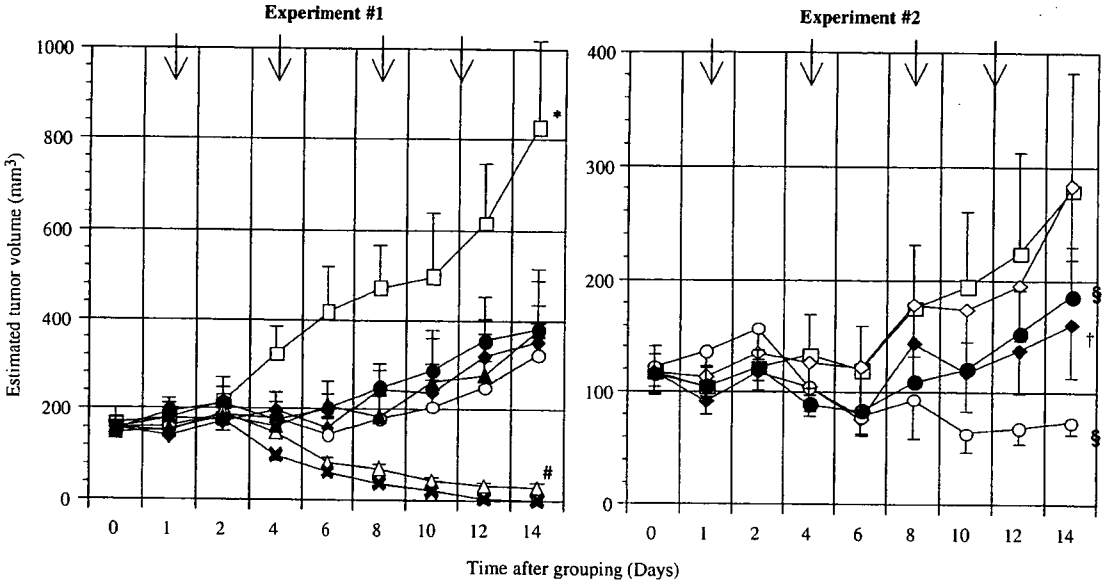

Fig. 4. Time-course change in estimated tumor volumes of subcutaneously transplanted human hepatocellular carcinoma tumors in nude mice. The mice received a subcutaneous injection of 640 (◆), 6400 (○), 64 000 (△), or 640 000 (✖) IU of pegylated IFN-α2b (PEG-IFN-α2b), or 6400 (●) or 64 000 (▲) IU of IFN-α2b, or medium alone (Control) (□) in Experiment #1, and of 640 (◆) or 6400 (○) IU of PEG-IFN-α2b, 640 (◇) or 6400 (●) IU of IFN-α2b, or medium alone (Control) (□) in Experiment #2, twice a week for two consecutive weeks. The arrows show the days of injection. *$P<0.001$, vs the other groups. #$P<0.0001$, vs IFN-α2b (64 000 IU). †$P<0.05$, vs control or IFN-α2b (640 IU). §$P<0.05$, vs Control. The experiments were repeated twice, and almost identical results were obtained. The figures represent average ± SE.

Table 2. Treatment of human HCC tumors subcutaneously transplanted in nude mice

Treatment group*	Number	Tumor weight (g)	Body weight (g)†
Experiment #1			
Control	8	0.436 ± 0.07‡	19.2 ± 0.6††
PEG-IFN-α2b (640 IU)	9	0.237 ± 0.05§	19.4 ± 0.4
PEG-IFN-α2b (6400 IU)	9	0.180 ± 0.03¶	19.9 ± 0.3
IFN-α2b (6400 IU)	6	0.259 ± 0.06§	19.4 ± 0.7
PEG-IFN-α2b (64 000 IU)	9	0.016 ± 0.01 ‖ ,#	19.0 ± 0.6
IFN-α2b (64 000 IU)	7	0.221 ± 0.06§	19.1 ± 0.5
PEG-IFN-α2b (640 000 IU)	9	0.0 ‖	19.6 ± 0.3
Experiment #2			
Control	8	0.160 ± 0.04	20.6 ± 0.5††
PEG-IFN-α2b (640 IU)	8	0.097 ± 0.02	20.1 ± 0.4
IFN-α2b (640 IU)	8	0.168 ± 0.03	20.7 ± 0.4
PEG-IFN-α2b (6400 IU)	7	0.050 ± 0.02¶	21.0 ± 0.3
IFN-α2b (6400 IU)	8	0.131 ± 0.03**	21.1 ± 0.3

*Cultured HAK-1B cells (1.0×10^7) were subcutaneously transplanted into nude mice. Five to seven days later when the largest diameter of the tumor reached approximately 5–10 mm, mice in each group were treated with twice per week sc injections of PEG-IFN-α2b, IFN-α2b, or culture medium. All mice were sacrificed on the 15th day. †Body weight on the 14th day. ‡Mean ± SE. §$P<0.05$ vs control. ¶$P<0.01$ vs control. ‖ $P<0.001$ vs control. #$P<0.01$ vs IFN-α2b (64 000 IU). **$P<0.03$ vs PEG-IFN-α2b (6400 IU). ††not significant vs the other groups. HCC, hepatocellular carcinoma; PEG-IFN-α2b, pegylated IFN-α2b.

PEG- and non-PEG-IFN-α2b ($P<0.05$, Fig 4). The tumors of the mice that received 6400 IU of PEG-IFN-α2b tended to be smaller in volume in both Experiments #1 and #2 ($P=0.068$ and 0.064, respectively), and the tumor was significantly lower in weight than that of IFN-α2b in Experiment #2 ($P<0.03$). At the end of the experiments, the estimated tumor volume in the mice that received 640 IU of PEG-IFN-α2b (3.2×10^4 IU/kg, about 1/3 of the clinical dose 9.6×10^4 IU/kg) in Experiments #1 and #2 became 42% and 58% of the Control, respectively. In the mice that received 640 000 IU of PEG-IFN-α2b, the tumors disappeared on the 14th day. PEG-IFN-α2b administration did not affect the body weight of the mice (Table 2).

Histological examination of the HAK-1B tumor specimens stained with HE revealed that the numbers of apoptotic cells in the mice treated with PEG-IFN-α2b (640–64 000 IU) or IFN-α2b (64 000 IU) were significantly higher than that of the Control, and the number increased dose dependently (Fig. 5A–C; Table 3). Immunostaining of vimentin fragment (V1) that is a marker for caspase-9 activation showed a positive reaction in the cytoplasm of the apoptotic cells (Fig. 5D).

No significant difference was observed in the number of blood vessels per unit area of the HAK-1B tumor between the Control and the PEG-IFN-α2b or IFN-α2b group (Table 3).

Immunohistochemical examination of BrdU uptake in HAK-1B tumors revealed that the BrdU labeling index was significantly higher in the Control than in the 6400 IU PEG-IFN-α2b or IFN-α2b groups (Fig. 5E, Table 3).

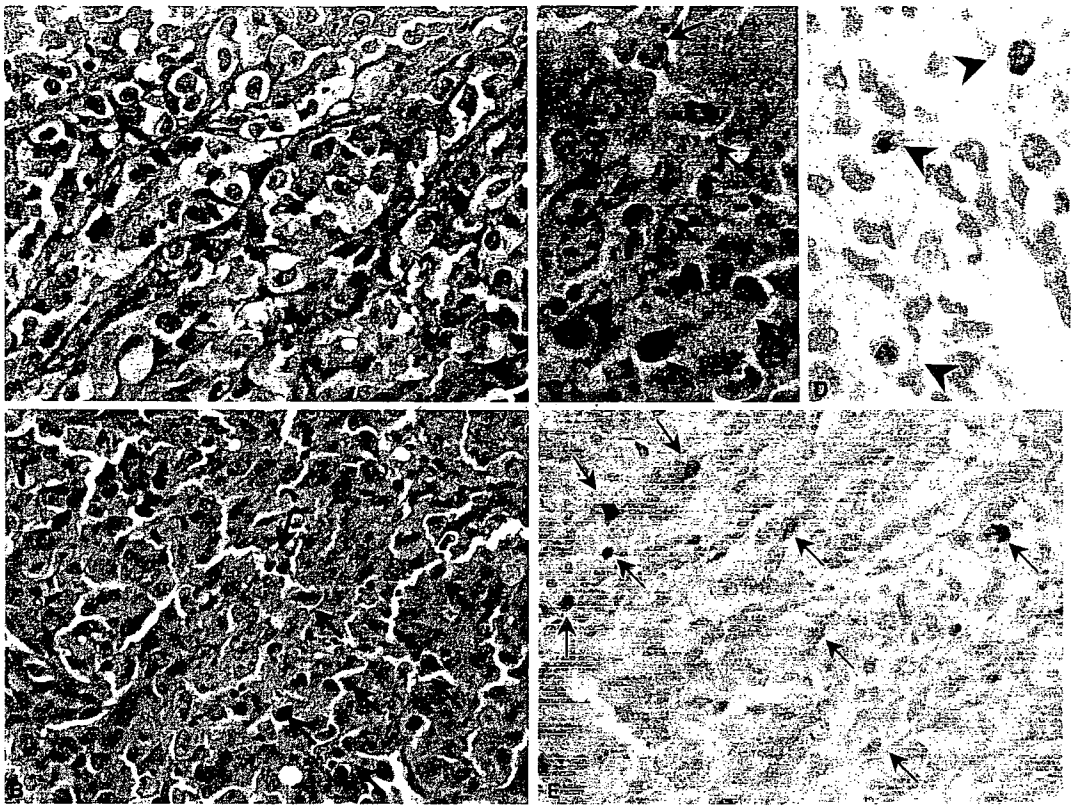

Fig. 5. Photomicrograph of subcutaneous human hepatocellular carcinoma tumor in nude mice that was developed after the injection of HAK-1B cells. (A) A control mouse that received culture medium alone. The tumor shows a thick trabecular arrangement of tumor cells and a sinusoid-like structure in the stroma. (B) A mouse that received a sc injection of 6400 IU pegylated IFN-α2b. There are many apoptotic tumor-cells (arrows, hematoxylin–eosin staining, × 200). (C) Higher magnification of B. It clearly shows apoptotic tumor cells characterized by shrinkage and eosinophilic change in the cytoplasm, chromatin condensation and/or fragmentation of nuclei (hematoxylin–eosin staining, × 400). (D) Immunostaining of vimentin fragment (V1). Positive reactions (red pigments) are shown in the shrunken cytoplasm of apoptotic cells as noted in C (counterstained with Mayer's hematoxylin, × 400). (E) Immunostaining of bromodeoxyuridine (BrdU). Many BrdU-positive cells (brown nuclear pigments) were found in a tumor of a control mouse (counterstained with Mayer's hematoxylin, × 200).

