

## Growth inhibitory effects of pegylated IFN- $\alpha$ 2b and 5-fluorouracil in combination on renal cell carcinoma cell lines *in vitro* and *in vivo*

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**Abstract.** We investigated the effects of pegylated IFN- $\alpha$ 2b (PEG-IFN- $\alpha$ 2b) alone and PEG-IFN- $\alpha$ 2b plus 5-fluorouracil (5-FU) *in vitro* on the proliferation of renal cell carcinoma (RCC) cell lines. After the transplantation of RCC cells into nude mice, we administered IFN (PEG-IFN- $\alpha$ 2b or IFN- $\alpha$ 2b) alone, 5-FU alone, or IFN (PEG-IFN- $\alpha$ 2b or IFN- $\alpha$ 2b) plus 5-FU; and investigated tumor volume, tumor weight, the numbers of apoptotic cells and artery-like blood vessels, relative mRNA expression levels of enzymes which relate to 5-FU metabolism, angiogenesis factor, and type I interferon receptor. RCC cells *in vitro* were generally and relatively resistant to the anti-proliferative effects of PEG-IFN- $\alpha$ 2b, but the addition of 5-FU augmented IFN-induced anti-proliferative effects with the induction of apoptosis. PEG-IFN- $\alpha$ 2b *in vivo* presented stronger anti-tumor effects than IFN- $\alpha$ 2b, and its combination with 5-FU augmented the effects. The significant anti-tumor effect of the combination treatment was the increase in apoptotic cell number, but there were no significant differences in the suppression of angiogenesis, expression of IFN receptor, and the actions of metabolic enzymes of 5-FU. In conclusion, PEG-IFN- $\alpha$ 2b presents stronger anti-tumor effects than non-pegylated IFN, and the effects are augmented in the combination with 5-FU. Our findings suggest the clinical usefulness of PEG-IFN- $\alpha$ 2b in the treatment of RCC.

### Introduction

Renal cell carcinoma (RCC) is highly resistant to conventional chemotherapy. The objective response rate is 6-9% for vinblastine and 5-8% for 5-fluorouracil (5-FU) (1). The response rates of treatment regimens using interleukin-2 are 6-31% (2), and the therapeutic response rates of interferon (IFN)- $\alpha$  are 4-33% in patients with metastatic RCC (3). The response rates of immunochemical therapies that utilize chemotherapeutic agents with IFN- $\alpha$  or interleukin-2 range between 8 and 39% (4). Immunochemical therapy is the best treatment for advanced RCC, but potential synergetic effects of the medicines as well as their mechanisms remain to be elucidated.

Wadler and Wienik (5) for the first time proposed a combination therapy of IFN- $\alpha$  and 5-FU in 1988 in their study using colon cancer cell lines. Later, this combination therapy was applied to various types of human malignancies including RCC and hepatocellular carcinoma (HCC). 5-FU has two major anti-tumor mechanisms: one involves its active metabolite 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP), inhibiting the activity of thymidylate synthase (TS) and consequently DNA synthesis; the other is related to the incorporation of 5-FU metabolite into RNA and DNA, thereby disrupting normal RNA processing and function. The sensitivity of cancer cells to 5-FU is often influenced by the enzymes affecting 5-FU metabolism, including TS, dihydropyrimidine dehydrogenase (DPD), orotate phosphoribosyl transferase (OPRT), thymidine phosphorylase (TP), uridine phosphorylase (UP) and thymidine kinase (TK).

PEG-IFN- $\alpha$ 2b, a new interferon, is a covalent conjugate of recombinant IFN- $\alpha$ 2b with monomethoxy polyethylene glycol (PEG) in a 1:1 molar ratio that produces a 31,000-Da molecule (6). 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- $\alpha$ 2b thereby increases patient exposure

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to IFN- $\alpha$ 2b and requires less frequent administration (6). Clinical trials in chronic hepatitis C patients have suggested that PEG-IFN- $\alpha$  preparations produce more potent therapeutic effects than IFN- $\alpha$  preparations (6-10). Yano *et al* (11) examined the *in vitro* and *in vivo* anti-tumor effects of PEG- and non-PEG-IFN- $\alpha$ 2b on human liver cancer cells, and they reported that the anti-tumor effect of PEG-IFN- $\alpha$ 2b was significantly more potent than that of non-PEG-IFN- $\alpha$ 2b. In addition, Motzer *et al* (12) conducted a phase I study of PEG-IFN- $\alpha$ 2b on advanced renal cancer patients, and reported that partial response was obtained in 5 (19%) patients. Yet, there have been few basic studies evaluating the efficacy of PEG-IFN- $\alpha$ 2b on RCC *in vitro* and *in vivo*.

Our current study examined the *in vitro* and *in vivo* anti-tumor effects of PEG-IFN- $\alpha$ 2b, IFN- $\alpha$ 2b, 5-FU, and the combination of one of the two IFNs and 5-FU, on RCC cell lines, using PEG-IFN- $\alpha$ 2b concentrations close to the clinical dosage. We also examined the effects of the therapies on apoptotic cells, artery-like blood vessels, the enzymes affecting 5-FU metabolism, vascular endothelial growth factor (VEGF), and type I IFN receptor subunits in human RCC tumors which were developed in nude mice.

## Materials and methods

**Cell lines and cell culture.** This study used 8 human RCC cell lines. KRC/Y (13) was established in our laboratory. KUR11 and KURM were donated by Professor K. Itoh of the Department of Immunology at our University. Caki-1, Caki-2, and ACHN were purchased from American Type Culture Collection. VMRC-RCW was purchased from Japan Health Sciences Foundation. OS-RC-2 was purchased from Riken Cell Bank (Tsukuba, Japan).

Culture medium for KRC/Y consisted of Dulbecco's modified Eagle's medium (Nissui Seiyaku Co., Tokyo, Japan) supplemented with heat-inactivated (56°C, 30 min) 5% fetal bovine serum (FBS, Bioserum, Vic, Australia), 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (Gibco BRL/Life Technologies Inc., Gaithersburg, MD). Culture medium for Caki-1, Caki-2, VMRC-RCW and ACHN consisted of modified Eagle's medium (Gibco); the medium for KUR11, KURM and OS-RC-2 consisted of RPMI-1640; and each medium was supplemented with 10% FBS, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin. Cells were cultured in an atmosphere of 5% CO<sub>2</sub> in air at 37°C. 5-FU was purchased from Kyowa Hakko K.K. (Tokyo, Japan).

