9 DiaZ. MO, Bohlander S, Allen G. Nomenclature of human interferon genes. *J. Interferon Res.* 1993; **13**: 243–4.
10 Henco K, Brosius J, Fujisawa A *et al.* Structural relationship of human interferon alpha genes and pseudogenes. *J. Mol. Biol.* 1985; **185**: 227–60.
11 Fish EN, Banerjee K, Stebbing N. Human leukocyte interferon subtypes have different antiproliferative and antiviral activities on human cells. *Biochem. Biophys. Res. Commun.* 1983; **112**: 537–46.
12 Foster GR, Rodrigues O, Ghouze F *et al.* Different relative activities of human cell-derived interferon-alpha subtypes: IFN-alpha 8 has very high antiviral potency. *J. Interferon Cytokine Res.* 1996; **16**: 1027–33.
13 Yamaoka T, Kojima S, Ichi S, Kashiwazaki Y, Koide T, Sokawa Y. Biologic and binding activities of IFN-alpha subtypes in ACHN human renal cell carcinoma cells and Daudi Burkitt's lymphoma cells. *J. Interferon Cytokine Res.* 1999; **19**: 1343–9.
14 Yanai Y, Sanou O, Yamamoto K, Yamauchi H, Ikegami H, Kurimoto M. The anti-tumor activities of interferon (IFN)-alpha in chronic myelogenous leukaemia (CML)-derived cell lines depends on the IFN-alpha subtypes. *Cancer Lett.* 2002; **185**: 173–9.
15 Yamamoto S, Yano H, Sanou O, Ikegami H, Kurimoto M, Kojiro M. Different antiviral activities of IFN-α subtypes in human liver cell lines: synergism between IFN-α2 and IFN-α8. *Hepatol. Res.* 2002; **24**: 99–106.
16 Foster GR, Masri SH, David R *et al.* IFN-alpha subtypes differentially affect human T cell motility. *J. Immunol.* 2004; **173**: 1663–70.
17 Grumbach IM, Fish EN, Uddin S *et al.* Activation of the Jak-Stat pathway in cells that exhibit selective sensitivity to the antiviral effects of IFN-beta compared with IFN-alpha. *J. Interferon Cytokine Res.* 1999; **19**: 797–801.
18 Yanai Y, Horie S, Yamamoto K *et al.* Characterization of the antitumor activities of IFN-alpha 8 on renal cell carcinoma cells in vitro. *J. Interferon Cytokine Res.* 2001; **21**: 1129–36.
19 Yonehara S, Yonehara-Takahashi M, Ishii A, Nagata S. Different binding of human interferon alpha 1 and alpha 2 to common receptors on human and bovine cells. Studies with recombination interferons produced in Escherichia coli. *J. Biol. Chem.* 1983; **258**: 9046–9.
20 Murakami T. Establishment and characterization of human hepatoma cell line (KIM-1). *Acta Hepatol. Jpn* 1984; **25**: 532–9.
21 Yano H, Kojiro M, Nakashima T. A new human hepatocellular carcinoma cell line (KYN-1) with a transformation to adenocarcinoma. *In Vitro Cell. Dev. Biol.* 1986; **22**: 637–46.
22 Yano H, Maruiwa M, Murakami T *et al.* A new human pleomorphic hepatocellular carcinoma cell line, KYN-2. *Acta Pathol. Jpn* 1988; **38**: 953–66.
23 Murakami T, Maruiwa M, Fukuda K, Kojiro M, Tanaka M, Tanikawa K. Characterization of a new human hepatoma cell line (KYN-3) derived from the ascites of the hepatoma patient [Abstract]. *Jpn. J. Cancer Res.* 1988 Proceedings of the Japanese Cancer Association: 292.
24 Yano H, Iemura A, Fukuda K, Mizoguchi A, Haramaki M, Kojiro M. Establishment of two distinct human hepatocellular carcinoma cell lines from a single nodule showing clonal dedifferentiation of cancer cells. *Hepatology* 1993; **18**: 320–7.
25 Haramaki M, Yano H, Iemura A *et al.* A new human hepatocellular carcinoma cell line (HAK-2) forms various structures in collagen gel matrices. *Hum. Cell* 1997; **10**: 183–92.
26 Murakami T, Yano H, Maruiwa M, Sugihara S, Kojiro M. Establishment and characterization of a human combined hepatocholangiocarcinoma cell line and its heterologous transplantation in nude mice. *Hepatology* 1987; **7**: 551–6.
27 Yano H, Iemura A, Haramaki M *et al.* A human combined hepatocellular and cholangiocarcinoma cell line (KMCH-2) that shows the features of hepatocellular carcinoma or cholangiocarcinoma under different growth conditions. *J. Hepatol.* 1996; **24**: 413–22.
28 Castelruiz Y, Larrea E, Boya P, Civeira MP, Prieto J. Interferon alfa subtypes and levels of type I interferons in the liver and peripheral mononuclear cells in patients with chronic hepatitis C and controls. *Hepatology* 1999; **29**: 1900–4.
29 Arora T, Floyd-Smith G, Espy MJ, Jelinek DF. Dissociation between IFN-alpha-induced anti-viral and growth signaling pathways. *J. Immunol.* 1999; **162**: 3289–97.
30 Aguet M, Grobke M, Dreiding P. Various human interferon alpha subclasses cross-react with common receptors: their binding affinities correlate with their specific biological activities. *Virology* 1984; **132**: 211–16.
31 Hisaka T, Yano H, Ogasawara S *et al.* Interferon-αCon1 suppresses proliferation of liver cancer cell lines in vitro and in vivo. *J. Hepatol.* 2004; **41**: 782–9.
32 Klein SB, Blatt LM, Taylor MW. Cell surface binding characteristics correlate with consensus type I interferon enhanced activity. *J. Interferon Cytokine Res.* 1996; **16**: 1–6.

Liver International 2006; 26: 964–975

Liver International
DOI: 10.1111/j.1478-3231.2006.01321.x

Clinical Studies

Growth inhibitory effects of pegylated IFN α-2b on human liver cancer cells *in vitro* and *in vivo*

Yano H, Ogasawara S, Momosaki S, Akiba J, Kojiro S, Fukahori S, Ishizaki H, Kuratomi K, Basaki Y, Oie S, Kuwano M, Kojiro M. Growth inhibitory effects of pegylated IFN α-2b on human liver cancer cells *in vitro* and *in vivo*. Liver International 2006: 26: 964–975

Abstract: *Purpose:* We investigated the effects of pegylated IFN-α2b (PEG-IFN-α2b) on the growth of human liver cancer cells. *Methods:* The effect of PEG-IFN-α2b on the proliferation of 13 liver cancer cell lines was investigated *in vitro*. Chronological changes in growth and IFN-α receptor-2 (IFNAR-2) expression were monitored in hepatocellular carcinoma (HCC) cells (HAK-1B) cultured with PEG-IFN-α2b. After HAK-1B cells were transplanted into nude mice, various doses of PEG-IFN-α2b or IFN-α2b were administered, and tumor volume, weight, histology, and IFNAR-2 expression were examined. *Results:* PEG-IFN-α2b inhibited the growth of nine cell lines with apoptosis in a dose- and time-dependent manner. Continuous contact with PEG-IFN-α2b induced time-dependent growth inhibition and down-regulation of IFNAR-2 expression. PEG-IFN-α2b induced a dose-dependent decrease in tumor volume and weight, a significant increase of apoptotic cells, and a decrease in IFNAR-2 expression in the tumor. The clinical dose for chronic hepatitis C was also effective. The antitumor effect of PEG-IFN-α2b was significantly stronger than that of non-PEG-IFN-α2b *in vivo*. *Conclusions:* Continuous contact with PEG-IFN-α2b induces strong antitumor effects and the down-regulation of IFNAR-2 in HCC cells. The data suggest potential clinical application of PEG-IFN-α2b for the prevention and treatment of HCC.