Table 3. Numbers of apoptotic cells, artery-like blood vessels, and BrdU-positive cells, and expression of IFNAR-2 subunit in human HCC tumors subcutaneously transplanted in nude mice

Treatment group*	Apoptosis†	Blood vessel‡	BrdU L.I.§	IFNAR-2¶
Experiment #1				
Control	21.3 ± 1.8 ‖	1.16 ± 0.20 ‖ ,**	15.5 ± 1.2 ‖	0.676 ± 0.10 ‖
PEG-IFN-α2b (640 IU)	28.4 ± 1.9#	1.04 ± 0.15	16.6 ± 3.1	0.410 ± 0.07#
PEG-IFN-α2b (6400 IU)	34.3 ± 5.0#	1.20 ± 0.19	10.2 ± 1.1#	0.451 ± 0.11
IFN-α2b (6400 IU)	24.0 ± 3.0	1.13 ± 0.16	9.4 ± 1.1#	0.656 ± 0.12
PEG-IFN-α2b (64 000 IU)	34.3 ± 5.3#	1.43 ± 0.39	ND	0.061 ± 0.06††
IFN-α2b (64 000 IU)	27.5 ± 2.5#	0.92 ± 0.20	10.6 ± 3.0	0.607 ± 0.11
PEG-IFN-α2b (640 000 IU)	ND	ND	ND	ND

*Cultured HAK-1B cells (1.0×10^7) were subcutaneously transplanted into nude mice. Five to seven days later when the largest diameter of the tumor reached approximately 5–10 mm, mice in each group were treated with twice per week sc injections of PEG-IFN-α2b, IFN-α2b, or culture medium. All mice were sacrificed on the 15th day. †The number of apoptotic cells was counted in ten 0.25 mm^2 areas in each section, and the average number per area in each group was obtained. ‡The number of the blood vessels in the tumor nodule was counted on each section, and the average number per area in each group was obtained. §The number of BrdU-positive cells was counted in ten 0.25 mm^2 areas in each section, and the average number per area in each group was obtained as the labeling index. ¶Values are expressed as pg/15 μg protein. ‖ Mean ± SE. #$P<0.05$, vs control. **Not significant, vs the other groups. ††Not significant ($P=0.068$) vs control. This is partly because only 2 samples were available for IFNAR-2 analysis in this group. BrdU, bromodeoxyuridine; IFNAR-2, IFN-α receptor-2; HCC, hepatocellular carcinoma; ND, not done; PEG-IFN-α2b, pegylated IFN-α2b.

The expression of the IFNAR-2 subunit tended to decrease in the PEG-IFN-α2b groups, and a significant difference was observed between the Control and the 640 IU PEG-IFN-α2b group ($P<0.05$).

Discussion

PEG-IFN-α2b induced a time-dependent antiproliferative effect in 10 cell lines in contact with 4096 IU/ml of PEG-IFN-α2b for 24–96 h and a dose-dependent antiproliferative effect in nine cell lines *in vitro* in the range of 16 and 4096 IU/ml. On an antiviral unit basis, the antiproliferative activity of PEG-IFN-α2b was not significantly different from that of non-PEG-IFN-α2b *in vitro* based on IC50 values. Compared with BALL-1 IFN-α that consists of the α2 subtype (about 75%) and the α8 subtype (25%) or with IFN-αCon1, both PEG-IFN-α2b and IFN-α2b showed low antiproliferative activity in terms of relative viable cell number and IC50 (11, 12). This is consistent with our recent finding, i.e., that the antiproliferative activity of the IFN-α2 subtype *in vitro* is relatively weak compared with other IFN-α subtypes such as α5, α10 and α8 (32). As a mechanism of antiproliferation, apoptosis induction was observed in 9 cell lines that received 1000 IU/ml of PEG-IFN-α2b (morphological changes occurred in 10 cell lines with 4096 IU/ml). IFN-αCon1 that possesses the most potent antiproliferative effect among the three IFN-α preparations induced apoptosis in all 13 HCC cell lines at a similar concentration (12). The four cell lines (HAK-2, HAK-3, HAK-4, and KMCH-2) in which PEG-IFN-α2b did not induce a dose-dependent antiproliferative effect showed the highest IC50 values (> 100 000 IU/ml) to IFN-α2 subtype among the 13 cell lines (32) and showed resistance to PEG-IFN-α2b-mediated apoptosis (HAK-2, HAK-3, and KMCH-2) and/or low cell surface IFNAR-2 expression (HAK-3, HAK-4, and KMCH-2) (11).

When IFN-α binds to its receptors, the IFN-receptor-complexes are internalized and degradated intracellularly (33, 34). It was demonstrated that IFN-α down-regulates type I IFN receptors in peripheral blood mononuclear cells (PBMC, (35–37)). Nakajima et al. (36) reported that the number of IFN receptors on PBMC in patients with chronic hepatitis B decreased to about 50% of the baseline with a fivefold increase in 2′,5′-oligoadenylate synthetase activity when the patients were treated with IFN for 2 or 4 weeks. To date, however, there have been no studies on the down-regulation of IFN receptors and its relationship with the antiproliferative effects of IFN-α in liver cancer cells. Human type I IFN receptor consists of two subunits, IFNAR-1 and IFNAR-2. IFNAR-2 is the binding subunit and is more important than IFNAR-1 for the expression of IFN activity (3, 38, 39). Therefore, the current study chronologically examined the relationship between the antiproliferative effect and the expression of the IFNAR-2 subunit in HAK-1B cells up to 240 h after the addition of PEG-IFN-α2b. We chose HAK-1B for this study because this cell line expresses the highest IFNAR-2 subunit expression among the 13 liver cancer cell lines (11) and because this cell line was also selected for the *in vivo* experiment, allowing comparison of results in the *in vitro* setting with those *in vivo*. The expression of IFNAR-2 subunit was significantly down-regulated at 3 h compared with the Control and then significantly up-regulated at 48 h. Expression then decreased in a time-dependent manner after 72 h, and the viable cell number continuously decreased with time. The down-regulation of IFNAR-2 was the specific change because another cell surface protein, i.e., EGF receptor, was not down-regulated at 240 h compared with Control. Therefore, at least for the HCC cell line, HAK-1B, in an *in vitro* setting, the IFNAR-2 subunit is down-regulated but an efficient antiproliferative effect is induced with continuous contact with PEG-IFN-α2b. Lau et al. (35) studied the binding characteristics of IFN-α to PBMC in patients with chronic hepatitis B virus infection and reported a possible increase in binding affinity of the remaining receptors as a reason for the continuous effects of IFN-α in long-term IFN therapy that reduces the number of IFN-α receptors. On the other hand, Dooley et al. (40) reported a decrease in binding affinity. Therefore, this point also needs to be further studied on HAK-1B and other HCC cell lines.

We then examined the *in vivo* antitumor effects of PEG-IFN-α2b on mice. Twice-a-week administration of PEG-IFN-α2b dose-dependently suppressed the growth of sc transplanted human HCC. The growth was effectively suppressed even at 1/3 of the clinical dose in patients with chronic hepatitis C, and the tumor size was reduced to 42–58% of the Control. This antiproliferative effect was equivalent to the effect of a consecutive 14-day administration of an approximately 1.3 times larger clinical dose of IFN-αCon1 (12). The antiproliferative effect of PEG-IFN-α2b *in vitro* is lower than IFN-αCon1; therefore, our *in vivo* finding would be understood as the serum half-time of IFN-α2b becoming longer due to pegylation, then PEG-IFN-α2b at a high concentrations remaining in the serum for a long time to affect tumor cells, resulting in much stronger antitumor effects. This consideration is also supported by our results, i.e., PEG-IFN-α2b and IFN-α2b *in vitro* presented the same antiproliferative effects; however, *in vivo*, IFN-α2b presented significantly weaker antitumor

effects than PEG-IFN-α2b. In addition, IFNAR-2 expression in the tumor of mice decreased, particularly in mice that received PEG-IFN-α2b. This was considered to be due to IFNAR-2 expression being down-regulated as a result of the long-term continuous action of PEG-IFN-α2b, and this is consistent with the *in vitro* findings. The above indicates that even if IFNAR-2 expression is down-regulated, antitumor effect does not decrease but, in fact, increases due to continuous action. In contrast, Krepler et al. (41) compared antitumor effects of PEG-IFN-α2a and non-PEG-IFN-α2a in a human melanoma SCID mouse xenotransplantation model and found no significant differences in tumor growth inhibition. We presume that this opposite result is attributable to the difference in the experimental conditions. For example, in their study, mice received extremely high doses of PEG- and non-PEG-IFN-α2a, i.e., mice received 900 μg of PEG-IFN-α2a (45 000 μg/kg) that is five or 10 times larger than the clinical dose for chronic hepatitis C patients (90–180 μg/body, 1.8–3.6 μg/kg). In addition, it is not clear whether the amounts of PEG- and non-PEG-IFN-α2a administrated to mice were equivalent in antiproliferative effects on the melanoma cells *in vitro*.

