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Original Article

Interferon alpha receptors are important for antiproliferative effect of interferon-α against human hepatocellular carcinoma cells

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Aim: Interferon (IFN)-α is a promising drug for the prevention and treatment of hepatocellular carcinoma (HCC). We reported that responders to IFN-α/5-fluorouracil combination therapy expressed higher IFN alpha receptor (IFNAR)2 in tumor. Herein we studied involvement of IFNARs in response to IFN-α in HCC cells.

Methods: IFN-α sensitivity and expression of IFNARs were studied in six HCC cell lines (HuH7, PLC/PRF/5, HLE, HLF, HepG2, Hep3B) using growth-inhibitory and RT-PCR, Western blot assays. Short interfering RNAs (SiRNAs) against IFNAR1 and 2 were used to analyze the role of the IFNARs in IFN-α's effect and signal transduction.

Results: The expressions of IFNAR1 and 2c mRNAs were higher in PLC/PRF/5 cells than those in other cell lines, and PLC/PRF/5 cells expressed abundant IFNAR2c on their cell membrane. When we examined the sensitivity of the HCC cell lines to the growth-inhibitory effect of IFN-α, PLC/PRF/5 exhibited a significant response, while the other cells were much more resistant. Knockdown of either IFNAR1 or 2 using siRNAs suppressed the IFN-α's signal transduction (2.5-fold), and decreased the growth-inhibitory effect (down by 69.9% and 67.3%).

Conclusion: The results suggest that the expression of IFNAR1 and IFNAR2c independently are important for the antiproliferative effect of IFN-α in HCC cells.

Key words: antiproliferative effect, hepatocellular carcinoma, interferon alpha receptor, interferon, short interfering RNA

INTRODUCTION

RECENT CLINICAL STUDIES suggested that combination chemotherapies with interferon (IFN), especially the combination of 5-fluorouracil (5-FU) and IFN-α, might be effective for patients with advanced hepatocellular carcinoma (HCC).[1–4] We reported that a combination of 5-FU and IFN-α therapy expanded the median overall survival period to 11.8 months in patients with highly advanced HCCs, and especially it reached 24.4 months in responders to the therapy (with a response rate in 43.6% of the patients).[5] At this same stage cancer patients die within 2.7 months with symptomatic therapy.[6] However, the effectiveness of IFN-α monotherapy for treatment of HCC is controversial.[7,8]

It has emerged that IFN-α exerts its antitumor effect by both a direct antiproliferative effect (through cell cycle arrest and induction of apoptosis) and indirectly (by immunomodulation or inhibition of tumor angiogenesis). This antiproliferative action is perhaps mediated by the interaction of IFN with multisubunit receptors – IFN alpha receptor (IFNAR) (including IFNAR1 and IFNAR2a [soluble subunit], 2b [short], 2c [long]) on the cell surface and further activation of the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway, which induces transcription of various genes.[9–11]

We reported that responders to IFN-α/5-FU combination therapy expressed higher IFNAR2 in HCC than non-responders.[5] Alternatively, Yano *et al.* reported that IFNAR2 expression does not correlate with responsiveness of HCC cell lines to IFN-α.[12] In this study we further investigated the roles of IFNAR1 and IFNAR2 in

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the direct growth-inhibitory effect of IFN-α, conducting a mechanistic study on HCC cell lines.

METHODS

Cell lines and specific reagents

THE HUMAN HCC cell lines HuH7, PLC/PRF/5, HLE, HLF and HepG2 were purchased from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and the Hep3B cell line was obtained from the Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). They were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 μg/mL streptomycin at 37°C in a humidified incubator with 5% CO_2 in air.

Human natural IFN-α (OIF) was obtained from Otsuka Pharmaceutical Co. (Tokyo, Japan). The IFN was prepared and assessed for experiments as described previously.[13] The concentration of the IFN was based on that used in previous studies.[13–16] Specific rabbit antihuman IFNAR2 (Otsuka Pharmaceutical), phosphorylated STAT (pSTAT)1 (Tyr701) (Cell Signaling Technology, Beverly, MA), pSTAT2 (Tyr689) (Upstate Biotechnology, Lake Placid, NY), pSTAT3 (Tyr705) (Cell Signaling Technology), actin (Sigma, St. Louis, MO, USA), and donkey antirabbit coupled to horseradish peroxidase (Amersham Biosciences, Buckinghamshire, UK) antibodies were used for Western blotting. Four different siRNAs predesigned to either human IFNAR1 or IFNAR2, and control non-silencing siRNA were purchased from Qiagen K.K. (Tokyo, Japan).

RNA extraction and RT-PCR analysis

Total RNA extraction was performed with a single-step method using Trizol Reagent (Invitrogen, Carlsbad, CA, USA), and complementary DNA (cDNA) was generated with avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA) as described previously.[17]

Conventional and quantitative real-time PCR (qRT-PCR) assays were performed using Genamp PCR System 9600 (Perkin-Elmer, Foster City, CA, USA) and the Light Cycler (Roche Diagnostics, Mannheim, Germany), as described in references.[17,18] The primer pair sequences were obtained from published sequences of IFNAR1, 2[12] and β-actin,[18] and synthesized from commercial sources. Conventional PCR conditions were the same as published previously.[12,18] qRT-PCR conditions for IFNAR1, 2 and β-actin were as follows: one cycle of denaturing at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 35 s, and the final extension at 72°C for 10 min (or annealing at 58°C for β-actin). In IFNAR2 qRT-PCR, we set one more additional step in each cycle with acquisition at 85°C for specific detection of the fluorescence of 481 bp product (IFNAR2c).

