PCI-6, MiaPaCa and ASPC-1), were all obtained from the Japanese Cancer Research Resources Bank. Cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS at 37°C.

Semiquantitative RT-PCR

RNA extraction was carried out with TRIzol reagent in a single-step method, and cDNA was generated with avian myeloblastosis virus reverse transcriptase (Promega Corp., Madison, WI), as described previously (14). Semiquantitative analysis for expression of MT3-MMP mRNA was performed by the multiplex reverse transcription-polymerase chain reaction (RT-PCR) technique, using a housekeeping gene, PBGD, as an internal standard (15,16). The PCR primers used for detection of MT3-MMP were synthesized as reported previously (17). To minimize the differences of inter-PCRs, PCR was performed with MT3-MMP and PBGD primers in an identical tube, under unsaturated conditions. PCR was performed in a 25μL reaction mixture containing 1μL of cDNA template, 1 x Perkin-Elmer PCR buffer, 1.5mM $MgCl_2$, 0.8mM deoxynucleotide triphosphates, 0.8μM of each primer for MT3-MMP, 80nM each for PBGD, and 1 unit of Taq DNA polymerase (AmpliTaq Gold; Roche Molecular Systems, Inc., Branchburg, NJ). The PCR primers used for detection of MT3-MMP and PBGD cDNAs were synthesized as reported previously (17,18). The conditions for multiplex PCR were as follows: one cycle of denaturing at 95°C for 12 min, followed by 35-40 cycles of 95°C for 1 min, and 72AC for 1 min, before a final extension at 72°C for 10 min.

Patients, Tissue Samples and Pathological Examination

Formalin-fixed, paraffin-embedded liver tissue specimens were prepared from 58 patients with HCC (44 males and 14 females; ranging in age from 36 to 77 years), who underwent hepatectomy at the Department of Surgery and Clinical Oncology, Osaka University between 1994 and 1999. All patients had either HCV infection (46 patients) or HBV infection (12 patients), and 3 patients had concomitant infection with HCV and HBV. Adjacent non-tumor liver tissues were either chronic hepatitis (n=24) or liver cirrhosis (n=34). Tissue sections (4μm thick) were deparaffinized in xylene, rehydrated and stained with hematoxylin and eosin solution. For non-tumor tissues, the presence of inflammation or cirrhotic nodules was checked. Tumor tissues were examined for the following characteristics; cell differentiation (well, moderately, poorly differentiated), number of tumors, capsular formation, capsular invasion, portal vein tumor thrombus, and hepatic vein invasion. Pathological diagnosis was established by one author (K.W.), who was blinded to the clinical background.

Reagents

Rabbit polyclonal anti-human MT3-MMP antibody was obtained from Fuji Co. (Tokyo, Japan). This was applied as the primary antibody at the concentration of 1:20.

Immunohistochemistry

Tissue sections (4μm thick) were deparaffinized in xylene and heat antigen retrieval was performed as described previously (12). The slides were then processed for immunohistochemistry using the Vectastain ABC-peroxidase kit (Vector Laboratories, Burlingame, CA) (13). In the step of primary antibody reaction, the slides were incubated with the MT3-MMP antibody for 1 hr at room temperature. For negative controls, non-immunized rabbit IgG (Vector Laboratories) or TBS (Tris-buffered saline) was used as a substitute for the primary antibody to verify the possibility of false-positive responses from non-specific binding of IgG or from the secondary antibody. For each section, the intensity of staining was scored on a scale from 0 to 2 where 0 represented no or faint staining, 1: moderate, and 2: strong staining. MT3-MMP expression levels were moderate in epithelial cells of the bile ducts and in the vascular epithelium. Accordingly, the vascular epithelium level of staining was used as an intrinsic control within the sample, which was designated arbitrarily as intensity level 1. In correlation of MT3-MMP protein immunoreactivity with clinicopathological characteristics and survival, the staining intensities were summarized, with intensity 0 and 1 labeled as "low expression", the intensity 2 as "high expression". Disease-free survival (DFS) and overall survival (OS) data were analyzed for 58 patients who had undergone curative surgery and could be followed-up. They were followed for a period of 2 to 95 months (mean, 23 months) by tumor markers (serum AFP, AFP-L3 or PIVKA-II) and/or imaging modalities. DFS was defined from the date of surgery to the date of tumor relapse. The postoperative DFS and OS curves were constructed by the Kaplan-Meier method.

MT3-MMP expression was often heterogeneous, and in 28 HCCs where tumor tissues were composed of two or more different histological types, the histological or immunohistological type that constituted the major volume of the tumor was selected as the representative type. Staining was repeated at least twice to avoid possible technical errors but essentially identical results were obtained. All slides were interpreted by one investigator (I.A.) in a blinded manner without knowledge of the clinical and pathological parameters. When the initial diagnosis was different, the final diagnosis was cooperatively determined using a multi-head microscope by two investigators (I.A. and M.K.).

Statistical Analysis

Statistical analysis was performed using the Statview J-5.0 program (Abacus Concepts, Inc. Berkeley, CA). The Chi-square test, Fisher's exact probability test and the log-rank test were used to examine the relationship between MT3-MMP expression and clin-

icopathological parameters or prognosis. A P value less than 0.05 denoted the presence of a statistically significant difference.

RESULTS

Expression of MT3-MMP mRNA in Epithelial Cancer Cell Lines

We investigated MT3-MMP mRNA expression in epithelial cancer cell lines by RT-PCR. MT3-MMP mRNA was expressed in all of the seven HCC cell lines. On the other hand, of other colon, stomach, esophagus and pancreatic cancer cell lines, MT3-MMP mRNA expression was observed in no colon cancer cell lines, in only one cell line of gastric and esophageal cancer, and in two pancreatic cancer cell lines. These results are summarized in **Table 1**.

Immunohistochemical Analysis for MT3-MMP

Localization and expression of MT3-MMP were investigated by immunohistochemistry in liver tissues from 58 patients with HCC. These exhibited immunostaining of cytoplasm and cellular membranes. **Figure 1** illustrates typical staining for MT3-MMP and corresponding intensity scores in the representative cancerous tissues. Heterogeneous staining was observed in some HCC and the intensity was evaluated by the major staining part.

In chronic hepatitis and liver cirrhosis tissues, 24/24 (100%) and 34/34 (100%) showed moderate to strong staining (intensity 1 or 2), respectively. MT3-MMP expression was not increased in chronic hepatitis compared with liver cirrhosis. In HCC tissues, the expression of MT3-MMP varied from strong to none or only faint. MT3-MMP was strongly demonstrated in cytoplasm and cellular membrane in capsular invasion of HCC **(Figure 1A)**, whereas it was faintly detected in non-capsular invasion of HCC **(Figure 1B)**. MT3-MMP expression levels were moderate in the vascular epithelium **(Figure 1C arrow)**. MT3-MMP expression was observed in 56/58 (97%) HCCs. The rate of strong staining, moderate staining, and faint staining was 1/2 (50%), 1/2 (50%), and 0/2 (0%) in well-differentiated HCC, 17/30 (57%), 12/30 (40%), 1/30 (3%) in moderately-differentiated HCC, and 13/26 (50%), 12/26 (46%), and 1/26 (4%) in poorly-differentiated HCC, respectively.

Correlation between MT3-MMP Expression and Clinicopathological Parameters

The patients with HCC were divided into a high expression group (intensity 2) and a low expression group (intensity 0 and 1), and the correlations between MT3-MMP expression and various clinicopathological parameters were examined **(Table 2)**. Among the clinicopathological factors studied (age, gender, tumor size, histological grade, presence or absence of hepatic vein invasion, presence or absence of portal vein tumor thrombus, number of tumors, capsular formation and capsular invasion), only the latter was significantly related to high MT3-MMP ex-

TABLE 1 MT3-MMP mRNA Expression in Human Cancer Cell Lines

Organ	Cell line	PBGD	MT3-MMP
Liver	HLE	+	+
	HLF	+	+
	HuH7	+	+
	PLC/PRF/5	+	+
	HepG2	+	+
	SKHep1	+	+
	PLL	+	+
Colon	Lovo	+	-
	HCT 116	+	-
	HT29	+	-
	DLD1	+	-
	SW 480	+	-
Stomach	MKN-1	+	+
	MKN-28	+	-
	MKN-74	+	-
	MKN-45	+	-
	KATO-III	+	-
Esophagus	TE-2R	+	-
	TE-2S	+	-
	TE-8	+	-
	TT	+	+
Pancreas	PANC1	+	+
	PSN-1	+	-
	PCI-6	+	+
	MiaPaCa	+	-
	ASPC-1	+	-

TABLE 2 Relationship between MT3-MMP Expression in HCC and Clinicopathological Parameters

	n	Intensity of MT3-MMP		
		low	high	p value
Age				
<60	33	15	18	NS
≥60	25	13	12	
Gender				
female	11	6	5	NS
male	47	22	25	
Tumor size				
<2cm	9	3	6	NS
≥2cm	49	25	24	
Histological grade				
well.	2	2	0	NS
mod./poor.	56	26	30	
Hepatic vein invasion				
yes	1	1	0	NS
no	57	27	30	
Portal vein tumor thrombus				
yes	8	6	2	NS
no	50	22	28	
Number of tumors				
multiple	19	9	10	NS
solitary	39	19	20	
Capsular formation				
yes	46	20	26	NS
no	12	8	4	
Capsular invasion				
yes	22	6	16	0.034
no	24	14	10	

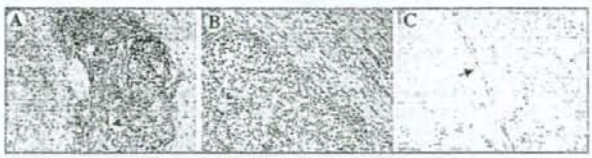

FIGURE 1 (A) High expression of MT3-MMP in HCC with capsular invasion (original magnification x200). **(B)** Low expression of MT3-MMP in HCC without capsular invasion (original magnification x200). **(C)** MT3-MMP expression was moderate (intensity 1) in the vascular epithelium (arrow; original magnification x400). Tissue processing, immunostaining and intensity evaluation were performed as described in "Methodology".

pression ($p=0.034$). The DFS and OS of the low expression group appeared better, but there was no significant difference between patients with high and low MT3-MMP expression in OS or DFS ($p>0.05$).