The induction of apoptosis is known as a mechanism of the *in vivo* antiproliferation activity of PEG-IFN-α2b. However, the induction of S-phase arrest that was observed *in vitro* was not clear *in vivo* in terms of the labeling index of BrdU. This was the same as the finding on the mechanism of the antiproliferation activity of IFN-αCon1 in mouse tumor (12). It is surmized that apoptosis holds the dominant position over the S-phase arrest when tumor cells *in vivo* were treated with IFN-α for 2 weeks. This point should be further investigated. Antiangiogenesis activity is one of the biological effects of IFN-α, and the administration of IFN-α to patients with vascular tumors, e.g., Kaposi sarcoma, results in the significant regression of tumor lesions (1, 4). IFN administration suppresses the growth of human tumor that was transplanted to mice through an antiangiogenesis effect (12, 31, 42–45). Tedjarati et al. (44) found that once per week injection of 7000 IU of PEG-IFN-α2b into nude mice bearing ip growing human ovarian carcinoma cells inhibited angiogenesis and tumor growth and that PEG-IFN-α2b administered at higher or lower doses was less effective. In our current study, however, significant antiangiogenesis effect was not observed at any dose. In another study that we conducted examining a consecutive 14-day administration of 5000 IU/mouse/day of BALL-1 IFN-α to mice that had subcutaneous transplantation of HAK-1B tumor, a slight increase in the number of blood vessels and an increase of the three angiogenesis factors were observed (46). On the other hand, another study that used 0.01 or 0.1 μg/mouse of IFN-αCon1 showed a significant decrease in the number of blood vessels (12). Further studies are necessary both *in vitro* and *in vivo* with various IFNs such as IFN-α and IFN-β to clarify whether the antiangiogenesis effects are attributable to the type of IFN. In addition, more studies are also needed to investigate the mechanism of antiproliferative effects including antiangiogenesis, the expression of IFNAR-2 and its relationship to antiproliferative effects using other HCC cells.

In the HCC cell line HAK-1B, continuous contact with PEG-IFN-α2b induced the down-regulation of IFNAR-2 and a potent antiproliferative effect that is stronger than the effects of non-PEG-IFN-α2b. The antitumor effect of PEG-IFN-α2b was expressed at approximately 1/3 of the clinical dose, and this suggests that PEG-IFN-α2b administration to patients with chronic hepatitis C would be effective in the prevention of hepatocarcinogenesis and the recurrence of HCC.

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HEPATOLOGY

Antiproliferative effects of 5-fluorouracil and interferon-alpha in combination on a hepatocellular carcinoma cell line *in vitro* and *in vivo*

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Abstract

Background and Aim: We investigated the antiproliferative effects of interferon-alpha (IFN-α) and 5-fluorouracil (5-FU) in combination on a hepatocellular carcinoma (HCC) cell line.

Method: In the *in vitro* study, IFN-α and/or 5-FU was added to the culture of the poorly differentiated-type HCC cell line, HAK-1B, and their antiproliferative effects and additional or synergic effects in combination treatment were examined. In the *in vivo* study, HAK-1B cells were transplanted into nude mice and the changes in tumor volume and weight, apoptosis, BrdU and cyclin A positive cells, and artery-like blood vessels were investigated. Expressions of angiogenesis factors and IFN-α receptor (IFNAR-2) were examined in the developed tumors.

Results: *In vitro* growth of HAK-1B cells was suppressed dose-dependently to 5-FU, but the addition of IFN-α did not induce additional or synergic effects. *In vivo* growth in terms of tumor diameter and weight was suppressed at most in the IFN-α + 5-FU (combination) group, that is, the tumor volume became 29.3% and the tumor weight became 54.7% of the control. In the combination group, numbers of BrdU-positive S-phase cells and cyclin A positive cells increased together with the increase in apoptotic cells, but there was no significant relation between the tumor shrinkage effects and angiogenesis factors or artery-like blood vessels. In the combination group, INFAR-2 decreased significantly in comparison to the other groups.

Conclusion: The synergic growth-suppression effects in the current *in vivo* study using the combination treatment are attributable to the enhanced induction of S-phase arrest and of apoptosis.

Key words: 5-fluorouracil, combination therapy, hepatocellular carcinoma, interferon-alpha.

INTRODUCTION

Hepatocellular carcinoma (HCC) is among the most frequent causes of death from malignancy, and the survival rate is very poor in patients with advanced HCC with complications such as portal vein tumor thrombosis (PVTT) or distant metastasis.[1–3] Previous studies have reported that patients with diffuse-type HCC who also have PVTT survive only 1–2 months if treatment is not effective.[4] For this reason, effective therapies that support patients' quality of life are sought.

Wadler and Wiernik,[5] for the first time, proposed a combination therapy of interferon-alpha (IFN-α) and 5-fluorouracil (5-FU) in 1988 in their study using colon cancer cell lines. Later, this combination therapy was applied to various types of human malignancies. Sakon *et al.*[6] applied this therapy to 11 HCC patients with non-resectable lesions and with tumor thrombi, and reported a remarkable decrease in tumor size and of tumor marker levels. IFN-α possesses antiviral activity, antiproliferative activity and various immunoregulatory activities. Examples of the immunoregulatory activities are: (i) stimulation of cytotoxic activities of lymphocytes and macrophages, and of natural killer cell activity; and (ii) induction of class I major histocompatibility complex antigens.[7] IFN-α has at least 14 different subtypes,

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each of which has different biological properties.[8-10] Our current study used OIF, a natural IFN-α that was induced by the Sendai virus on BALL-1 cells, composed of three subtypes; α2, α7 and α8.

Conversely, 5-FU is one of the most widely used chemotherapeutic agents in cancer treatment. Two routes have been proposed as the mechanism of action: (i) inhibition of RNA polymerase synthesis through a complicated metabolic process; and (ii) inhibition of DNA synthesis. Several researchers have reported the augmentation of suppressive effects on cell proliferation *in vitro* in the combination with IFN-α.[11-13] However, augmentation of antitumor effects *in vivo* in this combination and their mechanisms of action have not yet been fully investigated.[14] Our current study examined these points further by using a HCC cell line and nude mice.

METHOD

Materials

Natural human IFN-α (OIF) was kindly provided by Otsuka Pharmaceutical (Tokyo, Japan), and 5-FU was purchased from Kyowa Hakko (Tokyo, Japan). The HCC cell line, HAK-1B,[15] which was established in our laboratory was used in this study. HAK-1B was previously confirmed to retain morphological and functional characteristics of the original HCC. The cells were grown in culture medium consisting of Dulbecco's modified Eagle medium (Nissui Seiyaku, Japan) supplemented with 5% heat-inactivated (56°C, 30 min) fetal bovine serum (Bioserum, Canterbury, Australia), 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco BRL/Life Technologies, Gaithersburg, MD, USA) and 12 mM sodium bicarbonate in a humidified atmosphere of 5% CO_2 at 37°C. Nude mice were purchased from Clea Japan (Osaka, Japan).

Effects of IFN-α and 5-FU on the proliferation of HAK-1B cells

Effects of IFN-α and/or 5-FU on cell proliferation were examined by using colorimetric assays with 3-(4,5-dimethylthiazol-2yl-yl-)-2,5-dimethyl tetrazolium bromide (MTT) cell growth assay kits (Chemicon, Temecula, CA, USA), as described elsewhere.[16] Briefly, the HCC cells (HAK-1B, 2000 cells/well) were seeded on 96-well plates (Falcon, Becton Dickinson Labware, Tokyo, Japan), cultured for 24 h, and then the culture medium was changed to a new one containing IFN-α (0, 10 or 100 IU/mL), 5-FU (0, 1, 10 or 100 μM), or both 5-FU (0, 1, 10 or 100 μM) and IFN-α (0, 10 or 100 IU/mL) After 96 h of culture, the number of viable cells was examined.

Quantitative analysis of apoptotic cells induced by IFN-α or 5-FU *in vitro*

HAK-1B cells that were cultured with IFN-α (0, 10, 100 or 1000 IU/mL) or 5-FU (0, 1, 10 or 100 μM) for 72 h were stained with Annexin V-EGFP (enhanced green fluorescent protein) using Apoptosis Detection Kits (Medical & Biological Laboratories, Nagoya, Japan) according to the manufacturer's protocol. After staining, the cells were analyzed using a FACScan (Becton Dickinson Immunocytometry Systems), and the rate of Annexin V-EGFP-positive apoptotic cells was determined.

Effects of IFN-α and/or 5-FU on HCC cell proliferation in nude mice

This experiment was conducted according to the 'Guide for the Care and Use of Laboratory Animals' published and revised by the National Institute of Health in 1985. HAK-1B cells (1.0×10^7 cells/mouse) were transplanted subcutaneously to 4-week-old female BALB/c nu/nμ athymic nude mice ($n = 40$). The mice were divided into four groups ($n = 10$ each) on the seventh day when tumor size became 5–10 mm in diameter, and each group was assigned to one of the four treatments: (i) IFN-α alone; (ii) 5-FU alone; (iii) IFN-α + 5-FU (combination); and (iv) control.

A sustained injection pump (Alzet Micro-Osmotic Pump Model-1002, DURECT Cupertino, CA, USA) was subcutaneously placed in every mouse, and 5-FU (6 μL/day) was continuously administered for 14 days. IFN-α (100 μL/mouse) was subcutaneously injected once a day for 14 days to a point that was 2 cm or more away from the tumor. In the control and the IFN-α alone groups, saline as the replacement for 5-FU was continuously injected through the pump. In the control and 5-FU alone groups, saline as the replacement for IFN-α was injected subcutaneously. The dose of 5-FU (180 μg) in the ratio to the average bodyweight of a mouse (25 g) was 7 mg/kg and this is comparable to a clinical dose. The dose of IFN-α (OIF) for human HCC is 500×10^4 IU/day, and the actual dose for a patient weighing 50 kg is 1.0×10^5 IU/kg. For a nude mouse weighing 25 g, the comparable daily dose is 2500 IU/day/mouse, however, because of species difference, we used twice the dose in nude mice.