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Key words: apoptosis – hepatocellular carcinoma – nude mouse – pegylated interferon – receptor

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Received 17 March 2006,
accepted 17 May 2006

Interferon-α (IFN-α) is a multifunctional cytokine that possesses antiviral activity, antiproliferative activity, various immunoregulatory activities, antitelomerase activity, and antiangiogenesis activity (1–3). The antiviral activity of IFN-α has attracted much attention, and IFN-α preparations have been used in the treatment of hepatitis B virus- and hepatitis C virus (HCV)-related chronic hepatitis in many countries (4). Recently, IFN-α has been shown to possess highly suppressive effects on hepatocellular carcinogenesis and the recurrence of hepatocellular carcinoma (HCC) after curative treatment for HCC in patients with virus-related chronic hepatitis (5–10). However, the mechanisms of these actions have not yet been clarified. We previously reported that human lymphoblastoid IFN-α derived from Sendai virus-induced BALL-1 cells (BALL-1 IFN-α) directly suppressed the cell proliferation of 13 liver cancer cell lines to various degrees by inhibiting cell cycle progression with or without apoptosis *in vitro* (11). Recently, we also showed that consensus IFN-α at or close to the clinical dose used in treatment for HCV-related chronic hepatitis suppressed HCC growth in nude mice (12). This suggests that the direct antiproliferative action of IFN-α may be involved in the suppressive mechanisms of IFN-α on hepatocellular carcinogenesis. In clinical practice, IFN-α alone or in combination with other anticancer drugs such as 5-fluorouracil has been used in the treatment of malignant diseases including leukemia, renal cancer (4, 13) and advanced HCC (14).

Pegylated IFN α-2b (PEG-IFN-α2b) is a covalent conjugate of recombinant IFN-α2b with a monomethoxy polyethylene glycol (PEG) in a 1:1 molar ratio that produces a 31 000-Da molecule (15). PEG conjugation increases the size of the molecule. Therefore, the absorption of the pegylated molecule is slower, its serum half-life is longer, and its rate of clearance from the plasma is lower than that of the unmodified molecule. PEG-IFN-α2b thereby increases patient exposure to IFN-α2b and requires less frequent administration (15). Clinical trials in patients with chronic hepatitis C suggest that PEG-IFN-α preparations produce more potent therapeutic effects with or without ribavirin than do non-PEG-IFN-α preparations (15–20). However, whether or not PEG-IFN preparations are superior to non-PEG-IFN preparations in terms of suppressive effects on hepatocellular carcinogenesis and HCC growth has not been clarified. In addition, there have been no basic *in vitro* or *in vivo* studies that evaluate the efficacy of PEG-IFN-α2b on HCC cells. In the current study, we examined the *in vitro* and *in vivo* antitumor effects of PEG- and non-PEG-IFN-α2b on liver cancer cell lines by using several PEG-IFN-α2b concentrations including a low dose that is close to the clinical dose. We also examined the expression of type I IFN receptor 2 (IFNAR-2) subunit and its relationship with antitumor effects on HCC cells under the condition of continuous contact with PEG-IFN-α2b.

Materials and methods

Cell lines and cell culture

This study used 11 HCC cell lines (KIM-1, KYN-1, KYN-2, KYN-3, HAK-1A, HAK-1B, HAK-2, HAK-3, HAK-4, HAK-5, and HAK-6) and two human combined hepatocellular and cholangiocarcinoma (CHC) cell lines (KMCH-1 and KMCH-2). These HCC and CHC cell lines were originally established in our laboratory, and each cell line retains the morphological and functional features of the original tumor as described elsewhere (11, 21–29).

The cells were grown in Dulbecco's Modified Eagle Medium (Nissui Seiyaku Co., Tokyo, Japan) supplemented with 2.5% heat-inactivated (56 °C, 30 min) fetal bovine serum (FBS, Bioserum, Vic, Australia), 100 U/ml penicillin, 100 μg/ml streptomycin (GIBCO BRL/Life Technologies Inc., Gaithersburg, MD) and 12 mmol/l sodium bicarbonate, in a humidified atmosphere of 5% CO_2 in air at 37 °C.

IFN and reagents

PEG-IFN-α2b (PEG Intron®) and IFN-α2b (Intron®A) were kindly provided by Schering-Plough K.K. (Osaka, Japan). The specific activity of PEG-IFN-α2b was 6.4×10^7 IU/mg protein and that of IFN-α2b was 2.6×10^8 IU/mg protein.

Anti-bromodeoxyuridine (BrdU) antibody and fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (FITC-GAM) were purchased from BD Biosciences (San Jose, CA); control normal mouse IgG_1, from DAKO (Glostrup, Denmark); rat antibody against mouse endothelial cells (anti-CD34, clone MEC14.7), from Serotec Co., Oxford, UK; mouse monoclonal antibody against human α-smooth muscle actin (SMA) that cross-reacts with mouse α-SMA (clone 1A4), from Immunon (Pittsburgh, PA); rabbit antibody against vimentin fragment (V1) (caspase-9 activation state antibody (30)), from Medical & Biological Laboratories Co. Ltd. (Nagoya, Japan); mouse monoclonal antibody against human IFN α/β receptor chain 2, from Chemicon International Inc. (Temecula, CA); and mouse monoclonal antibody against human epidermal growth factor (EGF) receptor, from Upstate Biotechnology Incorporated (Lake Placid, NY).

Effects of PEG-IFN-α2b and IFN-α2b on the proliferation of HCC and CHC cell lines *in vitro*

The effects of PEG-IFN-α2b on the growth of the cultured cells were examined with colorimetry using 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay kits (Chemicon International Inc.) as described elsewhere (11, 12). Briefly, the cells ($1.5–8 \times 10^3$ cells per well) were seeded on 96-well plates (Nunc Inc., Roskilde, Denmark), cultured for 24 h, and the culture medium was changed to a new medium with or without PEG-IFN-α2b (16, 64, 256, 1024, or 4096 IU/ml). After culturing for 24, 48, 72 or 96 h, the number of viable cells was measured with ImmunoMini NJ-2300 (Nalge Nunc International, Tokyo, Japan) by setting the test wavelength at 570 nm and the reference wavelength at 630 nm. To keep the optical density within linear range, all experiments were performed while the cells were in the logarithmic growth phase. The effects of IFN-α2b on the growth of the cell lines were also examined in the same manner.

Morphological observation

For morphological observation under a light microscope, cultured cells were seeded on Lab-

Tek tissue culture chamber slides (Nunc Inc.), cultured with or without PEG-IFN-α2b (256, 1024 or 4096 IU/ml) for 72 h, fixed for 10 min in Carnoy's solution, and stained with hematoxylin–eosin (HE).

Quantitative analysis of PEG-IFN-α2b-induced apoptosis *in vitro*

The cells cultured with or without 1000 IU/ml PEG-IFN-α2b for 72 h were stained with the Annexin V-EGFP (enhanced green fluorescent protein) Apoptosis Detection Kits (Medical & Biological Laboratories Co., Ltd.) according to the manufacturer's protocol. After staining, the cells were analyzed using a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA), and Annexin V-EGFP-positive apoptotic cell rate was determined.

Effects of PEG-IFN-α2b on the proliferation and expression of the IFNAR-2 subunit

To investigate the expression of the IFNAR-2 subunit after continuous contact of PEG-IFN-α2b as well as its relationship with antiproliferative effects, HAK-1B cells were cultured with medium alone (Control) or medium containing 1000 IU/ml of PEG-IFN-α2b for 0, 3, 24, 48, 72, 96, 144, 192 or 240 h. The viable cell number and the cell surface expression of the IFNAR-2 subunit were examined. The cell surface expression of the IFNAR-2 subunit was analyzed using flow cytometry with the technique described elsewhere (11) with slight modification. Briefly, the cells were reacted with anti-IFN α/β receptor chain 2 antibody (final concentration, 2.5 μg/ml) or control antibody for 1 h, washed once, incubated with 4 μl of FITC-GAM for 30 min, washed once, fixed in 4% paraformaldehyde for 10 min, washed, and analyzed with a FACScan. The expression levels were compared according to the mean channel number. As an internal control to confirm that cell surface protein level on HAK-1B cells treated with or without 1000 IU/ml of PEG-IFN-α2b is constant, EGF receptor expression was measured on the cells cultured for 240 h in the same manner. After culturing for 72 h, cell cycle analysis was also performed in HAK-1B cells cultured with or without 1000 IU/ml of PEG-IFN-α2b with the technique described elsewhere (11). Briefly, cells were labeled with 10 mM BrdU (Sigma Chemical Co., St Louis, MO) for 30 min, harvested, fixed in 70% cold ethanol at 4 °C overnight, stained with anti-BrdU and propidium iodide (Sigma Chemical Co.), and analyzed by using a FACScan.