DISCUSSION

The relationship between MMPs and HCC progression has been studied by several groups (9-13). For example, carcinoma cells in early HCC invade portal tracts and/or fibrous bands with the participation of MMP-1 (9). Elevated levels of plasma MMP-9 and overexpression of its mRNA have been reported in HCC with invasive potential (10). MT1-MMP is also overexpressed in HCC with capsule infiltration and has been shown to cooperate with MMP-2 in the invasive process of cancer (13). These studies suggest that the evaluation of MMPs in HCC might be important from a clinical viewpoint to evaluate malignant potential. Thus, we undertook the present study to examine whether MT3-MMP expression is a valid biological indicator for HCC. Our findings included, 1) MT3-MMP expression was observed particularly in HCC cell lines, but not in other gastrointestinal cancer cell lines, and 2) high MT3-MMP expression in HCCs was correlated with capsular invasion, but did not influence OS or DFS.

Two major proteinases, MT1 and MT2-MMP, are widely expressed in normal organs as well as a variety of carcinoma tissues. In addition, MT3-MMP expression appears to be restricted in both normal and cancerous tissues (19,20). MT3-MMP was originally identified from an oral melanoma (21), and has also been detected in lung, placenta, brain, smooth muscle cells, and carcinoma of the kidney (21-25). Biochemically, MT3-MMP has been shown to be an MMP-2 activator and effective proteinase in degrading various ECM components, including native collagens (21,26,27). However, the biological consequence of MT3-MMP expression in human cancer has not been examined to date. Recently, Kitagawa *et al.* implicated the expression of MT3-MMP in the invasiveness of carcinoma of the kidney (24,25).

It is well known that capsular invasion and portal involvement are important factors in intrahepatic or distant metastasis of HCC. In this study, we observed a direct relationship between MT3-MMP expression and capsular invasion of HCC. This finding suggested that MT3-MMP plays a crucial role in the process of HCC progression, in addition to MT1-MMP, MMP-1, MMP-2 and MMP-9 (9-13). However, the detailed mechanisms of increased MT3-MMP expression in capsular invasive HCC are unknown at present. In this study, MT3-MMP expression was observed in 100% and 97% of HCC cell lines and HCC samples, respectively. Other studies reported that MT3-MMP was an important factor in MMP-2 activation (28,29). Giannelli *et al.* reported that MMP-2 was an important indicator of invasion of HCC (12). Our preliminary studies revealed that MMP-2 protein is overexpressed in HCC tissues. Moreover, RT-PCR analysis in our preliminary study suggested that expression of both MT3-MMP and MMP-2 mRNAs was detected in similar samples. We therefore postulate that, 1) isolated high MT3-MMP expression in HCC is associated with invasive potential, and 2) particularly in HCC, the activation of MMP-2 by MT3-MMP has a crucial role in capsular invasion.

On the other hand, patients with high MT3-MMP expression did not have significantly shorter DFS and OS compared with the low MT3-MMP expression group. One possible reason for this is that the total value of expression of all MMPs might play an important role in HCC progression. Consistent with this finding, Maatta *et al.* reported that MMP-9 was required for capsular invasion but did not impact on tumor recurrence and/or survival (13). Thus, the balanced expression of MMPs might be important in HCC progression. Recently, MT4-MMP, MT5-MMP and MT6-MMP have been identified. Future studies should investigate the role of total MMP levels in HCC and factors predicting the therapeutic effects of MMP inhibitors.

In conclusion, we have demonstrated in the present study the expression of MT3-MMP in HCC tissues as well as in liver tissues with viral hepatitis, and that the expression in HCC significantly correlated with capsular invasion.

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Combination therapy of interferon-α and 5-fluorouracil inhibits tumor angiogenesis in human hepatocellular carcinoma cells by regulating vascular endothelial growth factor and angiopoietins

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Abstract. We recently reported that interferon-α (IFN-α) and 5-fluorouracil (5-FU) combination therapy in advanced hepatocellular carcinoma (HCC) achieved excellent clinical results. However, the mechanism underlying this combination therapy remains to be elucidated. In this study, we examined the anti-tumor effects of IFN-α and 5-FU combination therapy *in vivo* and aimed to reveal its anti-angiogenic effects by investigating the expression of vascular endothelial growth factor (VEGF) and angiopoietins (Ang-1 and Ang-2). Human HCC cells, HuH7, were subcutaneously injected in nude mice. Ten days later, groups of mice received treatment with IFN-α alone, 5-FU alone, or with a combination of IFN-α and 5-FU for four weeks. Immunohistochemical examinations of proliferating cell nuclear antigen (PCNA), cell differentiation antigen 34 (CD34), Ang-1, -2 and VEGF, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay and quantification of VEGF, Ang-1 and-2 mRNA using real-time RT-PCR were performed. Results showed that IFN-α and 5-FU combination therapy significantly inhibited the growth of human HCC cells compared with the control group or single agent treatment. The combination therapy decreased PCNA-positive cells as well as microvessel density (MVD) and induced apoptosis of (TUNEL-positive cells) more than other treatment groups. Immunohistochemical analysis revealed that the combination therapy significantly decreased the expression of VEGF and Ang-2 and increased that of Ang-1. The ANG2/ANG1 mRNA expression ratio was significantly lower in the combination therapy group. In conclusion, our results suggested that IFN-α and 5-FU combination therapy has anti-proliferative and anti-angiogenic effects and can induce apoptosis *in vivo*. The synergistic and anti-angiogenic effects may in part be attributable to the regulation of Ang-1, -2 and VEGF.

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Abbreviations: 5-FU, 5-fluorouracil; Ang, angiopoietin; b-FGF, basic fibroblast growth factor; ELISA, enzyme-linked immunosorbent assay; FdUMP, fluorodeoxyuridine monophosphate; HCC, hepatocellular carcinoma; IFN, interferon; IL-8, interleukin-8; MMP, matrix metalloprotease; MVD, microvessel density; PCNA, proliferating cell nuclear antigen; PVTT, portal vein tumor thrombus; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling; TRAIL, tumor necrosis factor-related apoptosis inducing ligand; VEGF, vascular endothelial growth factor

Key words: hepatocellular carcinoma, angiopoietin, angiogenesis, interferon, chemotherapy

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide (1) and the fourth highest cause of cancer-related death in Japan. The development of new diagnostic modalities has brought about an earlier diagnosis of small HCC and new therapeutic modalities, such as microwave coagulation therapy and radiofrequency ablation therapy, have improved the prognosis of patients with small HCC. However, the prognosis of patients with advanced HCC, for example those with portal vein tumor thrombus (PVTT) or intrahepatic metastasis, is quite poor and a standard treatment regimen for advanced HCC has not yet been established (2). Chemotherapy is commonly used for the treatment of various malignancies. However, it is not suitable for HCC because of its resistance to anti-cancer drugs (3).

The interferons (IFNs) are a family of natural glycoproteins and regulatory cytokines with pleiotropic cellular functions, such as anti-viral, anti-proliferative and immunomodulatory activities (4-6). Furthermore, previous reports indicate that IFN-α and IFN-ß have anti-angiogenic activities and down-regulate the expression of pro-angiogenic molecules (7-12). The efficiency of IFN therapy for various malignancies has been investigated in several clinical trials and the results

indicate that it can be effective against some angioproliferative diseases and vascularized malignancies (13-15). In HCC, the results of IFN-α monotherapy are not satisfactory and its effects remain controversial (16). However, in combination with other anti-cancer drugs, promising results were reported by several investigators (17-20). In a series of studies, we also reported recently the excellent clinical efficiency of IFN-α and 5-fluorouracil (5-FU) combination therapy for advanced HCC with PVTT and intrahepatic metastasis (21-24). The exact mechanism of action of this combination therapy is still unclear. IFN-α enhanced the expression of thymidine phosphorylase in colon cancer cells and the accumulation of fluorodeoxyuridine monophosphate (FdUMP) by inhibition of thymidylate in leukemia cells (25). We previously showed that the expression of the IFN-α/ß receptor correlated with the growth-inhibitory activity of IFN-α and that IFN-α and 5-FU synergistically inhibited cell proliferation, induced cell cycle arrest (26,27) and induced apoptosis by regulating the expression of apoptosis-related molecules (28). We also reported that IFN-α exerted immunomodulatory properties and that tumor necrosis factor-related apoptosis inducing ligand (TRAIL) and its receptor pathway, partially contributed to the anti-tumor effects of IFN-α and 5-FU combination therapy (29).

The present study was designed to further explore the mechanism of action of IFN-α/5-FU combination therapy in HCC. For this purpose, we established an *in vivo* nude mouse model of HCC and examined the effect of the treatment on the expression of vascular endothelial growth factor (VEGF) and angiopoietins (Ang-1 and Ang-2).

Materials and methods

Cell line and culture conditions. The hepatocellular carcinoma cell line HuH7 was maintained as an adherent monolayer in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin mixture. Cell cultures were grown on plastic plates and incubated at 37˚C in a mixture of 5% CO_2 and 95% air.

Reagents. Purified human IFN-α was obtained from Otsuka Pharmaceutical Co. (Tokushima, Japan) and purified 5-FU was obtained from Kyowa Hokko Co. (Tokyo, Japan). We used the following primary antibodies; polyclonal rabbit anti-human VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal goat anti-human Ang-1 antibody (Santa Cruz, CA), polyclonal goat anti-human Ang-2 antibody (Santa Cruz, CA), monoclonal mouse anti-human proliferating cell nuclear antigen (PCNA) antibody cloned PC-10 (Dako, Glostrup, Denmark) and polyclonal rat anti-mouse cell differentiation antigen 34 (CD34) antibody (BD Biosciences, Flanklin Lakes, NJ).