Tumor size was measured in two directions using calipers, and tumor volume (mm^3) was estimated by using the equation: length × $(\text{width})^2 \times 0.5$. This measurement was performed every other day, and changes in average tumor volume in each group were recorded. The mice were killed and the tumor was resected on the next day after the completion of this 14-day treatment (on the 22nd day). Half of the resected tumor was fixed in formalin after the weight measurement, prepared into paraffin sections, and underwent hematoxylin and eosin (HE) staining and immunohistochemistry.

In the sections after HE staining, morphological features were observed and apoptotic cells and mitotic cells were counted. The number of cells showing characteristics of apoptosis such as cytoplasmic shrinkage, chromatin condensation and nuclear fragmentation were counted in 10 0.25 mm^2-areas within a HE-stained specimen where apoptotic cells were present at a relatively uniform density, and the average number per area was obtained. In addition, the TUNEL technique (ApopTag Peroxidase In Situ Apoptosis Detection Kits,

CHEMICON International, CA, USA) was used for the detection of apoptotic cells. Average number of TUNEL-positive cells per area was obtained, as described previously.

Immunohistochemistry

For the determination of HCC proliferation, 5 mg/kg of bromodeoxyuridine (BrdU, Sigma Chemical, St Louis, MO, USA) was intra-abdominally injected 1 h before killing. Mouse monoclonal anti-BrdU antibody for subcutaneous tumor was purchased from Becton Dickinson Immunohistochemistry USA (San Jose, CA, USA). Reactivity was detected by the BrdU immunohistochemistry system (Oncogene Research Products, EMD Bioscience, La Jolla, CA, USA).

Double immunohistochemical staining was performed by using antimouse endothelial cell (anti-CD34) antibody, antihuman alpha smooth muscle actin (α-SMA) antibody and histofine simple stain mouse MAX-PO (Rat) kits (Nichirei, Tokyo, Japan). Tumor sections were at first immunostained with antimouse endothelial cell antibody and by using HistoMouse MAX-PO (Rat) kits, and they were reacted with a mixture of 3,3-diaminobenzidine tetrahydrochloride (DAB). The second staining with antihuman α-SMA antibody was conducted by using HistoMouse-plus kits. The sections were stained with a mixture of a commercial chromogen (VIP), hydrogen peroxide (Vector VIP substance kit, Vector Laboratories, CA, USA) and hematoxylin for counterstaining. The number of blood vessels in a unit area (mm^2) of every section was calculated and its mean was obtained.

Cyclin A immunostaining was conducted according to a standard streptavidin-biotin method. Before conducting immunostaining, a heat-induced epitope retrieval method was used, that is, the sections were placed in a 0.01 M sodium citrate buffer, pH 6.0, and heated in a microwave oven for 30 min per cycle at 750 W. Antibody to cyclin A was purchased from Novocastra Laboratories (Newcastle-upon-Tyne, UK). Reactivity was detected by the Strp-ABComplex/HRP system by using-3′,3′-diaminobenzidine-tetrahydrochloride dehydrate as chromogen (DAKO, Glostrup, Denmark).

Enzyme-linked immunosorbent assay (ELISA)

Portions of the resected tumors were cut into pieces and an appropriate amount was homogenized in 500 μL of ice-cold Ca^{++}-and Mg^{++}-free phosphate-buffered saline containing 100 μg/mL phenymethylsulfonyl fluoride by using a pellet pestle. The mixture was centrifuged for 10 min (12 000 r.p.m., 4°C), and the supernatant was stored at −20°C until use. The amount of the tissue proteins was determined by using the BCA protein assay reagent (Pierce, Rockford, IL, USA). The concentration in the samples was determined by comparing their absorbance with a standard curve. The amount of bFGF, IL-8, VEGF and IFNAR-2 in the supernatant was measured by using ELISA kits. The kits for VEGF and IL-8 were supplied by Amersham Biosciences (Little Chalfont, UK), the kits for bFGF were from R&D (Minneapolis, MN, USA), and the kits for IFNAR-2 were from Otsuka Pharmaceutical.

Statistical analysis

ANOVA was used for the analysis of tumor volume and weight. The Mann–Whitney *U*-test was used for the between-groups analysis of intratumor artery-like blood vessels, BrdU labeling index, and expressions of apoptotic cells, mitotic cells, angiogenesis factors and IFNAR-2.

RESULTS

Effects of IFN-α and/or 5-FU on the proliferation of HAK-1B cells *in vitro*

The number of viable cells decreased along with the increase in the 5-FU dose. Addition of IFN-α at doses lower than 100 IU produced mild growth inhibitory effects (relative viable cell numbers >90%), although the relative viable cell number decreased to 42.6% when 1000 IU/mL of IFN-α was added to the culture (data not shown). In the combination treatment, the addition of IFN-α at doses lower than 100 IU did not produce additional or synergic effects (Fig. 1). In the 72 h culture, the number of Annexin V-EGFP-positive apoptotic cells increased dose-dependently to both IFN-α and 5-FU (Table 1). In the combination group, however, there was no additional or synergistic increase in the number of Annexin V-EGFP-positive apoptotic cells.

Effects of IFN-α and/or 5-FU on the HAK-1B cell proliferation in nude mice

The tumor started to shrink on the 10th day and the volume and weight of the tumor in the combination

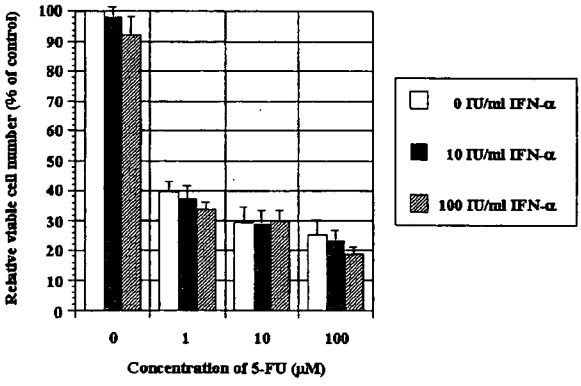

Figure 1 Effects of human natural interferon-alpha (IFN-α), 5-fluorouracil (5-FU) or IFN-α + 5-FU on the growth of HAK-1B cells in culture for 96 h.

Table 1 Apoptosis of HAK-1B cells induced by interferon-alpha (IFN-α) or 5-fluorouracil (5-FU) treatment *in vitro*

Treatment	Average† (mean ±SD)	*P*-value *versus* control	Treatment-control (%)	Degree of apoptosis§
Control	9.9 ±0.9			
IFN-α 10 IU	9.0 ±0.8	NS	−0.9	−
IFN-α 100 IU	14.5 ±0.7	<0.01	4.6	++
IFN-α 1000 IU	28.5 ±0.9	<0.001	18.6	+++
5-FU 1 μM	10.4 ±1.5	NS	0.5	+
5-FU 10 μM	18.8 ±2.6	<0.02	8.9	++
5-FU 100 μM	24.3 ±2.5	<0.01	14.4	+++
IFN-α 1000 IU + 5-FU 100 μM	29.9 ±0.3	<0.001	20.0	+++

§Degree of apoptosis was estimated as follows: the difference between Annexin-V positive rates in cells cultured with IFN-α or 5-FU were calculated and classified into four levels: −, no significant difference; +, <5%; ++, 5–10%; +++, 10–20%.

†Average of Annexin-V positive cell.

NS, not significant.

Table 2 Effect of human natural interferon-alpha (IFN-α), 5-fluorouracil (5-FU) or IFN-α + 5-FU on the weight of subcutaneous tumor of HAK-1B cells in nude mice

Treatment group	Tumor weight (mg)
Control	251.8 ±68.8
IFN-α alone	182.2 ±75.8*
5-FU alone	200.0 ±46.0*
IFN-α+5-FU	137.8 ±98.8*

*$P < 0.01$ *versus* control.

Figures represent mean ±SD. IFN-α alone group received 5000 IU/mouse injection every day. 5-FU alone group received continuous administration of 180 μg (7 mg/kg)/mouse/day. IFN-α + 5-FU group received both. Control group received injection and continuous administration of saline.

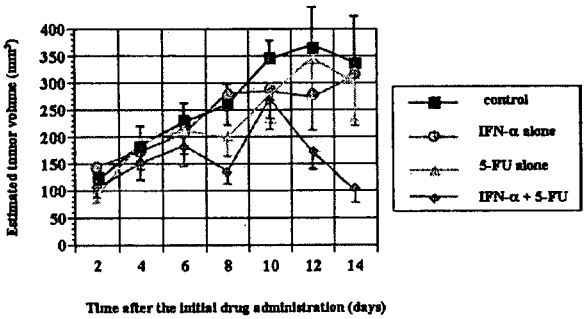

Figure 2 Chronological changes on the estimated volume of HAK-1B tumor that was developed subcutaneously on nude mice. The interferon-alpha (IFN-α) alone group (J) received subcutaneous injection of 5000 IU/day for 14 days. The 5-fluorouracil (5-FU) alone group (H) received continuous injection of 7 mg/kg/mouse/day through a subcutaneously placed osmotic pump. The IFN-α + 5-FU group (F) received 5000 IU of IFN-α and 7 mg/kg of 5-FU per day. The control group (B) received saline alone. *$P < 0.05$ *versus* control (by ANOVA); **$P < 0.005$ *versus* control; ***$P < 0.0001$ *versus* control.