**IFN and reagents.** PEG-IFN- $\alpha$ 2b (PEG Intron®) and IFN- $\alpha$ 2b (Intron® A) were provided by Schering-Plough K.K. (Osaka, Japan). The specific activity of PEG-IFN- $\alpha$ 2b was 6.4 $\times$ 10<sup>7</sup> IU/mg protein and that of IFN- $\alpha$ 2b was 2.6 $\times$ 10<sup>8</sup> IU/mg protein.

Rat antibody against mouse endothelial cells (anti-CD34, clone MEC14.7) was purchased from Serotec Co., Oxford, UK; and mouse monoclonal antibody against human  $\alpha$ -smooth muscle actin (SMA) that cross-reacts with mouse  $\alpha$ -SMA (clone 1A4), from Immunon (Pittsburgh, PA).

**Effects of PEG-IFN- $\alpha$ 2b on the proliferation of RCC cell lines *in vitro*.** The effects of PEG-IFN- $\alpha$ 2b or 5-FU on cell

proliferation were examined in colorimetric assays by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cell growth assay kits (Chemicon International Inc.) as described elsewhere (14). Briefly, the RCC cells (1.5-5 $\times$ 10<sup>3</sup> 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- $\alpha$ 2b (8, 32, 128, 512 or 2,048 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 to 570 nm and the reference wavelength to 630 nm. To keep the optical density within linear range, all experiments were performed when the cells were in the logarithmic growth phase. The effects of IFN- $\alpha$ 2b on the growth of VMRC-RCW cells were also examined in the same manner.

**Effects of combination therapy of PEG-IFN- $\alpha$ 2b and 5-FU on the proliferation of RCC cell lines *in vitro*.** RCC cells (VMRC-RCW, 3,000 cells/well) were seeded on 96-well plates (Nunc Inc.), cultured for 24 h, and then the culture medium was changed to a new medium containing PEG-IFN- $\alpha$ 2b alone (0, 160, 317, 625, 1,250, 2,500, 5,000 or 10,000 IU/ml); 5-FU alone (0, 0.6, 1.25, 2.5, 5 or 10  $\mu$ M); or both 5-FU (0, 0.6, 1.25, 2.5, 5, 10  $\mu$ M) and PEG-IFN- $\alpha$ 2b (0, 160, 317, 625, 1,250, 2,500, 5,000 or 10,000 IU/ml). After 96 h of culture, the number of viable cells was examined by MTT assay as described above.

The synergy of cooperative cytotoxicity was determined by the median-effect principle as described by Chou and Talalay (15). Data from each sample were analyzed by using CalcuSyn ver. 2 (Biosoft, Cambridge, UK).

**Morphological observation.** For morphological observation by light microscopy, 8 RCC cell lines were seeded on Lab-Tek tissue culture chamber slides (Nunc Inc.), cultured with or without PEG-IFN- $\alpha$ 2b (1,024, 4,098 or 8,192 IU/ml) for 72 h, fixed for 30 min in Carnoy's solution, and stained with hematoxylin and eosin (H&E).

In another experiment, one RCC cell line (VMRC-RCW, 8,000 cells/chamber) was seeded on Lab-Tek tissue culture chamber slides (Nunc Inc.), cultured with PEG-IFN- $\alpha$ 2b alone (0, 160, 317, 625, 1,250, 2,500, 5,000 IU/ml); 5-FU alone (0, 0.6, 1.25, 2.5, 5.0  $\mu$ M); PEG-IFN- $\alpha$ 2b (0, 160, 317, 625, 1,250, 2,500, 5,000 IU/ml) plus 5-FU (0, 0.6, 1.25, 2.5, 5.0  $\mu$ M), or PBS, for 72 h, fixed for 30 min in Carnoy's solution and H&E stained.

**Effects of PEG-IFN- $\alpha$ 2b and IFN- $\alpha$ 2b on RCC cell proliferation *in nude mice*.** Cultured VMRC-RCW cells (1.0 $\times$ 10<sup>7</sup> cells/mouse) were subcutaneously (s.c.) injected into the backs of 4-week-old female BALB/c athymic nude mice (n=62) (Clea Japan, Inc, Osaka, Japan). One week later when the largest diameter of the tumor reached ~10 mm (day 0), the mice were divided into 7 groups (n=8 or 9 each) in a manner to equalize the mean tumor diameter of each group. Each mouse received a subcutaneous injection of 0.1 ml of medium alone (control group), medium containing 640, 6,400, 64,000 or 640,000 IU of PEG-IFN- $\alpha$ 2b, or medium containing 640 or 6,400 IU of IFN- $\alpha$ 2b, twice a week for 2 consecutive weeks (on day 1, 4,