Subcutaneous xenograft model in nude mice. Specific, pathogen-free, female athymic nude mice (BAL B/c nu/nu, 4- to 6-week-old) were purchased from CLEA Japan, Inc. (Tokyo, Japan). The mice were maintained under specific pathogen-free conditions in accordance with the institutional guidelines of animal care. HuH7 cells were uniformly seeded into 15 cm dishes and after reaching 80-90% confluence, were briefly treated with 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA). Trypsinization was stopped with a medium containing 10% FBS (fetal bovine serum). The cells were washed once with free medium and then resuspended in free medium. The cells ($5x10^6$ cells/0.1 ml DMEM) were subcutaneously injected in the right flank of each mouse. The IFN/5-FU combination therapy was started after growth of the tumor to 5-7 mm in diameter (10 days after the injection of cells). The dose of IFN-α was based on the results of previous studies (12,30) and was adjusted so as to match the schedules of IFN-α used recently in clinical studies (20,23). The doses and schedules of 5-FU represent the widely used standard clinical regimen (31-33). Mice were randomly assigned to one of the four groups as follows; (a) mice of the first group were administered a subcutaneous (SC) injection of IFN-α (20,000 units/body) three times per week, (b) mice of the second group were administered an intraperitoneal (IP) injection of 5-FU (30 mg/kg) three times per week, (c) mice of the third group were admministered an SC injection of IFN-α (20,000 units/body) and an IP injection of 5-FU (30 mg/kg) three times per week and (d) mice of the fourth group were administered SC and IP injections of phosphate buffered saline for the control group three times per week. There were eight mice in each group. Tumor volume was measured twice a week and was calculated using the following formula; (longest diameter) x (shortest diameter)^2 x 0.5. Four weeks after the initial treatment, all mice from each group were sacrificed and tumors were harvested for examination. One part of the tumor was fixed in 10% buffered formalin for immunohistochemical staining, the other part was embedded in optimal cutting temperature (OCT) compound for frozen sectioning and stored at -80˚C. The remainder of the tumor was later placed in RNA (Qiagen, Hilden, Germany) for RNA isolation.

Immunohistochemistry detection of PCNA, VEGF, Ang-1 and Ang-2. Formalin-fixed paraffin-embedded sections were used for immunohistochemical identification of PCNA, VEGF, Ang-1 and Ang-2. Sections measuring 5 μm in thickness were deparaffinized in xylene and rehydrated in a graded series of ethanol baths. The immunostaining procedure was performed using Vectastain ABC peroxidase kits (Vector Labs, Burlingame, CA) as described previously (34). Briefly, after deparaffinization and rehydration, the sections were treated with an antigen retrieval procedure in 0.01 M sodium citrate buffer (pH 6.0) for 40 min at 95˚C and then incubated in methanol containing 0.3% hydrogen peroxide for 20 min at room temperature to block endogenous peroxidase. All primary antibodies; mouse anti-PCNA (diluted 1:400), rabbit anti-VEGF (diluted 1:100), goat anti-Ang-1 (diluted 1:50) and goat anti-Ang-2 (diluted 1:50), were incubated overnight at 4˚C. After the sections were incubated with biotinylated secondary antibody and peroxidase-conjugated streptavidin, peroxidase reactions were developed with 3,3'-diaminobenzidine tetrachloride (Wako Pure Chemical Industries). For a positive control, we used tissue of a placenta, which expressed VEGF, Ang-1 and Ang-2 proteins (35), was incubated in each staining procedure. For the negative control, non-immunoreactive rabbit IgG or Tris-buffered saline were

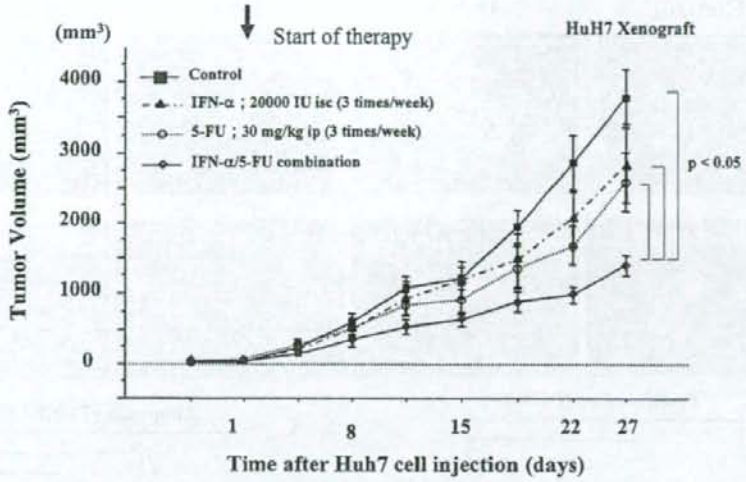

Figure 1. Effect of IFN-α/5-FU therapy in nude mice (Volume of tumor, V = L x W²/2). HuH7 cells were subcutaneously injected into nude mice (n=8, each group). Treatment was initiated when tumors grew to a size of 5-7 mm in diameter (10 days after injection of cells). Nude mice were not treated (■), treated by SC injection of IFN-α (20,000 units/mouse) alone (▲), treated by IP injection of 5-FU (30 mg/kg) alone (○), or treated by a combination of IFN-α and 5-FU (◇). Data are mean volume of tumors calculated by the following formula; (longest diameter) x (shortest diameter) 2 x 0.5. Data are mean ± SEM. The tumor volume of the combined therapy group was significantly decreased compared with other groups ($p<0.05$).

applied instead of the primary antibody. The intensity of immunohistochemical staining of VEGF, Ang-1 and Ang-2 was evaluated using MacSCOPE software (Mitani corp., Japan). For quantification of cell proliferation, five microscopic fields were randomly selected at high power magnification (x200) and the average counts of PCNA-positive cells were determined.

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 (27). 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 and in methanol containing 0.3% hydrogen peroxide for 20 min to block endogenous peroxidase. The remaining procedures were performed according to the instructions provided by the manufacturer. For the quantification of apoptosis, five microscopic fields were randomly selected at high power magnification (x200) and the average counts of TUNEL-positive cells were calculated.

Identification of microvessel density by CD34 immunohistochemistry. Frozen sections (8 μm thickness) were fixed in cold acetone for 10 min at -20˚C. The sections were washed in PBS three times for 5 min each and were incubated in methanol with 0.3% hydrogen peroxide for endogenous peroxidase block. Subsequent procedures were the same as for paraffin-embedded sections. Rat polyclonal anti-mouse CD34 antibody (diluted 1:20, BD Bioscience, San Jose, CA) was used as the primary antibody for the detection of tumor vessels. Ten microscopic fields were randomly selected at x100 magnification and the average counts of CD34-positive vessels were determined as the microvessel density (MVD) of an individual tumor.

RNA extraction and quantitative real-time RT-PCR. Total RNA was extracted from frozen tissues via a single step method using TRIzol reagent (Life Technologies, Gaithersburg, MD). Total RNA (1 μg) was used for reverse transcription and complementary DNA (cDNA) was generated using the Reverse transcription system (Promega, Madison, WI) as described previously (30). Quantification of mRNA expression of VEGF, ANG1 and ANG2 was performed using a real-time thermal cycler, Light Cycler® and detection system (Roche Diagnostics, Mannheim, Germany). For detection of the amplification products, LightCycler-DNA master SYBR green I (Boehringer Mannheim, Mannheim, Germany) was used as described previously (29). Briefly, a 20 μl reaction volume containing 2 μl of cDNA and 0.2 μmol/l of each primer was applied to a glass capillary. The primers used were as follows; human VEGF (forward, 5'-AAGCCATCCTGTG TGCCCCTGATG-3'; reverse, 5'-GCGAATTCCTCCTGCC CGGCTCAC-3'), human ANG1 (forward, 5'-AAATGGAA GGAAAACACAAGGAA-3'; reverse 5'-ATCTGCACAGT CTCTAAATGGT-3'), human ANG2 (forward, 5'-GACGGC TGTGATGATAGAAATAGG-3'; reverse, 5'-GACTGTAG TTGGATGATGTGCTTC-3') and human ß-actin (forward, 5'-GAAAATCTGGCACCACACCTT-3'; reverse, 5'-GTTG AAGGTAGTTTCGTGGAT-3'). PCR cycle conditions were set as described previously (35). The annealing temperatures of ANG1, ANG2, VEGF and ß-actin were 53˚C, 51˚C, 56˚C

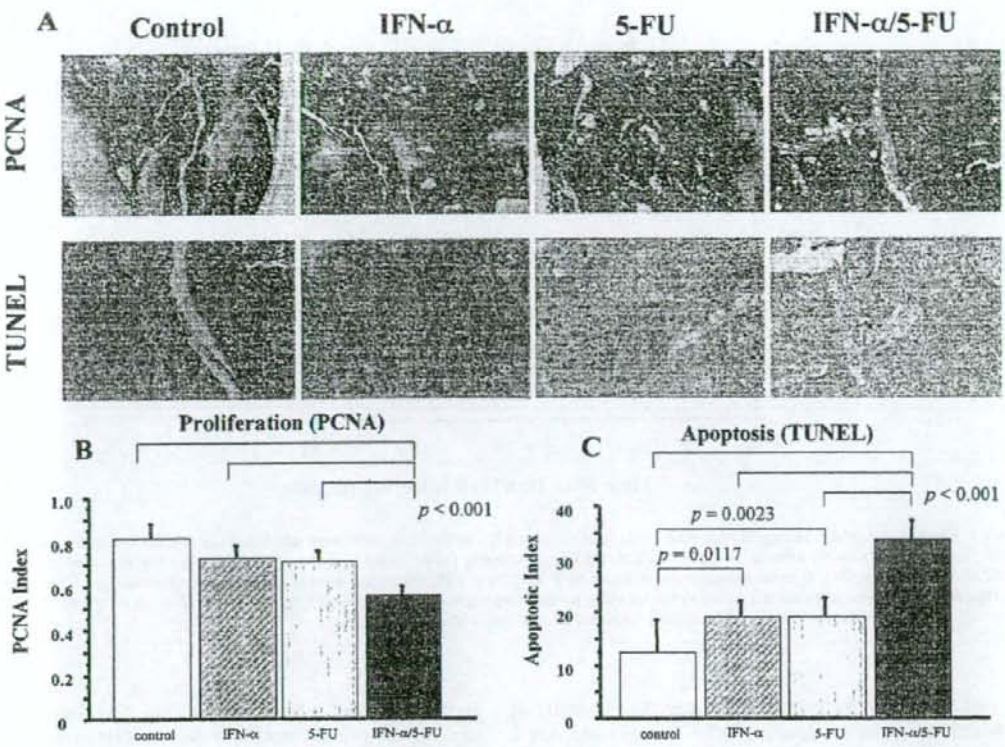

Figure 2. (A) Immunohistochemical analyses of PCNA (cell proliferation) and TUNEL (cancer cell apoptosis). Twenty-seven days after initial treatment, tumors were harvested from control mice or mice treated with IFN-α (20,000 units/mouse) alone, treated with 5-FU (30 mg/kg) alone or treated with a combination of IFN-α and 5-FU. (B) Cell proliferation in each treatment group. Quantification of cell proliferation was expressed as the percentage of total cancer cells per field that were PCNA-positive in 5 random microscopic fields at high power magnification (x200). Numbers represent mean ± SD. In combined therapy groups, the percentage of PCNA-positive cells was significantly decreased compared with the other groups ($p<0.001$). (C) Apoptosis of cancer cells. For quantification of apoptosis, the average number of TUNEL-positive cells was calculated in 5 random microscopic fields at high power magnification (x200). Data are mean ± SD. In the group treated with IFN-α or 5-FU alone, the number of TUNEL-positive cells was increased *versus* control ($p=0.0117$ and 0.0023, respectively). Apoptosis was significantly induced in tumors of mice of the combination therapy group, *versus* the other groups ($p<0.001$).