Table 3 Number of apoptotic cells, mitotic cells, BrdU-positive cells and cyclin A-positive cells in the tumor of HAK-1B cells in nude mice

Treatment group	Apoptotic cells	Mitotic cells	BrdU-positive cells	Cyclin A-positive cells
Control	9.7 ±1.1	58.6 ±30.6	11.0 ±3.93	38.1 ±7.8
IFN-α alone	10.5 ±3.5	50.0 ±30.2	14.2 ±7.8	45.1 ±13.3
5-FU alone	22.9 ±2.5*	52.8 ±20.3	82.9 ±50.4**	74.6 ±6.0*
IFN-α+5-FU	14.5 ±1.9**	26.3 ±14.5**	95.4 ±63.5*	96.9 ±13.8*

*$P < 0.01$ *versus* control; **$P < 0.02$ *versus* control.

5-FU, 5-fluorouracil; IFN-α, interferon-alpha.

group after the completion of treatment became 29.3% (Fig. 2) and 54.7% (Table 2), respectively, of the control. Conversely, the IFN-α or 5-FU alone groups did not show a remarkable decrease in the volume.

The number of apoptotic cells increased significantly in the combination group ($P < 0.02$) and the 5-FU alone group ($P < 0.01$) than in the control (Fig. 3, Table 3). In the combination group, mitotic cell numbers significantly decreased in comparison to the other groups ($P < 0.001$). The number of BrdU-positive cells significantly increased in the 5-FU alone group ($P < 0.02$) and the combination group ($P < 0.01$) in

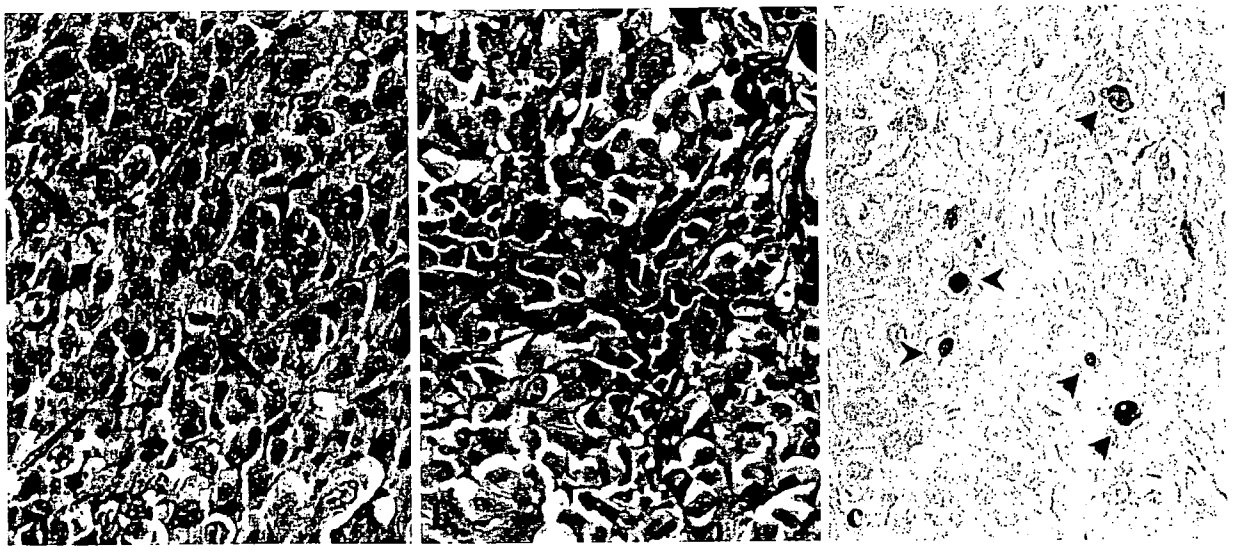

Figure 3 Photomicrograph of subcutaneous human hepatocellular carcinoma (HCC) tumors developed after injection of HAK-1B cells in nude mice. (a) A tumor in a control mouse showing thick trabecular arrangement of tumor cells with some mitotic figures (thick arrows). (b) A tumor in a mouse that received interferon-alpha (IFN-α) plus 5-fluorouracil (5-FU). There are many apoptotic tumor cells (thin arrows) characterized by cytoplasmic shrinkage and nuclear chromatic condensation. (c) The same tumor as shown in (b). There are some TUNEL-positive apoptotic cells showing brown nuclei (stained by TUNEL).

Table 4 Enzyme-linked immunosorbent assay (ELISA) of angiogenesis factors and artery-like blood vessel in the tumor of HAK-1B cells in nude mice

Treatment group	Levels in the tumor lysate (pg/mL)			Artery-like blood vessel
	bFGF	IL-8	VEGF	
Control	256.0 ±185.0	338.3 ±54.8	72.4 ±33.2	12.4 ±3.8
IFN-α alone	427.6 ±119.6*	687.9 ±117.6	141.4 ±129.0	15.2 ±1.7**
5-FU alone	172.3 ±185.0	441.5 ±183.6	75.6 ±57.0	9.6 ±3.1***
IFN-α+5-FU	309.4 ±186.5	608.7 ±228.1*	63.0 ±33.1	10.6 ±1.8****

*$P < 0.02$ *versus* control; **$P < 0.04$ *versus* control; ***$P < 0.05$ *versus* control; ****$P < 0.001$ *versus* control. Figures represent mean ± SD. IFN-α alone group received 5000 IU/mouse injection every day. 5-FU alone group received continuous administration of 180 μg (7 mg/kg)/mouse/day. IFN-α + 5-FU group received both. Control group received injection and continuous administration of saline.

5-FU, 5-fluorouracil; IFN-α, interferon-alpha.

comparison to the control (Fig. 4, Table 3). Cyclin A positive cells increased in the IFN-α alone group, the 5-FU alone group and then the combination group (Fig. 5, Table 3). In particular, the combination group presented twice or more than twice the number of positive cells than the control ($P < 0.01$). The combination group showed a synergic increase in the positive cells in comparison to the 5-FU alone group.

Density of artery-like blood vessels and expression of angiogenesis factors

In the IFN-α alone group, density of artery-like blood vessels increased (Table 4) and the expressions of angiogenesis factors increased (bFGF $P < 0.02$, VEGF and IL-8 not significant). In contrast, the combination group and the 5-FU alone group showed decreases in the density of artery-like blood vessels, but their relationship with angiogenesis factors was not clear.

IFNAR-2 expression *in vivo*

IFNAR-2 expression decreased in the IFN-α alone group and the combination group more than in the control, and the decrease was significant in the combination group ($P < 0.03$ *vs* control, Table 5).

DISCUSSION

Some studies reported that IFN directly suppresses tumor proliferation and at the same time augments the

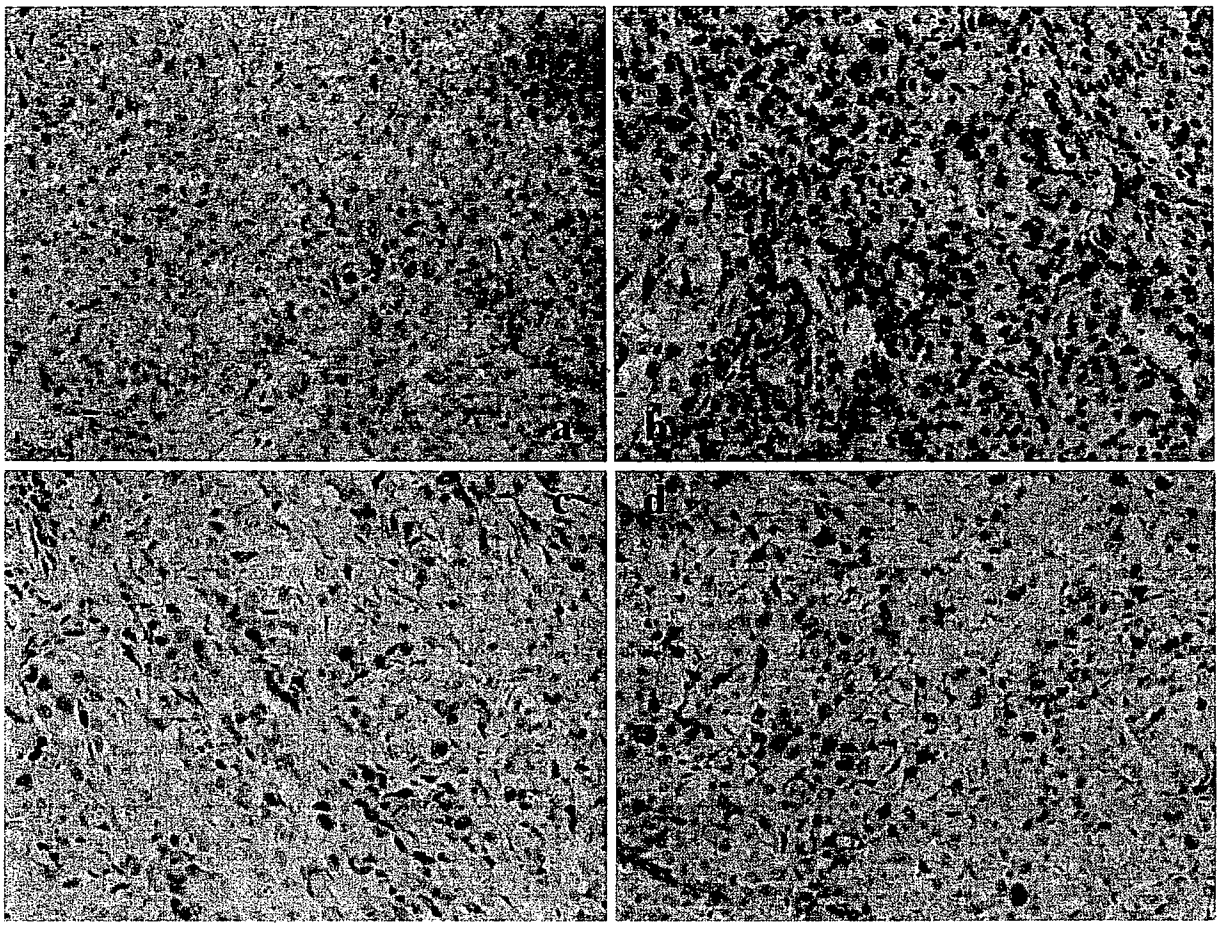

Figure 4 BrdU-positive cells in each of the three treatment groups: (a) control; (b) interferon-alpha (IFN-α) alone; (c) 5-fluorouracil (5-FU) alone; (d) IFN-α plus 5-FU (combination). The 5-FU alone group and the combination group had a larger number of positive cells.

