

Significant antitumor activity of cationic multilamellar liposomes containing human interferon- β gene in combination with 5-fluorouracil against human renal cell carcinoma

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Abstract. Immunotherapy is one of the most effective treatments against metastatic renal cell carcinoma (RCC). However, the response rate is not high. Therefore, more effective therapies are necessary for patients with metastatic RCC. We previously reported on the significant antitumor activity of cationic multilamellar liposome containing human interferon- β (huIFN- β) gene (IAB-1) against RCC. We then examined the antitumor effect of IAB-1 in combination with anticancer drugs against RCC. The cytotoxicity of IAB-1 alone, and in combination with anticancer drugs, cisplatin, adriamycin, 5-fluorouracil, gemcitabine, paclitaxel and irinotecan hydrochloride against the human RCC cell line NC65 was examined by the colorimetric method using tetrazolium salt. For the *in vivo* study, we used NC65 cells inoculated into the severe combined immunodeficiency mouse. The results showed that the *in vitro* combination therapy with IAB-1 and 5-FU was more cytotoxic than IAB-1 alone. However, synergistic cytotoxicity was not observed when combined with IAB-1 and other anticancer drugs. NC65 tumors transfected with IAB-1 in mice were smaller than those

receiving an injection of empty liposome or the recombinant huIFN- β protein. Treatment with IAB-1 in combination with 5-FU resulted in significant anticancer activity. IAB-1 enhanced the activity of thymidine phosphorylase (TP), which converts 5-FU to the active metabolite, FdUMP. In contrast, IAB-1 decreased the activity of thymidylate synthase (TS), which is a target enzyme of 5-FU. In conclusion, these findings indicate that a combination of IAB-1 and 5-FU may have enhanced antitumor activity against human RCC, suggesting its potential clinical application. The mechanism of enhanced cytotoxicity by combination therapy with IAB-1 and 5-FU may up-regulate TP activity and down-regulate TS activity.

Introduction

Metastatic renal cell carcinoma (RCC) is resistant to conventional chemotherapy and radiotherapy. Immunotherapy including interferon (IFN) and interleukin 2 (IL-2) is a relatively effective treatment against metastatic RCC. However, the response rate is ~15%.

Many investigators have attempted to enhance the effectiveness of IFNs by combining them with other biological agents, but the effects were inadequate (1-5). Gene therapy trials using cytokine genes such as granulocyte macrophage-colony stimulating factor (6,7) or IL-2 (8,9) have commenced in patients with metastatic RCC. Although these therapies appear to be safe, their efficacy has yet to be proven. Therefore, the development of novel strategies for metastatic RCC is desirable.

The RCC cells treated with cationic multilamellar liposome containing human interferon- β gene (IAB-1) constantly secreted significant amounts of human interferon- β (huIFN- β) protein (10). Apoptosis was observed in cells treated with IAB-1, but the recombinant huIFN- β protein failed to induce apoptosis. NC65 tumors (human RCC cell line) (11) transfected with IAB-1 in mice were significantly smaller than those receiving an injection of empty liposome or recombinant huIFN- β protein. To find a more effective therapy against metastatic RCC, we examined the antitumor effect of IAB-1 in combination with anticancer drugs against RCC.

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Abbreviations: ADR, adriamycin; CDDP, cisplatin; CPT-11, irinotecan hydrochloride; DPD, dihydropyrimidine dehydrogenase; E.L., empty liposome; 5-FU, 5-fluorouracil; GEM, gemcitabine; IAB-1, cationic multilamellar liposome containing human interferon- β gene; IFN, interferon; OPRT, orotate phosphoribosyl-transferase; PBS, phosphate-buffered saline; PTX, paclitaxel; RCC, renal cell carcinoma; SCID, severe combined immunodeficiency; TP, thymidine phosphorylase; TS, thymidylate synthase

Key words: cationic multilamellar liposome containing human interferon- β gene, 5-fluorouracil, gene therapy, renal cell carcinoma

Materials and methods

Cell line. The NC65 human RCC cell line was maintained in RPMI-1640 medium (Life Technologies Inc., Gaithersburg, MD) supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin (Life Technologies Inc.) and 10% fetal bovine serum (Life Technologies Inc., Bio-cult, Glasgow, Scotland, UK).