Cell preparation and Western blot analysis

For separation of the membrane fraction for detection of IFNAR2 by Western blot, we used the method described previously with minor modifications.[19] Briefly, the cells were solubilized with 1.5% Triton X-114 (Sigma) in TBS on ice for 15 min. The extract was separated by centrifugation, and the supernatant was warmed for 2 min at 37°C and centrifuged at 12 000 r.p.m. at 22°C. The membrane fraction (lower, hydrophobic phase) was diluted with 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]) and assayed for Western blotting as described in our previous studies.[15,17]

For study of pSTAT proteins, the cells were cultured in a medium containing 0.5% FBS for two days, then in FBS-free medium for 2 h. They were then incubated in medium with or without IFN-α for 20 min, and the cells were lyzed in RIPA buffer with 1 mM sodium orthovanadate and probed on Western blotting.

Cell growth curves

The cells were uniformly seeded in 12-well dishes (7×10^3 cells/well for HLE, HLF; 2×10^4 cells/well for HuH7, HepG2, Hep3B; 4×10^4 cells/well for PLC/PRF/5). From the following and every other day, the medium was replaced with fresh medium with or without IFN-α (500 IU/mL), and/or adherent cells were counted using the Celltac semiautomatic analyzer (Nihon Kohden, Tokyo).[17]

Knockdown of IFNARs with siRNA

The subconfluent (50–80%) growing PLC/PRF/5 cells were transiently transfected with 5 nM siRNAs using HiPerFect Reagent (Qiagen K.K.) according to the manufacturer's instructions. Knockdown efficacy for IFNAR1 or IFNAR2 were examined for 24 h or 48 h after transfection, respectively, using qRT-PCR or Western blot analyses as described above. The suitable siRNAs were selected and used for further experiments based on the highest knockdown efficacy for each IFNAR (data not shown). For the cell proliferation experiment the medium with or without IFN-α was applied 48 h after

siRNA transfection, and the cell numbers were counted after 48 h with the Celltac semiautomatic analyzer. This observation time (48 h) was chosen based on cell confluency. In all experiments the non-silencing siRNA was used as a control.

RESULTS

Expression of IFNARs in HCC cell lines

WE EXAMINED THE expressions of IFNARs in the cell lines at transcriptional level. Although the IFNAR1 mRNA was similar in all cells by conventional PCR (Fig. 1A upper), it was higher in the PLC/PRF/5 cell line by qRT-PCR (lower table). As shown in Figure 1B, the cells expressed clear bands of full length IFNAR2c subunit (481 bp) and faint bands of short subunit – IFNAR2b (713 bp), while there is no expression of the 350 bp band that corresponds to IFNAR2a (soluble subunit). IFNAR2c expression was slightly higher in PLC/PRF/5 compared to other cell lines, which was confirmed by qRT-PCR (Fig. 1B, lower table). Because the cell surface IFNAR2c subunit is suggested to be important in the binding of IFN-α,[20,21] we examined the IFNAR2c protein on cell membrane. As shown in Figure 1C, only PLC/PRF/5 cells displayed prominent expression of the IFNAR2c in their membrane fraction.

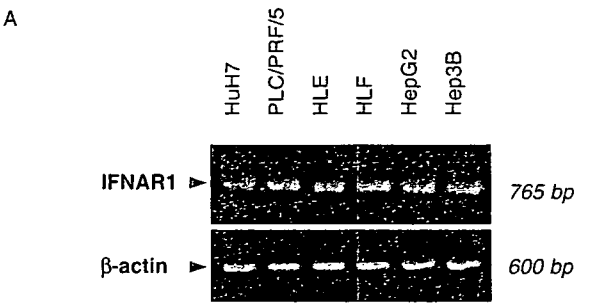
Growth-inhibitory effect of IFN-α on HCC cells

Next, we examined the sensitivity of HCC cells to IFN-α. As shown in Figure 2, IFN-α (500 U/mL) showed strong growth-inhibition in PLC/PRF/5 cells (closed squire curve, 90.8% growth-inhibition at 8th day), while it did not show a significant effect in other cell lines (growth-inhibition ranged between 7.6 and 35.8%). As reported in our previous study with a dose escalation experiment, the 50% growth-inhibitory concentration (IC50) of IFN-α was 6600 IU/mL for PLC/PRF/5, and higher than 25 000 IU/mL for the other cell lines[13] (data not shown).

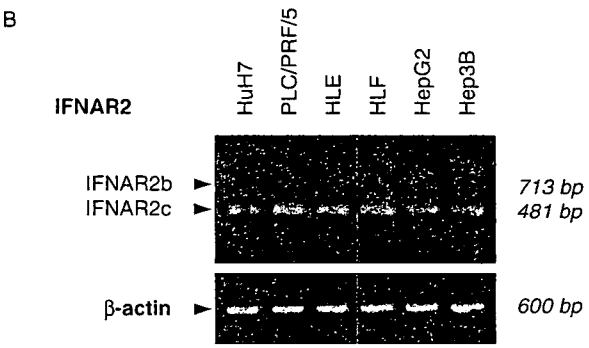
Impact of IFNAR1 and 2 on IFN signaling and antiproliferative effect

To clarify the role of IFNARs in the IFN-α growth-inhibitory effect, we performed knockdown of the receptors with siRNAs designed to IFNAR1 or 2, and studied the IFN-α's signaling and antiproliferative effect on the PLC/PRF/5 cell line, which expresses the IFNARs and exhibits a response to IFN-α (Figs 1,2).

