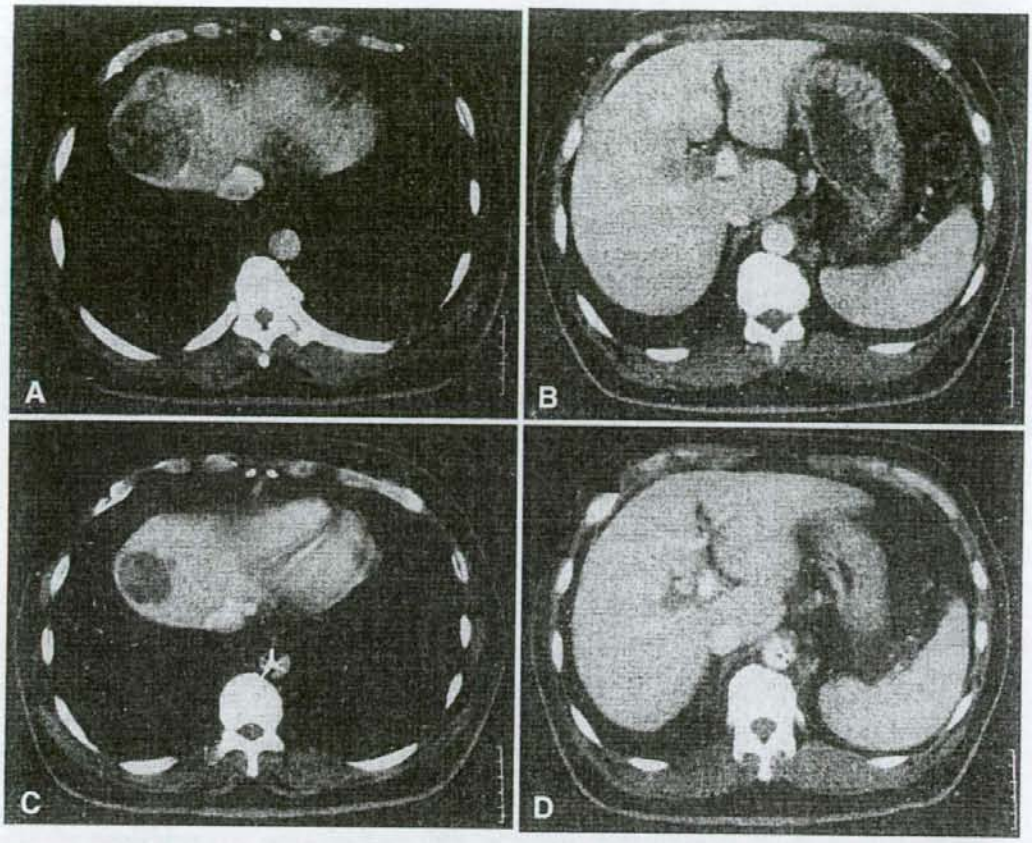

Fig. 2a–d. Computed tomography of the abdomen before the initiation of IFN-α/5FU combination therapy showed **a** hepatocellular carcinoma involved with satellite nodules around the main nodule, and **b** portal vein thrombosis. **c** After three cycles of IFN-α/5FU combination therapy, the main hepatocellular carcinoma nodule had markedly decreased in size and the number of satellite lesions had decreased, and **d** portal vein thrombosis had diminished

Before the initial treatment, this patient's hepatitis C virus (HCV) genotype was group 1b and HCV RNA concentration in serum was 280 kIU/ml. After three courses of the therapy, HCV RNA was not detected by reverse-transcription-polymerase chain reaction (RT-PCR). After the hepatectomy, the viral load of HCV RNA was positive. However, after one more course of the combination therapy, HCV RNA was not detected again. The serum ALT level was less than 100 U/l during the course of the treatment. During the clinical course, a gastric ulcer occurred. The catheter had fallen out of the gastroduodenal artery (GDA) and had become inserted into the common hepatic artery; the catheter was re-inserted into the GDA and fixed in an appropriate position.

The patient is alive 58 months after the first IFN-α/5-FU combination chemotherapy, with some recurrent nodules in the liver.

Discussion

In Japan, intraarterial perfusion chemotherapy is the most common treatment for advanced HCC which can not be treated by either surgery or embolization is contraindicated. This treatment is thought to deliver a higher concentration of chemotherapeutic drug to the tumor cells in the whole liver and to induce a greater antitumor effect that systemic chemotherapy. Nevertheless, the response rate of the hepatic arterial infusion chemotherapy has been still unsatisfactory.[12]

In general, HCCs are resistant to anticancer drugs.[13] However, recent studies, including those from our group, have demonstrated an excellent clinical response to the combination therapy of IFN-α and 5-FU in HCC patients complicated with Vp3–4.[9,14,15] We have previously reported