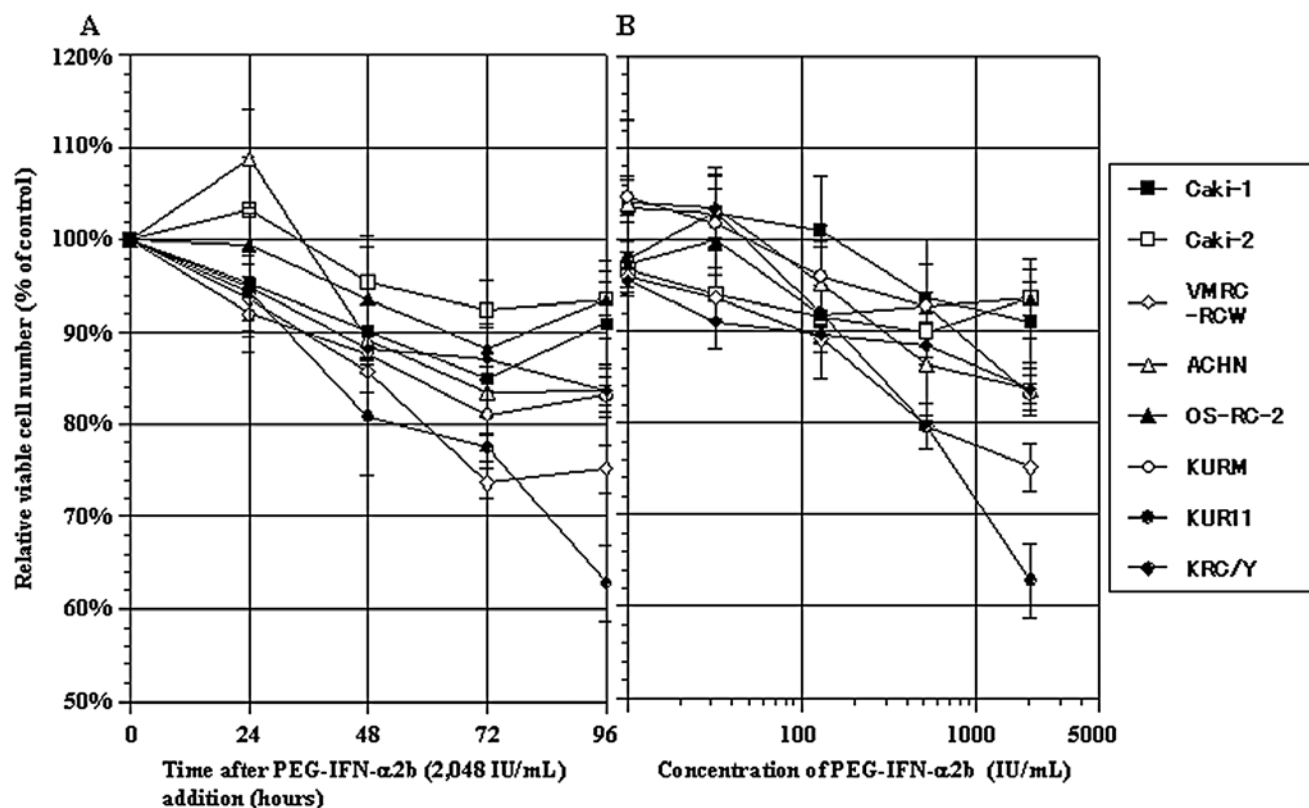


Figure 1. Anti-proliferative effects of PEG-IFN-α2b. (A) Chronological changes in relative viable cell number (% of the control) after adding 2,048 IU/ml of PEG-IFN-α2b. Growth was significantly suppressed over time in 2 cell lines (KUR11 and KRC/Y). (B) Ninety-six hours after adding 8, 32, 128, 512 or 2,048 IU/ml of PEG-IFN-α2b. Cell proliferation was suppressed in a dose-dependent manner in 6 cell lines (all but Caki-2 and OS-RC-2). Eight samples were used in each experiment. The experiment was repeated at least three times for each cell line. The values represent the average  $\pm$  SE.

8 and 11). The clinical daily dose of IFN-α2b for human RCC is  $600 \times 10^4$  IU/body ( $1.2 \times 10^5$  IU/kg), and this is approximately four times the lowest dose ( $3.2 \times 10^4$  IU/kg) used in this experiment. Tumor size was measured in two directions by using calipers on the first and second days of s.c. injection (day 1 and 2) and then once every 2 days until day 14, and tumor volume ( $\text{mm}^3$ ) was estimated by using the equation: Length  $\times$  (Width) $^2 \times 0.5$ . Mouse body weight was measured on day 0, 8 and 14. On day 15, all mice were sacrificed and the tumors were removed.

The animals received human care according to 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).

**Effects of combination therapy of PEG-IFN-α2b and 5-FU on RCC cell proliferation in nude mice.** VMRC-RCW cells ( $7.5 \times 10^6$  cells/mouse) were subcutaneously injected into 4-week-old female BALB/c athymic nude mice ( $n=58$ ). The mice were divided into 7 groups ( $n=8$  or 9 each) on day 7 when tumor size reached  $\sim 10$  mm in diameter, and each group was assigned to one of the 7 treatments: i) PEG-IFN-α2b alone (6,400 IU); ii) IFN-α2b alone (6,400 IU); iii) low dose 5-FU alone (160  $\mu\text{g}$ ); iv) high dose 5-FU alone (320  $\mu\text{g}$ ); v) combination therapy of PEG-IFN-α2b (6,400 IU) and low

dose 5-FU; vi) combination therapy of IFN-α2b (6,400 IU) and low dose 5-FU; and (vii) control.

5-FU was administered intra-abdominally every day for 2 consecutive weeks. The dose of 5-FU (160  $\mu\text{g}$ /mouse, 8 mg/kg) is comparable to the clinical dose.

Tumor size measurement and IFN administration were performed in the same manner as described above. On day 15, all mice were sacrificed and each tumor was removed. After the tumor weight was measured, half of the obtained tumors were used for histological examination and the other half were used for quantitative real-time RT-PCR.

The number of cells showing characteristics of apoptosis such as cytoplasmic shrinkage, chromatin condensation and nuclear fragmentation was counted in ten 0.25  $\text{mm}^2$  areas within an H&E-stained specimen, and the average number per area was obtained. The TUNEL technique (ApopTag<sup>®</sup> Peroxidase In Situ Apoptosis Detection Kits, Chemicon International, CA, USA) was also used to detect apoptotic cells. The average number of TUNEL-positive cells per area was obtained as described above.

**Immunohistochemistry.** Double immunohistochemical staining was performed by using anti-mouse endothelial cell (anti-CD34) antibody, anti-human  $\alpha$  smooth muscle actin ( $\alpha$ -SMA) antibody and histofine simple stain mouse Max-Po (Rat) kits (Nichirei, Tokyo, Japan) as described elsewhere (16). We



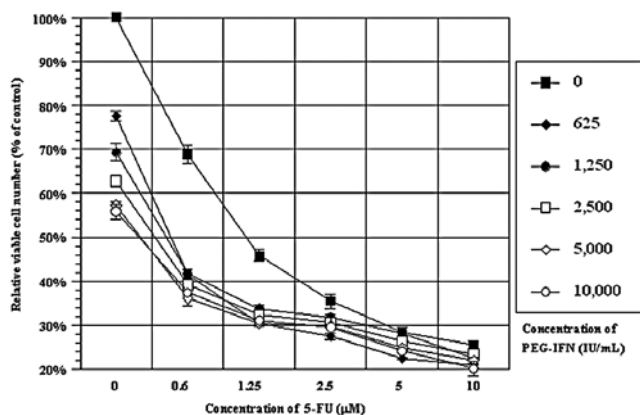


Figure 2. Anti-proliferative effects of the combination therapy of PEG-IFN- $\alpha$ 2b and 5-FU on VMRC-RCW cells in a 96-h culture. The relative viable cell number decreased dose-dependently. Two samples were used in each experiment. The experiment was repeated three times. The values represent the average  $\pm$  SE. PEG-IFN, PEG-IFN- $\alpha$ 2b.

calculated the number of artery-like blood vessels in the entire area of each section and obtained for each the mean number per mm<sup>2</sup>.