Reagents. IAB-1 is a plasmid DNA/lipid complex composed of plasmid (pSV2IFN β) that contains the SV40 early promoter, the huIFN- β coding sequence (12), and positively charged liposomes [N-(α -trimethyl ammonioacetyl)-didodecyl-D-glutamine chloride, dilauroyl phosphatidyl-choline, and dioleoyl phosphatidylethanolamine at a molar ratio of 1:2:2] (13-17). The IAB-1 was dissolved in phosphate-buffered saline (PBS) at a final concentration of 50 nmol of lipids/ μ l with 1.0 μ g of plasmid DNA/ μ l. The empty liposome (50 nmol of lipids/ μ l) contained no plasmid DNA.

The recombinant huIFN- β protein (IFN- β Mochida, 2.0×10^5 IU/mg) was provided by Mochida Pharmaceutical Inc., Tokyo, Japan.

Anticancer drugs. Cisplatin (CDDP), adriamycin (ADR), 5-fluorouracil (5-FU), gemcitabine (GEM), paclitaxel (PTX) and irinotecan hydrochloride (CPT-11) were used in this experiment.

In vitro cytotoxicity assay. Cytotoxicity was evaluated by a colorimetric method using 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8, Nacalai Tesque, Kyoto, Japan) (18). Briefly, NC65 cells suspended in culture medium were placed in a 24-well plate at a density of 2.5×10^4 cells/ml per well and cultured at 37°C in a humidified atmosphere of 5% CO₂ overnight. An aliquot of diluted IAB-1 suspension (from 0.001 to 0.10 μ l/ml), empty liposome suspension (from 0.001 to 0.10 μ l/ml), or huIFN- β protein (from 100 to 10,000 IU/mg) were added to each well, and then anticancer drugs were added. After 24 h of incubation, the medium was exchanged for 500 μ l of fresh medium and the cells were incubated for an additional 48 h. The culture supernatant was replaced by 500 μ l of completely fresh medium containing 0.5 μ mol of tetrazolium salt. After incubation for 1 h, the culture supernatant was harvested and absorbance (A) was measured at 450 nm. Cytotoxicity was calculated as: cytotoxicity (%) [1-(absorbance of experimental wells/average absorbance of control wells)] x 100.

Animal care. We purchased female severe combined immunodeficiency (SCID) mice, 8-9 weeks of age from CLEA Japan (Osaka, Japan). The animals were fed irradiated mouse chow and autoclaved reverse osmosis-treated water. The Committee for Animal Research, Kyoto Prefectural University of Medicine, permitted this experimental procedure.

In vivo study. NC65 cells (6×10^6) were subcutaneously injected with a mixture of 50 μ l Matrigel (Becton-Dickinson, NJ) and 50 μ l RPMI-1640 without antibiotics or serum into the right flanks of the SCID mice. Seven days after the tumor

cell injection, the tumor had developed to 5-7 mm in length and 5 mm in width. Then, 30 μ l of IAB-1 (1.5 μ mol of lipid with 30 μ g of plasmid DNA), 30 μ l of recombinant huIFN- β protein (6000 IU), 30 μ l of empty liposome (1.5 μ mol of lipid), or 30 μ l of PBS were injected into the established tumors on days 0, 2, 7, 9, 14, 16, 21 and 23 (twice a week for four courses). 5-FU (800 μ g) was injected into the peritoneal cavities on days 6, 13, 20 and 27 (once a week for four courses).

Tumor diameters were scaled with a digital caliper. The tumor volume was calculated as: volume = $a \times b^2/2$, where a, long diameter and b, short diameter.

Measurement of thymidylate synthase (TS) activity. The activity of TS was determined by the 5-fluoro-2'-deoxyuridine 5'-monophosphate (FdUMP) binding assay combined with gel filtration as previously described in the References (19,20). NC65 cells were sonicated in homogenized buffer (50 mM Tris-HCl, 1 mM EDTA and 5 mM MgCl₂ pH 7.4) at maximum output (Sonifier cell disruptor 350: SmithKline), and centrifuged at 105,500 g at 4°C for 60 min in a Beckman ultracentrifuge (model TL-100). The supernatant was divided into several tubes and frozen at -80°C until use.