and 58˚C, respectively. A quantitative analysis of mRNA was performed using LightCycler® analysis software (Roche Diagnostics). The expression level of each angiogenic factor was normalized to the level of ß-actin mRNA. We compared the ratio of ANG1/ß-actin, ANG2/ß-actin, VEGF/ß-actin and ANG2/ANG1 between each treatment group.

Statistical analysis. Data are expressed as mean ± SD or SEM. Statistical analysis was performed using the StatView J-4.5 program (Abacus Concepts, Inc., Berkeley, CA). The tumor volume of each treatment group was compared by ANOVA. The unpaired Student's t-test was used to examine the difference in cell proliferation, apoptosis, MVD and expression of VEGF, Ang-1, Ang-2 proteins and an mRNA ratio between each group. A p-level <0.05 was considered statistically significant.

Results

IFN-α and 5-FU combination therapy for HCC xenografts. The growth curve of the implanted tumor in each group is shown in Fig. 1. On day 27, the tumor volume of the control group was 3.8±1.2 cm^3 and those of the single agent IFN-α and 5-FU groups were 2.8±1.6 and 2.5±1.2 cm^3 (mean ± SEM), respectively. While the single agent therapy reduced the tumor volume compared with the control group, these differences were not statistically significant. The tumor volumes of the combined therapy group were 1.4±0.4 cm^3 and were significantly smaller in size than those of the other groups ($p<0.05$). The body weights of mice after removing xenografts on the 27th day in the control, IFN-α alone, 5-FU alone and the combination group were 14.8±1.2, 14.3±2.6, 14.3±1.5 and 14.4±1.8 g, respectively (mean ± SD). There were no significant differences between the weight of the mice in each group.

IFN-α and 5-FU combination therapy inhibits tumor cell proliferation and angiogenesis and induces apoptosis. Examining cell proliferation, PCNA-positive cells in the control group was 81.6%, while the percentage with IFN-α or 5-FU treatment alone was 72.5% and 70.8%, respectively. In the combination therapy group, the cell proliferation was

Table I. MVD and the expression of angiogenic factors in each treatment.

Group	MVD	VEGF	Ang-1	Ang-2
Control	29.6±2.9	35.4±3.2	18.8±4.3	25.5±1.3
IFN-α (20,000 units)	18.1±2.9[a]	22.3±8.2[a]	23.6±7.1	19.8±3.3[a]
5-FU (30 mg/kg)	22.0±3.8[a]	30.4±6.2	30.2±2.4[a]	15.0±9.1
IFN-α/5-FU	10.3±2.1[b]	15.1±7.6[c]	41.5±5.7[d]	8.8±8.6[e]

The data showed mean ± SD. [a]p<0.05 compared with tumors of control mice. [b]p<0.001 compared with tumors of control mice, mice treated with IFN-α or 5-FU alone. [c]p<0.003 compared with tumors of control mice and p<0.03 compared with tumors of mice treated with 5-FU alone. [d]p<0.02 compared with tumors of control mice, mice treated with IFN-α or 5-FU alone. [e]p<0.02 compared with tumors of control mice, mice treated with IFN-α alone.

significantly inhibited in comparison with control or single therapy groups, with a percentage of PCNA-positive cells of 55.4%. The average number of TUNEL-positive cells at high power magnification (x200) in each treatment group; the control, IFN-α alone, 5-FU alone and combination of IFN-α and 5-FU was 12.4, 19.1, 19.2 and 33.7, respectively, indicating that the combination therapy induced significant apoptosis of tumor cells (p<0.001) (Fig. 2).

The MVDs of tumors in the control group were 29.6±2.9, in the IFN-α alone group 18.1±2.9, in the 5-FU alone group 22.0±3.8 and in the combination therapy group 10.3±2.1, respectively. MVD was not significantly reduced in the group treated by 5-FU alone but was in the group treated by IFN-α alone or by the combination of IFN-α and 5-FU. Furthermore, MVD in the combined therapy group was significantly reduced relative to the other groups (Table I and Fig. 3).

Immunohistochemical analysis of angiogenic factors. We evaluated the protein expression of tumors in each treatment group by immunohistochemistry. Representative samples of immunohistochemical staining of Ang-1, Ang-2 and VEGF are shown in Fig. 3. The expression of Ang-2 and VEGF were significantly decreased in tumors of mice treated with IFN-α and 5-FU compared with tumors of control mice or from mice treated with IFN-α or 5-FU alone. The expression of Ang-1 was significantly increased in tumors of the IFN-α and 5-FU combination therapy group (Table I).

ANG2/ANG1 mRNA expression ratio in tumors of mice treated with IFN-α and 5-FU. ANG1, ANG2 and VEGF mRNA levels in the combination therapy group were 2.17±1.66, 1.13±0.78 and 1.46±0.66, respectively. ANG2 and VEGF mRNA levels in the combination therapy group were lower than that of IFN-α or 5-FU alone, but these differences were not significant. The ANG2/ANG1 mRNA expression ratio was significantly lower in the combination therapy group compared with the group treated by IFN-α alone or the control group (Fig. 4).

Discussion

In the present study, we investigated the mechanism of the anti-tumor effect of IFN-α and 5-FU combination therapy using a nude mouse xenograft model. The administration of IFN-α combined with 5-FU three times per week significantly inhibited the growth of human hepatocellular carcinoma cells injected subcutaneously into nude mice. Interferon monotherapy or combination therapy with various chemotherapeutic agents in other solid malignancies is well documented in various *in vivo* models (7,9,12,30). As reported previously, daily or three times weekly injections of IFN-α was necessary to produce therapeutic effects. With regard to the dosage, a total dose per week of 35,000 to 70,000 units of IFN-α inhibited tumor growth and angiogenesis of xenografts in nude mice (30). In HCC, Hisaka *et al* (36) reported that a subcutaneous injection of 10,000-1,000,000 units of IFN-α decreased tumor volume *in vivo* in a dose-dependent fashion. In the group with a daily administration of 10,000 units of IFN-α, the volume of the xenograft of human HCC cells was reduced to about 60% of the control. Therefore, we determined that the schedule for treatment with IFN-α would be three times per week, since this was recently used clinically and the dose would be 20,000 units/body. The maximum tolerated dose of 5-FU in nude mice was 60 mg/kg, in a schedule of three injections every 4 days (32). The standard and widely used regimen for 5-FU is 20-50 mg/kg per injection and a total dosage per week of about 100 mg/kg (31,33). We determined that 5-FU would be administered IP three times per week at a dose of 30 mg/kg. In our study, single agent treatment (SC injection of IFN-α or IP injection of 5-FU alone) inhibited tumor growth compared with the control group, but the difference was not significant. We confirmed that IFN-α and 5-FU combination therapy significantly inhibited tumor growth compared with other groups. However, an orthotopic model by placing the cells in the hepatic parenchyma might be necessary to reveal the mechanisms of anti-angiogenic effects of IFN/5-FU combination therapy. The dosage and schedule of IFN-α and 5-FU used in our study were standard, clinically used and the estimated volume of the tumors after using a combined therapy for 4 weeks was 38% of those of the control group. In another study of IFN-α monotherapy, comparatively higher doses of IFN-α were needed to reduce tumor volumes to half of those of the control group (36). These phenomena emphasize the high anti-tumor effects of IFN-α and 5-FU combination therapy.