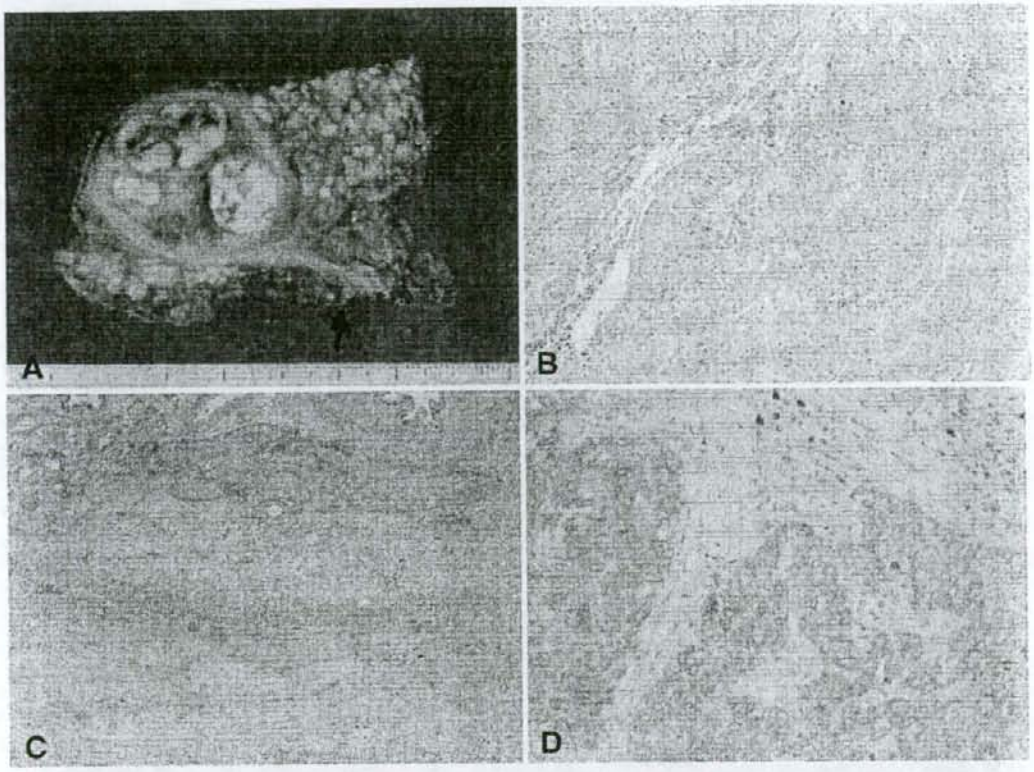

Fig. 3. **a** Surgical specimen of the liver shows that the portal vein thrombosis had become organized (*arrow*). **b** Histological examination shows residual viable hepatocellular carcinoma cells in the main nodule, and **c** no evidence of tumor cells in the portal vein thrombosis. **d** Immunohistochemical staining for type I IFN receptors (IFNAR) in the hepatocellular carcinoma tissues of the patient who received IFN-α/5FU combination therapy. The resected surgical specimen shows high IFNAR expression. Immunohistochemistry was performed according to our previous report.[21] **b** ×100; **c** ×40, H&E; **d** ×200

the efficacy of IFN-α and 5-FU combination therapy against advanced HCC, based on its high response rate and low incidence of side effects.[9] In our experience, in approximately half of 55 patients who were deemed unresectable because of multiple intrahepatic metastases and major portal vein thrombosis, IFN-α/5-FU combination chemotherapy was effective.[10]

Although the exact mechanism of action of this combination therapy has not yet been established, it has been reported that, in colon carcinoma cells, IFN-α enhances the expression of thymidine phosphorylase, which converts 5-FU to an active metabolite and enhances the DNA damage caused by 5-FU.[16,17] We also demonstrated previously that IFN-α and 5-FU synergistically reduced tumor cell proliferation through cell-cycle arrest[18] and that IFN-α also exerted immunomodulatory effects by stimulating natural killer (NK) cells and monocytes through the upregulation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which induces apoptosis in HCC cells.[19]

IFN-α exerts its multiple functions through type I IFN receptors (IFNAR). We also demonstrated the importance of this receptor for antitumor effects.[20] We estimated the expression of IFNAR in tumor tissues by means of immunohistochemistry[21] in 13 HCC patients who received IFN-α/5-FU combination chemotherapy;[10] in the HCC patients who received IFN-α/5-FU combination therapy, the expression of IFNAR in tumor tissue was significantly higher in clinical responders than in nonresponders.[10] It should be noted that none of the responders were negative for IFNAR expression. It is conceivable that the existence of IFNAR is a minimal requirement for an effective response to IFN-α/5-FU combination therapy. In the patient described in the present report, there was high IFNAR expression in the tumor tissue (Fig. 3d).

The complete remission in this patient was thought to be due to the following factors: (1) high sensitivity of the HCC to 5-FU; (2) IFN-α exerting its multiple antitumor effects through positive IFNAR in tumor cells; (3) appropriate

surgical removal of the tumor performed with the right timing; and (4) the use of an appropriate drug delivery system into the liver through an intraarterial catheter. Mainly, we think that the chemotherapeutic effects of IFN-α/5-FU prevented intrahepatic metastasis and improved the prognosis. Futhermore, in this patient, the elimination of HCV by the IFN therapy may have been a contributing factor in suppressing the occurrence of multicentric HCC.

Most chemotherapy regimens for HCC, such as those including doxorubicin or cisplatin, cause severe leucopenia or thrombocytopenia, leading to discontinuation of the therapy. On the other hand, there are few reported adverse myelosupressive effects of the IFN-α/5-FU combination therapy that would lead to its discontinuation. However, an adverse effect of IFN-α/5-FU combination therapy is catheter trouble, because 5-FU is administered through the catheter intraarterially. Catheter troubles – such as a subcutaneous seroma or hematoma forming around the implanted reservoir; gastrointestinal ulcer, or inflammation due to dislocation of the catheter; and pseudoaneurism in the femoral or subclavian artery – often occur and lead to discontinuation of the therapy. In the present patient, the catheter dropped out to the common hepatic artery and it caused a gastric ulcer as 5-FU flowed into the gastric mucosa via the right gastric artery. Fortunately, the dislocation of the catheter was easily corrected, and the patient was able to continue the therapy.