As shown in Figure 3A, the selected siRNAs suppressed the expression of the correspondent IFNAR1



	HuH7	PLC/PRF/5	HLE	HLF	HepG2	Hep3B
IFNAR1 / β-actin	0.51±0.06	1.81±0.35	0.59±0.10	0.69±0.19	1.10±0.41	1.25±0.26



	HuH7	PLC/PRF/5	HLE	HLF	HepG2	Hep3B
IFNAR2c / β-actin	1.52±1.05	2.33±0.92	0.64±0.38	0.63±0.41	0.98±0.50	0.81±0.50

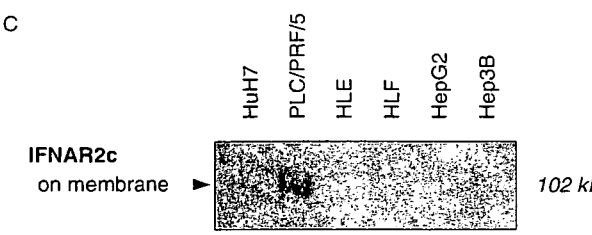


Figure 1 Expressions of IFNAR1 and 2 in HCC cell lines. (A) By conventional PCR assay, the IFNAR1 was expressed in all cell lines in similar levels; while with qRT-PCR, the expression of the IFNAR1 (shown in table) was higher in PLC/PRF/5 than those of the other cells. The IFNAR2's expression was examined in transcriptional (B) and protein (C) levels. PLC/PRF/5 cells expressed higher IFNAR2c (full length subunit) mRNA and displayed a prominent band of the IFNAR2c in their cell membrane. Protein load in each lane was equal by Ponso-S staining (data not shown). Figures are representative of similar results in repeat experiments.

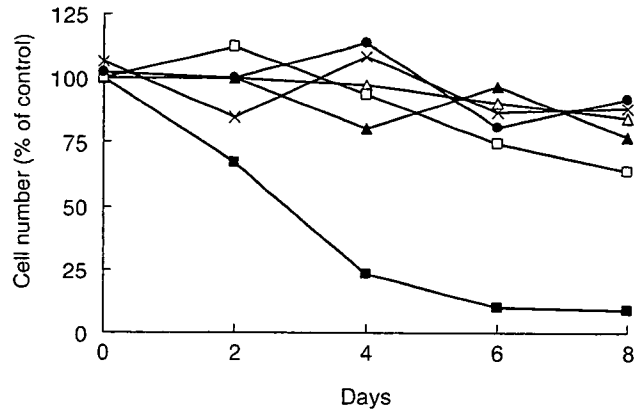


Figure 2 Growth-inhibitory effects of IFN-α in HCC cell lines. The curves were drawn for cells treated or untreated with 500 IU/mL IFN-α over eight days, and are shown as mean cell number percentages of untreated control from three independent experiments. IFN induced strong cell growth suppression in PLC/PRF/5 cells (closed squire curve, ■). However, the other cell lines (HuH7 *, HLE △, HLF □, HepG2 ▲ and Hep3B ●) were much more resistant to IFN-α.

and 2 down to 19.9% (upper right panel, by qRT-PCR) and 32.9% (lower right panel, by Western blot) of the corresponding controls. Since the IFN-α's signal transduction involves tyrosine phosphorylation of STAT1, 2, 3, we examined activation of the STATs with IFN-α after the knockdown. Inductions of pSTAT1 and 3, but not that of pSTAT2, were decreased with the receptor knockdown (Fig. 3B). pSTAT1 and 3 were suppressed 2.8-fold and 2.4-fold in IFNAR1 knockdown, and 2.3- and 2.4-fold, respectively, in IFNAR2 knockdown than in controls. Expression of base STATs was not changed by the knockdown and IFN induction (data not shown).

When we examined the growth-inhibitory effect of IFN-α 48 h after the IFNARs knockdowns, the effect was decreased in knockdown cells compared with non-silencing siRNA transfected controls (Fig. 3C). The viabilities of IFN-treated control cells were 60.7% and 62.1%, while they were 88.2% and 87.6%, respectively, in IFNAR1 and IFNAR2 knockdown cells. This means that the IFNAR1 and 2 siRNAs suppressed IFN-α's effect by 69.9% and 67.3%, respectively.