**cDNA preparation and quantitative real-time RT-PCR.** Total RNA was extracted using RNA-Bee™ (Tel-Test, Inc., TX) and reverse transcribed using Superscript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, CA) according to the manufacturer's instructions. Quantitative real-time RT-PCR was performed with an ABI PRISM 7300 (Applied Biosystems, Foster City, CA). We examined 6 enzymes related to 5-FU metabolism, i.e., TS, TP, DPD, OPRT, UP and

TK. The sequences of the primers and probes for the 6 enzymes are listed elsewhere (17). The sequences for VEGF were 5'-CCATGAACCTTTCTGCTGTCTTGG-3' as the forward primer, 5'-CTGCGCTGATAGACATCCATGA-3' as the reverse primer, and 5'-TGCTCTACCTCCACCATGC CAAGT-3' as the probe. The sequences of the primers and probes for VEGFR-1, IFNAR-1, IFNAR-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Applied Biosystems.

**Statistical analysis.** We used two-factorial ANOVA for the comparisons of tumor volume, tumor weight, number of apoptotic cells, number of artery-like blood vessels, and relative levels of mRNAs related to 5-FU metabolism.

## Results

**Effects of PEG-IFN- $\alpha$ 2b on the proliferation of RCC cell lines *in vitro*.** After adding 2,048 IU/ml of PEG-IFN- $\alpha$ 2b, the relative viable cell number of the cultured 8 cell lines was suppressed in a time-dependent manner until 72 h, but at 96 h, suppression was noted in only 2 cell lines (KRC/Y and KUR11). On the other hand, with different doses of PEG-IFN- $\alpha$ 2b, the relative viable cell number at 96 h was suppressed in the 6 cell lines, i.e., VMRC-RCW, KRC/Y, KURM, KUR11, ACHN and Caki-1. In the 8 cell lines, IC<sub>50</sub> was not reached for either time- and dose-dependent suppressions, but the most sensitive case was KUR11 with the dose of 2,048 IU/ml at 96 h, i.e., the relative viable cell number was 62.7% of the control (Fig. 1).

In the VMRC-RCW cell line, the anti-tumor effects of PEG-IFN- $\alpha$ 2b and IFN- $\alpha$ 2b were not markedly different.

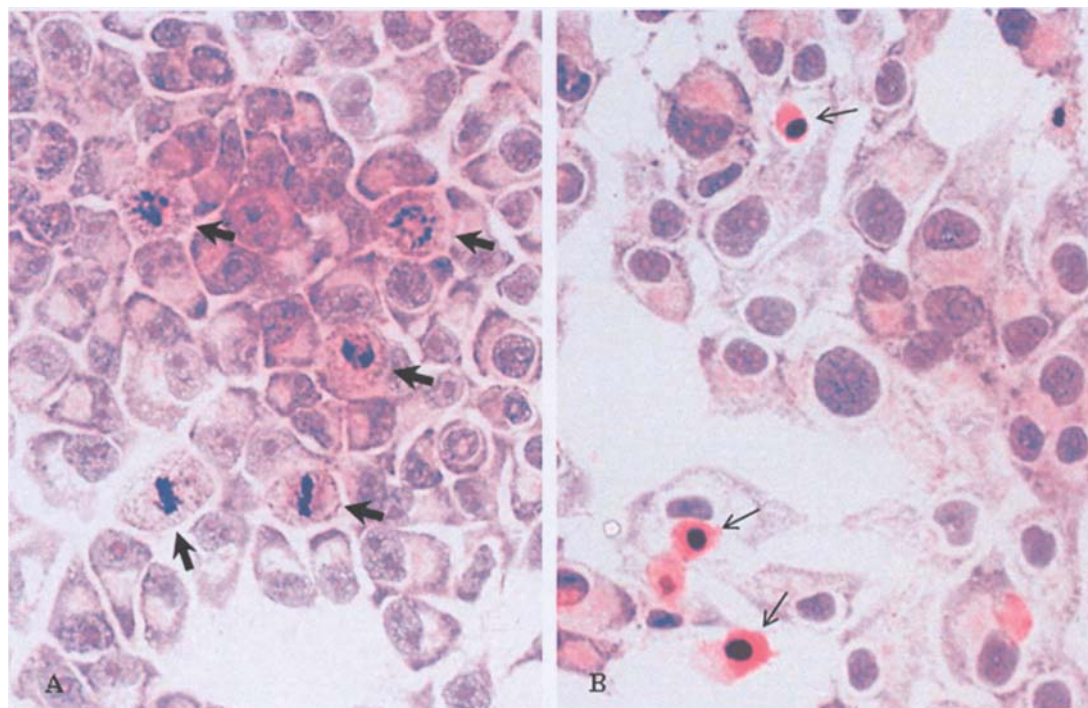


Figure 3. Photomicrograph of VMRC-RCW cells cultured for 72 h on a Lab-Tek Chamber Slide. (A) Without PEG-IFN- $\alpha$ 2b in culture medium. Mitotic figures (thick arrows) were noted. (B) With 2,500 IU/mL of PEG-IFN- $\alpha$ 2b and 2.5  $\mu$ M of 5-FU in culture medium. Apoptotic cells (thin arrows) characterized by cytoplasmic shrinkage and chromatic condensation were noted (H&E staining, x200).

Table I. Effects of PEG-IFN- $\alpha$ 2b and IFN- $\alpha$ 2b on RCC cell proliferation in nude mice.

Treatment group	Number	Tumor weight (g, mean $\pm$ SE)	Body weight (g, mean $\pm$ SE on day 15)
Control (culture medium)	9	1.835 $\pm$ 0.132	17.122 $\pm$ 0.362
IFN- $\alpha$ 2b (640 IU)	9	1.735 $\pm$ 0.177	16.089 $\pm$ 0.599
IFN- $\alpha$ 2b (6,400 IU)	9	1.455 $\pm$ 0.140	16.667 $\pm$ 0.420
PEG-IFN- $\alpha$ 2b (640 IU)	9	1.267 $\pm$ 0.072 <sup>a,c</sup>	16.156 $\pm$ 0.308
PEG-IFN- $\alpha$ 2b (6,400 IU)	9	1.160 $\pm$ 0.075 <sup>b</sup>	15.244 $\pm$ 0.313
PEG-IFN- $\alpha$ 2b (64,000 IU)	9	0.920 $\pm$ 0.126 <sup>b</sup>	16.922 $\pm$ 0.601
PEG-IFN- $\alpha$ 2b (640,000 IU)	8	0.444 $\pm$ 0.077 <sup>b</sup>	17.638 $\pm$ 0.717

Cultured VMRC-RCW cells were subcutaneously transplanted in each nude mouse ( $1.0 \times 10^7$ /mouse). Seven days later, when the largest diameter of the tumor reached  $\sim 10$  mm, mice were treated twice per week with s.c. injection of PEG-IFN- $\alpha$ 2b, IFN- $\alpha$ 2b, or culture medium. All mice were sacrificed on day 15. <sup>a</sup> $p < 0.01$  and <sup>b</sup> $p < 0.001$  vs. control; <sup>c</sup> $p < 0.05$  vs. the same concentration of IFN.