In conclusion, IFN-α/5-FU combination therapy is a useful treatment for HCC in patients who have multiple intrahepatic metastases and portal vein thrombosis. In addition to this therapy, combined modality therapy, including, for example, surgical resection, can sometimes have a dramatic therapeutic effect, leading to tumor markers dropping to normal levels. Randomized controlled trials would be need to clarify the efficacy of this therapy, not only as the treatment for advanced HCC but also as the adjuvant therapy after curative resection.

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Role of the Fas/FasL pathway in combination therapy with interferon-α and fluorouracil against hepatocellular carcinoma in vitro ☆,☆☆

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***Background/Aims*: Several studies have reported the efficacy of combination therapy of interferon (IFN) α and 5-fluorouracil (5-FU) for hepatocellular carcinoma (HCC). However, the mechanism underlying the clinical anti-tumor effects of this treatment is not well understood. The aim of this study was to determine the role of Fas/FasL signaling in the anti-tumor effect of this combination therapy.**

***Methods and Results*: We used six human hepatoma cell lines, three of which are known Fas-expressing cells. Growth of Fas-positive hepatoma cell lines was inhibited by an agonistic anti-Fas antibody in a dose-dependent manner, and these effects were enhanced by IFNα or 5-FU alone, but even more so by combination therapy using both agents. Annexin-V assay implicated apoptosis as the main mechanism underlying these growth inhibitory effects, although changes in Fas expression regulated by IFNα and/or 5-FU did not correlate with increased apoptosis. Caspase-3 activation was exclusively increased by IFNα/5-FU combination treatment, which was compatible with enhancement of the synergistic apoptotic effect, and other caspases and apoptotic factors (FLIP, BCL-xl, and Bax) were also regulated by IFNα/5-FU. ^{51}Cr-release assay revealed that pretreatment with IFN activated cytotoxicity of peripheral blood mononuclear cells (PBMCs) against HCC cells. The largest interaction was observed when both PBMC and HCC cells were pretreated with the combination of IFNα/5-FU. These cytotoxicities were markedly inhibited by a neutralizing anti-Fas antibody.**

***Conclusions*: Our results indicated that IFNα/5-FU combination treatment enhances the induction of apoptosis and the cytotoxic effect of PBMCs via the Fas/FasL pathway. The Fas/FasL pathway seems, at least in part, to contribute to the anti-tumor effects of IFNα/5-FU against HCCs.**

***Keywords*: Hepatocellular carcinoma; Combination therapy; Interferon-α; 5-Fluorouracil; Apoptosis; Fas/FasL pathway; Caspase-3**

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1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common solid tumors [1]. The prognosis for patients with HCC remains poor and most die within several months after diagnosis, particularly in advanced cases with tumor thrombosis in the major portal vein (Vp3-4) [2–4]. Chemotherapy is the traditional first choice

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for the treatment of unresectable solid tumors; however, these drugs are not effective in promoting tumor regression and prolonging survival in HCC [5,6]. In addition, conventional therapeutic modalities such as transcatheter arterial embolization, radiofrequency ablation and microwave coagulation therapy are not recommended when portal vein tumor thrombosis (PVTT) is present because of low efficacy and potential complications [7,8]. Therefore, a new effective modality is needed to treat advanced HCC, especially in those cases with portal vein involvement.

Interferon (IFN) has a variety of biological properties including immunomodulation and anti-tumor activity. The anti-tumor effect of IFN against HCC was tested in several studies. From a randomized controlled trial, Llovet et al. [9] concluded that IFN used alone provides no clinical benefit for HCC patients with respect to tumor progression rate and survival. However, several other investigators reported a strong anti-tumor activity for IFN in HCC, when used in combination with some other chemotherapeutic agents. Urabe et al. [10] found that treatment with a combination of subcutaneous IFNα injection and intra-arterial infusion of 5-fluorouracil (FU), cisplatin and methotrexate for HCC with PVTT achieved a response rate of 46.7%. In addition, Patt et al. [11] reported that combination treatment with FU and IFN promoted anti-tumor has activity in HCC and could be tolerated even by cirrhotic patients. We also previously reported the beneficial results of subcutaneous IFNα injection and intra-arterial 5-FU infusion against HCC with PVTT [12–14]. This therapy showed an anti-tumor effect with a response rate approaching 50%, including several complete remissions of the tumor and prolonged survival without major adverse effects. From these results, we proposed that the combination chemotherapy of IFNα and 5-FU should become a standard therapy for advanced HCC.

We have already reported the synergistic effects of IFNα and 5-FU in influencing cell-cycle progression into the S phase via $p27^{Kip1}$, inducing apoptosis by downregulating Bcl-xl, and modulating the immune response via the TRAIL/TRAIL-receptor pathway [15–17]. The present study is an extension of this previous work, to investigate the role of the Fas/FasL pathway in the IFNα/5-FU treatment effect. Fas/FasL signaling participates in an apoptosis-inducing mechanism related to cytotoxic T Lymphocytes (CTL) and natural killer (NK) cells, which was implicated as a major pathway of T-cell-mediated cytotoxicity and a mediator of apoptosis via an IFN-stimulated gene [18]. In addition, we also investigated the mechanism underlying the apoptosis-enhancing effect of IFNα/5-FU that acts via the Fas/FasL pathway.