DISCUSSION

IFN-α EXERTS ITS effect through specific cell surface receptors (IFNARs), which subsequently activate the JAK/STAT pathway and result in the transcription of various genes realizing multiple effects.[9-11] Previously Yano *et al.* reported that IFNAR expression at the transcriptional and protein levels, which were detected with conventional PCR and flow cytometry methods, does not correlate with responsiveness of HCC cell lines to IFN-α.[12] Consistently, when we employed conventional PCR analysis, the IFNAR mRNAs were expressed at similar levels in our studied cell lines. However, when we quantified the expressions of IFNAR1 mRNA using qRT-PCR, as well as when we examined the expression of cell membrane IFNAR2c subunit using Western blot analysis, the expressions were significantly higher in IFN-sensitive PLC/PRF/5 cell line (Fig. 1). The subunits of IFNAR2 appear to have different functions, and cell surface full length IFNAR2 (IFNAR2c) is reported as functional and involved in IFN binding and signal transduction.[20] Furthermore, IFNAR2 expressions in mRNA and protein levels had some variation, suggesting its possible regulation in post-transcriptional or membrane presentation levels. Correspondingly, the relationship between the antiproliferative effect of IFN-α and mRNA expression levels of IFNARs was not clear in 'less sensitive' cell lines (HuH7, HLE, HLF, HepG2 and Hep3B), perhaps due to insufficient expression of IFNARs, especially the absence of IFNAR2c protein on their cell surface. Furthermore, IFN signal transduction (phosphorylation of STAT1, 3) was also greater in IFN-sensitive cell line (data not shown). Examination of IFNAR1 expression in protein level was unfeasible due to the lack of suitable antibody.

Generally, IFN-α exerts its growth-inhibitory effect by induction of cell cycle arrest and apoptosis in cancer cells.[9,10] The mechanism of strong antiproliferative effect of IFN-α in PLC/PRF/5 cells was studied previously. The studies showed an increase of S phase population of PLC/PRF/5 cells treated with IFN-α for 24 h.[14,22] Moreover, the proportion of apoptosis induced by IFN-α treatment for 3–10 days was 4–6.1% (vs 1–1.3% in untreated control cells).[14,15]

To verify the role of the IFNARs in IFN-α's antitumor effect, we reduced the receptors with siRNAs against IFNAR1 or 2 and examined the signal transduction and growth-inhibitory effect. From the results, knockdown of either IFNAR1 or 2 significantly decreased the signaling as well as antiproliferative effect of IFN-α (Fig. 3). Although we could perform only partial knockdown of the receptors, the decrease of the signaling and growth-inhibitory effect was almost parallel to the knockdown level. Alternatively, when we performed the neutralizing experiments using an anti-IFNAR2 antibody binding to the intracellular part of the receptor, the IFN-α's effect and signal transduction were suppressed, confirming