Effects of PEG-IFN-α2b and IFN-α2b on HCC cell proliferation in nude mice

Cultured HAK-1B (10^7 cells/mouse) was subcutaneously (sc) injected into the backs of 5-week-old female BALB/c athymic nude mice (Clea Japan Inc., Osaka, Japan). Five to seven days later when the largest diameter of the tumor reached approximately 5–10 mm (Day 0), the mice were divided into seven groups ($n = 6$–9 each) in the first experiment (Experiment #1) and into five groups ($n = 7$ or 8 each) in the second experiment (Experiment #2) in a manner to equalize the mean tumor diameter of every group. In Experiment #1, each mouse received a sc injection of 0.1 ml of medium alone (Control), medium containing 640, 6400, 64 000, or 640 000 IU of PEG-IFN-α2b, or medium containing 6400 or 64 000 IU of IFN-α2b, twice a week for two consecutive weeks (Day 1, Day 4, Day 8, and Day 11). Experiment #2 was conducted in the same manner but 640 and 6400 IU/mouse of PEG-IFN-α2b and the same doses of IFN-α2b were used. The aim of Experiment #2 was to examine the reproducibility of the antitumor effects of PEG-IFN-α2b at low concentrations and to compare this activity to that of IFN-α2b. The clinical dose of PEG-IFN-α2b in chronic hepatitis C treatment is 9.6×10^4 IU/kg and is three times the lowest dose (3.2×10^4 IU/kg) in the experiment. During this 2-week period, tumor size was measured in two directions using calipers on the first and second days of sc injection (Day 1 and Day 2) and then once every 2 days until Day 14, and tumor volume (mm^3) was estimated using the equation 'length × (width)^2 × 0.5'. Mouse body weight was measured on Day 0, Day 8, and Day 14. On Day 15, the mice were sacrificed and the tumors were resected and weighed and used for morphological studies (e.g., HE staining and immunohistochemistry) and ELISA analysis. Every mouse received an intraperitoneal injection of 1 mg of BrdU 30 min before sacrifice.

The animals received human care according to the criteria outlined in the 'Guide for the Care and Use of Laboratory Animals' prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985).

Morphological examination of the subcutaneous tumors of nude mice

The number of cells showing the characteristics of apoptosis (e.g., cytoplasmic shrinkage, chromatin condensation, and nuclear fragmentation) was counted in ten 0.25 mm^2 areas within an HE-stained specimen of the first experiment, and the

average number per area was obtained. The appearance of apoptotic cells was confirmed by the immunohistochemical detection of vimentin fragment (V1), i.e., a marker for caspase-9 activation (30), with the specific antibody and HistoMouse™-plus kits (Zymed Laboratories Inc., CA). The specimens were also immunostained for incorporated BrdU using BrdU Staining Kits (Oncogene Research Products, Boston, MA), and the average number of positive cells per area was obtained as described above. In addition, double-immunostaining was performed with anti-mouse endothelial cell antibody, anti-human α-SMA antibody, Histofine simple stain mouse MAX-PO (Rat) kits (Nichirei, Tokyo, Japan), and HistoMouse™-plus kits to detect artery-like blood vessels as described in our previous report (12, 31). The number of blood vessels in the tumor nodule was counted on each specimen. The size of the counted area was traced and measured using TurboCAD software (IMSI, Novato, CA). From the number of vessels per unit area (mm^2) obtained, the group mean was obtained for group comparison.

ELISA

The tumors were cut into pieces, and an appropriate amount was homogenized in 500 µl of ice-cold Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline containing 100 µg/ml phenymethylsulfonyl fluoride using a pellet pestle. The mixture was centrifuged for 10 min (12000 g, 4 °C), and the supernatant was stored at −20 °C until use. The amount of the IFNAR-2 subunit in the supernatant was measured using ELISA kits (Otsuka Pharmaceutical Co. Ltd., Tokyo, Japan). The amount of tissue protein was determined using a BCA protein assay reagent (Pierce, Rockford, IL).

Statistics

Comparisons of estimated tumor volume and colorimetric cell growth were performed using two-factor factorial ANOVA and Student's *t*-test, respectively. The other data comparisons were performed using the Mann–Whitney *U*-test.

Results

Effects of PEG-IFN-α2b on liver cancer cell proliferation *in vitro*

Twenty-four hours after the addition of 4096 IU/ml of PEG-IFN-α2b, mild increase in the relative viable cell number occurred in 10 cell lines (all cell lines except HAK-1B, HAK-6, and KMCH-1). However, after 72 h or later, a 10% or more decrease in the cell number occurred in 12 cell lines (Fig. 1A). In HAK-3, proliferation was not suppressed but slightly promoted up to 96 h of PEG-IFN-α2b contact. In HAK-2 and HAK-4, proliferation was suppressed up to 72 h and the cell number reached a plateau thereafter. In the other 10 cell lines, proliferation was suppressed to varying degrees up to 96 h.

Ninety-six hours after the addition of PEG-IFN-α2b, the relative viable cell number was suppressed in nine cell lines (all cell lines except HAK-2, HAK-3, HAK-4, and KMCH-2) in a dose-dependent manner (Fig. 1B). In four cell lines (KYN-2, HAK-1B, KYN-1, and KIM-1), the number was suppressed to 50% or less with 4096 IU/ml of PEG-IFN-α2b, and the 50% inhibitory concentration (IC50) was 831.8 IU/ml for KYN-2, 839.0 IU/ml for HAK-1B, 1298.6 IU/ml for KYN-1, and 3396.4 IU/ml for KIM-1. The IC50 of non-PEG-IFN-α2b in the four cell lines was 918.5, 627.7, 1237.7, and 2617.8 IU/ml, respectively, which was not significantly different from that of PEG-IFN-α2b (paired Student's *t*-test). No relationship was detected between the histological differentiation level of the original tumor and sensitivity to the antiproliferative effect of PEG-IFN-α2b.

Seventy-two hours after adding 4096 IU/ml of PEG-IFN-α2b, 10 cell lines (all cell lines except HAK-2, HAK-3, and KMCH-2) presented characteristics of apoptosis, e.g., cytoplasmic shrinkage, chromatin condensation, and nuclear fragmentation, to various degrees (Fig. 2).

Quantitative analysis of Annexin V-EGFP-positive apoptotic cells revealed that the appearance of apoptosis was significantly higher in the cultures with 1000 IU/ml of PEG-IFN-α2b than those without PEG-IFN-α2b in nine cell lines (Table 1).

Effects of PEG-IFN-α2b on the proliferation and expression of IFNAR-2 *in vitro*

With continuous contact of PEG-IFN-α2b up to 240 h, the expression of IFNAR-2 in HAK-1B cells was significantly down-regulated at 3 h compared with the Control, then significantly up-regulated at 48 h, and significantly down-regulated in the period between 96 and 240 h (Fig. 3A). To check the specificity of the down-regulation of IFNAR-2 expression, the expression of EGF receptor was also analyzed at 240 h. The mean channel numbers of HAK-1B cells cultured with and without 1000 IU/ml of PEG-IFN-α2b were 10.1 ± 0.4 and 10.6 ± 0.9, respectively, and there was no significant difference in EGF recep-