2. Materials and methods

2.1. Cells

Human HCC cell lines (HuH7, PLC/PRF/5, HLE, HLF and HepG2) were obtained from the Japan Cancer Research Resources Bank (JCRB) (Osaka, Japan) and the human HCC cell line, Hep3B, was obtained from the Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). These cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin at 37 °C in a humidified incubator with 5% CO_2 in air. A non-tumorigenic SV40-immortalized human liver epithelial cell line (THLE-2) was obtained from American Type Culture Collection (Manassas, VA). THLE-2 cells were maintained as an adherent monolayer in Bronchial epithelial medium (BEGM bullet kit, Combrex, NJ) from which remove the gentamicin/amphotericin and epinephrine and to which add extra 5 ng/ml EGF, 70 ng/ml phosphoethanolamine and 10% fetal bovine serum (FBS).

2.2. Reagents

Purified human IFNα was obtained from Otsuka Pharmaceutical Co. (Tokushima, Japan), and 5-FU was kindly provided by Kyowa Hakko Co. (Tokyo, Japan). Antibodies against Fas (UB-2, CH-11 and ZB4) were obtained from Medical and Biological Laboratories (Nagoya, Japan). Caspase-3-specific inhibitor (Z-DEVD-FMK), caspase-8-specific inhibitor (Z-IETD-FMK) and caspase-9-specific inhibitor (Z-LEHD-FMK) were purchased from Calbiochem (San Diego, CA).

2.3. Flow cytometric analysis of Fas expression

HCC cells were characterized for their surface expression of Fas receptors by flow cytometry. Cells (1×10^6) were incubated with 2.5 μg/ml of anti-Fas antibody (IgG, UB-2) for 30 min at 4 °C. After washing with PBS, the cells were analyzed on a FACScan (BD Transduction Laboratories, Lexington, KY), and data were processed using Cell Quest™ software (BD Transduction Laboratories).

2.4. Cell growth assay

Cell growth was assessed by the 3-(4-,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, 3×10^4 cells were seeded on a 96-well plate in 100 μl of medium and left overnight to adhere. Several concentrations of the test drugs in 100 μl volumes were added, and the cells were incubated for 48 h. After treatment, 10 μl of MTT solution was added to each well and incubated for another 4 h at 37 °C. Then 100 μl of acid–isopropanol was added, and after 24 h at 4 °C, reduced MTT was measured spectrophotometrically in a dual-beam microtiter plate reader at 570 nm with a 650 nm reference.

2.5. Flow cytometric analysis of annexin V-FITC binding

The binding of annexin V-FITC was used as a sensitive method for measuring apoptosis, according to a modification of a previously described method [19]. Briefly, after treatment with IFNα/5-FU and/or anti-Fas antibody CH-11, the cultured cells (1×10^6) were incubated with binding buffer (10 mM Hepes, 140 mM NaCl and 2.5 mM $CaCl_2$, pH 7.4) containing saturating concentrations of annexin V-FITC (BioVision Research Products, Mountain View, CA) and propidium iodide (PI) for 15 min at room temperature. After incubation, the cells were pelleted and analyzed on a FACScan (BD), and data were processed using Cell Quest™ software (BD).

2.6. Caspase activity

After treatment with the test drugs and/or anti-Fas antibody, cytosolic extracts were prepared using lysis buffer. The caspase activity in the cell cytosol was measured using a Caspase Colorimetric Protease Assay Kit (MBL) as per the instructions provided by the manufacturer. This assay is based on the spectrophotometric detection of the chromophore, *p*-nitroanilide after cleavage from the labeled substrate. Caspase-3, -8 and -9 assay kits were used in this study.

2.7. Real-time PCR

The LightCycler PCR and detection system (Roche Diagnostics, Mannheim, Germany) was used for amplification and quantification. For detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the amplification products of some apoptotic factors, the LightCycler DNA Master SYBR Green I (Boehringer Mannheim, Mannheim, Germany) was used. Real-time PCRs were performed in a sample mixture containing each primer (final concentration, 0.2 μM), 1× LC-DNA Master SYBR Green I, 4 mM of $MgCl_2$, and 2 μl of cDNA as a template using the following primers: human GAPDH (forward: 5′-CAACTACATGGTTTACATGTTC-3′, reverse: 5′-GCCAGTGGACTCCACGAC-3′); Bcl-xl (forward: 5′-GTAAACTGGGGTCGCATTGT-3′, reverse: 5′-TGGATCCAAGGCTCTAG GTG-3′), and Bax (forward: 5′-CCAGCTGCCTTGGACTGT-3′, reverse: 5′-ACCCCCTCAAGACCACTCTT-3′) yielding products of 182, 146 and 135 bp, respectively [20]. The GAPDH PCR cycle conditions were set up as follows: one cycle of 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 10 s and 72 °C for 20 s. Fluorescence was acquired at the end of every 72 °C extension phase. The annealing temperatures for Bcl-xl and Bax were 60 and 61 °C, respectively. Quantitative analysis of data was performed using the LightCycler™ analysis software (Roche Diagnostics).

2.8. Western blot analysis

The sub-confluent growing cells were washed with PBS (Sigma) and lysed in an ice-cold RIPA buffer [25 mM Tris (pH 7.5), 50 mM NaCl, 0.5% sodium deoxycholate, 2% Nonidet P-40, 0.2% SDS, 1 mM phenylmethylsulfonyl fluoride and 500 KIE/ml "Trasylol"® proteinase inhibitor (Bayer Leverkusen, Germany)]. Total protein concentration was determined using the Bradford protein assay (Bio-Rad, Hercules, CA) and Western blot analysis was performed as described in our previous study [17]. The antibodies were used in dilutions of 1:100 for FLIPS/L (sc-5276; Santa Cruz Biotechnology, Santa Cruz, CA), 1:1000 for actin (A-2066; Sigma), and 1:2000 for secondary donkey anti-rabbit (NA934V; Amersham Biosciences, Buckinghamshire, UK) antibodies. The expression of proteins was evaluated by measuring the optical densities of protein bands, using the National Institute of Health Image analysis software version 1.61 and the expression value was calculated relative to that of actin.