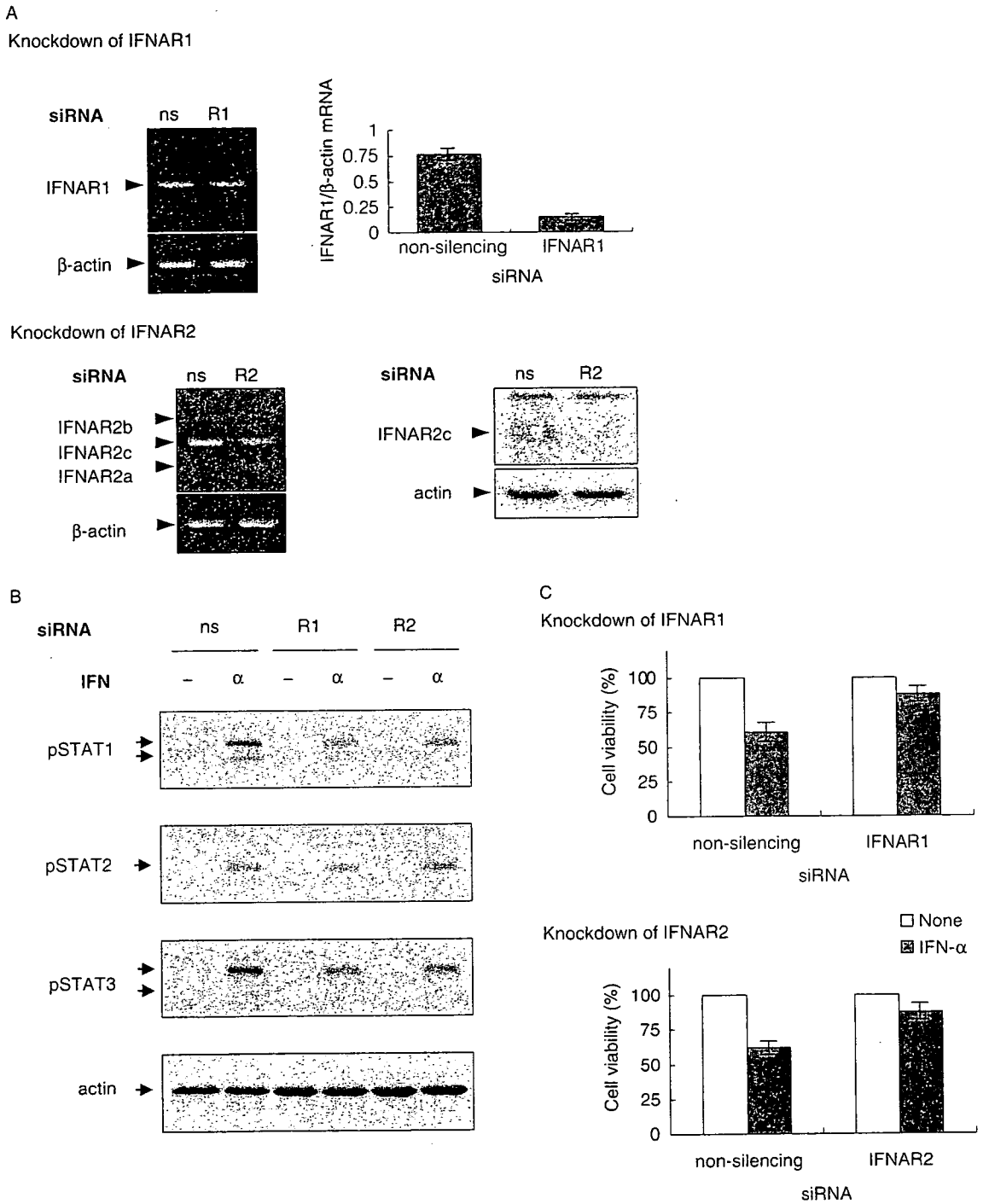


Figure 3 Knockdown of IFNAR1 and IFNAR2 using siRNAs. (A) The siRNAs suppressed the expressions of the correspondent IFNAR1 (left) and 2 (right) down to 19.9% and 32.9% of the controls. The expression of the internal control – β-actin – was the same in either non-silencing or IFNAR siRNA transfectants. (B) IFN-α signal transduction (inductions of pSTAT1 and 3, but not that of pSTAT2) was decreased with the receptor knockdown. (C) IFN-α's antiproliferative effect was decreased with IFNAR knockdown compared with non-silencing siRNA transfected controls, and it was suppressed by 69.9% and 67.3% of the primary antiproliferative effect on control cells by IFNAR1 and 2 siRNA. Data are representative of similar results in repeat experiments or mean of three independent experiments. ns, non-silencing; R1, IFNAR1; R2, IFNAR2.

the importance of IFNAR2 in IFN's effect (Damdinsuren *et al.*, 2006, unpublished data). Taken together, the existence of high IFNARs may induce subsequent intensive signal transduction, and this is considered to be one of the essential factors for the success of the IFN-α antitumor action.

From a clinical perspective, our findings may be important for two reasons. First, it is vital that patients be appropriately selected for IFN-α combination chemotherapy. The therapy may cause side-effects, including neutropenia, thrombocytopenia or renal insufficiency, and crucial survival time is lost in non-responders, possibly precluding their chance to undertake other treatment options.[1-3,23,24] Thus, accurate prediction of sensitivity to first-line chemotherapy is critical. Previously we and other groups have reported that HCCs express varied levels of IFNAR2 protein.[25,26] Also, we showed that IFNAR2 expression in tumors correlates significantly with response to IFN-α/5-FU combination therapy in patients with advanced HCC.[5] Therefore, the present *in vitro* results support the above suggestion that IFNAR2 (and also IFNAR1) expression might be a suitable predictor of response to IFN-based chemotherapy.

The second important point is that our findings suggest a new strategy for increasing response rates to IFN combination chemotherapy. It might be possible with upregulation of IFNAR expression in cancers lacking the receptor, for example by IFNAR gene transfer. We previously described that IFNAR2c-transfected HCC cells treated with combination IFN-α/5-FU (or with IFN-α alone) underwent apoptosis to a greater extent than control non-transfected cells.[15] Wagner *et al.* recently demonstrated the increase of antitumor effect of IFN-α in cancer cells using IFNAR2c gene transfection.[27] This strategy might also be practical for other malignancies, for instance melanomas, renal cell carcinomas and lymphomas, for which the IFN therapy is useful.[28]

In conclusion, our results suggest that the expressions of IFNAR1 and IFNAR2c independently play an important role in the antiproliferative effect of IFN-α in HCC cells.

ACKNOWLEDGMENTS

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Original Paper

Overexpression of MT3-MMP in Hepatocellular Carcinoma Correlates with Capsular Invasion

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ABSTRACT

Background/Aims: Extracellular matrix-degrading matrix metalloproteinases (MMPs) are invariably up-regulated in epithelial cancers and are key agonists of angiogenesis, invasion and metastasis. Recent studies have shown high levels of various MMPs, including MT1-MMP, MMP-1, MMP-2 and MMP-9, and their involvement in tumor progression in human hepatocellular carcinoma (HCC). However, the expression and role of MT3-MMP in HCC remains unclear.

Methodology: We examined the immunohistochemical expression of MT3-MMP in surgically resected HCCs (n=58), hepatitis C virus (HCV) and hepatitis B virus (HBV)-related chronic hepatitis (n=34) and cirrhosis (n=24).

Results: MT3-MMP expression was observed in all non-cancerous liver tissues. In HCCs, 52% (30/58) of patients showed high MT3-MMP expression while the remaining 48% (28/58) of patients showed low expression. A clinicopathological survey demonstrated a significant correlation between high MT3-MMP expression and capsular invasion of carcinoma (p=0.034) although there was no correlation between high MT3-MMP expression in HCC and overall survival or disease-free survival.

Conclusions: MT3-MMP was expressed not only in chronic hepatitis and liver cirrhosis, but also in HCC, and high MT3-MMP expression correlated significantly with capsular invasion of carcinoma.