Our results demonstrated a significant decrease in PCNA-positive proliferating cells and an increase in TUNEL-

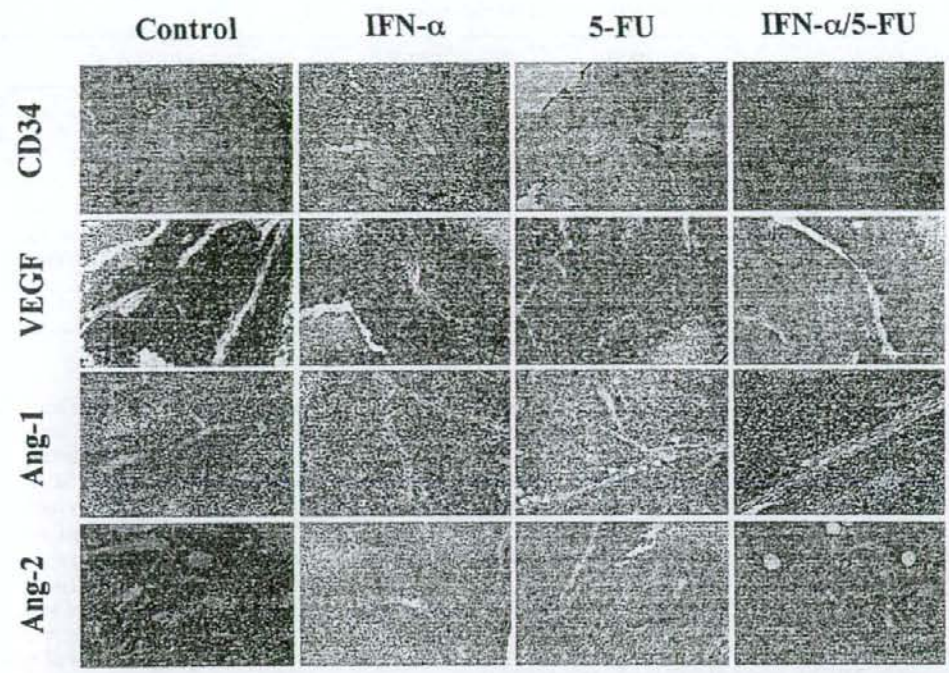

Figure 3. Immunohistochemical analyses of CD34 (endothelial cells) and VEGF, Ang-1 and Ang-2 (angiogenic factors). The sections were immunostained for expression of CD34 (to show MVD), VEGF, Ang-1 and Ang-2. Tumors of mice treated with combined IFN-α and 5-FU showed a significant decrease of MVD. The expression of VEGF and Ang-2 significantly decreased and the expression of Ang-1 significantly increased in tumors of combination therapy mice. Representative samples are shown (x100).

positive apoptotic cells in the combination therapy group, in agreement with our previous studies (27,28). IFN-α has an anti-proliferative effect and the combination of IFN-α and 5-FU synergistically induces cell cycle arrest and up-regulation of p27Kip1 *in vitro* (27). In our recent study, the IFN-α and 5-FU combination therapy induced apoptosis and up-regulated the expression of various apoptosis-regulated proteins, including Bcl-2, Bcl-xl and Bax (28). Kojiro *et al* reported that anti-proliferative effects of IFN-α and 5-FU in combination on a hepatocellular carcinoma cell line were attributable to the enhanced induction of S-phase arrest and apoptosis (37). These results are consistant with our present results.

We also examined the anti-angiogenic effects of IFN-α and 5-FU combination therapy, because angiogenesis is essential for tumor growth and metastasis (38) and HCC is one of the most hypervascular tumors. IFN-α has anti-angiogenic properties in clinical tumors such as Kaposi's sarcomas (15), infantile hemangiomas (13) and some vascular-rich malignancies, melanoma, renal cell carcinoma and neuro-endocrine tumors (14). Immunohistochemical analysis showed a significant decrease in CD34-positive cells (and therefore MVD) in the combination treatment group. Both *in vitro* and *in vivo*, IFN-α inhibited the transcription and production of pro-angiogenic molecules. Previous studies showed that IFN-α decreased the production of major pro-angiogenic factors such as VEGF (7,12), b-FGF (11), MMP-2 and MMP-9 (8,9), and IL-8 (10). Marschall *et al* (7) previously reported that the therapeutic effects of IFN-α on neuro-endocrine tumor cells were based on Sp1- and/or Sp3-mediated inhibition of VEGF transcription both *in vivo* and *in vitro*. In pancreatic cancer cells, IFN-α combined with the chemo-therapeutic agent gemcitabine, induced apoptosis of tumor-associated endothelial cells and decreased the local production of pro-angiogenic molecules from tumor cells (12).

The present data confirmed that the use of a combination therapy in an *in vivo* mouse model resulted in significant reductions in VEGF and Ang-2 protein expression and an increase in Ang-1 protein expression. We reported previously that cooperation between Ang-2 and VEGF plays an important role in enhancing the formation of new blood vessels in hepatic metastases of colorectal cancer (35). Furthermore, VEGF and Ang-2 have been shown to play an important role in angiogenesis in HCC, in our reports and those of others (34,39-41). Angiopoietins have been identified as a new family of endothelial growth factors and comprises ligands for the vascular endothelium-specific tyrosine kinase receptor Tie2 (42-44). Ang-1, which is an agonist of Tie2 and induces its phosphorylation, serves as a survival factor for endothelial cells and promotes recruitment of pericytes and smooth muscle cells. Therefore, Ang-1 is thought to help maintain and stabilize vascular networks (45). Ang-2 is a biological antagonist of Ang-1 and reduces vascular stability, blocking the stabilizing action of Ang-1. However, in the presence of

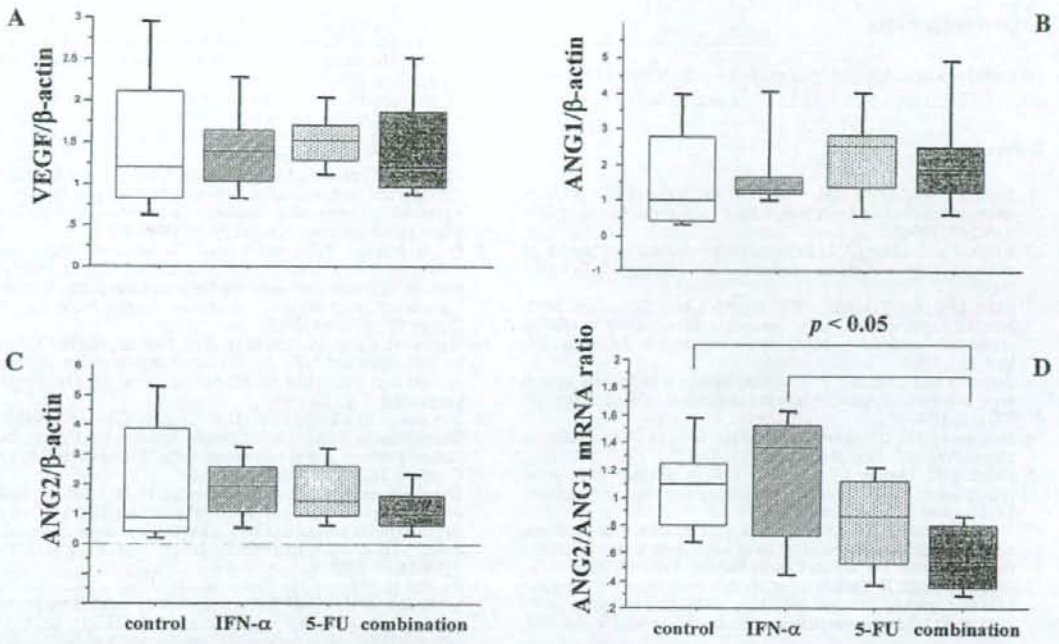

Figure 4. Expression of mRNA encoding VEGF, Ang-1 and Ang-2 and expression ratio of ANG2/ANG1 mRNA in tumors of control mice, mice treated with IFN-α alone, 5-FU alone and a combination of IFN-α and 5-FU. The mRNA expression levels were normalized to ß-actin. Data are displayed in box plots, with mean values represented by the horizontal lines inside the boxes. Mean values are as follows: (A) VEGF: control, 1.51±0.90; IFN-α alone, 1.43±0.55; 5-FU alone, 1.53±0.35; IFN-α/5-FU, 1.46±0.66; (B) ANG1: control, 1.64±1.53; IFN-α alone, 1.79±1.35; 5-FU alone, 2.31±1.28; IFN-α/5-FU, 2.17±1.66; (C) ANG2: control, 2.03±2.15; IFN-α alone, 1.89±1.02; 5-FU alone, 1.71±1.03; IFN-α/5-FU, 1.13±0.78; D, mRNA ratio of ANG2/ANG1: control, 1.08±0.37; IFN-α alone, 1.15±0.24; 5-FU alone, 0.83±0.34; IFN-α/5-FU, 0.57±0.24. Data are mean ± SD. The combination therapy resulted in a significant reduction of ANG2/ANG1 mRNA ratio compared with the control and IFN-α alone (p=0.0087 or 0.046, respectively).

VEGF, Ang-2 induces vascular sprouting and angiogenesis (46). Ang-2 is markedly expressed in organs that undergo vascular remodeling, such as the ovaries and placenta (35). Furthermore, several studies reported similar findings in various malignancies including HCC and that the expression levels of Ang-2 protein and mRNA correlate with clinicopathological factors in HCC (39-41).

Our results showed that the combination therapy increased the mRNA levels of Ang-1 and decreased those of Ang-2. The difference in Ang-1 and Ang-2 levels *in vivo* was not significant. However, the Ang-2/Ang-1 mRNA ratio was significantly decreased by systemic administration of IFN-α and 5-FU. Although there is a discrepancy between the proteins and mRNA, the balance between Ang-1 and Ang-2 mRNA expressions is most important because the high Ang-2/Ang-1 mRNA ratios in HCC were closely associated with portal vein invasion, tumor diameter, the MVD levels of HCC and the poor prognosis(41). The exact mechanism of regulation of angiopoietins remains unknown. IFN-α exerts most of its biological activity by altering the level of gene expression in target cells. IFN regulates oncogene expression resulting in the regulation of both transcriptional and post-transcriptional events (47). The transcriptional regulation of angiopoietins is not well characterized. Using ovarian cancer cells, Zhang *et al* (48) reported that tumor-derived VEGF up-regulates Ang-2 in host stroma endothelial cells. Potente *et al* (49) recently reported that Ang-2 was exclusively regulated by forkhead box O (Foxo) 1. The Foxo subclass of transcriptional factors plays an important role in the control of cell growth, development and survival. Dephosphorylation of Foxo factors leads to the activation or repression of apoptosis- and cell cycle-related genes such as Bim, p27Kip1, MnSOD, or GADD45 (50, 51). We previously reported that the synergistic effect of IFN-α and 5-FU was in part attributable to alterations in cell cycle progression via up-regulation of p27Kip1 (27). We speculate that the IFN-α and 5-FU combined therapy may induce the regulation of angiopoietins, via regulation of Foxo, as well as up-regulation of p27Kip1. Further studies are needed to identify the mechanism of the transcriptional or post-transcriptional regulation of angiopoietins by IFN-α and 5-FU combined therapy.

In conclusion, we confirmed that the IFN-α and 5-FU combined therapy had anti-proliferative and anti-angiogenic effects and induced apoptosis, in human HCC cells using a nude mouse xenograft model. The synergistic and anti-angiogenic effects of IFN-α and 5-FU may contribute to the anti-tumor effect against HCC, through the regulation of VEGF and angiopoietins.