The supernatant was incubated with [³H]-FdUMP as well as 5,10-CH₂-FH₄ at 30°C for 20 min. The mixture was gel-filtered using a PD-10 column (Pharmacia Biotech, Uppsala, Sweden) to separate TS-bound from free [³H]-FdUMP. The sample was eluted with PBS (-) and the total radioactivity of the fractions containing protein was measured. The protein content of the supernatant was measured using the BCA protein assay reagent (Pierce Chemical Co., Rockford, IL, USA).

Measurement of dihydropyrimidine dehydrogenase (DPD) activity. The NC65 cells were homogenized in 4 volumes of 50 mM Tris-HCl (pH 8.0) containing 5 mM 2-mercaptoethanol, 25 mM KCl and 5 mM MgCl₂. The homogenate was centrifuged at 105,000 g for 1 h at 4°C, and the supernatant fluid was used to measure the DPD activity (21). The assay mixture, in a final volume of 0.25 ml, consisted of 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 25 mM NaF, 50 mM nicotinamide, 5 mM adenosine triphosphate (ATP), 1 mM NHDPH, [6-³H] 5-FU (0.2 μ Ci and 20 μ M) and the enzyme extract (0.1 ml). The mixture was incubated for 30 min at 37°C and the reaction was stopped by heating at 100°C in a water bath. After centrifugation at 3000 rpm, the supernatant (0.1 ml) was treated with 0.01 ml of 2 M KOH for 30 min at room temperature. The mixture was treated with 0.005 ml of 2 M PCA and centrifuged. An aliquot (20 μ l) of the supernatant was spotted onto a thin layer chromatography plate (Merck silica gel 60F254 precoated plate, 2.5x10 cm, thickness 0.25 mm) and developed with a mixture of chloroform, methanol and acetic acid (17:3:1, v/v/v). The spots of 2-fluoro- β -alanine, 2-fluoro- β -ureidopropionic acid and 5-FU degradation products, were scraped into vials and mixed with 10 ml of ACS-II scintillation fluid (Amersham, Buckinghamshire, UK). The radioactivity was measured in a Wallac 1410 liquid scintillation counter (Pharmacia, Uppsala, Sweden).

Measurement of orotate phosphoribosyltransferase (OPRT) activity. The NC65 cells were sonicated in homogenized