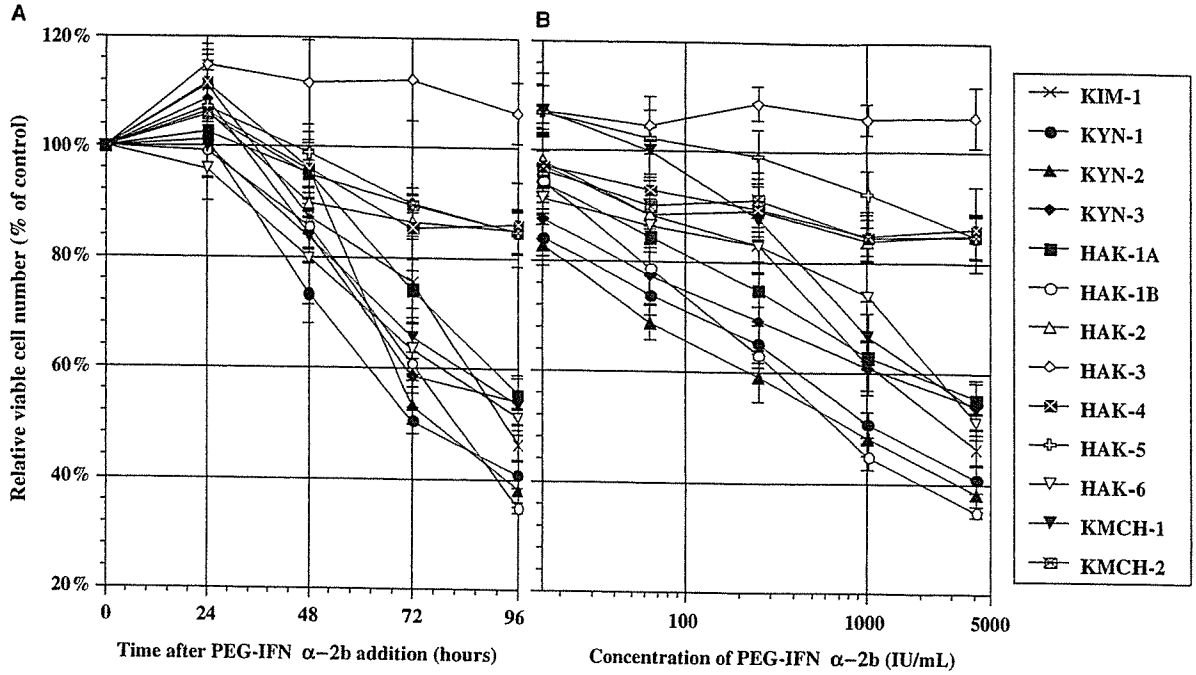

Fig. 1. Antiproliferative effect of pegylated IFN-α2b (PEG-IFN-α2b). (A) Chronological changes in relative viable cell number (% of the control) after adding 4096 IU/ml of PEG-IFN-α2b. Growth was suppressed with time in 10 cell lines. (B) 96 h after adding 16, 64, 256, 1024, or 4096 IU/ml of PEG-IFN-α2b. Cell proliferation was suppressed in a dose-dependent manner in nine cell lines. The suppression was significant ($P<0.001$–0.05) in the ranges of 16–4096 IU/ml of PEG-IFN-α2b in KYN-1, KYN-2, and HAK-6; 64–4096 IU/ml in KYN-3, HAK-1A, HAK-1B, and HAK-2; 256–4096 IU/ml in KIM-1 and KMCH-1; 1024–4096 IU/ml in KMCH-2; and at 4096 IU/ml in HAK-5 (Student's *t*-test). Eight samples were used in each experiment ($n = 8$). The experiment was repeated at least three times for each cell line. The figures represent average ± SE of the experiments.

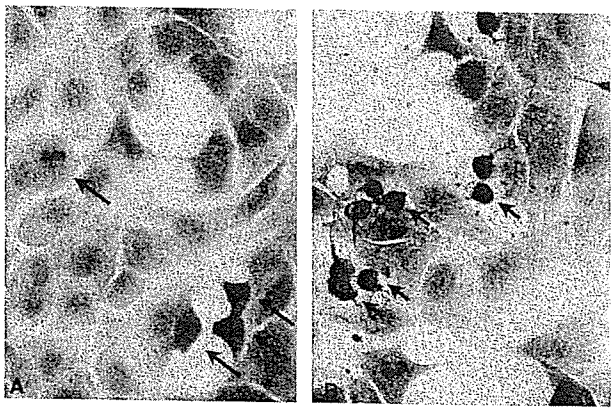

Fig. 2. Photomicrograph of HAK-1B cells cultured for 72 h on a Lab-Tek Chamber slide. (A) Without pegylated IFN-α2b (PEG-IFN-α2b) in culture medium. Some mitotic figures (long arrows) were noted. (B) With 4096 IU/ml of PEG-IFN-α2b in culture medium. Apoptotic cells (short arrows) characterized by cytoplasmic shrinkage, chromatic condensation and nuclear fragmentation were noted (hematoxylin–eosin staining, ×200).

Table 1. Quantitative analysis of apoptosis induced by PEG-IFN-α2b in 13 liver cancer cell lines

	Annexin V-EGFP-positive apoptotic cells (%)	
Cell line	Control	PEG-IFN-α2b
KIM-1	5.9 ± 0.2	28.0 ± 0.7†
KYN-1	4.7 ± 1.0	6.6 ± 0.5
KYN-2	0.6 ± 0.1	3.1 ± 0.7†
KYN-3	14.2 ± 2.4	21.5 ± 1.0*
HAK-1A	8.6 ± 0.3	14.8 ± 0.4†
HAK-1B	5.4 ± 0.4	25.0 ± 0.5†
HAK-2	0.5 ± 0.1	0.2 ± 0.0
HAK-3	3.2 ± 0.4	4.7 ± 0.6
HAK-4	4.6 ± 1.0	9.2 ± 0.3†
HAK-5	5.8 ± 0.1	9.1 ± 0.3†
HAK-6	13.6 ± 0.7	31.4 ± 0.4†
KMCH-1	2.9 ± 0.1	15.8 ± 0.5†
KMCH-2	5.3 ± 0.4	3.6 ± 0.6

Cells were cultured with medium alone (Control) or medium with 1000 IU/ml of PEG-IFN-α2b. Apoptosis was measured by Annexin V-EGFP staining. The rates of Annexin V-EGFP-positive apoptotic cell were shown as average ± SE. Five samples were used in each experiment. *$P<0.05$, vs corresponding control value. †$P<0.01$, vs corresponding control value. PEG-IFN-α2b, pegylated IFN-α2b; EGFP, enhanced green fluorescent protein.

tor expression. The relative viable cell number decreased in a time-dependent manner up to 240 h (Fig. 3A).

The cell cycle analysis shows that the number of HAK-1B cells at the S phase and G_2/M phase increased and decreased, respectively, with continuous contact of PEG-IFN-α2b, and this indicates the induction of S-phase arrest by PEG-IFN-α2b (Fig. 3B). In addition, the number of cells at the preG_1 phase increased with continuous contact of PEG-IFN-α2b, and this indicated the induction of apoptosis.