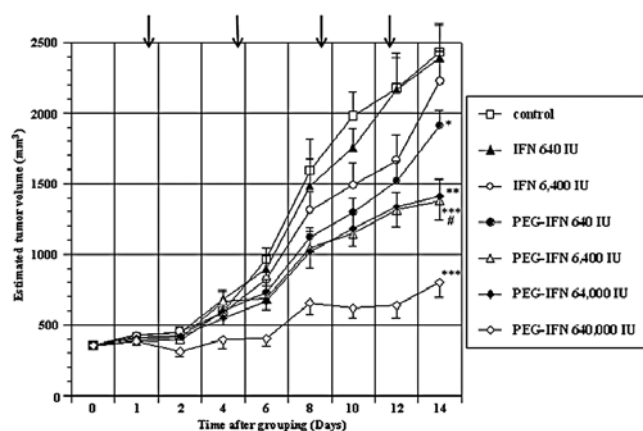


Figure 4. Chronological changes in the estimated volume of subcutaneously transplanted RCC tumors (VMRC-RCW cells,  $1.0 \times 10^7$ ) in nude mice according to the treatment dose. Seven days after the transplantation, when the largest tumor diameter reached  $\sim 10$  mm (day 0), mice were divided into 7 groups (n=8 or 9, each). The arrows show the days of treatment. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  vs. control; # $p < 0.01$  vs. the same dose of IFN- $\alpha$ 2b (6,400 IU). The values represent the average  $\pm$  SE. PEG-IFN, PEG-IFN- $\alpha$ 2b.

**Effects of the combination treatment of PEG-IFN- $\alpha$ 2b and 5-FU on the growth of the VMRC-RCW cell line in vitro.** Without 5-FU, the relative viable cell number did not decrease to 50% or lower of the control even when the highest dose of PEG-IFN- $\alpha$ 2b (5,000 IU/ml) was added to the culture. When 5-FU (0.6  $\mu$ M) was used in combination, the relative viable cell number was suppressed to 41.6% even when PEG-IFN- $\alpha$ 2b was at the lowest dose (625 IU/ml, Fig. 2). The anti-proliferative effect of these two agents was additive, not synergistic.

**Morphological examination in vitro.** The 8 cell lines presented such apoptotic features as cytoplasmic shrinkage and chromatin condensation in a varying degree and in a dose-dependent manner at 72 h after adding PEG-IFN- $\alpha$ 2b. For the combination treatment of PEG-IFN- $\alpha$ 2b and 5-FU, more apoptotic cells were observed than in the PEG-IFN- $\alpha$ 2b

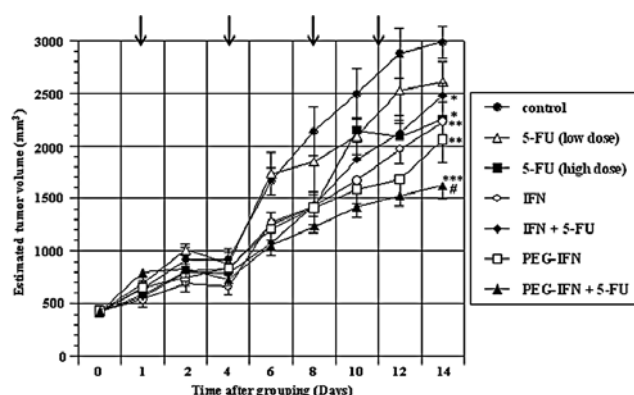


Figure 5. Chronological changes in the estimated volume of subcutaneously transplanted RCC tumors (VMRC-RCW cells,  $7.5 \times 10^6$ ) in nude mice according to the treatment method. Seven days after the transplantation, when the largest tumor diameter reached  $\sim 10$  mm (day 0), mice were divided into 7 groups (n=8 or 9, each), i.e., PEG-IFN- $\alpha$ 2b (6,400 IU) alone; IFN- $\alpha$ 2b (6,400 IU) alone; combination of 5-FU and PEG-IFN- $\alpha$ 2b (6,400 IU) or IFN- $\alpha$ 2b (6,400 IU); 5-FU alone (low or high dose); and culture medium alone (control). The arrows show the days of treatment. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  vs. control; # $p < 0.001$  vs. IFN- $\alpha$ 2b and 5-FU. The values represent the average  $\pm$  SE. PEG-IFN, PEG-IFN- $\alpha$ 2b.

alone-treated cells, and the apoptotic cells increased dose-dependently to PEG-IFN- $\alpha$ 2b plus 5-FU (Fig. 3).

**Effects of PEG-IFN- $\alpha$ 2b on RCC cell proliferation in nude mice.** Chronological changes in estimated tumor volume after IFN administration to nude mice are summarized in Fig. 4. Dose-dependent suppression of tumor volume was observed in mice receiving PEG-IFN- $\alpha$ 2b. The estimated tumor volume on day 14 in the mice receiving 6,400 IU of PEG-IFN- $\alpha$ 2b became 61.9% of the mice receiving the same dose of IFN- $\alpha$ 2b ( $p < 0.01$ ) and 56.8% of the control ( $p < 0.001$ ). The tumor weight on day 15 in the mice receiving 6,400 IU of PEG-IFN- $\alpha$ 2b became 63.2% of the control ( $p < 0.001$ , Table I).

Significant differences in the estimated tumor volume were observed between each PEG-IFN- $\alpha$ 2b group (640, 6,400,



Table II. Effects of combination therapy of PEG-IFN- $\alpha$ 2b and 5-FU on RCC cell proliferation in nude mice.

Treatment group	Number	Tumor weight (g, mean $\pm$ SE)	Body weight (g, mean $\pm$ SE on day 15)
Control (culture medium)	8	2.255 $\pm$ 0.102	17.188 $\pm$ 0.578
5-FU (low dose)	9	2.430 $\pm$ 0.185	16.778 $\pm$ 0.595
5-FU (high dose)	7	1.603 $\pm$ 0.107 <sup>c</sup>	15.686 $\pm$ 0.814
IFN- $\alpha$ 2b alone	8	1.812 $\pm$ 0.084 <sup>b</sup>	16.363 $\pm$ 0.692
IFN- $\alpha$ 2b + 5-FU	8	1.917 $\pm$ 0.170	16.344 $\pm$ 0.426
PEG-IFN- $\alpha$ 2b	8	1.771 $\pm$ 0.172 <sup>a</sup>	15.963 $\pm$ 0.459
PEG-IFN- $\alpha$ 2b + 5-FU	9	1.742 $\pm$ 0.194 <sup>a</sup>	15.767 $\pm$ 0.621

Cultured VMRC-RCW cells were subcutaneously transplanted in each nude mouse ( $7.5 \times 10^6$ /mouse). Seven days later, when the largest diameter of the tumor reached  $\sim 10$  mm, mice were treated with s.c. injection of IFNs and/or intraperitoneal injection of 5-fluorouracil (5-FU) daily. All mice were sacrificed on day 15. The concentration of both PEG-IFN- $\alpha$ 2b and IFN- $\alpha$ 2b was 6,400 IU/ml. <sup>a</sup> $p < 0.05$ , <sup>b</sup> $p < 0.01$  and <sup>c</sup> $p < 0.001$  vs. control.