KEY WORDS:
MT3-MMP; HCC; Capsular invasion; Immunohistochemistry

ABBREVIATIONS:
Disease-Free Survival (DFS); Dulbecco's Modified Eagle Medium (DMEM); Extracellular Matrix (ECM); Fetal Bovine Serum (FBS); Hepatitis B Virus (HBV); Hepatocellular Carcinoma (HCC); Hepatitis C Virus (HCV); Matrix Metalloproteinases (MMPs); Overall Survival (OS)

INTRODUCTION

Hepatocellular carcinoma (HCC) ranks as the fifth most common cancer in the world and in Southeast Asia and Africa the incidence of this cancer is about 30 per 100,000 males per year (1). The prognosis of patients with HCC is poor, because most HCC patients develop tumor recurrence, which occurs at a rate of 20-40% within 1 year, and about 80% within 5 years after therapy, even if curative treatment is performed (2-5).

In HCCs, it is well known that infiltration into the tumor capsule and portal involvement are important histopathological characteristics predicting worse clinical outcomes of HCC (2-5). Portal involvement, a crucial route of intrahepatic metastasis, requires destruction of the basement membrane because approximately 80% of HCCs are encapsulated by fibrous tissue and nearly all HCCs arise from extracellular matrix (ECM)-rich diseased liver (6,7). Thus, ECM degradation might play a major role in HCC invasion and progression to metastasis.

Proteinase plays a major role in ECM degradation. Four types of proteinase have been identified, including asparagine proteinase, cysteine proteinase, serine proteinase and matrix metalloproteinases (MMPs) (8). MMPs are of great significance for degradation of the ECM in cancers that have extensive decomposing activity (8). Recent studies have shown increased levels of MMPs and implications for tumor progression in human HCC including MT1-MMP, MMP-1, MMP-2 and MMP-9 (9-13). However, little is known about the expression and role of MT3-MMP in HCC. The present study was designed to determine the expression of MT3-MMP in human HCC cell lines and in human HCC, and to define the relationship between its expression and clinicopathological features.

METHODOLOGY

Epithelial Cancer Cell Lines

Seven HCC cell lines (HLE, HLF, HuH7, PLC/PRF/5, HepG2, SKHep1, and PLL), five colorectal carcinoma cell lines (Lovo, HCT116, HT29, DLD and SW48), five gastric carcinoma cell lines (MKN-1, MKN-28, MKN-74, MKN-45 and KATO-III), four esophageal carcinoma cell lines (TE-2R, TE-2S, TE-8 and TT), and five pancreatic carcinoma cell lines (PANC1, PSN-1,

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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	

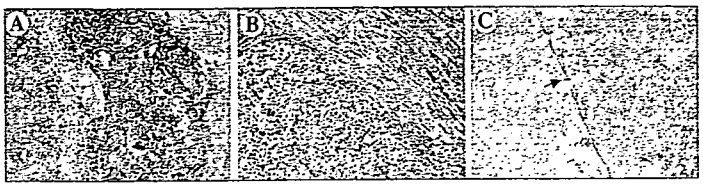

FIGURE 1 (A) High expression of MT3-MMP in HCC with capsular invasion (original magnification x200). **(B)** Low expression of MT3-MMP in HCC without capsular invasion (original magnification x200). **(C)** MT3-MMP expression was moderate (intensity 1) in the vascular epithelium (arrow; original magnification 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

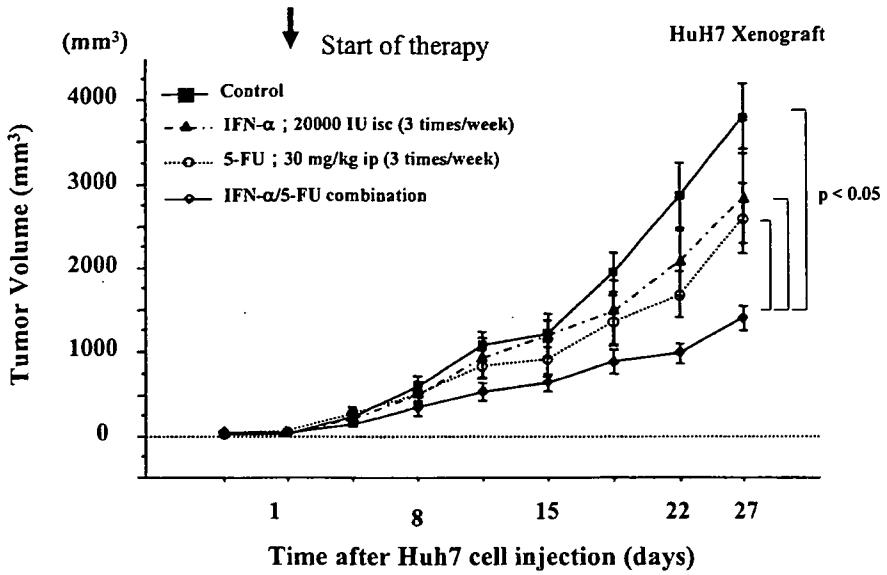

Figure 1. Effect of IFN-α/5-FU therapy in nude mice (Volume of tumor, V = L x W²/2). HuH7 cells were subcutaneously injected into nude mice (n=8, each group). Treatment was initiated when tumors grew to a size of 5-7 mm in diameter (10 days after injection of cells). Nude mice were not treated (■), treated 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