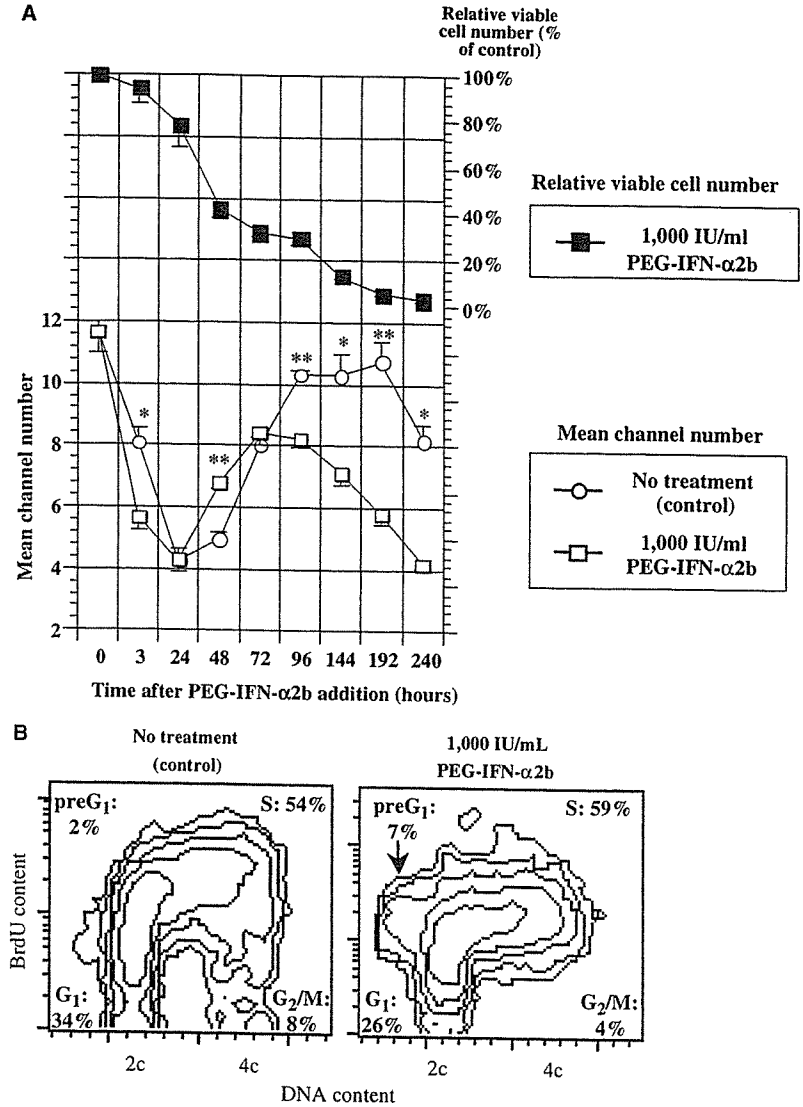

Fig. 3. Effects of 1000 IU/ml of pegylated IFN-α2b (PEG-IFN-α2b) on growth and IFN-α receptor-2 (IFNAR-2) expression in HAK-1B cells. (A) Time-course changes in relative viable cell number (% of Control) and IFNAR-2 expression before and after 1000 IU/ml of PEG-IFN-α2b addition. Cells reacted with anti-IFNAR-2 antibody or normal mouse IgG (control antibody) were stained with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin and analyzed using flow cytometry. The expression levels were compared according to the mean channel numbers that were calculated as the difference between the mean channel number in the cells stained with anti-IFNAR-2 antibody and that stained with normal mouse IgG (control antibody). The figures represent the average ± SE of at least two independent experiments, and each experiment used three to five samples for each measurement. $^{*}P<0.01$, vs continuous PEG-IFN-α2b contact. $^{**}P<0.001$, vs continuous PEG-IFN-α2b contact. (B) Cell cycle analysis. HAK-1B cells were cultured with 1000 IU/ml of PEG-IFN-α2b or medium alone (Control) for 72 h. The cells were labeled with 10 mM bromodeoxyuridine (BrdU) for 30 min, fixed, stained with anti-BrdU and propidium iodide, and analyzed using a FACScan. The contour plots are shown. The arrow shows the area of the preG$_1$ phase. The experiments were repeated twice, and almost identical results were obtained.

Effects of PEG-IFN-α2b on HCC cell proliferation in nude mice

Chronological changes in estimated tumor volume after subcutaneous injection of cultured HAK-1B cells to nude mice are summarized in Fig. 4. Dose-dependent suppression of tumor volume was observed in mice receiving PEG-IFN-α2b. In Experiment #1, a significant difference in the changes in tumor volume and tumor weight was observed between the Control mice and the mice that received 640, 6400, 64 000, or 640 000 IU of PEG-IFN-α2b or 6400 or 64 000 IU of IFN-α2b ($P<0.001$ by two-factor factorial ANOVA; and $P<0.05$–0.001 by the Mann–Whitney U-test) and between 64 000 IU of PEG- and non-PEG-IFN-α2b ($P<0.0001$ and $P<0.01$, Fig. 4 and Table 2). In Experiment #2, significant difference in tumor volume change was observed between the Control mice and the mice that received 640 or 6400 IU of PEG-IFN-α2b or 6400 IU of IFN-α2b and between 640 IU of

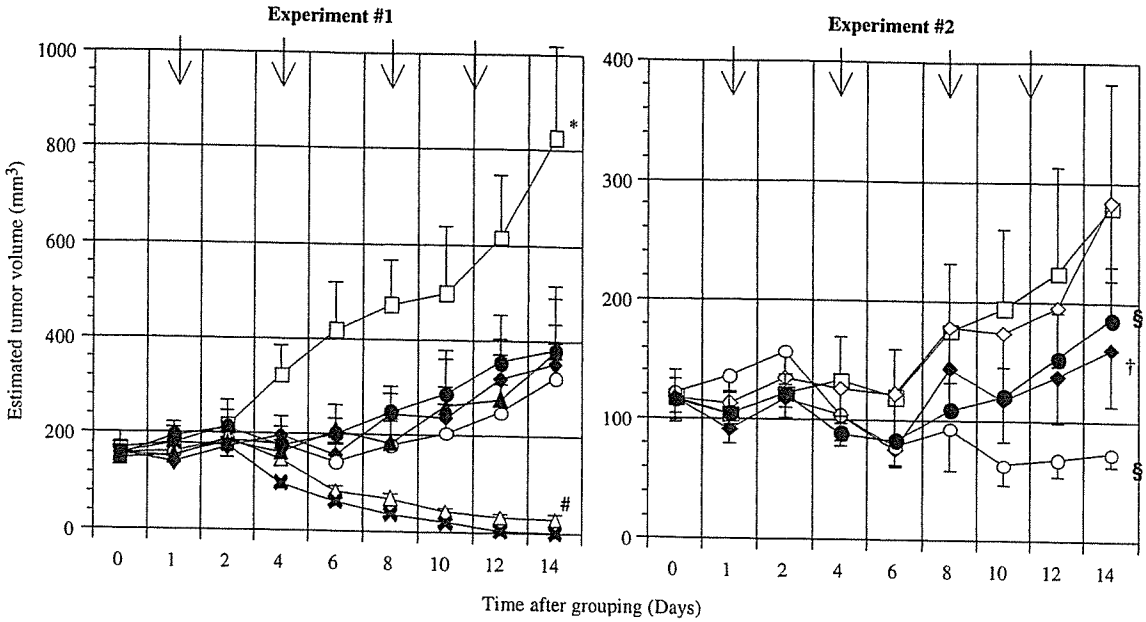

Fig. 4. Time-course change in estimated tumor volumes of subcutaneously transplanted human hepatocellular carcinoma tumors in nude mice. The mice received a subcutaneous injection of 640 (♦), 6400 (○), 64 000 (△), or 640 000 (✖) IU of pegylated IFN-α2b (PEG-IFN-α2b), or 6400 (●) or 64 000 (▲) IU of IFN-α2b, 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).

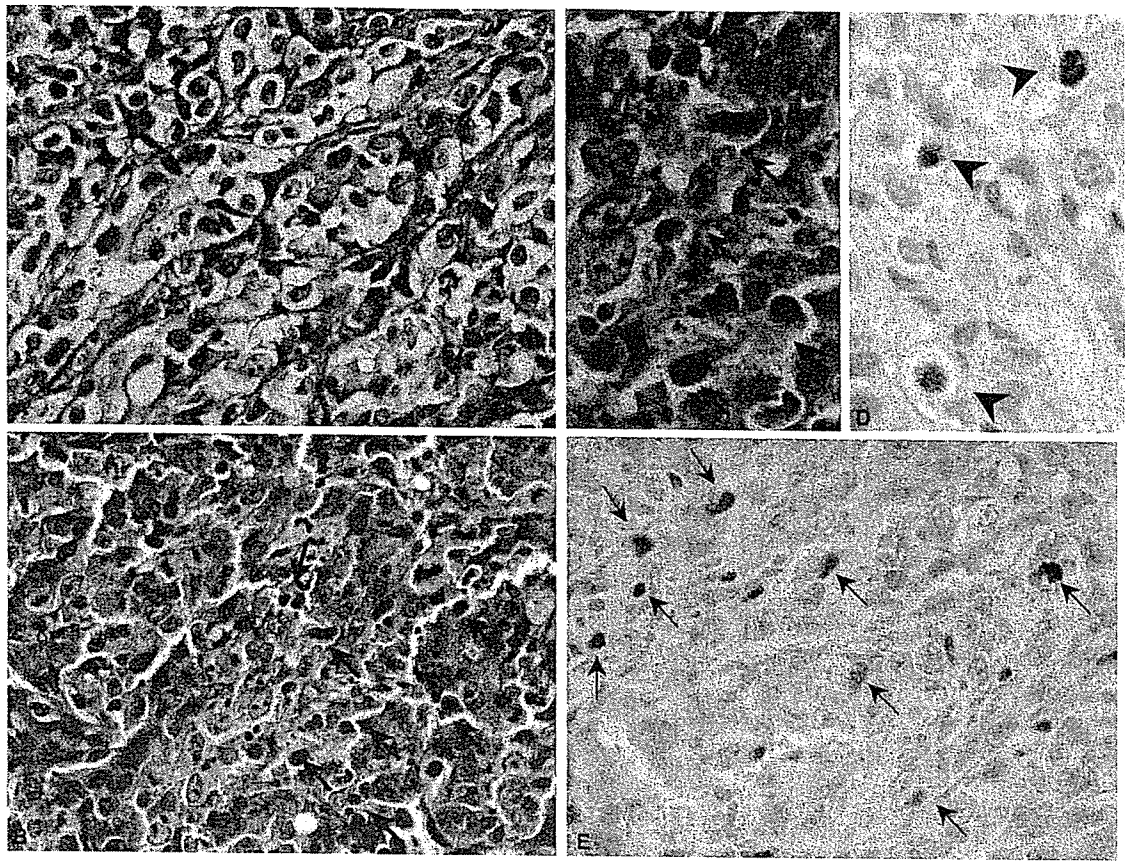

Fig. 5. Photomicrograph of subcutaneous human hepatocellular carcinoma tumor in nude mice that was developed after the injection of HAK-1B cells. (A) A control mouse that received culture medium alone. The tumor shows a thick trabecular arrangement of tumor cells and a sinusoid-like structure in the 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.