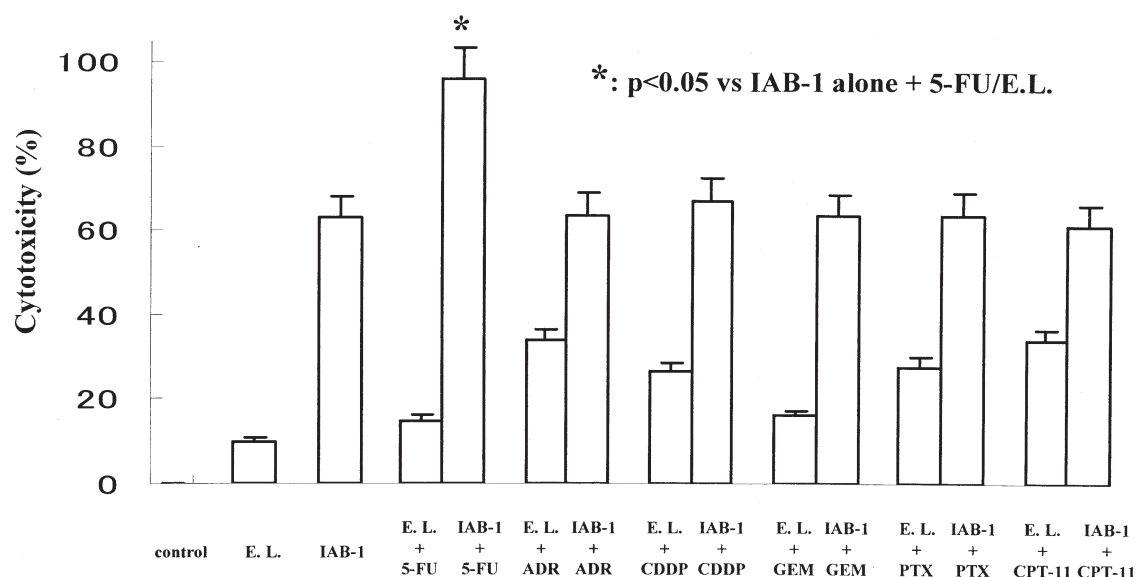


Figure 1. Cytotoxicity of IAB-1 in combination with anticancer drugs against NC65 cells *in vitro*. Cytotoxicity was evaluated by a colorimetric method using tetrazolium salt. NC65 cells placed in a 24-well plate at a density of 2.5×10^4 cells/ml were cultured overnight at 37°C . Medium (control), empty liposome (E.L.) (0.10 $\mu\text{l/ml}$), IAB-1 (0.10 $\mu\text{l/ml}$), CDDP (1 μM), ADR (0.01 μM), 5-FU (10 μM), GEM (0.1 μM), PTX (0.05 μM), CPT-11 (0.005 μM) and/or Taxol (0.05 μM) were added. After incubation for 24 h, the medium was exchanged for 500 μl of fresh medium. After incubation for a further 48 h, the cytotoxic effect was evaluated. Results derived from seven different experiments are expressed as means \pm SD. * $p < 0.05$; IAB-1 alone versus IAB-1 + 5-FU/E.L.

buffer (50 mM Tris-HCl, pH 7.4, and 5 mM 2-mercaptoethanol) at maximum output using a Sonifier cell disruptor 350 (SmithKline, London, UK). They were centrifuged at $105,000 \times$ gravity at 4°C for 60 min in a model TL-100 (Beckman, Fullerton, CA) ultracentrifuge. The supernatants from each sample were divided into several tubes and frozen at -80°C until use.

The test supernatant was incubated with 10 μM [$6\text{-}^3\text{H}$] 5-FU (74 kBq), 50 mM Tris-HCl, pH 8.0, 50 mM MgCl_2 , 10 mM NaF and 4 mM phosphoribosylpyrophosphate at 37°C for 30 min. Incubation was terminated by adding 2 M perchloric acid, followed by centrifugation at 3000 rpm for 10 min. The supernatant (100 μl) was then neutralized with 30 μl 2 M KOH solution and 20 μl aliquots were subjected to silica gel 60 F_{254} thin layer chromatography (2.5 \times 10 cm and 0.25 mm thick) with a mixture of chloroform, methanol and acetic acid (17:3:1 volume per volume per volume) as the mobile phase. Spots of 5-fluorouridine 5'-monophosphate were scraped into vials and extracted with 0.1 ml 4 M HCl. The extracts were mixed with 10 ml ACS-II (Amersham) scintillation fluid. Radioactivity was measured in a Wallac 1410 (Pharmacia) liquid scintillation counter. The protein content of the test supernatant was measured using the BCA protein assay reagent (Pierce Chemical Co.). OPRT activity was calculated per mg protein. Internal standards were used to compare assays. We analyzed the samples at the same time. This method made it possible to estimate OPRT activity >0.05 pmol/mg protein per minute. Repeat measurements yielded almost the same results.

Measurement of thymidine phosphorylase (TP) activity by the thin layer chromatography method. The NC65 cells were homogenized in 4 volumes of 50 mM Tris-HCl, pH 8.0, containing 5 mM 2-mercaptoethanol, 25 mM KCl and 5 mM MgCl_2 and were centrifuged at $105,000 \times g$ at 4°C for 60 min

in an ultracentrifuge (Model TL-100; Beckman Instruments, Fullerton, CA). The supernatants from each sample were divided into several tubes and frozen at -80°C until use.

The reaction mixture, in a total volume of 100 μl , consisted of 100 mM sodium phosphate buffer, pH 6.4; β -mercaptoethanol; 0.5 mM [^3H]thymidine; and the supernatant (50 μl). The mixture was incubated for 20 min at 37°C , and the reaction was stopped by heating at 100°C in a water bath. After centrifugation at 14,000 rpm for 2 min, the supernatant (20 μl) was applied to a polyethylenimine cellulose TLS sheet and was developed with distilled water. The spots corresponding to the substrate and product were visualized under ultraviolet light and excised, and the level of radioactivity in each was measured. Internal standards were used to compare assays. This method made it possible to estimate TP activity >0.01 nmol/mg protein per minute. We analyzed the samples at the same time.