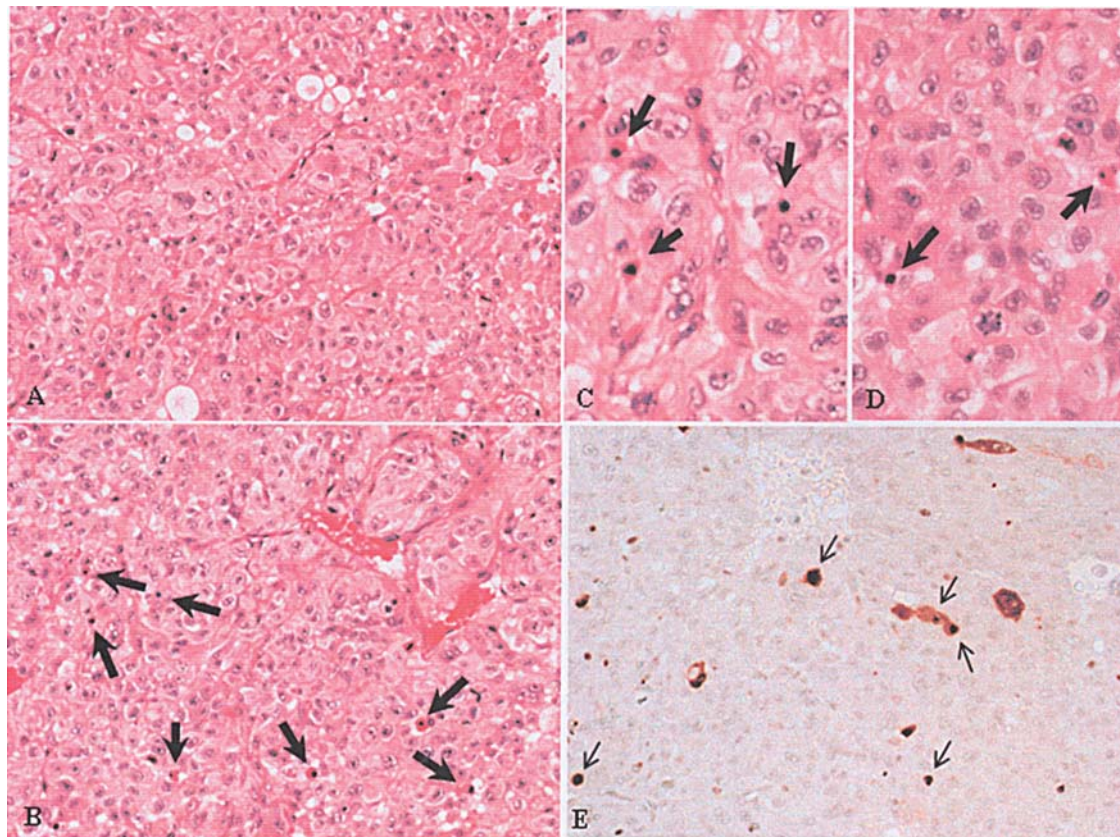


Figure 6. Photomicrograph of a subcutaneous human RCC tumor in nude mice, which developed after the injection of VMRC-RCW cells. (A) A control mouse that received culture medium alone. The tumor showed a thick trabecular arrangement of tumor cells and thin fibrous connective tissues and capillary vessels in the stroma. (B) A mouse that received PEG-IFN- $\alpha$ 2b and 5-FU. There were many apoptotic tumor-cells (thick arrows, H&E staining, x200). (C and D) Higher magnifications of B (x400). Apoptotic tumor-cells characterized by shrinkage and eosinophilic change in the cytoplasm and chromatin condensation are shown (thick arrows, H&E staining). (E) TUNEL-positive apoptotic cells showing brown nuclei (thin arrows, TUNEL staining, x200).

64,000, 640,000 IU) and the control ( $p < 0.05$  to  $p < 0.001$ , Fig. 3). There was no significant difference between 640 or 6,400 IU of the IFN- $\alpha$ 2b group and the control. There were no significant differences in body weight of the mice among the groups.

*Effects of the combination therapy of PEG-IFN- $\alpha$ 2b and 5-FU on RCC cell proliferation in nude mice.* Chronological changes in estimated tumor volume are shown in Fig. 5. The tumor volume on day 14 for the combination therapy of PEG-IFN- $\alpha$ 2b and 5-FU was 54.2% of the control ( $p < 0.0001$ ).

Table III. Relative mRNA expression levels of the enzymes related with 5-FU metabolism, VEGF, VEGFR-1 and type I IFN receptor subunits.

Treatment group	DPD	TP	TK	TS	UP	OPRT	VEGF	VEGFR-1	IFNAR-1	IFNAR-2
5-FU (low dose)	129	101	68	182	59	48	116	135	122	94
5-FU (high dose)	72	50	52	40 <sup>a</sup>	86	41	30	93	59	90
IFN- $\alpha$ 2b	110	71	82	119	100	103	97	134	108	174
IFN- $\alpha$ 2b + 5-FU	95	80	85	74	53	106	56	111	63	44 <sup>a,d</sup>
PEG-IFN- $\alpha$ 2b	648 <sup>b,d</sup>	420 <sup>a</sup>	313	297 <sup>b,d</sup>	76	124	366 <sup>a,d</sup>	277	159	217
PEG-IFN- $\alpha$ 2b + 5-FU	159 <sup>c</sup>	143 <sup>c</sup>	162	129	129	86	251 <sup>a,e</sup>	138	91	141

mRNA levels were examined by quantitative real-time RT-PCR and normalized with GAPDH. The values of relative mRNA expression level represent the average of the ratio to the level of control in each group. <sup>a</sup> $p < 0.05$  and <sup>b</sup> $p < 0.01$  vs. control; <sup>c</sup> $p < 0.05$  vs. PEG-IFN- $\alpha$ 2b; <sup>d</sup> $p < 0.05$  vs. IFN- $\alpha$ 2b; and <sup>e</sup> $p < 0.01$  vs. IFN- $\alpha$ 2b plus 5-FU. DPD, dihydropyrimidine dehydrogenase; TP, thymidine phosphorylase; TK, thymidine kinase; TS, thymidylate synthase; UP, uridine phosphorylase; OPRT, orotate phosphoribosyl transferase; VEGF, vascular endothelial growth factor; VEGFR-1, VEGF receptor 1; IFNAR-1, type I interferon receptor subunit 1; and IFNAR-2, type I interferon receptor subunit 2.