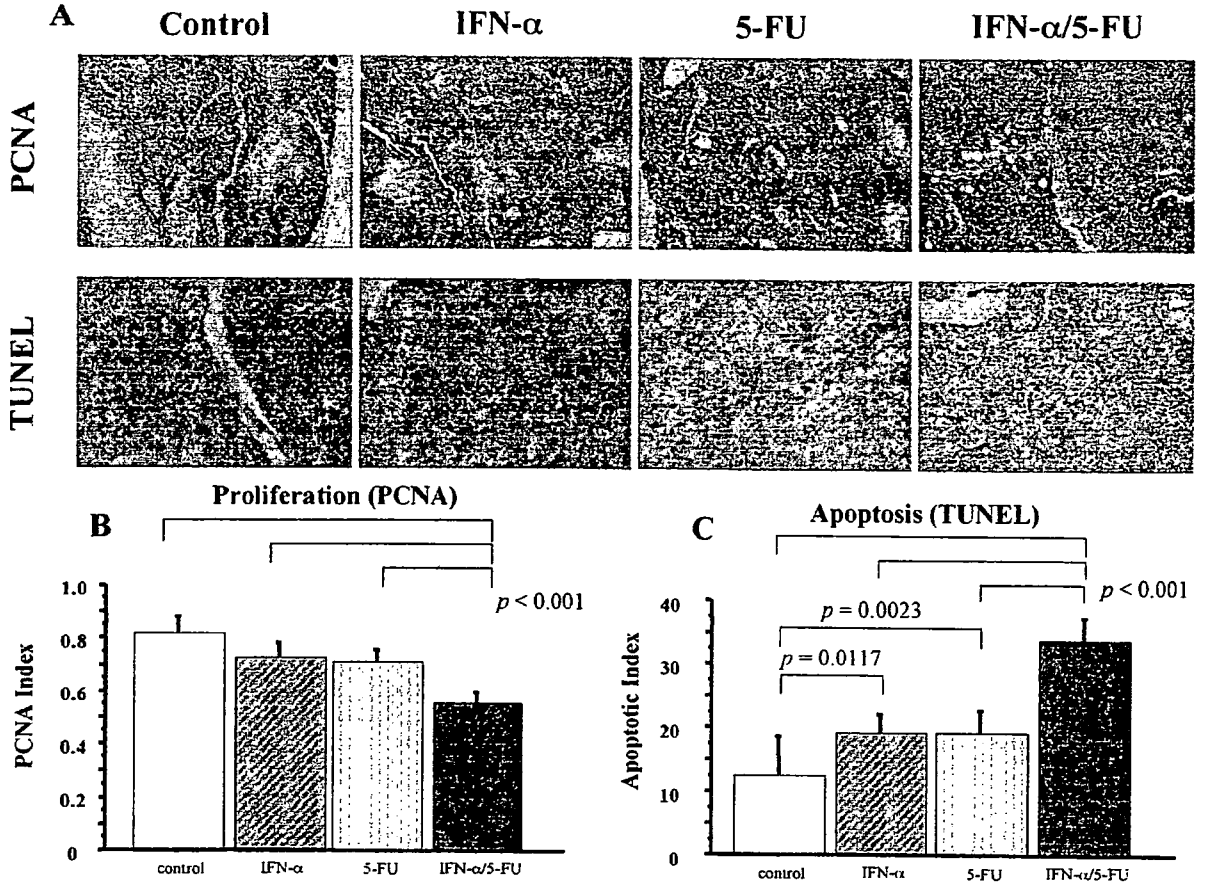

Figure 2. (A) Immunohistochemical analyses of PCNA (cell proliferation) and TUNEL (cancer cell apoptosis). Twenty-seven days after initial treatment, tumors were harvested from control mice or mice treated with IFN-α (20,000 units/mouse) alone, treated with 5-FU (30 mg/kg) alone or treated with a combination 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-