Table 5 Enzyme-linked immunosorbent assay (ELISA) of the interferon-alpha (IFN-α) receptor IFNAR-2

Treatment group	IFNAR-2 levels in the tumor lysate (pg/mL)
Control	58.6 ± 30.6
IFN-α alone	49.9 ± 30.1
5-FU alone	52.8 ± 20.3
IFN-α + 5-FU	26.3 ± 14.5*

*$P < 0.03$ *versus* Control. IFN-α alone group received 5000 IU/mouse injection every day. 5-FU alone group received continuous administration of 180 μg (7 mg/kg)/mouse/day. IFN-α + 5-FU group received both. Control group received injection and continuous administration of saline.

5-FU, 5-fluorouracil.

suppressive effects of 5-FU on tumor growth, including apoptosis induction.[17,18] As the mechanism of this augmentation, several researchers reported that IFN-α acts on the metabolic pathway of 5-FU. Kase *et al.*[13] indicated that IFN-α enhances the antitumor effects by accelerating the metabolization of 5-FU into 5-fluorouridine (FUR). Schwartz *et al.*[11] considered that IFN-α increases the intracellular levels of the active metabolite of 5-FU through an increase of the activities of uridine phospholylase and pyrimidine nucleoside phospholylase. IFN is reported to augment the anabolism of 5-FU to its active metabolite fluorodeoxyuridine (FdUMP) which inhibits thymidine synthase (TS).[12] Guglielmi *et al.*[19] also showed that IFN-β enhanced the amount of tritiated 5-FU incorporated into nucleic acid. In our current study, suppression of tumor proliferation *in vitro* due to apoptosis induction was observed in both the IFN-α and 5-FU treatments, but a synergic effect in the combination treatment was not observed until the 96th hour of the culture. Conversely, in the experiment using nude mice, the IFN-α + 5-FU group showed a significant decrease in tumor volume in comparison to the control, IFN-α alone group and 5-FU

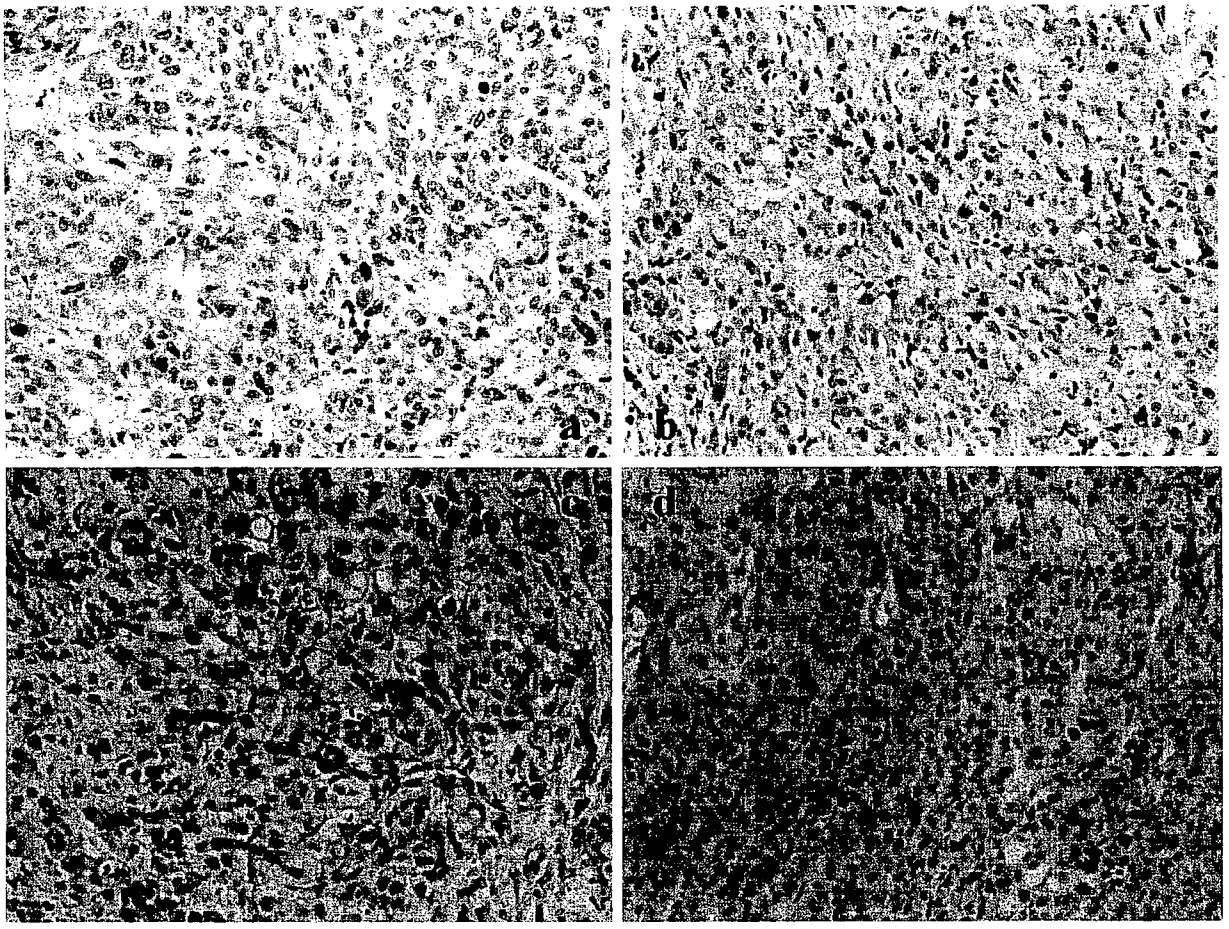

Figure 5 Cyclin A-positive cells in each of the three treatment groups: (a) control; (b) interferon-alpha (IFN-α) alone; (c) 5-fluorouracil (5-FU) alone; (d) IFN-α plus 5-FU (combination). The 5-FU alone group and the combination group had a larger number of positive cells.

alone group after the completion of treatment. The tumor volume in the combination group was 29.3% of the volume of the control, while the volumes in the IFN-α alone group and 5-FU alone group were almost the same as those in the control. The weight of the resected tumor in the combination group was 54.7% of the weight of the control. The discrepancy between tumor volume and weight may be attributed to the presence of necrotic areas in some tumors.

The IFN-α + 5-FU group also showed an increase in apoptotic cells and BrdU-positive cells and a decrease in mitotic count. These points indicate that the synergic suppressive effects on tumor proliferation would be expressed through apoptosis induction and arrest of the cell cycle at the S-phase. It is assumed that even in the 5-FU alone group, apoptosis and S-phase arrest would be induced but at a milder level than in the combination group and these levels were not sufficient enough to suppress tumor proliferation. Growth suppression effects of 5-FU via the cell cycle (e.g. G_0/G_1-phase arrest, G_1/S-phase arrest and G_2/M-phase arrest) have been reported on various cells.[20–25] In HAK-1B cells, 5-FU is thought to induce S-phase arrest. In our experiment, the 5-FU group and the combination group showed an increase of cyclin A-positive cells. Takeda *et al.*[20] examined cyclin A levels in several human colon cancer cell lines under 5-FU treatment and showed S-phase arrest and an increase in the cyclin A level that were dose-dependent to 5-FU. Their findings together with Kawashima *et al.*'s report[26] suggest the involvement of 5-FU in the changes of cyclin A level and cell cycle in the 5-FU alone group and the combination group. In the combination group, the addition of IFN-α, which potentially has the same growth inhibitory effects as 5-FU, resulted in synergic suppression effects on proliferation.

Synergic effects were observed in the *in vivo* experiment, whereas no additional or synergic effects were found in the *in vitro* combination group. In our *in vitro* study, the cells were exposed to the test drugs for 96 h, and during the same period our *in vivo* mice study showed no synergic decrease in the estimated tumor volume. In order to have synergic effects, longer exposure to IFN-α and 5-FU would be necessary or there would have to be an involvement of certain *in vivo*-specific actions such as the suppression of angiogenesis.

Angiogenesis is an essential factor in the growth and metastasis of solid tumors. Major angiogenesis factors are VEGF, bFGF and IL-8.[27,28] Solid tumors cannot grow beyond a few millimeters in size without angiogenesis.[29,30] There are several studies showing that IFN-α inhibits angiogenesis by downregulating angiogenesis factors such as VEGF, bFGF and IL-8.[31-35] For example, Dinney *et al.*[32] systematically administered IFN-α to a nude mice model of bladder tumor and reported the decrease of *in vivo* expression of bFGF and of blood vessel density in the tumors, which then resulted in the shrinkage of tumor size. In contrast, Hong *et al.*[36] found no relationship between the suppression of angiogenesis due to IFN and the expression of bFGF or VEGF. In the present study, IFN-α did not inhibit, but slightly promoted, angiogenesis through upregulating angiogenesis factors under the current experimental conditions. The reason for these contrary findings is unclear, and the action of IFN on angiogenesis of HCC tumors should be further evaluated with caution by using different types and doses of IFN and different HCC cell lines. In contrast, the 5-FU group and the combination group had a significant decrease in blood vessel counts in the tumor compared to the control. Decrease in bFGF expression was observed in the 5-FU group, suggesting that the antiangiogenesis effects of 5-FU may be mediated by downregulation of bFGF. In the combination group, a significant decrease in angiogenesis factor expression was not observed, and IL-8 significantly increased. Although the true mechanism is unknown, the following two possibilities should be considered: (i) suppression of another unknown angiogenesis factor would be involved in the mechanism of antiangiogenesis action of the combination treatment; and (ii) 5-FU or combination treatment could directly act on the blood vessels and inhibit neovascularization.