Acknowledgements

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Combined intra-arterial 5-fluorouracil and subcutaneous interferon-alpha therapy for highly advanced hepatocellular carcinoma

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Because of the difficulties of low sensitivity for anticancer agents and giving sufficient dose because of poor liver function, chemotherapy may not play a central role for treatment of hepatocellular carcinoma (HCC) patients, especially those with liver cirrhosis. However, chemotherapy must be one of the important possibilities of multimodal treatment for advanced HCC, for which hepatic resection, percutaneous ablation, transcatheter arterial embolization and other general therapies would not be effective or even possible. Also, intra-arterial perfusion chemotherapy is a common therapy for HCC and it is not difficult to maintain; but the effective rate is not sufficient. Recently, the combination therapy of s.c. interferon (IFN)-α and intra-arterial 5-fluorouracil (5-FU) showed an outstandingly effective rate for intractable HCC (with portal vein thrombosis). In addition, recent preclinical and clinical studies have revealed that the mechanism of combination therapy may concern direct antitumor effects (through cell-cycle arrest and induction of apoptosis) and indirect actions (through immunocompetent cells and anti-angiogenic effect). For the further advance of HCC treatment and prognosis, this therapy might be a promising treatment modality and is expected to develop. In this review, we summarize recent clinical and preclinical data regarding IFN-α and 5-FU combination therapy and discuss the further prospects of this therapy.

Key words: 5-fluorouracil, antitumor effect, chemotherapy, hepatocellular carcinoma, interferon-α

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is one of the most common malignancies worldwide, with an estimated number of more than half a million new cases per year, most of which occur in Asia and Africa.[1] Recently, a trend of increasing rates of HCC has been reported from several developed countries in North America, Europe and Asia, adding to the increasing incidence in Japan over the past 40 years.

Many investigators have reported a putative link between the development of HCC and chronic viral infection and/or liver cirrhosis. Hepatic cirrhosis is observed in 80% of patients with HCC, and the major risk factor for HCC is infection with hepatitis B virus (HBV) or C virus (HCV), 20–70% or 10–70%, respectively, depending on geographic location.[1] In studies on the Japanese population, HBV-related or anti-HCV-positive HCC accounted for 14% and 81%, respectively, of cases in 2003,[2] showing that HCC in Japan is mainly related to HCV infection.

The prognosis of HCC is generally poor. In 2001, the death rates from primary liver cancer were 27.3 in men (third leading cause of death from malignant neoplasms) and 8.8 in women (fifth leading cause) per 100 000 cancer deaths in Japan.[2] Curative therapies such as hepatic resection, liver transplantation, transcatheter arterial embolization or percutaneous ablation have led to improvement in the survival of patients with HCC. However, the majority of patients are still diagnosed at an inoperable advanced stages and/or have recurrence or metastasis after therapy, and their prognoses remain extremely poor.[1,3] Almost all patients with unresectable tumors, especially those with tumor thrombi in the major branches of the portal vein (Vp3–4), die within several months with poor quality of life (QOL) due to liver failure, intractable ascites or esophageal bleeding. Also, tumor cells may spread out through the portal tract, resulting in extensive intrahepatic metastases.

For such highly advanced HCC, surgical resection, use of transcatheter chemoembolization and systematic chemotherapy have been reported, but the results were

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Table 1 Clinical studies of 5-fluorouracil (5-FU) and interferon (IFN)-α alone for hepatocellular carcinoma (HCC)

Author	Regimen	Response CR + PR/total (%)	Survival over-all (responders)	Patient characteristics
Ansfield (1971)[11]	5-FU i.a.	3/11 (27%)		Unresectable
Ramming (1976)[12]	5-FU i.a. 10 mg/kg/day	1/7 (14%)		
Link *et al.* (1977)[13]	5-FU i.v. p.o.	0/21 (0%)		Unresectable
Lin (1988)[14]	5-FU	2/21 (9.5%)		Unresectable, stage II
Doci *et al.* (1988)[4]	5-FU i.a.	2/9 (22%)		Advanced
Stehlin *et al.* (1988)[5]	5-FU i.a.	5/30 (16%)		Advanced
Ueno (2002)[15]	5-FU i.v. continuous 300 mg/m²-	0/20 (0%)	12 weeks	
Sachs (1985)[16]	IFN-α (12, 50 MU/m², i.m., 3 times/ week)	0/30 (0%)		
Lai *et al.* (1989)[17]	IFN-α (9–18 MU/m² i.m. daily or 25–50 MU/m², i.m., 3 times/week)	red 25–50% in 12% and >50% in 10% (22%)	8.3 weeks	Inoperable
GTSG (1990)[18]	IFN-α (?MU/m², i.m., 3 times/week)	2/28 (7%)	22 weeks	
Lai *et al.* (1993)[19]	IFN-α (50 MU/m², i.m., 3 times/week)	11/35 (31%)	14.5 weeks	Inoperable
Llovet *et al.* (2000)[20]	IFN-α (3 MU, 3 times/week)	2/30 (7%)	Around 13 m	Advanced
Yuen *et al.* (2003)[21]	IFN-α (10–50 MU/m², i.a. embolization)	11/18 (61%)	15.9 months	Inoperable

CR, complete response; PR, partial response.

unsatisfactory.[4–7] In addition, conventional therapies such as percutaneous ethanol injection, microwave coagulation therapy and transcatheter arterial embolization are not generally indicated due to lack of efficacy and possible complications.[8]

CLINICAL PERSPECTIVE OF COMBINED INTRA-ARTERIAL 5-FLUOROURACIL AND S.C. INTERFERON-α THERAPY

Summary of clinical trails of single chemotherapeutic agents and/or in combinations for advanced HCC

ALTHOUGH VARIOUS CHEMOTHERAPIES have been used for the treatment of advanced HCC, it could not play a central role for HCC patients, especially those with liver cirrhosis, because of low sensitivity to the anticancer agents and difficulty in giving a sufficient dose due to poor liver function.[8,9] However, chemotherapy must be one of the important possibilities of multimodal treatment for advanced HCC, for which hepatic resection and other general therapies would not be effective.

Several randomized controlled trials (RCT) have assessed the role of systemic or intra-arterial chemotherapy using different anti-neoplastic agents (doxorubicin [DOX], cisplatin [CDDP], mitomycin, 5-fluorouracil [5-FU] and others) either alone or in combination on tumor progression and survival. These trials described an overall partial response (PR) rate less than 20%, and complete response (CR) rates were negligible. Therefore, most investigators advise against using chemotherapy as a single therapy.[10]

The pyrimidine anti-metabolite FU was the first reported chemotherapeutic agent tested in the treatment of HCC (Table 1, upper part).[11,12,14] Ueno *et al.* conducted a phase I clinical study to evaluate the maximum tolerated dose of 5-FU administered by 5-day continuous infusion every 4 weeks in patients with HCC.[15] The maximum tolerated dose for this continuous infusion of 5-FU in HCC patients was 500 mg/m²/day. As summarized in Table 1, the treatment schedules, dosage and durations have varied among reports studying 5-FU monotherapy. Response rates (RR) of the monotherapy ranged 0–27%, with a median survival of only <13.8 months. Also, systemic administration of 5-FU showed no response at all for HCC patients.[13,15]

The antitumor effects of interferon (IFN)-α therapy in HCC remain controversial (Table 1, lower part).[16,18,21] Two RCT from Hong Kong showed that high doses of IFN-α were better than no treatment or DOX administration with RR in 22–31% and average survival for 8.3–14.5 weeks in patients with inoperable HCC.[17,19] However, an RCT of Western patients failed to show benefit of an average dose of IFN-α (RR 7%) and the treatment was associated with high rates of severe side-effects leading to treatment discontinuation.[20]

Taken together, the data from published studies show that treatment with either IFN-α or 5-FU as a single agent is of little, if any, benefit in patients with advanced

HCC. However, it has to be kept in mind that a small proportion of patients may present a PR to the treatments and, thus, it could be appropriate to test the usefulness of this agent in a multidrug approach. In that sense, recent reports have described encouraging results when combining IFN-α with the administration of 5-FU alone and with CDDP or DOX. However, because of the rate of severe side-effects, the cost of 5-FU with DOX is possibly higher than in 5-FU with CDDP.[22]

Other combination chemotherapies with or without IFN-α

Systemic combination therapy with IFN-α and DOX was found to be ineffective with an RR ranging 3–17%.[23,24] Using intra-arterial chemotherapy with methotrexate, 5-FU, CDDP and s.c. IFN-α administration, Urabe *et al.* reported an RR of 47% (7/15) in patients with Vp3.[25] Chung *et al.* presented a PR in 33% (6/18) of patients with major portal veinous tumor thrombus (PVTT) or distant metastases, who received systemic combination therapy with IFN-α and CDDP.[26] Recent clinical studies reported that the RR in combination therapy with IFN-α and multiple anticancer agents including CDDP, 5-FU and DOX were 26% (13/50),[27] 15.4% (4/26)[28] and 20.9% (19/91)[29] for advanced HCC. However, treatment was unlikely to be tolerated by patients with HCC and cirrhosis. Moreover, the other studies discussed hepatic arterial infusion chemotherapy using low-dose CDDP (3–7 mg/m^2 or 10 mg per day) and 5-FU, which may be a useful alternative for the treatment of patients with complicated PVT or recurrence of HCC with RR in 29% (9/31),[30] 48% (23/48),[31] 47% (18/37)[32] and 33% (6/18).[33]

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.

Clinical trails of combination therapy with 5-FU and IFN-α

The use of a combination of 5-FU and IFN-α had controversial results in patients with gastrointestinal malignancies. Although Wadler *et al.* suggested that the addition of IFN-α to 5-FU improved the activity of the latter in colorectal cancer patients,[34] phase III trials of the combination in that malignancy,[35] and phase II trials in advanced carcinoid and pancreatic cancer, were negative.[36,37] Despite these results, the use of the combination for HCC seemed warranted in view of the association of HCC with HBV or HCV, by virtue of the concurrent anti-neoplastic and antiviral effects of IFN-α and the potential synergism between IFN-α and 5-FU.