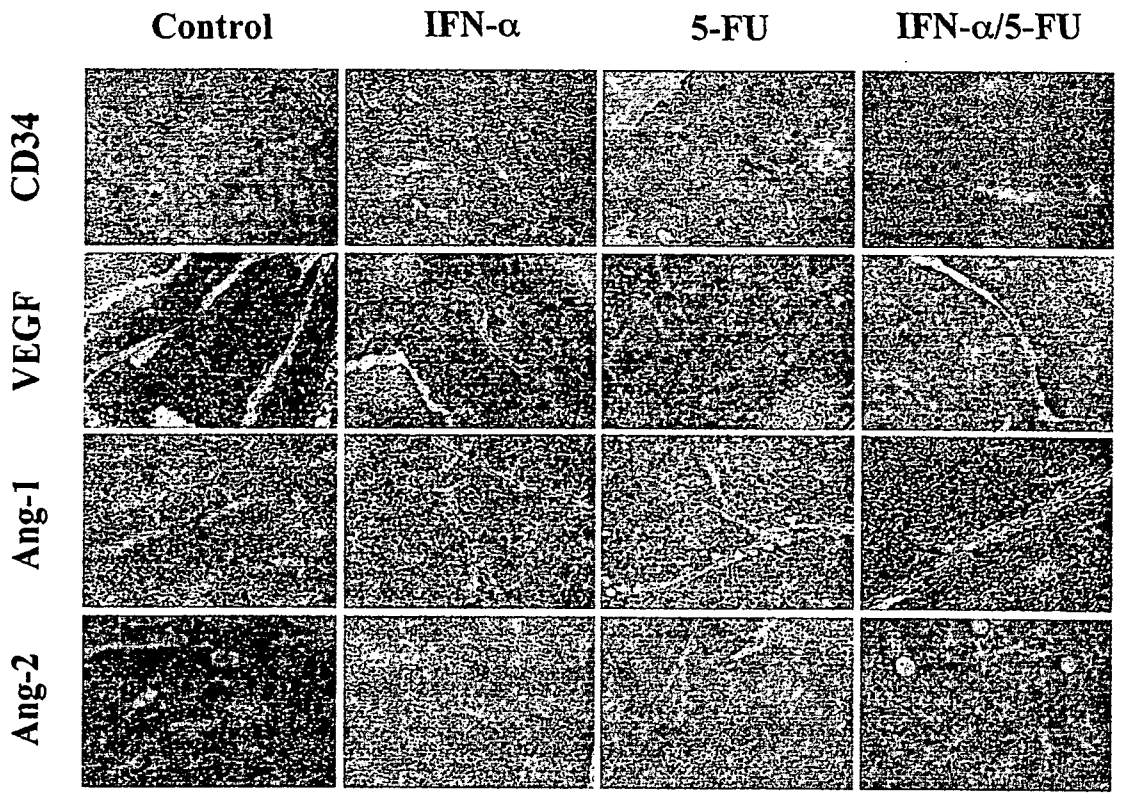

Figure 3. Immunohistochemical analyses of CD34 (endothelial cells) and VEGF, Ang-1 and Ang-2 (angiogenic factors). The sections were immunostained for expression of CD34 (to show MVD), VEGF, Ang-1 and Ang-2. Tumors of mice treated with combined IFN-α and 5-FU showed a significant decrease of MVD. The 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

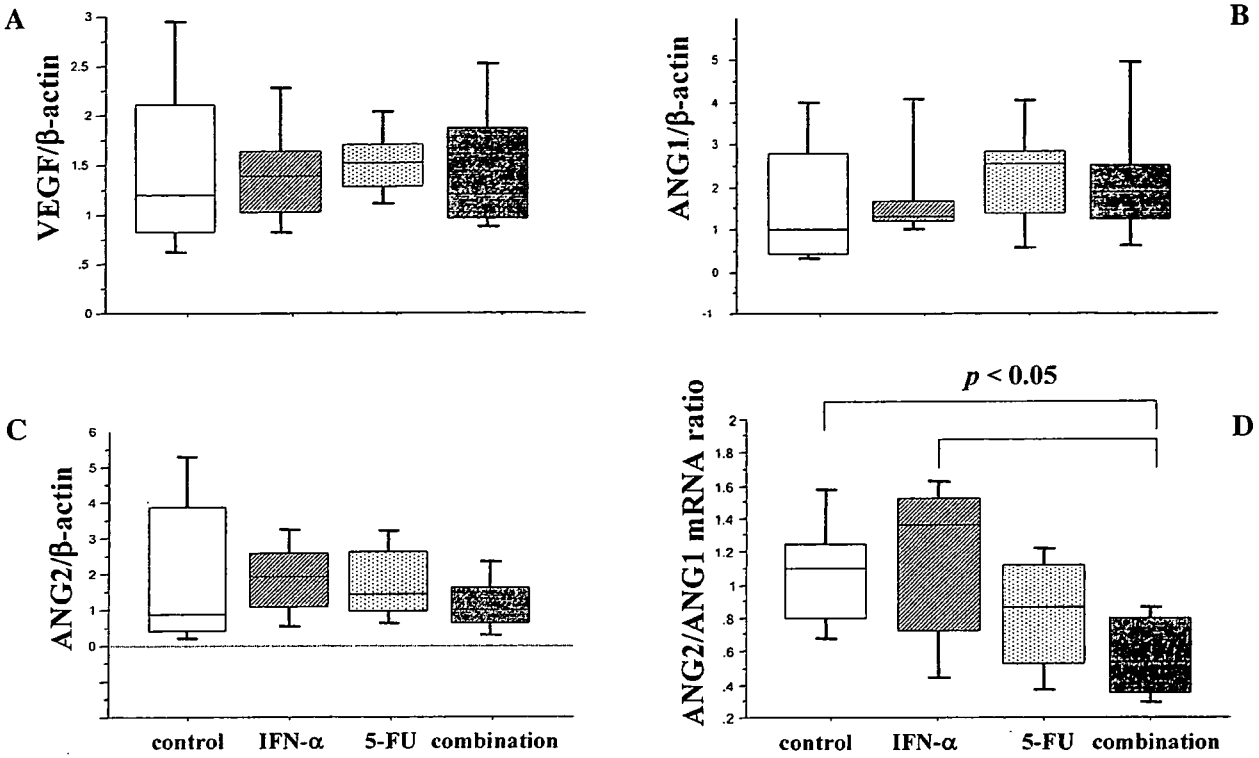

Figure 4. Expression of mRNA encoding VEGF, Ang-1 and Ang-2 and expression ratio of ANG2/ANG1 mRNA in tumors of control mice, mice treated with IFN-α alone, 5-FU alone and a combination of IFN-α and 5-FU. The mRNA expression levels were normalized to ß-actin. Data are displayed in box plots, with mean 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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