2.9. Cytotoxicity assay

Target cells (1×10^6) were labeled with 40 μCi $Na^{51}CrO_4$ for 45 min at 37 °C. ^{51}Cr-labeled target cells (1×10^4) and effector cells (peripheral blood mononuclear cells, PBMCs) were mixed in U-bottomed wells of a 96-well microplate at the indicated *E/T* ratios. After 8 h of incubation, the cell-free supernatants were collected and counted on a gamma counter. The percent-specific cytotoxicity was calculated using the formula: [100 × (experimental release − spontaneous release)/(total release − spontaneous release)]. Total or spontaneous release was determined in the presence of 1% NP-40 or medium alone. For blocking, anti-Fas mAb ZB-4 was added at a final concentration of 500 ng/ml before the cytotoxicity assay for 1 h in accordance with the manufacturer's instructions.

2.10. Magnetic sorting

PBMCs obtained from a healthy volunteer were prepared by Ficoll–Hypaque centrifugation. $CD4^+$ T cells, $CD8^+$ T cells, and $CD4^-CD8^-$ cells were isolated from PBMCs by using anti-CD4 and anti-CD8 immunomagnetic beads and a Magnetic Cell Sorter (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of each subset was estimated at >95% by flow cytometry.

2.11. TUNEL assay

To detect apoptosis, we used the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method, using the Apop Tag in situ apoptosis detection Kit (Chemicon International, Inc., Temecula, CA) as described previously [17]. This method can detect fragmented DNA ends of apoptotic cells. Briefly, the paraffin-embedded sections were deparaffinized in xylene and rehydrated in a graded series of ethanol baths. The sections were treated with 20 μg/ml of proteinase K in distilled water for 10 min at room temperature. The adherent cultured THLE-2 cells were fixed in 1% paraformaldehyde for 10 min. To block endogenous peroxidase, the slides were incubated in methanol containing 0.3% hydrogen peroxide for 20 min. The remaining procedures were performed according to the instructions provided by the manufacturer. For quantification of apoptosis, five microscopic fields were randomly selected at high power magnification (200×) and the average counts of TUNEL-positive cells were calculated.

2.12. Statistical analysis

Statistical analysis was performed using the StatView J-5.0 program (Abacus Concepts, Inc., Berkeley, CA). Data are expressed as means ± SD. Differences between groups were examined for statistical significance using the Dunnett method and Student's *t*-test. $P < 0.05$ denotes a statistically significant difference.

3. Results

3.1. Fas expression in human hepatoma cell lines

Flow cytometry using an anti-Fas antibody (UB-2) revealed expression of Fas receptor on the cell surface in three of the six cell lines (HLE, HLF and HepG2), but not on HuH7, PLC/PRF/5 and Hep3B (Fig. 1).

3.2. Response to agonistic anti-Fas antibody with dose escalation

We confirmed the response described above using the agonistic anti-Fas monoclonal antibody, CH-11, which is used widely to replace FasL in vitro. The 48-h MTT assay showed that CH-11 treatment inhibited the growth of three Fas-positive hepatoma cell lines (HLE, HLF and HepG2) in a dose-dependent manner. In contrast, no Fas-negative cell lines (HuH7, PLC/PRF/5 and Hep3B) were growth-responsive to CH-11 (Fig. 2a). Dose dependency of IFNα and 5-FU was examined with various CH-11 concentrations. This effect was synergistic and observed in the combination of CH-11 and 5-FU in the doses of 0–0.5 μg/ml of 5-FU. There was seen little difference between 0.5 and 1 μg/ml of 5-FU (Fig. 2b).

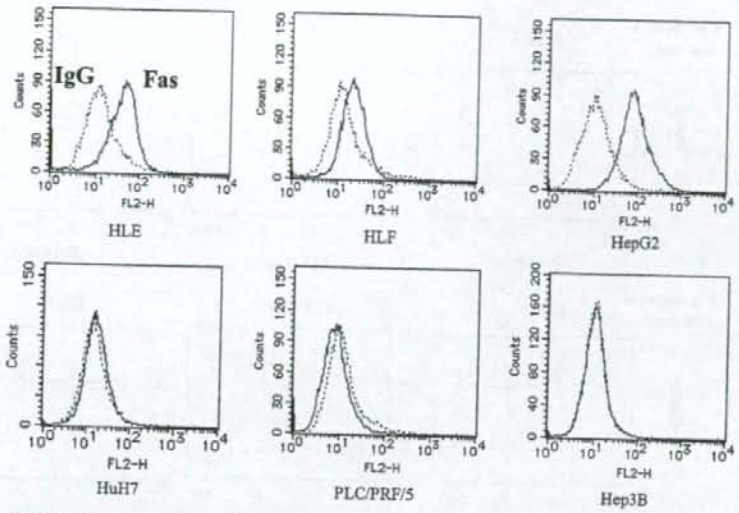

Fig. 1. Fas expression in six hepatoma cell lines assessed using flow cytometry with an anti-human Fas antibody (IgG, clone UB2). Histogram with dotted line represents cells stained with secondary antibody alone; histogram with solid line is those cells stained with anti-Fas antibody. Fas was expressed on the surface of three of the six cell lines (HLE, HLF and HepG2). All experiments were performed three times independently.