Recent studies have indicated the beneficial effects of combined intra-arterial 5-FU and s.c. IFN-α therapy (we abbreviated this treatment as FAIT) for advanced HCC. For the further advance of HCC treatment and prognosis, this therapy might be a promising treatment modality and is expected to develop. Up to date, four clinical studies have been published using intra-arterial 5-FU, and two studies using systemic 5-FU, combined with s.c. IFN-α therapy for advanced HCC (Table 2). First, Patt *et al.* reported that IFN-α in combination with systemic 5-FU injection showed 21% RR in patients with unresectable advanced HCC and low α-fetoprotein (AFP) levels.[38] In the phase II study conducted by the same group, the antitumor response of this combination was assessed as a PR in four patients (14.3%) and minor response (tumor regression of 25–49%) in two (7.1%) of 28 patients with HCC.[39] The median survival duration of all patients was 15.5 months.

In 1997, we experienced a patient with advanced HCC and lung metastasis, who was successfully treated with a combination therapy of IFN-α and tegafur-uracil (UFT), an oral anti-neoplastic drug consisting of uracil and tegafur (a prodrug of 5-FU).[40] Thereafter we studied the effect of FAIT for patients with advanced HCC with PVT in major branches.[22] We reported for the first time that FAIT showed CR (two cases) or PR (three cases) in five patients from the subsequent eight patients (objective RR, 63%) treated with this therapy.[23]

In our second prospective clinical trail we enrolled 55 patients with advanced HCC with major PVTT.[41] The treatment was done on an outpatient basis. An intra-arterial catheter was inserted through the subclavian or femoral artery with a s.c. implanted drug-delivery system. Each patient was treated with natural IFN-α (OIF, Otsuka Pharmaceutical, Tokyo, Japan) and intra-arterial infusion of 5-FU (Kyowa Hakko, Tokyo, Japan) as shown in Figure 1. Of the 55 patients, eight (14.5%) were evaluated as CR, 16 (29.1%) as PR, four (7.3%) as no change (NC) and 27 (49.1%) as progressive disease (PD), with an objective response in 43.6% of the patients. The median progression-free and survival periods of the patients were 5.2 and 11.8 months, respectively. The median survival periods of CR/PR cases (responders) was 24.4 months, while that of NC/PD cases (non-responders) was 5.4 months. The 1- and 3-year survival rates of responders were 82.9% and 30.9%, respectively, and those of non-responders were 13.1% and 0%, respectively, with a significant difference between the groups ($P = 0.0001$).

Table 2 Clinical studies of 5-FU and IFN-α in combinations for HCC

Author	Regimen	Response CR + PR/total (%)	Survival over-all (responders)	Patient characteristics
Patt *et al.* (1993)[38]	IFN-α (5 MU, i.m., 3 times/ week) 5-FU (750 mg/m², continuous i.v., 5 days)	6/28 (21%)		
Sakon *et al.* (2002)[32]	IFN-α (5 MU, i.m., 3 times/ week) 5-FU (450–500 mg/ day, continuous i.a., 2 weeks)	5/8 (63%)	n/a	Vp3 multiple
Patt *et al.* (2003)[39]	IFN-α (4 MU/m², s.c., 3 times/ week) 5-FU (200 mg/m²/day, continuous i.v., 21 days)	4/28 (14%)	15.5 months	tumor node metastasis (TNM) stage >III
Ota *et al.* (2005)[41]	IFN-α (5 MU, i.m., 3 times/ week) 5-FU (300 mg/m²/day, continuous i.a., 2 weeks)	24/55 (44%)	11.8 months (24.4)	Vp3 or 4, IM3
Enjoji *et al.* (2005)[42]	IFN-α (3 MU, s.c., 3 times/ week) 5-FU (500 mg/day, i.a., 5 days)	6/28 (21.5%)		Advanced HCC with IM or portal vein thrombosis
Obi *et al.* (2006)[43]	IFN-α (5 MU, i.m., 3 times/ week) 5-FU (500 mg/day, continuous i.a., 2 weeks)	61/116 (53%)	6.9 months?	Portal venous invasion
Nagano *et al.* (2006)[45]	IFN-α (5 MU, i.m., 3 times/ week) 5-FU (300 mg/m²/day, continuous i.a., 2 weeks)			Resectable advanced HCC (Vp3) as a postoperative adjuvant

Enjoji *et al.* published that the overall RR was 21.5% (6/28) in patients with advanced HCC received FAIT.[36] The study by Obi *et al.* of 116 patients with advanced HCC with PVTT reported that 19 (16.4%) patients had CR, 42 (36.2%) had PR, two (1.7%) had SD, and 53 (45.7%) had PD, resulting in 52.6% RR.[37] The average duration of complete and partial responses was 13.6 and 4.8 months, respectively.

The RR of i.v. 5-FU and IFN-α therapy was lower than that of FAIT (Table 2). Perhaps the major dissimilarities of the above studies were in the different administrations of 5-FU in different patient populations; there are no evidences of unlike responses to the therapies.

Taken together, the efficacy of FAIT for patients with highly advanced HCC ranged 21.5–63% (overall RR, 46.4%; 96/207), which was better than the previous reports with other combination chemotherapies for patients of similar stage (see above). Generally, the prognosis of such patients is extremely poor and survival is generally limited to a few months after diagnosis, despite multimodal therapies, even in cases suitable for surgical resection.[44] The FAIT markedly decreased tumor size and levels of tumor markers with an encouraging RR and prolonged survival time in the responders. Furthermore, the clinical response completely reflected the survival benefits.

FAIT as an adjuvant therapy after curative operation

From our clinical study on 30 patients treated with FAIT (n = 15) or without FAIT (m = 15) as adjuvant therapy after curative hepatectomy, disease-free survival was 11 of 15 patients during 5–55 months, and survival with recurrence in two patients for 13 and 48 months in the FAIT group.[45] In the group that did not receive adjuvant FAIT, almost all patients (11/15) died of recurrent cancer in the residual liver or had lung and lymph node metastasis within 2 years. The overall survival rates at 1 and 3 years were 100% and 74%, respectively, in patients with FAIT, and 41% and 22%, respectively, in the controls without FAIT ($P = 0.0031$).

With respect to postoperative adjuvant therapy for HCC, a recent review mentioned that systemic chemotherapy, hepatic-artery chemotherapy or TAE, as well as combinations of these therapies did not improve overall or disease-free survival after potentially curative surgery for localized HCC.[46] Compared with previous studies, the clinical outcome using FAIT as a postoperative adjuvant was excellent and highly satisfactory in terms of disease-free and overall survival rates.

The study suggested that FAIT is not only promising for treatment of highly advanced HCC, and also effec-

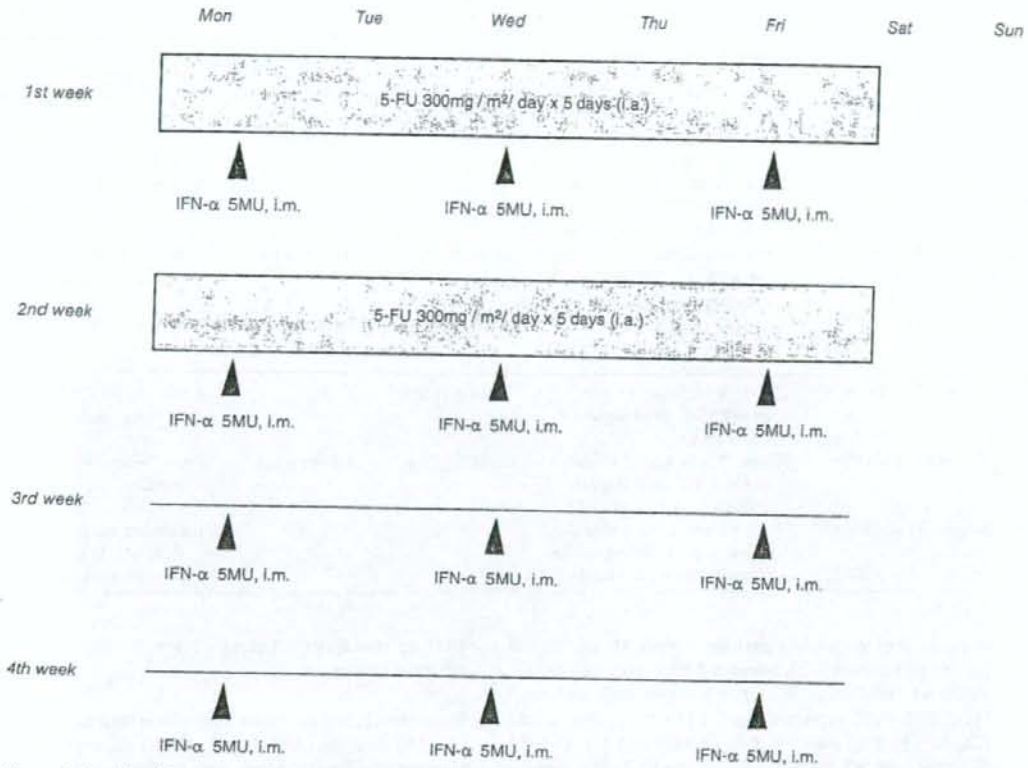

Figure 1 Combined intra-arterial 5-fluorouracil (5-FU) and s.c. interferon (IFN)-α therapy (FAIT) protocol; one cycle.

tive for prevention of recurrence in residual liver as a postoperative adjuvant treatment in resectable tumors.