Results

***In vitro* treatment with IAB-1 in combination with anticancer drugs against NC65 cells.** Treatment with IAB-1 at 0.1 $\mu\text{l/ml}$ against the NC65 cells resulted in strong cytotoxicity, compared with the 1000 IU/ml recombinant huIFN- β protein (data not shown). We used CDDP, ADR, 5-FU, GEM, PTX and CPT-11 as anticancer agents for combination therapy. The cytotoxicity induced by IAB-1 in combination with CDDP, ADR, GEM, PTX or CPT-11 was almost the same as that induced by IAB-1 alone. However, treatment with IAB-1 and 5-FU resulted in significant anticancer activity, compared with IAB-1 alone (Fig. 1). Similar results were observed, when IAB-1 and 5-FU were used at different concentrations (Fig. 2). These results suggest that the combination treatment of NC65 cells with IAB-1 and 5-FU demonstrates synergistic cytotoxicity.

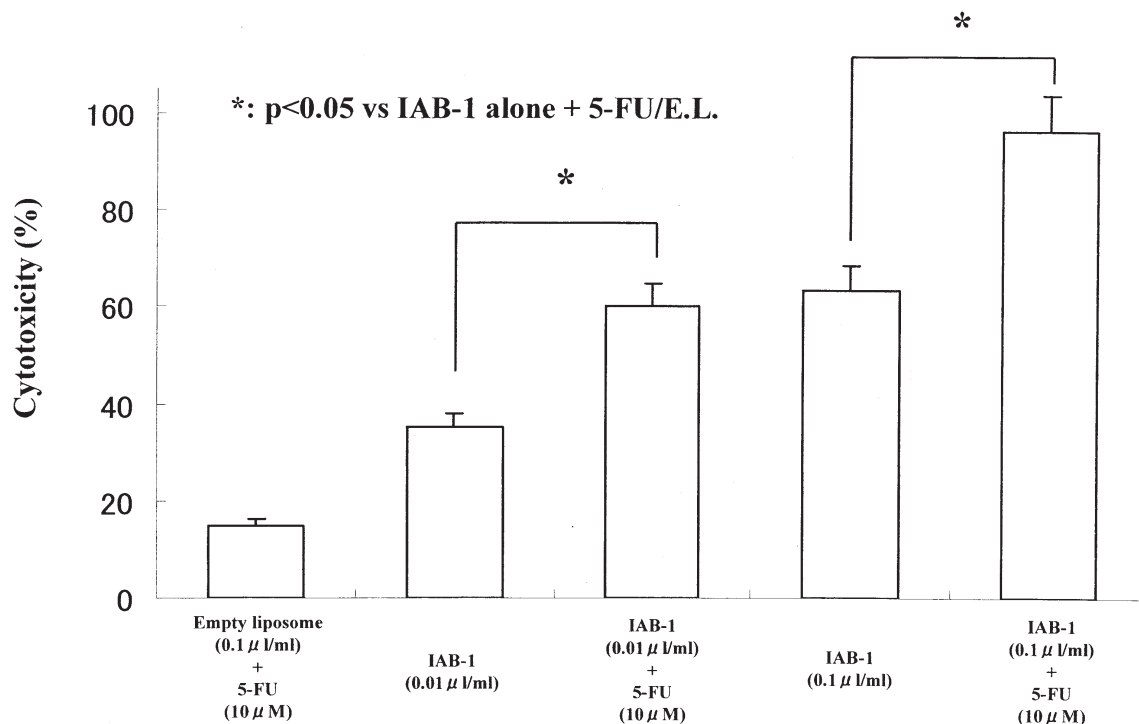


Figure 2. Cytotoxicity of IAB-1 in combination with 5-FU against NC65 cells *in vitro*. NC65 cells were treated with empty liposome (0.10 μl/ml), IAB-1 (0.01 μl/ml), IAB-1 (0.10 μl/ml) and/or 5-FU (10 μM) for 24 h. The medium was aspirated and the NC65 cells were washed with RPMI-1640 medium. Cytotoxicity was assessed by a two-day assay. * $p < 0.05$; IAB-1 alone versus IAB-1 + 5-FU/E.L.