3.3. Influence of IFNα and/or 5-FU on apoptosis induced by agonistic anti-Fas antibody

We next evaluated the effects of IFNα, 5-FU and combination treatments on growth inhibition induced by CH-11 using the MTT assay. In Fas-expressing cell lines, the inhibitory effect of CH-11 was enhanced with IFNα or 5-FU alone, but the maximum effect was observed with a combination treatment of both agents (Fig. 3a). In the HepG2 cells, the anti-proliferating effect of CH-11 alone was $26.7 \pm 1.8\%$, and the effect of either IFNα or 5-FU used alone was $18.0 \pm 4.7\%$, which did represent a significant enhancement. However, the combination treatment (CH-11 + IFNα/5-FU) yielded a markedly increased effect of $83.8 \pm 6.3\%$ ($P = 0.01$). Without CH-11, none of the agents, whether used alone or in combination, had any anti-proliferative effects. Similar results were obtained in the other Fas-positive cell lines, HLE and HLF.

To confirm these results, we performed an annexin-V assay to detect Fas-mediated apoptosis. Results with the HepG2 cells were comparable with those from the MTT assay (Fig. 3b, Table 1), in that an increase in apoptotic cell numbers induced by CH-11 was found with stimulation by IFNα alone, 5-FU alone, and particularly strongly with the combination treatment. In contrast, the effects of CH-11 and the influence of IFNα/5-FU were not observed with the three Fas-negative cell lines (HuH7, PLC/PRF/5 and Hep3B) in both the MTT and annexin-V assay (Fig. 3a; MTT assay, data not shown; annexin-V assay).

3.4. Regulation of Fas expression by IFNα and/or 5-FU

To investigate the mechanism underlying the upregulation of Fas-mediated apoptosis, we analyzed the relationship between the change in Fas expression and the regulation of apoptosis. Out of the six cell lines, 5-FU increased Fas expression in the HepG2 cells only, while IFNα also increased Fas in the HuH7 and PLC/PRF/5 cells (Fig. 4). No additional effects were seen with the combination of IFNα and 5-FU compared with each drug used alone. In the other three hepatoma cell lines (HLE, HLF and Hep3B), neither IFNα nor 5-FU affected the level of cell surface Fas.

3.5. Caspase activation after stimulation with agonistic anti-Fas antibody and/or IFNα/5-FU

Results indicated that the change in Fas expression seen in this study would not be related to the CH-11 and IFNα/5-FU-mediated effects on apoptosis. Therefore, we next tested for variations in caspase activity in the HepG2 cells, as the representative Fas-positive cell line, using a caspase colorimetric protease assay kit (Fig. 5a). Caspase-3 activity (downstream of caspase cascades) was increased after 12 h of CH-11 stimulation. IFNα and 5-FU alone enhanced this upregulation, with the combination treatment having a further effect (Fig. 5a); 5-FU alone, IFNα alone and combination therapy did not affect caspase-3 activity without CH-11. Caspase-8 activity also

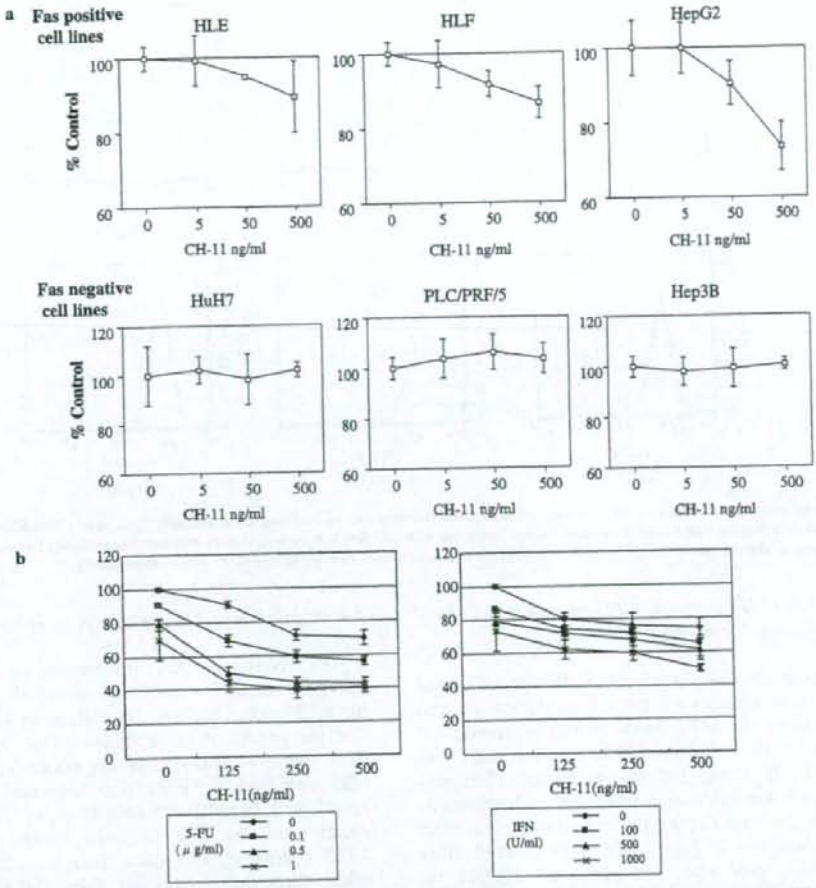

Fig. 2. Susceptibility of six hepatoma cell lines to agonistic anti-Fas monoclonal antibody CH-11-mediated apoptosis was measured by MTT assay. (a) Each cell line was incubated with CH-11 at various concentrations for 48 h. Three Fas-positive cell lines (HLE, HLF and HepG2) were naive to CH-11 in a dose–response manner. Fas-negative cell lines (HuH7, PLC/PRF/5 and Hep3B) were resistant to Fas-mediated apoptosis. (b) Dose dependency of combination treatment was examined. CH-11 and 5-FU or IFNα were added to HepG2 cells with indicated doses. Results are expressed as percent of cell growth of each untreated cell. Data represent means ± SD of at least triplicate samples. Similar results were observed in three independent experiments.