Adverse effects of FAIT in HCC patients

Although 5-FU and IFN-α is known to result in multiple adverse effects, the occurrence of life-threatening side-effects among advanced HCC patients (even with cirrhosis) receiving FAIT was rare from the published data.[22,41,43,45] Non-hematological side-effects, such as fever, chills and flu-like syndrome, mostly in grades 1 and 2, were commonly (90–100% of the patients) observed but were easily controlled by non-steroidal anti-inflammatory drugs. In 20–50% of the patients, grade 1 generalized fatigue and nausea (5.5–50%) were observed. In a lesser percentage of patients (–5%), diarrhea, depression, gastric ulceration and skin reaction occurred. Only one case of grade 3 stomatitis and another of grade 3 depression, were noted and the latter patient discontinued the therapy due to the side-effects.[43] Myelosuppressive adverse effects were particularly important in patients. Decreased numbers of leukocytes or platelets were found in 30–80% of patients, and 5.5–9.1% of patients developed grade 3 leukopenia, thrombocytopenia or anemia (1.8%), but none resulted in termination of the therapy or required granulocyte-colony-stimulating factor administration, as reported. All were manageable. No complications resulting from the arterial catheter were reported. In addition, from our experience, the QOL of HCC patients was good. Moreover, they had no symptoms related to liver dysfunction. No hospital admission was necessary to receive FAIT. In studies with i.v. 5-FU and s.c. IFN-α reported by Patt *et al.*, grade 3 or 4 toxicity occurred in leukopenia (8.8%), anemia (5.9%), thrombocytopenia (11.8%) and mucositis (41.2%) of treated patients and were similar to those with FAIT.[38,39]

In our experience, only one case of the occurrence of interstitial pneumonia during FAIT was reported.[47] The patient with advanced HCC died 32 days after start of the therapy due to respiratory failure, suspected to have been caused by interstitial pneumonia, after steroid pulse therapy was started. The association of IFN-α with the development of interstitial pneumonia has been reported.[48] However, the prognosis of IFN-induced interstitial pneumonia has mostly been favorable when the medication was discontinued. It has been postulated that interstitial pneumonia induced by FAIT may be therapy-resistant. Thus, interstitial pneumonia in these patients should be carefully managed.

MECHANISM OF ANTITUMOR EFFECTS OF IFN-α AND 5-FU COMBINATION THERAPY

TO ADVANCE THE effect of FAIT and to increase the RR, it is necessary to understand the mechanism of action of IFN-α, 5-FU alone and in combination. Tumor cell resistance seems to be an important reason for failure of IFN-α and chemotherapy. It is therefore appropriate to consider what specific mechanisms might be involved in antitumor activity of those agents in HCC cells.

Action mechanism of 5-FU

5-Fluorouracil is an analog of uracil with a fluorine atom at the C-5 position in place of hydrogen. It rapidly enters the cell using the same facilitated transport mechanism as uracil. 5-FU is converted intracellularly to several active metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP). These active metabolites incorporate into RNA disrupting its synthesis, and inhibit the nucleotide (DNA) synthetic enzyme thymidylate synthase (TS).[49] The rate-limiting enzyme in 5-FU catabolism is dihydropyrimidine dehydrogenase (DPD), which converts 5-FU to dihydrofluorouracil (DHFU). More than 80% of administered 5-FU is normally catabolized primarily in the liver, where DPD is abundantly expressed.[50] It is known that 5-FU induces dysfunction of RNA by FUTP or inhibition of DNA synthesis by FdUMP; however, it has not been demonstrated clearly that which mainly contributes to antitumor effects of clinically administrated 5-FU. Also, when 5-FU concentration is high (10–100 mM), it preferably induces dysfunction of RNA, while at a lower concentration (0.5–1.0 mM) DNA synthesis can be inhibited.[51] Based on pharmacological characteristics of 5-FU, various studies have been conducted to find the optimal way of 5-FU administration. As a result, experimental studies have revealed that 5-FU is a time-dependent chemotherapeutic agent.[52] That is, antitumor effects of 5-FU are poor even at high concentration when it contacts tumor cells for a short time, while enhanced antitumor effects can be obtained even at low concentration when it contacts tumor cells for a long time. In addition, many prospective randomized clinical studies have demonstrated that both lower hematological toxicities[53] and higher antitumor effects[54,55] can be achieved with continuous infusion of 5-FU as compared with single bolus injection, which has been confirmed in a recent meta-analysis study.[56] Altogether, maintenance of a certain concentration of 5-FU in liver tumor for a long time by continuous hepatic artery infusion for 5 days, in ours and other clinical studies, has been established as the optimal way of 5-FU administration.[21,41,43,45]

Action mechanism of IFN-α

The actions of type I IFN, which includes IFN-α, -β -ω, are mediated by their interaction with a multisubunit cell-surface receptor, IFN-α receptor (IFNAR)1 and IFNAR2 (long, short and soluble subunits).[57,58] After IFN bind to the receptors, IFNAR-associated tyrosine kinases (JAK), including JAK1 and Tyk2, are activated, followed by phosphorylation of signal transducer and activator of transcription factor (STAT)1, 2, 3 and 5. Phosphorylated STAT (pSTAT) form hetero- or homodimers with an IFN-stimulated gene factor 3 (ISGF3), and that transfer into the nucleus where they induce the transcription of numerous IFN-responsive genes, which ultimately results in the biological effects of the IFN-α treatment, including antiviral, growth inhibitory, apoptotic, anti-angiogenic and immunomodulatory effects.[58]

Theoretically, the patients may respond to IFN therapy for a variety of reasons. The IFN-α may have direct effects on the tumor cells, for example, it may be cytotoxic, affect the proliferation of the tumor cells or induce cellular differentiation. Alternatively, or in addition, the IFN-α may have indirect effects; for example, on host immune functions, the tumor stromal cells or the vascularization of the tumor.[58] Some of these possibilities have been investigated in HCC.

Hepatocellular carcinoma cells differ greatly in their sensitivity to growth-inhibitory effect of IFN-α.[59–62] In IFN-sensitive cells, the IFN-α treatment resulted in a time- and dose-dependent reduction of proliferation.

Interferon-α-induced events that lead to cell cycle arrest have also been studied in HCC cell lines.[59,60] IFN-α treatment inhibits growth of HCC cells by specifically mainly delaying S-phase progression, most likely

because of inhibition of cyclin A induction, resulting in decreased activity of the associated Cdk2 and Cdc2 kinases.[60]

Several studies have investigated the role of IFN-α in apoptotic events, and in some instances IFN-α have now been shown to exert limited proapoptotic activity that is unrelated to its cell growth inhibitory action.[62,63]

Interferons also have profound effects on a number of immunological functions such as natural killer (NK)-cell activity, T-cell cytotoxicity and macrophage function, and induction of class I major histocompatibility complex antigens.[58] Another possible mechanism is via its antiangiogenesis activity; IFN-α has been shown to inhibit HCC angiogenesis in various experimental settings.[64,65]

Interestingly, a negative regulator of the IFN signal transduction, SOCS-1, was found to be silenced by methylation in human HCC.[66] In addition, our unpublished results showed a clear relationship between sensitivity to IFN-α and IFNAR expression; the expression rate of IFNAR2 in HCC was higher than in esophageal, gastric, colorectal, cholangiocarcinoma and pancreatic cancer samples.[67] Therefore, the antitumor effect of IFN may be better than those in other gastrointestinal cancers. Also, the expressions of IFNAR with subsequent activation of STAT were important for antiproliferative effect of IFN-α in HCC cells.[68]

Cooperative effect of 5-FU and IFN-α in HCC cells

From clinical data, the combination of 5-FU and IFN-α seems to have some synergism. Also, synergistic cooperative effects were clearly observed in experiments on HCC cell lines.[61,69] Also, Kondo *et al.* and Moriyama *et al.* found that IFN-α markedly increases susceptibility to 5-FU, respectively, in three of four, and five of eight human HCC cell lines.[70,71] Besides the above, several experimental studies have demonstrated that IFN-α enhanced the cytotoxic effect of 5-FU in other cultured malignant cells.[34]

The mechanism underlying the ability of IFN-α to strengthen the anticancer effect of 5-FU has been studied previously. Possible mechanisms of the cooperative effect pathways are schematically summarized in Figure 2. IFN-α enhances the conversion of 5-FU to an active metabolite, FdUMP, through an increase of thymidine phosphorylase (TP)[72] and a suppression of DPD in HCC.[73] Increased levels of FdUMP inhibit TS activity, resulting in an increase in DNA double-strand breaks.[74] In addition, Braybrooke *et al.* have demonstrated that a single dose of IFN-α could upregulate TP in peripheral blood lymphocytes within few hours of the administration and that the effect could be sustained for at least several days.[75] However, it is not clear for HCC.

We have investigated the mechanism of the cooperative effect of the IFN-α and 5-FU on HCC cells, and published in serial in our previous papers. A study by Eguchi *et al.* showed that augmentation of antitumor activity of 5-FU by IFN-α was associated with upregulation of p27Kip1, by delaying the progression of G1 to S phase in IFNAR2 expressing the HCC cell line.[67] Also, a possible explanation for the synergistic or additive effects was suggested by up- or downregulation of the Bcl-2 protein family, especially Bcl-xL, which was correlated with the incidence of apoptosis.[71] In these direct actions, IFNAR2 on tumor cells has been shown to be important and working as a "gatekeeper" of the cooperative action in this combination.[41,68,71,76] We reported that the modulation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor-mediated cytotoxic pathway could partially contribute to the anti-HCC effect of IFN-α/5-FU combination therapy.[77] Recently, we also demonstrated contribution of the Fas/FasL pathway in this combination.[78] Another possible mechanism is via its antiangiogenesis activity; the combination has been shown to inhibit cooperatively tumor angiogenesis in HCC (unpubl. data). This antiangiogenesis activity may be clinically important because we observed reduced tumor blood flow demonstrated by dynamic computed tomography (CT) scan as an initial finding leading to clinical response. It was reported that IFN-α induces p53, which enhances apoptotic responses to 5-FU.[79]

Taken together, these *in vitro* findings provide supportive evidence for the beneficial effect of combination therapy with IFN-α and 5-FU on HCC. Also, using gene profiling, several genes showed distinct gene expression profiles in the responsive cells and others. Further investigation of these genes may elucidate underlying molecular mechanisms, enabling us to improve the efficacy of this combination therapy.

FUTURE DIRECTIONS TO IMPROVE THE EFFICACY OF FAIT

Prediction of response to the therapy

HOWEVER EFFECTIVE FAIT is for advanced HCC with significant prolongation of survival, it did not have any survival benefits for non-responders. Of the patients receiving FAIT, 37–78.5% (overall, 53.6%) did not respond and average survival time was only a few months, which is similar to patients symptomatically