The effects of IFN-α are mediated through the interaction with the specific cell surface receptor, type I IFN receptor. This receptor consists of two chains, IFNAR-1 and IFNAR-2, and they can be present in different forms.[35,37-40] Yano *et al.*[14] reported strong expression of IFNAR-2 on the cell surface of HAK-1B. In our current study, the IFNAR-2 expression level decreased slightly in the IFN-α alone group and significantly in the combination group. This decrease in the combination group indicates that the ligand-receptor reaction occurs more frequently and this could induce ligand-induced downregulation of the receptor, as previously indicated by Lau *et al.*[41] and Maxwell *et al.*[42] Whether 5-FU could influence the ligand-receptor reaction should be further examined to clarify this point.

The combination of IFN-α and 5-FU did not produce additional or synergic antitumor effects *in vitro*, whereas the combination enhanced the antitumor effects *in vivo*. This *in vivo* mechanism of action would be related to the S-phase arrest in the cell cycle and the induction of apoptosis.

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インターフェロンの抗腫瘍効果

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索引用語：肝細胞癌，インターフェロン，PEG-インターフェロン，アポトーシス

1 はじめに

インターフェロン(IFN)は，抗ウイルス作用，細胞増殖抑制作用，血管新生抑制作用，免疫応答調節作用，MHC抗原発現調節作用など，多彩な生物活性を有するサイトカインであり，α，β，ωからなるⅠ型IFNと，γからなるⅡ型IFNに大きく分類される[1]．αやβなどのⅠ型IFNは，C型慢性肝炎などのウイルス性疾患，慢性骨髄性白血病や腎細胞癌などの腫瘍性疾患の治療薬として臨床的に使用されている[2]．

C型慢性肝炎は，長期的経過で肝硬変・肝細胞癌へと進展する例が多く，肝病変の進展阻止は重要な問題である．近年，C型慢性肝炎あるいは肝硬変に対してIFN療法を行うことにより，ウイルスの完全排除と関係なく，肝機能の改善や肝発癌率の低下が誘導されることが報告されている[3~5]．さらに，肝細胞癌の切除術後の再発防止に対するIFN投与の有用性や[6,7]，進行肝癌の治療に対するIFNと抗癌剤の併用療法の有用性[8,9]も報告されている．このように肝癌発生予防や治療に対するIFNの有用性が臨床的に明らかにされつつあるが，その作用メカニズムはいまだ明らかにされていない．臨床的に明らかな肝細胞癌の合併を認めないにもかかわらず，血清のα-fetoprotein (AFP)の持続高値を呈するC型慢性肝炎患者は，肝細胞癌発症のハイリスク群に位置する[10]．最近，これらの患者に対して，肝庇護剤を投与した場合，肝機能の改善はみられても，血清AFP値の低下は見られないのに対し，IFNを投与すると肝機能の改善と血清AFP値も有意に低下すると報告されている[11]．このAFP低下の機序も不明だが，もしかするとIFNが，臨床的に不顕性な肝癌細胞に直接的に作用を示し，その結果AFPの低下を誘導しているのかもしれない．このように，IFNによる直接的な増殖抑制作用が，IFNによる肝癌発症，再発抑制や肝癌治療にも関連している可能性が十分考えられる．

筆者らは，IFNの肝癌細胞に対する作用を明らかにするために，種々のIFNによる増殖

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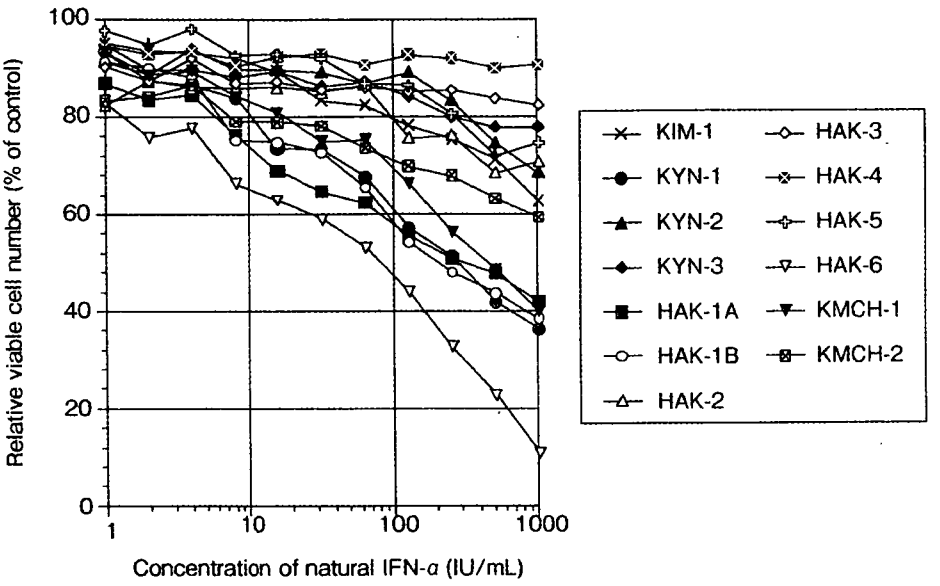

図1　ヒト天然型IFN-α製剤(OIF®)の13種類の肝癌細胞株に対する増殖抑制作用
13種類の肝癌細胞株を1～1,024 U/mLのIFN-α添加培地で培養し，96時間目にIFN-α非添加培養(コントロール)と比較した生細胞数の割合(%)を示す．

抑制作用やその機序解明などに関して，培養肝癌細胞を使用し検討を行っているが[12～17]，本稿では，そのデータの一部を以下に紹介する．

2 ヒト天然型IFN-αの肝癌細胞株に対するin vitroの増殖抑制作用

われわれは，当教室で独自に樹立・維持されている11種類の分化度の異なる肝細胞癌細胞株と2種類の混合型肝癌株の合計13株の肝癌の細胞株を使用して実験を行った．IFN-α，-βの作用発現には，AR-1鎖とAR-2鎖から構成されるⅠ型IFNレセプターの発現が前提となるが，これらの発現をmRNAレベルで確認し，AR2鎖に関しては，蛋白レベルでも，13株中12株の肝癌細胞に種々の程度に発現を確認している[12]．天然型IFN-α(1～1,024 IU/mL，OIF®)を13種類の肝癌細胞株に対し24～96時間接触させると，大部分の細胞株で時間依存性の細胞増殖抑制作用が認められた[12]．また，IFN-α接触後96時間目では，すべての細胞株で種々の程度に濃度依存性に細胞増殖が抑制された(図1)．IFN-αの増殖抑制作用に対する感受性と細胞株のオリジナル腫瘍の組織学的異型度との間に関連性は認めなかった．また，細胞表面のAR-2鎖の発現とIFN-αによる増殖抑制作用との関連性に関しては，AR-2鎖の発現が極端に低い細胞は，増殖抑制作用が乏しかったが，ある程度以上の発現がある細胞では，発現と増殖抑制作用とは必ずしも相関していなかった[12]．

3 IFN-αサブタイプの肝癌細胞株に対するin vitroの増殖抑制作用

IFN-αには，少なくとも13種類のサブタイプ遺伝子(α 1, 2, 4, 5, 6, 7, 8, 10, 13, 14, 16, 17, 21)がある．各サブタイプ分子が構造的に極めて類似しており，共通のレセプター分子を介して細胞内にシグナルを送るにもかかわらず，抗ウイルス作用，細胞増殖抑制作用などの生物学的作用や標的細胞特異性にはサブ

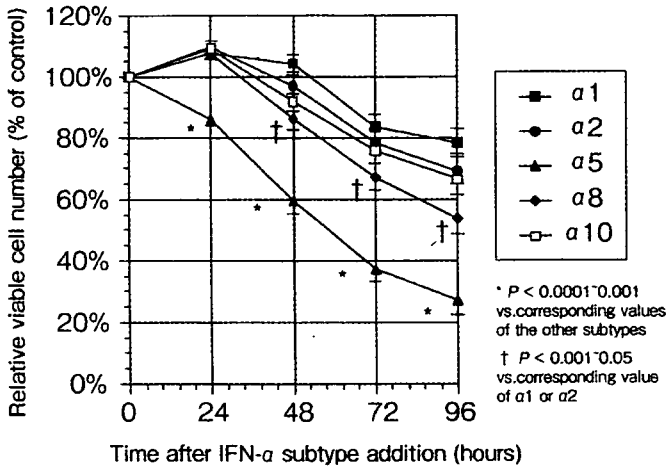

図2　5種類のIFN-αサブタイプ(α1, α2, α5, α8, α10)の肝癌細胞株に対する経時的な増殖抑制作用

13種類の肝癌細胞株を1,024 IU/mLの各種IFN-αサブタイプ添加培地で24, 48, 72あるいは96時間培養し，各種IFN-αサブタイプの各時点におけるIFN-α非添加培養(コントロール)と比較した生細胞数の割合(%)を算出した．さらに，IFN-αサブタイプ毎に13株の生細胞数の割合の平均値を算出しプロットしたものを図に示す．IFN-α5の平均値がいずれの測定時間でも最も低く，最も強い増殖抑制作用を示していた．値は，平均値±標準誤差を示す．