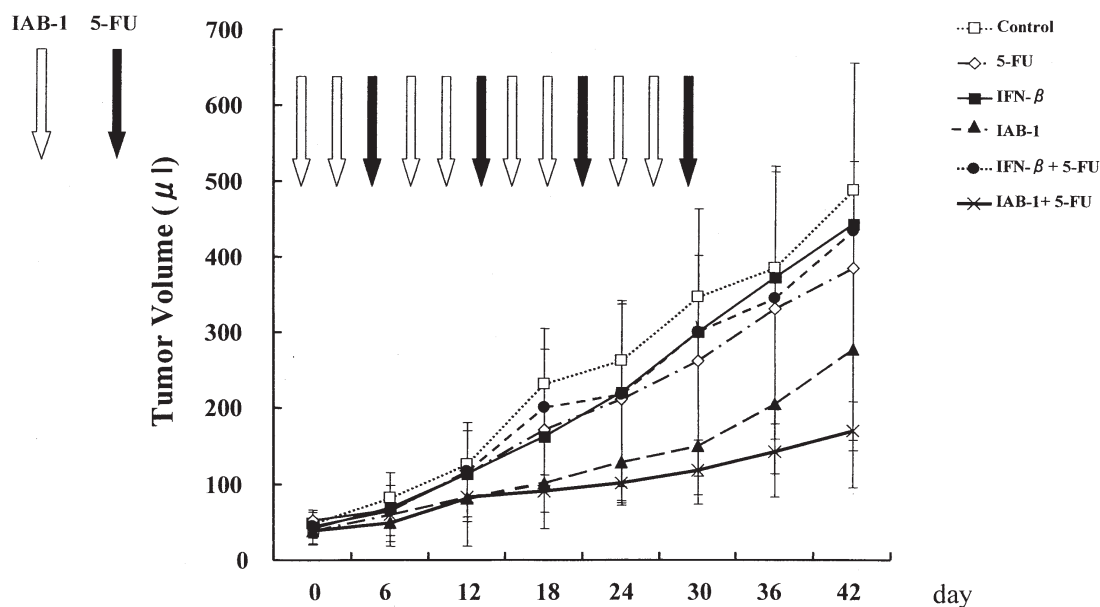


Figure 3. *In vivo* treatment with IAB-1 in combination with 5-FU against NC65 tumors. Seven days before the first treatment, 6 million NC65 cells were subcutaneously injected into the right flank of SCID mice. Then, 30 μl of IAB-1 (1.5 μmol of lipid with 30 μg of plasmid DNA), 30 μl of recombinant huIFN-β protein (6000 IU), 30 μl of empty liposome (1.5 μmol of lipid), or 30 μl of PBS (control) were injected into established tumors on days 0, 2, 7, 9, 14, 16, 21 and 23 (twice a week for four courses). 5-FU (800 μg) was injected into the peritoneal cavities on days 6, 13, 20 and 27 (once a week for four courses). The tumor volume growth curves are shown. $p < 0.05$; IAB-1 versus control, 5-FU, and IFN-β and $p < 0.05$; IAB-1 + 5-FU versus control, 5-FU, IFN-β and IFN-β + 5-FU.

Effect of the treatment sequence with IAB-1 and 5-FU on synergistic cytotoxicity. The simultaneous treatment of NC65 cells with IAB-1 and 5-FU resulted in synergistic cytotoxicity. The effect of sequential treatment with IAB-1 and 5-FU was compared with the treatment of the two agents together. NC65

cells were treated for 24 h with one agent, the medium was exchanged, the second agent was added for 24 h and the cells were tested for viability. The results show that synergy was obtained irrespective of the treatment sequence (data not shown). Similar results were observed when IAB-1 and 5-FU