Acknowledgements

We thank Ms. Akemi Fujiyoshi and Dr. Akiko Takayama for their assistance in our experiments. This study was supported in part by the Sarah Cousins Memorial Fund, Boston, Massachusetts, and by a Grant-in-Aid from the Ministry of Health, Labor and Welfare of Japan (#17200501).

References

1. Stark G R, Kerr I M, Williams B R, Silverman R H, Schreiber R D. How cells respond to interferons. Annu Rev Biochem 1998; 67: 227–64.
2. Xu D, Erickson S, Szeps M, et al. Interferon α down-regulates telomerase reverse transcriptase and telomerase activity in human malignant and nonmalignant hematopoietic cells. Blood 2000; 96: 4313–8.
3. Pestka S, Langer J A, Zoon K C, Samuel C E. Interferons and their actions. Annu Rev Biochem 1987; 56: 727–77.
4. Gutterman J U. Cytokine therapeutics: lessons from interferon α. Proc Natl Acad Sci USA 1994; 91: 1198–205.
5. Ikeda K, Saitoh S, Arase Y, et al. Effect of interferon therapy on hepatocellular carcinogenesis in patients with chronic hepatitis type C: a long-term observation study of 1643 patients using statistical bias correction with proportional hazard analysis. Hepatology 1999; 29: 1124–30.
6. Ikeda K, Arase Y, Saitoh S, et al. Interferon beta prevents recurrence of hepatocellular carcinoma after complete resection or ablation of the primary tumor-A prospective randomized study of hepatitis C virus-related liver cancer. Hepatology 2000; 32: 228–32.
7. Mazzella G, Accogli E, Sottili S, et al. Alpha interferon treatment may prevent hepatocellular carcinoma in HCV-related liver cirrhosis. J Hepatol 1996; 24: 141–7.

8. Nishiguchi S, Kuroki T, Nakatani S, et al. Randomised trial of effects of interferon-α on incidence of hepatocellular carcinoma in chronic active hepatitis C with cirrhosis. Lancet 1995; 346: 1051–5.
9. Nishiguchi S, Tamori A, Kubo S. Effect of long-term postoperative interferon therapy on intrahepatic recurrence and survival rate after resection of hepatitis C virus-related hepatocellular carcinoma. Intervirology 2005; 48: 71–5.
10. Sakaguchi Y, Kudo M, Fukunaga T, Minami Y, Chung H, Kawasaki T. Low-dose, long-term, intermittent interferon-alpha-2b therapy after radical treatment by radiofrequency ablation delays clinical recurrence in patients with hepatitis C virus-related hepatocellular carcinoma. Intervirology 2005; 48: 64–70.
11. Yano H, Iemura A, Haramaki M, et al. Interferon alfa receptor expression and growth inhibition by interferon alfa in human liver cancer cell lines. Hepatology 1999; 29: 1708–17.
12. Hisaka T, Yano H, Ogasawara S, et al. Interferon-αCon1 suppresses proliferation of liver cancer cell lines *in vitro* and *in vivo*. J Hepatol 2004; 41: 782–9.
13. Negrier S, Escudier B, Lasset C, et al. Recombinant human interleukin-2, recombinant human interferon alfa-2a, or both in metastatic renal-cell carcinoma. Groupe Francais d'Immunotherapie. N Engl J Med 1998; 338: 1272–8.
14. Sakon M, Nagano H, Dono K, et al. Combined intraarterial 5-fluorouracil and subcutaneous interferon-alpha therapy for advanced hepatocellular carcinoma with tumor thrombi in the major portal branches. Cancer 2002; 94: 435–42.
15. Baker D E. Pegylated interferon plus ribavirin for the treatment of chronic hepatitis C. Rev Gastroenterol Disord 2003; 3: 93–109.
16. Reddy K R, Wright T L, Pockros P J, et al. Efficacy and safety of pegylated (40-kd) interferon α-2a compared with interferon α-2a in noncirrhotic patients with chronic hepatitis C. Hepatology 2001; 33: 433–8.
17. Lindsay K L, Trepo C, Heintges T, et al. A randomized, double-blind trial comparing pegylated interferon alfa-2b to interferon alfa-2b as initial treatment for chronic hepatitis C. Hepatology 2001; 34: 395–403.
18. Craxi A, Licata A. Clinical trial results of peginterferons in combination with ribavirin. Semin Liver Dis 2003; 23(Suppl. 1): 35–46.
19. Lee S D, Yu M L, Cheng P N, et al. Comparison of a 6-month course peginterferon α-2b plus ribavirin and interferon α-2b plus ribavirin in treating Chinese patients with chronic hepatitis C in Taiwan. J Viral Hepatol 2005; 12: 283–91.
20. Bruno S, Camma C, Di Marco V, et al. Peginterferon alfa-2b plus ribavirin for naive patients with genotype 1 chronic hepatitis C: a randomized controlled trial. J Hepatol 2004; 41: 474–81.
21. Utsunomiya I, Iemura A, Yano H, Akiba J, Kojiro M. Establishment and characterization of a new human hepatocellular carcinoma cell line, HAK-3, and its response to growth factors. Int J Oncol 1999; 15: 669–75.
22. Murakami T. Establishment and characterization of human hepatoma cell line (KIM-1). Acta Hepatol Jpn 1984; 25: 532–9.
23. Murakami T, Maruiwa M, Fukuda K, Kojiro M, Tanaka M, Tanikawa K. Characterization of a new human hepatoma cell line (KYN-3) derived from the ascites of the hepatoma patient [Abstract]. Jpn J Cancer Res 1988;Proceedings of the Japanese Cancer Association: 292.
24. Murakami T, Yano H, Maruiwa M, Sugihara S, Kojiro M. Establishment and characterization of a human combined hepatocholangiocarcinoma cell line and its heterologous transplantation in nude mice. Hepatology 1987; 7: 551–6.
25. Haramaki M, Yano H, Iemura A, et al. A new human hepatocellular carcinoma cell line (HAK-2) forms various structures in collagen gel matrices. Hum Cell 1997; 10: 183–92.
26. Yano H, Iemura A, Fukuda K, Mizoguchi A, Haramaki M, Kojiro M. Establishment of two distinct human hepatocellular carcinoma cell lines from a single nodule showing clonal dedifferentiation of cancer cells. Hepatology 1993; 18: 320–7.
27. Yano H, Iemura A, Haramaki M, et al. A human combined hepatocellular and cholangiocarcinoma cell line (KMCH-2) that shows the features of hepatocellular carcinoma or cholangiocarcinoma under different growth conditions. J Hepatol 1996; 24: 413–22.
28. Yano H, Kojiro M, Nakashima T. A new human hepatocellular carcinoma cell line (KYN-1) with a transformation to adenocarcinoma. In Vitro Cell Dev Biol 1986; 22: 637–46.
29. Yano H, Maruiwa M, Murakami T, et al. A new human pleomorphic hepatocellular carcinoma cell line, KYN-2. Acta Pathol Jpn 1988; 38: 953–66.
30. Nakanishi K, Maruyama M, Shibata T, Morishima N. Identification of a caspase-9 substrate and detection of its cleavage in programmed cell death during mouse development. J Biol Chem 2001; 276: 41237–44.
31. Takemoto Y, Yano H, Momosaki S, et al. Antiproliferative effects of interferon-αCon1 on ovarian clear cell adenocarcinoma *in vitro* and *in vivo*. Clin Cancer Res 2004; 10: 7418–26.
32. Yano H, Yanai Y, Momosaki S, et al. Growth inhibitory effects of interferon-α subtypes vary according to human liver cancer cell lines. J Gastroenterol Hepatol 2006, in press.
33. Evans T, Secher D. Kinetics of internalisation and degradation of surface-bound interferon in human lymphoblastoid cells. EMBO J 1984; 3: 2975–8.
34. Zoon K C, Zur Nedden D, Hu R, Arnheiter H. Analysis of the steady state binding, internalization, and degradation of human interferon-α2. J Biol Chem 1986; 261: 4993–6.
35. Lau J Y, Sheron N, Morris A G, Bomford A B, Alexander G J, Williams R. Interferon-α receptor expression and regulation in chronic hepatitis B virus infection. Hepatology 1991; 13: 332–8.
36. Nakajima S, Kuroki T, Shintani M, et al. Changes in interferon receptors on peripheral blood mononuclear cells from patients with chronic hepatitis B being treated with interferon. Hepatology 1990; 12: 1261–5.
37. Tochizawa S, Akamatsu S, Sugiyama Y, et al. A flow cytometric method for determination of the interferon receptor IFNAR2 subunit in peripheral blood leukocyte subsets. J Pharmacol Toxicol Methods 2004; 50: 59–66.
38. Lutfalla G, Holland S J, Cinato E, et al. Mutant U5A cells are complemented by an interferon-αβ receptor subunit generated by alternative processing of a new member of a cytokine receptor gene cluster. EMBO J 1995; 14: 5100–8.
39. Domanski P, Witte M, Kellum M, et al. Cloning and expression of a long form of the β subunit of the interferon αβ receptor that is required for signaling. J Biol Chem 1995; 270: 21606–11.
40. Dooley J S, Vergalla J, Hoofnagle J H, Zoon K C, Munson P J, Jones E A. Specific binding of human alpha interferon to high-affinity cell-surface binding sites on peripheral blood mononuclear cells. J Lab Clin Med 1989; 113: 623–31.
41. Krepler C, Certa U, Wacheck V, Jansen B, Wolff K, Pehamberger H. Pegylated and conventional interferon-α