increased with CH-11 stimulation compared with the control and further increased with CH-11 + IFNα/5-FU; there was no significant difference between stimulation of CH-11 + IFNα and the combination. On the other hand, caspase-9 activity showed a different tendency from the data for caspase-3 and -8. Stimulation with CH-11+5-FU or CH-11 + IFNα/5-FU slightly increased the caspase-9 activity, but the effects were much less pronounced than for caspase-3 and caspase-8, and they were not significant (Fig. 5a). To confirm the significance of caspase activities in the apoptotic effect in Fas/FasL system, MTT assay using specific caspase inhibitors was performed. All specific caspase inhibitors blocked the apoptotic effect of CH-11 with IFNα/5-FU totally or partially in the dose-dependent manner (data not shown). Caspase-3-specific inhibitor (Z-DEVD-FMK) and caspase-8-specific inhibitor (Z-IETD-FMK) almost completely blocked CH-11 induced apoptosis enhanced by IFNα/5-FU (Fig. 5b). Caspase-9-specific inhibitor (Z-LEHD-FMK) showed only partial blocking effect. These results were compatible to the results of caspase assay (Fig. 5a). Colorimetric caspase assay using specific caspase inhibitors showed Z-DEVD-FMK blocked caspase-3 activation induced by CH-11 and IFNα/5-FU (Fig. 5c).

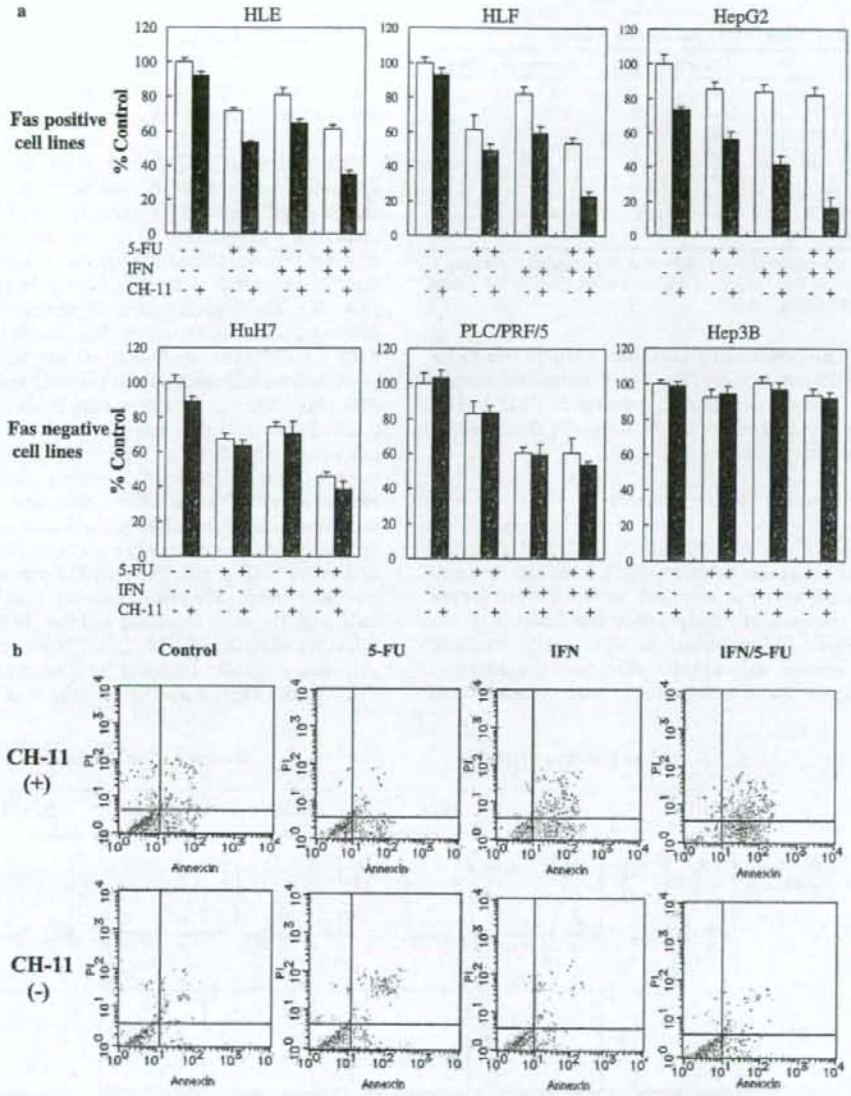

Fig. 3. (a) Effects of IFNα and/or 5-FU on Fas-mediated apoptosis in six hepatoma cell lines measured by MTT assay. All cells were incubated with IFNα (500 U/ml) and/or 5-FU (0.5 μg/ml) and with agonistic anti-Fas monoclonal antibody CH-11 (500 ng/ml) (□) or without CH-11 (■) for 48 h. The susceptibility of Fas-positive hepatoma cells to Fas-mediated apoptosis was significantly enhanced by IFNα or 5-FU alone, and further so in the combination treatment. Results were expressed as percent of cell viability of untreated cells. Data represent means ± SD values of at least triplicate samples. Similar results were observed in three independent experiments. (b) Apoptotic cells were determined using the annexin-V assay (HepG2). [This figure appears in colour on the web.]