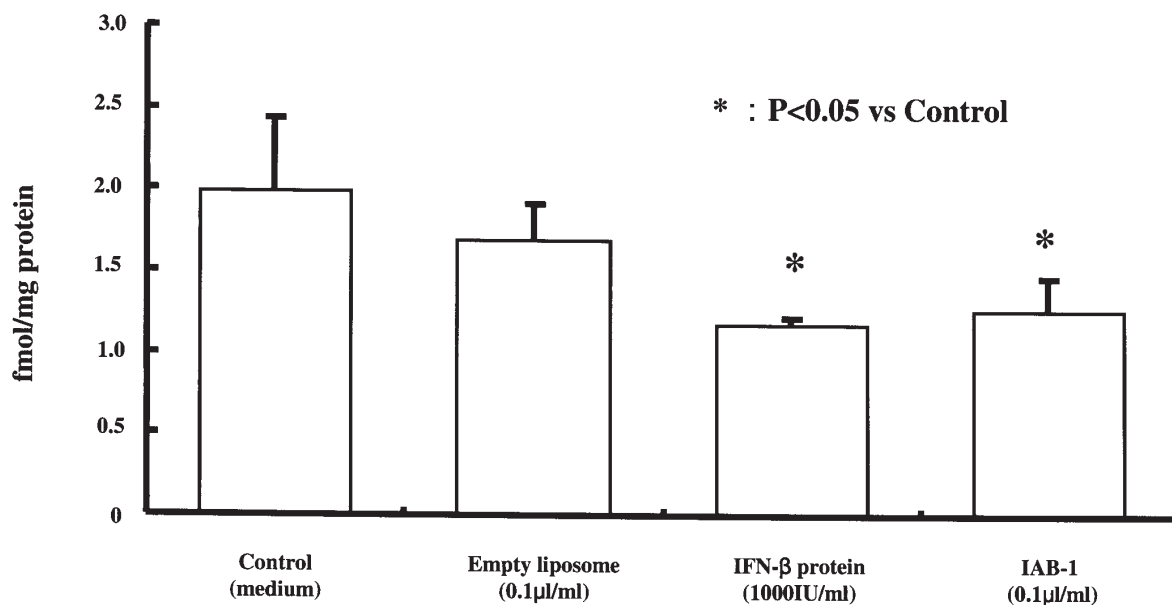


Figure 4. Suppressed TS activity in NC65 cells by IAB-1. TS activity was examined by the FdUMP binding assay. TS activity in the NC65 cells was decreased by IAB-1 or IFN-β protein.

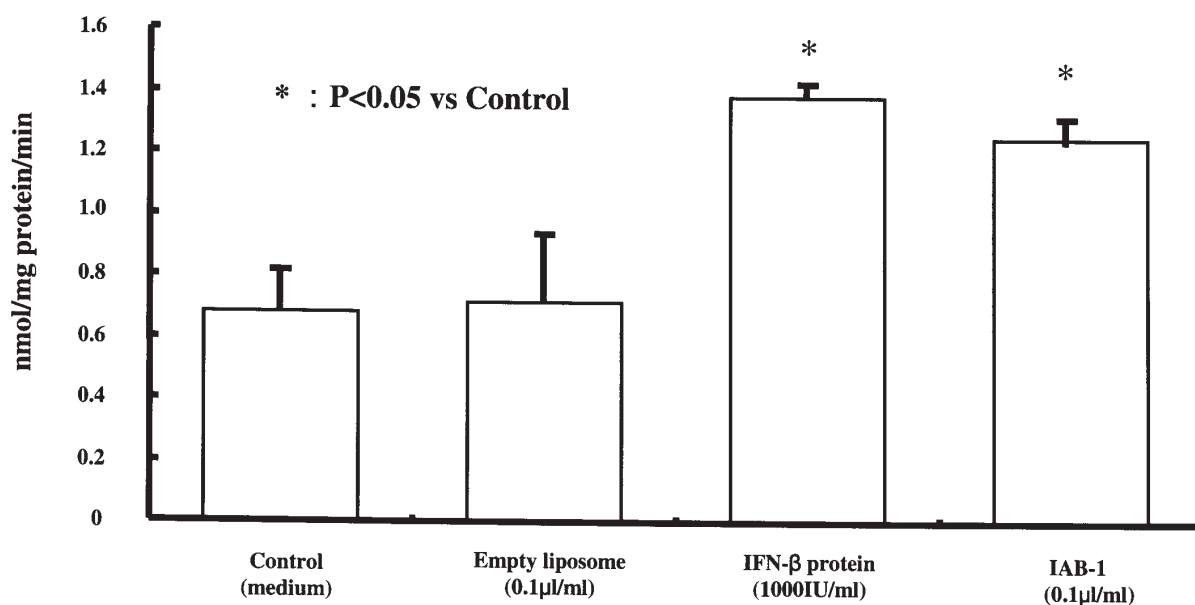


Figure 5. Enhanced TP activity in NC65 cells by IAB-1. TP activity was measured by a thin layer chromatography assay. TP activity in the NC65 cells was enhanced by IAB-1 or IFN-β protein.

were used at different concentrations. These findings show that the sequence of treatment with IAB-1 and 5-FU is not critical for obtaining maximal cytotoxicity in RCC cells.