induce comparable transcriptional responses and inhibition of tumor growth in a human melanoma SCID mouse xenotransplantation model. J Invest Dermatol 2004; 123: 664–9.

42. Dinney C P, Bielenberg D R, Perrotte P, et al. Inhibition of basic fibroblast growth factor expression, angiogenesis, and growth of human bladder carcinoma in mice by systemic interferon-alpha administration. Cancer Res 1998; 58: 808–14.
43. Hong Y K, Chung D S, Joe Y A, et al. Efficient inhibition of *in vivo* human malignant glioma growth and angiogenesis by interferon-beta treatment at early stage of tumor development. Clin Cancer Res 2000; 6: 3354–60.
44. Tedjarati S, Baker C H, Apte S, et al. Synergistic therapy of human ovarian carcinoma implanted orthotopically in nude mice by optimal biological dose of pegylated interferon alpha combined with paclitaxel. Clin Cancer Res 2002; 8: 2413–22.
45. Singh R K, Gutman M, Llansa N, Fidler I J. Interferon-beta prevents the upregulation of interleukin-8 expression in human melanoma cells. J Interferon Cytokine Res 1996; 16: 577–84.
46. Kojiro S, Yano H, Ogasawara S, et al. Antiproliferative effects of 5-fluorouracil and interferon-alpha in combination on a hepatocellular carcinoma cell line *in vitro* and *in vivo*. J Gastroenterol Hepatol 2006; 21: 129–37.

Journal of Gastroenterology and Hepatology (2006) **21**, 129–137 DOI: 10.1111/j.1440-1746.2005.04154.x

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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Accepted for publication 15 June 2005.

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

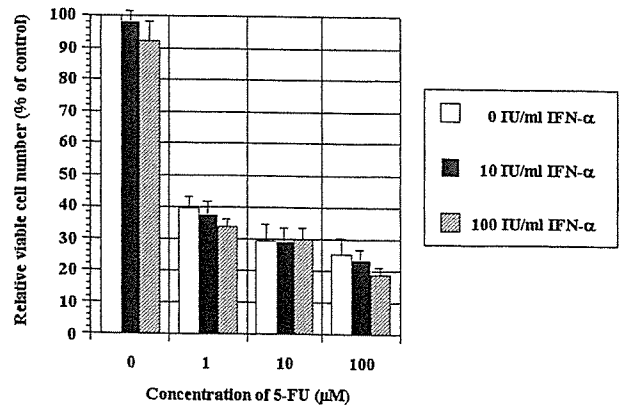

Figure 1 Effects of human natural interferon-alpha (IFN-α), 5-fluorouracil (5-FU) or IFN-α + 5-FU on the growth of HAK-1B cells in culture for 96 h.

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.

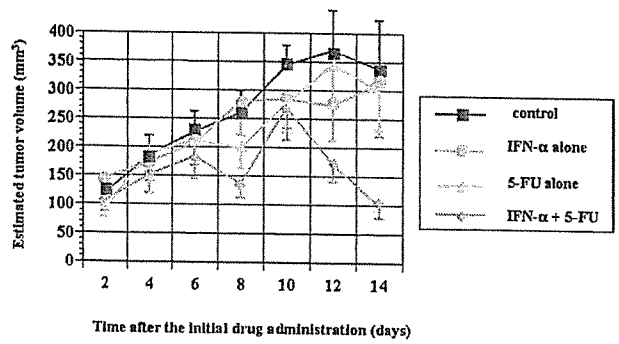

Figure 2 Chronological changes on the estimated volume of HAK-1B tumor that was developed subcutaneously on nude mice. The interferon-alpha (IFN-α) alone group (J) received subcutaneous injection of 5000 IU/day for 14 days. The 5-fluorouracil (5-FU) alone group (H) received continuous injection of 7 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

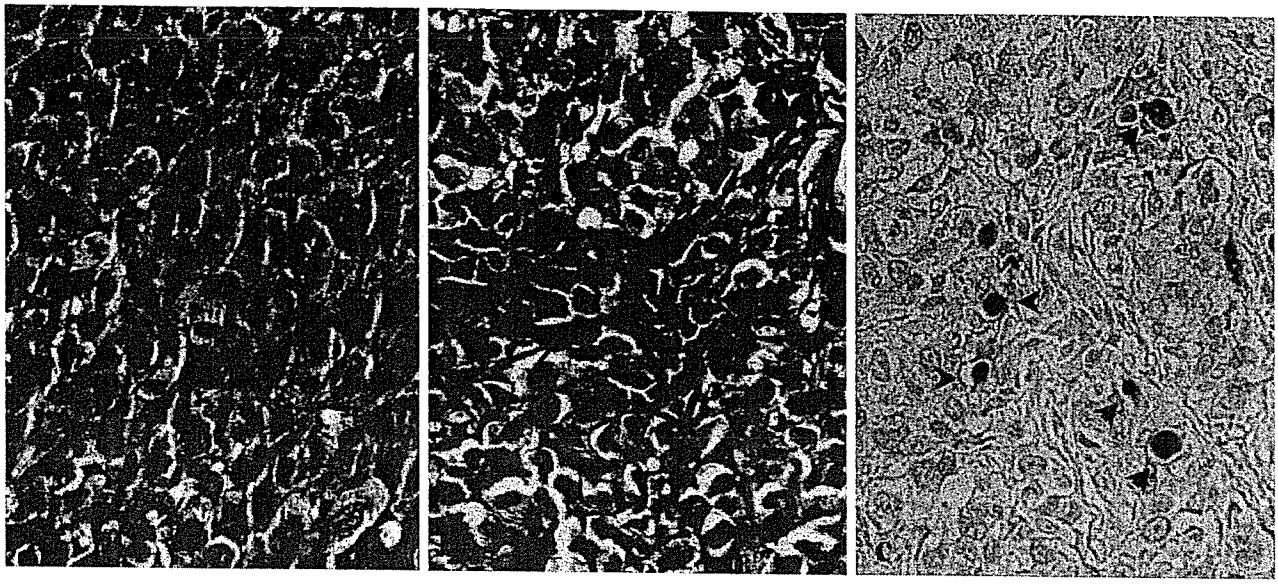

Figure 3 Photomicrograph of subcutaneous human hepatocellular carcinoma (HCC) tumors developed after injection of HAK-1B cells in nude mice. (a) A tumor in a control mouse showing thick trabecular arrangement of tumor cells with some mitotic figures (thick arrows). (b) A tumor in a mouse that received 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)			
	bFGF	IL-8	VEGF	Artery-like blood vessel
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