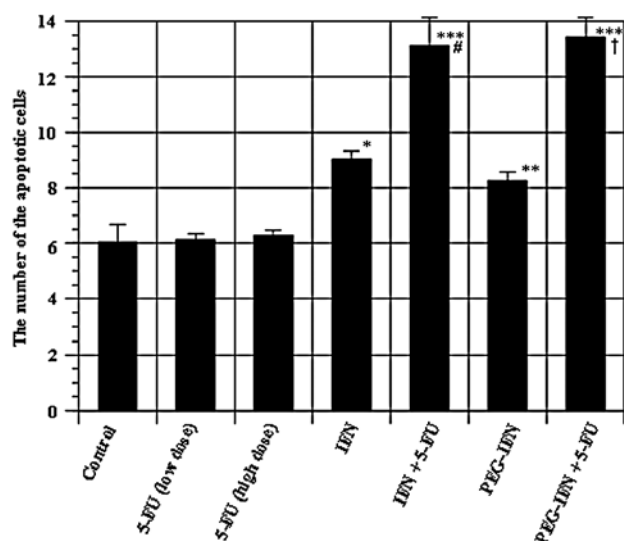


Figure 7. Number of apoptotic cells in the tumors. The number was counted in ten 0.25 mm<sup>2</sup> areas in each section, and the average number per area in each group was obtained. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  vs. control; # $p < 0.05$  vs. IFN- $\alpha$ 2b; † $p < 0.001$  vs PEG-IFN- $\alpha$ 2b. PEG-IFN, PEG-IFN- $\alpha$ 2b.

The tumor weights of the mice on day 15 were significantly different between the control and the 5-FU high dose group, each IFN alone group, and the combination group of PEG-IFN- $\alpha$ 2b and 5-FU. The two types of IFNs and/or 5-FU did not affect the body weight of the mice (Table II).

Histological examination of the RCC tumor specimens stained with H&E revealed that the number of apoptotic cells was significantly higher in the mice treated with 6,400 IU of PEG-IFN- $\alpha$ 2b ( $p < 0.01$ ) or 6,400 IU of IFN- $\alpha$ 2b ( $p < 0.05$ ) in comparison to the control (Fig. 6A-D). The incidence of apoptosis in TUNEL-stained sections showed the same tendencies as those obtained in the H&E-stained sections (Fig. 6E). The number of apoptotic cells significantly increased in the mouse tumors treated with the combination

therapy in comparison to the control (for each IFN,  $p < 0.0001$ ). The number also significantly increased with the combination treatment of PEG-IFN- $\alpha$ 2b and 5-FU in comparison to PEG-IFN- $\alpha$ 2b alone ( $p < 0.0001$ ), and with the combination of IFN- $\alpha$ 2b and 5-FU in comparison to IFN- $\alpha$ 2b alone ( $p < 0.05$ , Fig. 7).

The results of quantitative real-time RT-PCR are shown in Table III. The VEGF mRNA levels increased significantly in the PEG-IFN- $\alpha$ 2b alone group ( $p < 0.05$  vs. control,  $p < 0.05$  vs. IFN- $\alpha$ 2b) and in the combination (PEG-IFN- $\alpha$ 2b plus 5-FU) group ( $p < 0.05$  vs. control,  $p < 0.01$  vs. IFN- $\alpha$ 2b plus 5-FU). There were also significant increases in the expression levels of DPD ( $p < 0.01$ ), TP ( $p < 0.05$ ), and TS ( $p < 0.05$ ) in the PEG-IFN- $\alpha$ 2b alone group in comparison to the control. On the other hand, significant decreases were observed in the expression levels of DPD ( $p < 0.05$ ) and TP ( $p < 0.05$ ) in the combination (PEG-IFN- $\alpha$ 2b plus 5-FU) group in comparison to the PEG-IFN- $\alpha$ 2b alone group. In addition, the TS mRNA levels in the PEG-IFN- $\alpha$ 2b group increased in comparison to the IFN- $\alpha$ 2b group ( $p < 0.05$ ). The relative mRNA levels of IFN- $\alpha$ 2b receptors in the combination group were lower than the levels of the IFN alone group.

The number of artery-like blood vessels increased slightly in comparison to the control in the groups receiving IFN- $\alpha$ 2b alone, PEG-IFN- $\alpha$ 2b alone, or the combination therapies; and there were no significant differences among the 7 groups (Fig. 8).

## Discussion

Shang *et al* (18) examined 5 RCC cell lines and reported that the greatest decrease in the viable cell number after adding 1,600 IU/ml of Sumiferon to the cultures was 42% (58% of the control). On the other hand, Vyas *et al* (19) comparatively examined the anti-tumor effects of PEG-IFN- $\alpha$ 2b and IFN- $\alpha$ 2b by using an RCC cell line, ACHN, and reported that the addition of 1,033 IU/ml of PEG-IFN- $\alpha$ 2b suppressed the viable cell number to 50% of the control. Our current

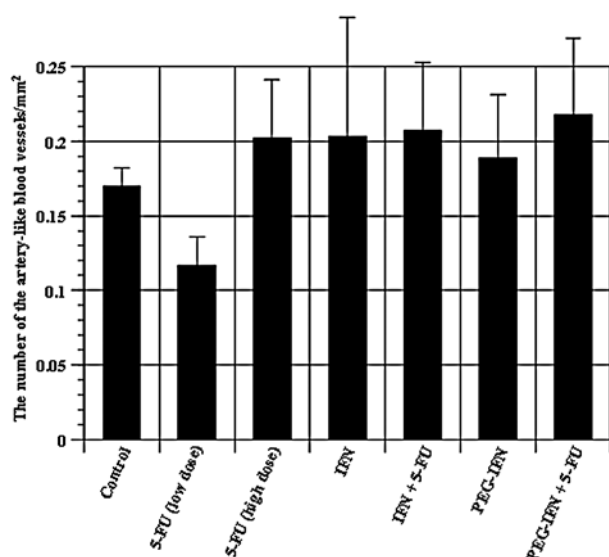


Figure 8. Number of artery-like blood vessels in the tumors. The number was counted in the whole area of each section, and mean number per mm<sup>2</sup> was obtained. Each figure shows the average  $\pm$  SE. PEG-IFN: PEG-IFN- $\alpha$ 2b.

experiment used concentration levels close to those of Shang *et al*, and similar anti-tumor effects were obtained. However, the level of suppression in our study did not reach 50% of the control even though the concentrations were higher than those of Vyas *et al*. The reasons for these disparate findings are not clear, however, they may be related to the different cell density in the experiments, different measurement methods, and possible changes in cell characteristics due to cultures. Vyas *et al* (19) also reported that the anti-tumor effects of PEG-IFN- $\alpha$ 2b and IFN- $\alpha$ 2b were not markedly different as we demonstrated in the present study.