In vivo treatment with IAB-1 in combination with 5-FU against NC65 cells. We examined the antitumor effect of IAB-1 in combination with 5-FU *in vivo*. The NC65 tumors transfected with IAB-1 in mice were significantly smaller than those receiving an injection of empty liposome or the recombinant huIFN-β protein (Fig. 3). Similar to the result

in vitro, treatment with IAB-1 in combination with 5-FU resulted in significant anticancer activity, compared with IAB-1 alone.

Effects of IAB-1 on TS, DPD, OPRT and TP activities in NC65 cells. The properties of important enzymes in the metabolic pathways of 5-FU, TS, DPD, OPRT and TP in the cell lysate treated with IAB-1 were measured. TS is a target enzyme of 5-FU, while IAB-1 down-regulated TS activity (Fig. 4). DPD is the rate-limiting enzyme in the pathway of

5-FU catabolism, while IAB-1 had no effect on DPD activity (data not shown). OPRT and TP convert 5-FU to the active metabolite, FdUMP. Although IAB-1 had no effect on OPRT activity, IAB-1 up-regulated TP activity (Fig. 5). These results suggest that IAB-1 enhances 5-FU cytotoxicity by down-regulating TS activity and up-regulating TP activity.

Discussion

RCC accounts for ~2% of all cancer cases worldwide. Metastatic disease is often present at the time of diagnosis of RCC, and its poor response to chemotherapy and radiotherapy determines its poor prognosis. Cytokines, IFN- α , - β , - γ and IL-2 are used for patients with RCC. However, the response rate is ~15%. We have reported that significant and continuous huIFN- β production was detected in the culture medium of NC65 cells after transfection with IAB-1 (10). Apoptosis was observed in cells treated with IAB-1, but the recombinant huIFN- β protein failed to induce apoptosis. IAB-1 and the recombinant huIFN- β protein may have different mechanisms concerning antitumor effects.

As treatment for metastatic RCC, combination therapy with cytokines, IFN, IL-2 and anticancer drugs, 5-FU and GEM is used. Some investigators have reported that the combination therapy was more effective than cytokine therapy alone (23-27). Experimental studies have demonstrated some evidence of synergism between chemotherapeutic agents, notably 5-FU and recombinant IFN- α (28). However, the addition of 5-FU to recombinant IFN- α does not have sufficient antitumor activity against metastatic RCC. The combination of IFN- β gene therapy and 5-FU resulted in a significant tumor regression in esophageal and colorectal cancer cells (29,30). We examined the antitumor effect of IAB-1 and anticancer agents including 5-FU.

CDDP, ADR, GEM, PTX and CPT-11 did not enhance IAB-1 cytotoxicity against RCC. Combination therapy with IAB-1 and 5-FU was more cytotoxic than IAB-1 alone. These results suggest that combination therapy with IAB-1 and 5-FU is more beneficial for the treatment against RCC.

We then examined the mechanisms responsible for the significant antitumor activity of IAB-1 and 5-FU against RCC. 5-FU is a prodrug that is converted by several enzymes including OPRT and TP to the active metabolite, FdUMP, which binds to TS and inhibits DNA synthesis. 5-FU is catabolized mainly by DPD. This study demonstrated that IAB-1 enhanced TP but suppressed TS activity. Therefore, IAB-1 may augment 5-FU cytotoxicity by these effects.

In vivo, tumors transfected with IAB-1 and 5-FU in mice were smaller than those treated by IAB-1 alone. In addition, the body weight of mice treated with IAB-1 and 5-FU as controls did not change during the treatments (data not shown). Therefore, it may be safe to use combination therapy with IAB-1 and 5-FU clinically.

The overall response rate of patients with RCC to immunotherapy and molecular-targeted therapy has gradually improved. However, drug resistance and metastatic RCC remain important issues and more effective therapies are necessary for these patients. The current study demonstrates that combination treatment with IAB-1 and 5-FU resulted in enhanced cytotoxicity in RCC cells. These findings suggest

that combination treatment with IAB-1 and 5-FU is useful in patients with RCC as a new form of therapy with more selective cytotoxicity and less collateral toxicity.

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