3.6. Regulation of FLIP

Several factors involved in apoptosis were next examined at the mRNA expression level. The expression of FLIP (FLICE/caspase-8 inhibitory protein), which is an inhibitor of caspase-8 [21,22], was markedly decreased by treatment with IFNα or 5-FU, as shown by the FLIP/GAPDH ratio, compared with untreated

Table 1
Comparison of results of MTT and annexin-V assays

	Cell toxicity (%)	Annexin (+) cells (%)
Control	0.0	5.8
5-FU	14.4	9.5
IFN	16.2	10.0
IFN + 5FU	18.0	19.7
CH-11	26.7	27.8
CH-11 + 5FU	43.8	37.6
CH-11 + IFN	58.0	62.4
CH-11 + IFN + 5FU	83.8	93.5

The two independent assays showed similar tendency. Annexin-V assay indicated that the growth inhibition effect noted in MTT assay was caused by apoptosis.

cells with the combination treatment again providing the most significant decrease (Fig. 6a). Western blot analysis was performed to examine the change of FLIP at protein level. Expression of FLIP long was decreased by the combination treatment (Fig. 6b).

3.7. Regulation of apoptotic factors

Caspase-9 acts through mitochondria and is regulated by certain apoptotic factors [23]. Although the levels of caspase-9 were not increased, we checked the expression of the apoptotic factors Bax and Bcl-xl (Fig. 6c). The IFNα/5-FU combination significantly increased Bax expression, although the effect was not dramatic. Each of the above treatments in turn reduced Bcl-xl expression, although in this case the IFNα/5-FU combination produced no enhancement.

3.8. Involvement of the Fas/FasL pathway in IFNα/5-FU-induced PBMC cytotoxicity against HCC cells

We performed ^{51}Cr-release assay to evaluate the interaction between PBMC and hepatoma cell lines via the Fas/FasL pathway by which IFNα/5-FU appear to exert their influence (Fig. 7). In the Fas-positive HepG2 cells, we first established the optimal E/T ratio, which is directly proportional to the increase in released ^{51}Cr (Fig. 7a). The blocking effect of neutralizing anti-Fas antibody, ZB-4, is shown in Fig. 7b. Based on these data, an E/T ratio of 20 was chosen, to produce the most distinct difference in the presence and absence of ZB4 (Fig. 7c). Fig. 7c shows that IFNα increased the released ^{51}Cr and that this enhanced cytotoxicity was blocked by ZB4. Next, we tried to identify the main proponent of the cytotoxic effect using a magnetic sorting technique. $CD4^+$ cells, $CD8^+$ cells, and $CD4^-CD8^-$ cells were isolated from PBMCs and used as effecter cells in the ^{51}Cr-release assay after pretreatment with IFNα and/or 5-FU (Fig. 7d). The HepG2 target cells received no pretreatment. The results show that the $CD4^-CD8^-$ cells were the most cytotoxic and that IFNα enhanced this effect more than 5-fold. This IFNα-induced cytotoxicity was markedly inhibited by ZB4. Lastly, we pretreated both effecter and target cells with IFNα/5-FU

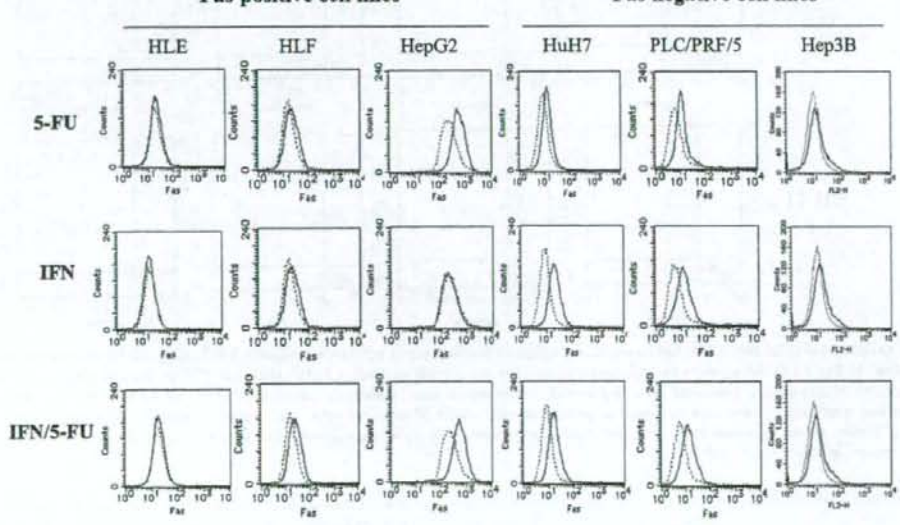

Fig. 4. Regulation of Fas expression induced by IFNα and/or 5-FU. Adherent cells were incubated with IFNα (500 U/ml) and/or 5-FU (0.5 μg/ml) for 24 h. Cell surface Fas was detected by flow cytometry using a mouse monoclonal anti-human Fas IgG (UB2). Histogram with dotted line shows untreated; histogram with solid line represents the drug-treated cells.