Some medical institutions administer the combination therapy of IFN- $\alpha$  and 5-FU in the treatment for advanced RCC. Sella *et al* (20) reported that the combination of IFN- $\alpha$  and chemotherapy (5-FU and mitomycin C) resulted in a significant clinical effect on RCC patients. Our results support the anti-tumor effects reported by Sella *et al* regarding the combination of IFN- $\alpha$  and chemotherapy. Moreover, in the present study, the combination of PEG-IFN- $\alpha$ 2b and 5-FU exhibited enhanced anti-tumor effects in comparison to the combination of IFN- $\alpha$ 2b and 5-FU *in vivo*.

The induction of apoptosis is a mechanism of the anti-tumor effects of IFN- $\alpha$ 2b, and Vyas *et al* (19) reported a dose-dependent increase in apoptotic cell number for PEG-IFN- $\alpha$ 2b in cell cultures. Shang *et al* (18) found that apoptosis induction by IFN- $\alpha$ 2b was not significant even at a dose of 5,000 IU/ml. On the other hand, 5-FU induced apoptosis in a dose-dependent manner, and 50 and 100 IU/ml of IFN- $\alpha$ 2b was able to promote 5-FU-induced apoptosis in RCC cells. In our current study, the number of apoptotic cells *in vitro* increased proportionally to the dose of PEG-IFN- $\alpha$ 2b and to the doses of PEG-IFN- $\alpha$ 2b plus 5-FU in the combination treatment. The apoptotic cell number in the tumors also increased dose-dependently in the IFN- $\alpha$ 2b alone group and PEG-IFN- $\alpha$ 2b alone group, and the number in each group further increased with the combination of 5-FU. This indicates that PEG-IFN-

$\alpha$ 2b and IFN- $\alpha$ 2b induced apoptosis and the combination with 5-FU induced apoptosis more extensively. Comparing the two combination treatments, i.e., PEG-IFN- $\alpha$ 2b plus 5-FU vs. IFN- $\alpha$ 2b plus 5-FU, the estimated tumor volume was significantly smaller in the PEG-IFN- $\alpha$ 2b plus 5-FU group, but the number of apoptotic cells did not differ markedly between the groups. Shang *et al* (18) revealed that IFN- $\alpha$ 2b caused cell cycle arrest at G1 in ACHN cells and at G2/M in Caki-1 cells in their flow cytometric analyses, and this suggests that cell cycle arrest could be the reason why there was no remarkable difference in the number of apoptotic cells in our results.

Anti-angiogenesis activity is a biological effect of IFN- $\alpha$ 2b, and it has been shown that IFN- $\alpha$ 2b inhibits angiogenesis by down-regulating angiogenesis factors. For example, Dinney *et al* (21) systematically administered IFN- $\alpha$  in a nude mouse model of bladder tumor and reported a decrease in *in vivo* blood vessel density in the tumors, which then resulted in the shrinkage of the tumor size. On the other hand, Kojiro *et al* (16) reported that there was no significant relation between the tumor shrinkage effects and angiogenesis factors or artery-like blood vessels when IFN- $\alpha$  and 5-FU were administered in combination to nude mice receiving transplantation of HCC cells. In our current study, the mRNA expression of VEGF and the number of artery-like blood vessels in the tumors were not suppressed in the PEG-IFN- $\alpha$ 2b alone group and the PEG-IFN- $\alpha$ 2b plus 5-FU group, but the estimated tumor volume of the PEG-IFN- $\alpha$ 2b plus 5-FU group was the most suppressed among the groups. The reason for these contrary findings is unclear. Angiogenesis plays an important role in the proliferation and metastasis of solid tumors such as renal cancer, therefore the relation between angiogenesis factors and anti-tumor effects should be investigated in future studies by using different IFN preparations and other RCC cell lines.

It has been reported that IFN directly suppresses tumor proliferation and at the same time augments the suppressive effects of 5-FU on tumor growth, including the induction of apoptosis (15,22). In regards to the mechanism of this augmentation, several researchers reported that IFN- $\alpha$  acts on the metabolic pathway of 5-FU (23,24). Low levels of TS and DPD and high levels of OPRT, TP, UP and TK render cancer cells sensitive to 5-FU. In our results, the enzymes related to 5-FU metabolism, except OPRT, slightly increased (not significantly) in comparison to the control. Therefore, the activity of 5-FU-related enzymes were not related to the anti-tumor effects shown in our PEG-IFN- $\alpha$ 2b plus 5-FU group.

IFN- $\alpha$ 2b exerts its actions through a specific cell surface receptor, Type I IFN receptor, which 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- $\alpha$ 2b activity (25-27). Oie *et al* (17) examined the expression of type I IFN receptor mRNA in 6 HCC cell lines treated with 5-FU. They showed that the expression of type I IFN receptor was markedly increased in the 3 cell lines whose proliferation was suppressed synergistically by the administration of 5-FU and IFN- $\alpha$  than in the other 3 cell lines whose proliferation was suppressed in an additive manner. In our current study, expression of IFNAR-1 and IFNAR-2 increased in the IFN- $\alpha$ 2b alone and PEG-IFN- $\alpha$ 2b alone groups, whereas the expression levels were markedly lower



in the combination groups of IFN- $\alpha$  plus 5-FU than in the IFN alone groups. These findings differ from those of Oie *et al* which could be the reason why the effects of our combination treatment were additive and not synergistic.

Our results confirmed that in the treatment of RCC, PEG-IFN- $\alpha$ 2b presents more potent anti-tumor effects than conventional non-pegylated IFN- $\alpha$ 2b, and the effects are augmented when 5-FU is used in combination. The most probable mechanism of this potent effect is apoptosis induction, and the target molecules that induce apoptosis will be determined in future studies. We expect that the addition of another agent to the combination of IFN- $\alpha$ 2b and 5-FU would result in more potent anti-tumor effects in the treatment of RCC.

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