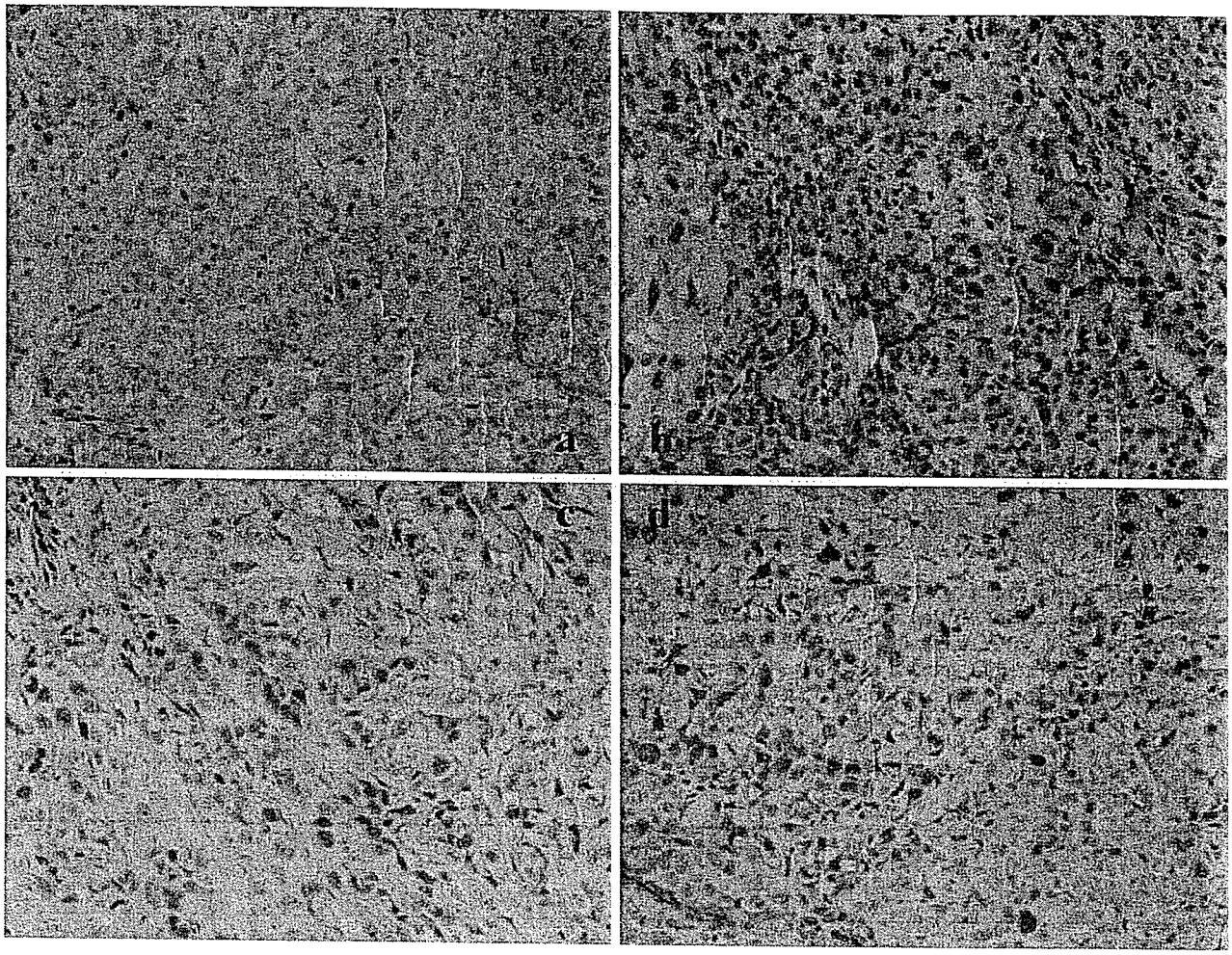

Figure 4 BrdU-positive cells in each of the three treatment groups: (a) control; (b) interferon-alpha (IFN-α) alone; (c) 5-fluorouracil (5-FU) alone; (d) IFN-α plus 5-FU (combination). The 5-FU alone group and the combination group had a larger number of positive cells.

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

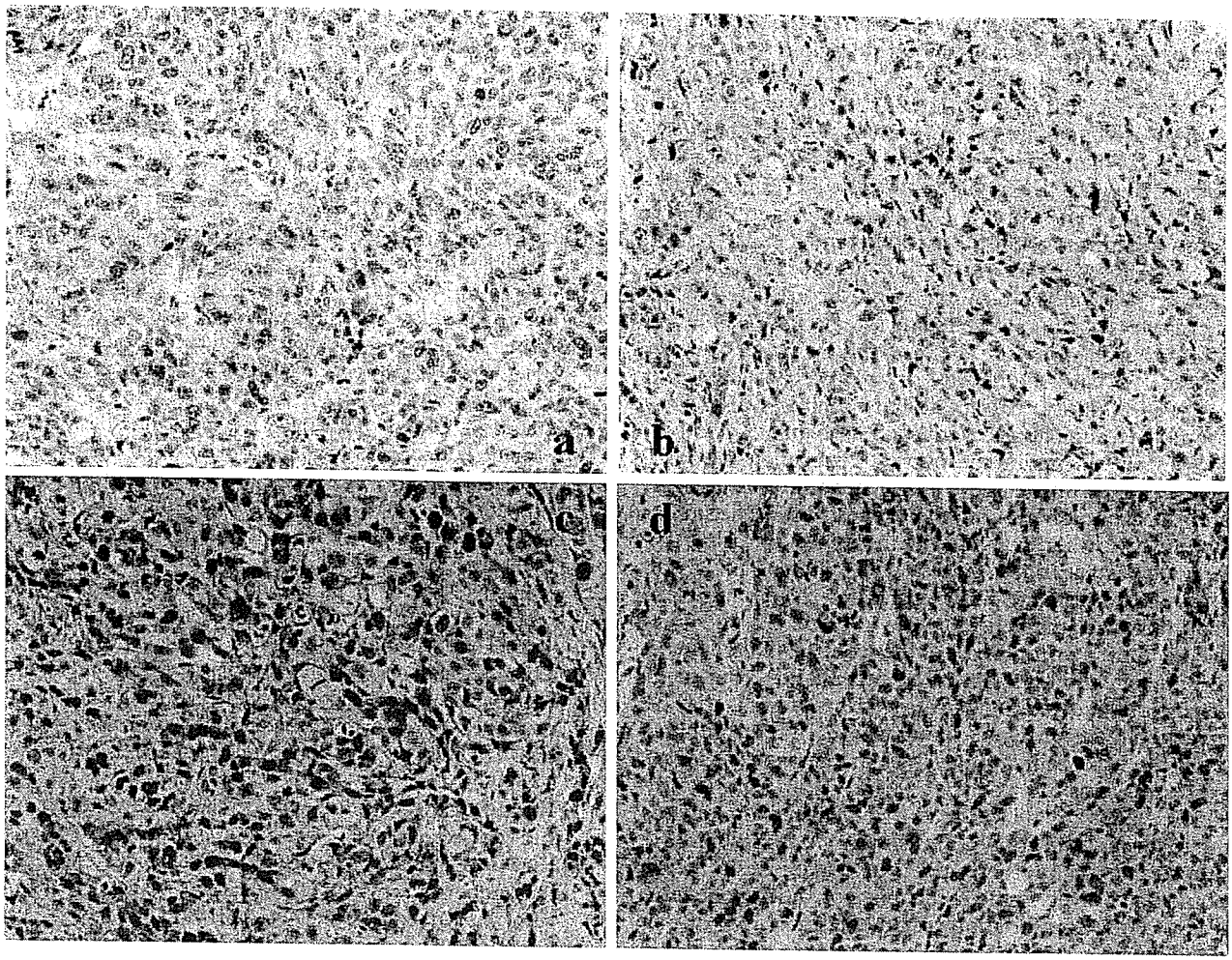

Figure 5 Cyclin A-positive cells in each of the three treatment groups: (a) control; (b) interferon-alpha (IFN-α) alone; (c) 5-fluorouracil (5-FU) alone; (d) IFN-α plus 5-FU (combination). The 5-FU alone group and the combination group had a larger number of positive cells.

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.