タイプ間で差があると言われている[18]．上記実験に使用した天然型IFN-αの成分は，約75%がα2で，残り25%がα8で構成されている．IFN-αのサブタイプによって肝癌細胞の増殖抑制作用に違いがないか，5種類のリコンビナントIFN-α1, 2, 5, 8, 10を使用し検討を行った．その結果，各IFN-αサブタイプの増殖抑制作用に対して13種類の細胞株は，それぞれ異なる感受性を示したが，特にα5とα8に高感受性を示す細胞株が多いことが判明した[14]．サブタイプ別の経時的な増殖抑制効果を13株の平均値で比較すると，α5が終始効果が最も強く，次いでα8, α10, α2, α1の順であった(図2)．α5の増殖抑制効果は，他のサブタイプより早期に出現し接触後24時間目から認められた．現在使用されているIFN-α製剤は，製剤毎に含有するIFN-αのサブタイプが異なり，したがって，肝癌細胞の増殖抑制能も異なる可能性が考えられる．次に，この点に関して検討を行った．

4　IFN-α製剤およびIFN-β製剤間の肝癌細胞株に対するin vitroの増殖抑制作用と増殖抑制機序の比較

最初に実験に使用したヒト天然型IFN-α(OIF®)に加え，コンセンサスIFN (rIFN-α Con1, Advaferon®)，IFN-α2b (Intron® A)，ペグ(PEG)-IFN-α2b (PegIntron®)の合計4種類のIFN-α製剤と，1種類の天然型IFN-β製剤(FERRON®)の13種類に肝癌細胞株に対する増殖抑制作用を比較検討した．1,024 IU/mLの各種IFN添加培地で96時間培養後に，IFN非添加培養(コントロール)と比べ生細胞数の割合が50%以下まで低下した細胞株の数は，ヒト天然型IFN-αでは，5株[12]，コンセンサスIFNでは，7株[17]，IFN-α2bとPEG-IFN-α2bでは，いずれも2株[15]，

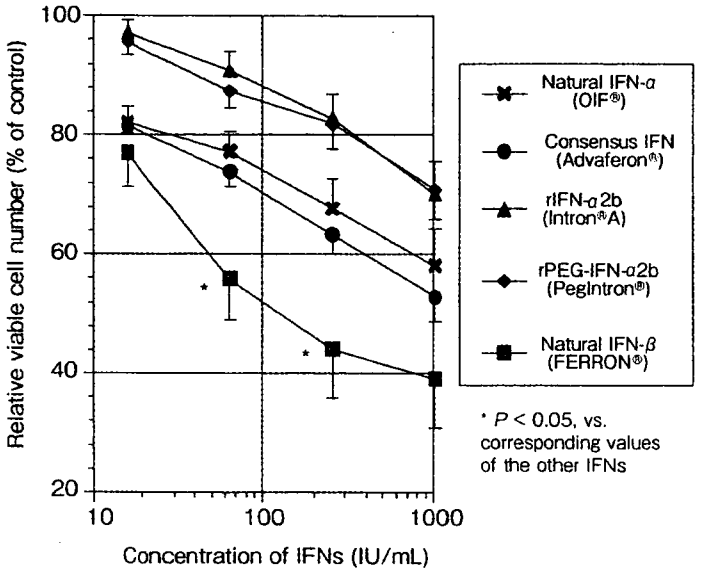

図3　4種類のIFN-α製剤(ヒト天然型IFN-α，コンセンサスIFN，IFN-α 2b，PEG-IFN-α 2b)と1種類の天然型IFN-β製剤の肝癌細胞株に対する増殖抑制作用

13種類の肝癌細胞株を16～1,024 IU/mLの各種IFN製剤添加培地で96時間培養し，各種IFN製剤に関してIFN-α非添加培養(コントロール)と比較した生細胞数の割合(%)を算出した．さらに，IFN製剤毎に13株の生細胞数の割合の平均値を算出しプロットしたものを図に示す．IFN-βの平均値がいずれの測定時間でも最も低く，最も強い増殖抑制作用を示していた．値は，平均値±標準誤差を示す．

天然型IFN-βでは，10株であった(未発表データ)．これらの細胞株の50%増殖抑制濃度(IC50)は，ヒト天然型IFN-αでは，86～466 IU/mL，コンセンサスIFNでは，128～804 IU/mL (0.128～0.804 ng/mL)，IFN-α 2bでは，628～919 IU/mL，PEG-IFN-α 2bでは，832～839 IU/mL，天然型IFN-βでは，15～153 IU/mLであり，IFN-α 2bとPEG-IFN-α 2bのIC50値は，他より高く，逆にIFN-βのIC50値は最も低かった．IFNの製剤別の増殖抑制作用を13株の平均値で比較すると，天然型IFN-β，コンセンサスIFN，天然型IFN-α，PEG-IFN-α 2b・IFN-α 2bの順に強い作用を認めた(図3)．特に，天然型IFN-βでは，経時的に増殖抑制作用が増大し，接触96時間後では，低濃度でも比較的強い増殖抑制効果が見られた．PEG-IFN-α 2bやIFN-α 2bの増殖抑制作用が比較的低いと言う結果は，上記の5種類のサブタイプの中でIFN-α 2の増殖抑制作用が比較的低いという結果とよく一致していた．

各種IFNを肝癌細胞の培地に添加し，48から72時間培養し細胞形態を観察すると細胞質の縮小や核の濃縮・核の断片化など，アポトーシスに特徴的な細胞像の出現が認められた．またこのような細胞からDNAを採取しアガロースゲル内で電気泳動すると，アポトーシスの生化学的なマーカーであるDNA ladderの形成が認められた(図4)．アポトーシス誘導は，使用したIFNの種類，濃度，そして細胞株により差を認めるものの，最低でも13株中10株で認められた[12, 15, 17]．IFN-α誘導性アポトーシスでは，caspase-9，caspase-8，caspase-7，caspase-3の活性化と伴に

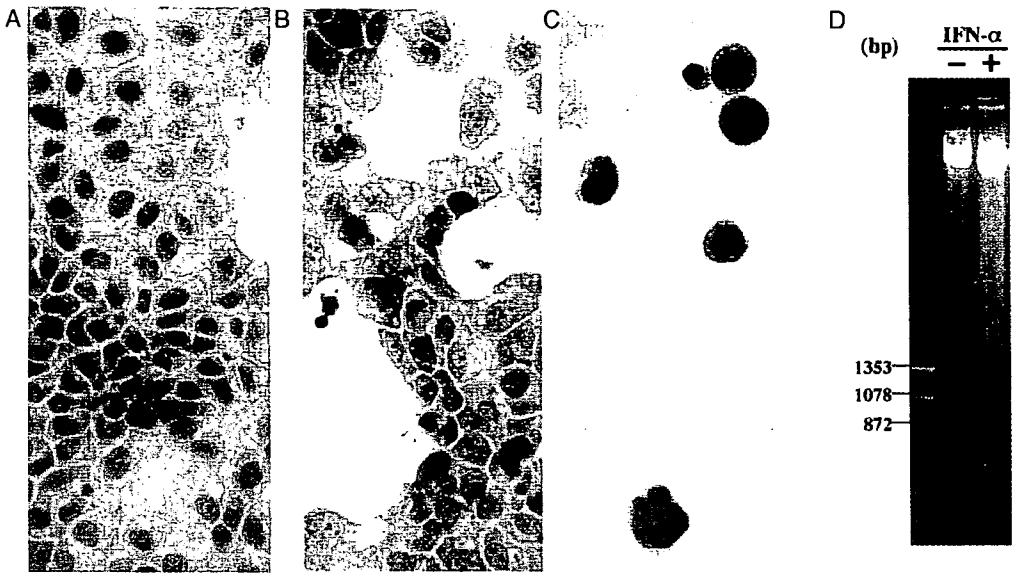

図4　ヒト天然型IFN-αの肝癌細胞株に対するアポトーシス誘導
A～CにIFN-α添加あるいは非添加培養時の肝細胞癌細胞株KIM-1株の細胞形態を示す(A：IFN-α非添加培養，200倍；B：1,000 IU/mL IFN-α添加培養，200倍；C：Bの強拡大，400倍，ヘマトキシリン・エオジン染色)．Dは，IFN-α添加によりアポトーシスの生化学的指標であるDNAラダーの出現を認める．

cytochrome cやSmac/DIABLOのミトコンドリアから細胞質への放出がみられ，ミトコンドリア系のアポトーシス誘導経路の関与が示唆されるが[13]，TRAILやTRAIL-R1, -R2などの発現亢進も見られており(未発表データ)デスリガンド-デスレセプターを介した経路の関与も考えられ，今後更なる検討が必要である．アポトーシス誘導以外の増殖抑制の機序としてすべての細胞株で細胞周期の進行停止誘導が認められ，S期での停止誘導が11株，G_2/M期での停止誘導が1株，G_1期での停止誘導が1株で認められた[12]．

5　IFN-α製剤製剤の肝癌細胞株に対するin vivoの増殖抑制作用と増殖抑制機序

肝細胞癌細胞株HAK-1B[19]をヌードマウスの皮下に接種し，約1週間後5～10 mmの腫瘍径の腫瘍が形成された時点から，各種IFN-α製剤を投与し*in vivo*における増殖抑制作用の検討を行った．IFN-αあるいはコンセンサスIFN-αに関しては，C型慢性肝炎患者の治療に使用される投与量にほぼ相当する量(臨床量)(天然型IFN-α：4,000 IU/mouse，2.0×10^5 IU/kg；コンセンサスIFN：0.01 μg/mouse，0.5 μg/kg)，その10倍量あるいは100倍量を14日間連日マウスの皮下に接種し，腫瘍の経時的な推定体積や，15日目に摘出された腫瘍の組織像を比較検討した．その結果，14日目の腫瘍体積は，IFN-αの臨床量投与によりIFN-αを投与しなかったマウス(コントロール)に比べ，天然型IFNで30％前後(未発表データ)，コンセンサスIFNで40％前後減少し[17]，100倍量投与したマウスではいずれのIFNでも腫瘍がほぼ消失した．コンセンサスIFNを投与されたマウスの腫瘍組織では，肝癌細胞のアポトーシス数が増加し，腫瘍内血管の減少も認めら