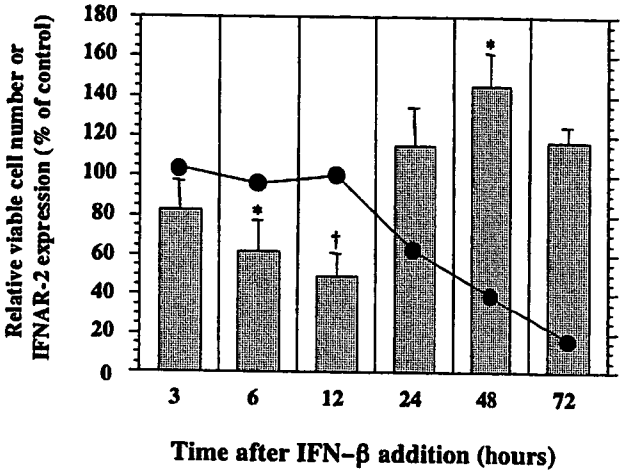

FIG. 2. Effects of 1000 IU/mL IFN-β. Time course changes in the relative viable cell number (circles, % of control) and IFNAR-2 expression in HAK-1B cells before and after the addition of 1000 IU/mL IFN-β ($n = 5$ at each measurement). The figures represent the average ± SE. *$p < 0.05$ vs. corresponding control value; †$p < 0.005$ vs. corresponding control value. The experiment was repeated three times.

liferative effects of three IFN-α preparations by using the same cell lines and methods.[20,21,24] Comparison between previous findings on IFN-α preparations and the current findings on IFN-β showed that (1) IFN-β induced a time-dependent antiproliferative effect in the largest number of cell lines, (2) in the 96-h culture with 1024 IU/mL IFN-β, the relative viable cell number became lower than 50% in 9 cell lines, and this was also the largest number of cell lines in our IFN studies, and (3) in 6 of the 9 cell lines, growth suppression occurred at a dose <100 IU/mL, and the IC_{50} of the 9 cell lines ranged between 14.8 and 260.1 IU/mL, lower than the levels for the 3 IFN-α preparations.[20,21,24] For example, in HAK-1B cells, the IC_{50s} of the 3 IFN-α preparations (i.e., BALL-1 lymphoblastoid IFN-α, consensus IFN-α, and PEG-IFN-α2b)[20,21,24] were 14, 43, and 55 times higher, respectively, than the IC50 of IFN-β. In various cell lines, such as melanoma and glioma, IFN-β presents a stronger antiproliferative effect than IFN-α even though IFN-α and IFN-β bind to the same heterodimeric receptor.[41–43] Leaman et al.[44] conducted oligonucleotide array analysis on 2 melanoma cell lines and showed that IFN-β was more potent than IFN-α2 in the induction of IFN-stimulated gene (ISG) expression. In HCC cell lines, there have been different findings, that is, IFN-β induced a stronger antiproliferative effect and higher ISG expression than IFN-α,[29,30] and neither IFN-α nor IFN-β significantly inhibited the growth of liver cancer cells.[28] We presume that this difference is attributable to differences in the IFN preparations and experiment methods, and particularly to differences in the cell lines used in the experiments. The 4 cell lines (HAK-2, HAK-3, HAK-4, KMCH-2) whose relative viable cell numbers in the current study were higher than 50% after 96-h exposure to 4096 IU/mL IFN-β were found to be also relatively insensitive to the antiproliferative effect of 3 IFN-α preparations.[20,21,24] IFN-β-mediated apoptosis induction was observed as an antiproliferative mechanism in 10 cell lines but not in the other 3 cell lines (HAK-3, HAK-4, KMCH-2). The low sensitivity or insensitivity to the antiproliferative effect of IFN-β in the 4 cell lines (HAK-2, HAK-3, HAK-4, KMCH-2) would be attributable to the resistance to IFN-β-mediated apoptosis or low cell surface IFNAR-2 expression (HAK-3, HAK-4, KMCH-2).[20]

IFN-β induces a stronger antiproliferative effect and higher ISG expression than IFN-α. It has been reported that the different biologic effects of IFN-β from those of IFN-α2 may be mediated by the formation of a uniquely stable type I IFN receptor complex, greater affinity for the type I receptor complex, involvement of other receptor components, and the activation of additional signaling pathways.[45–47] These findings suggest

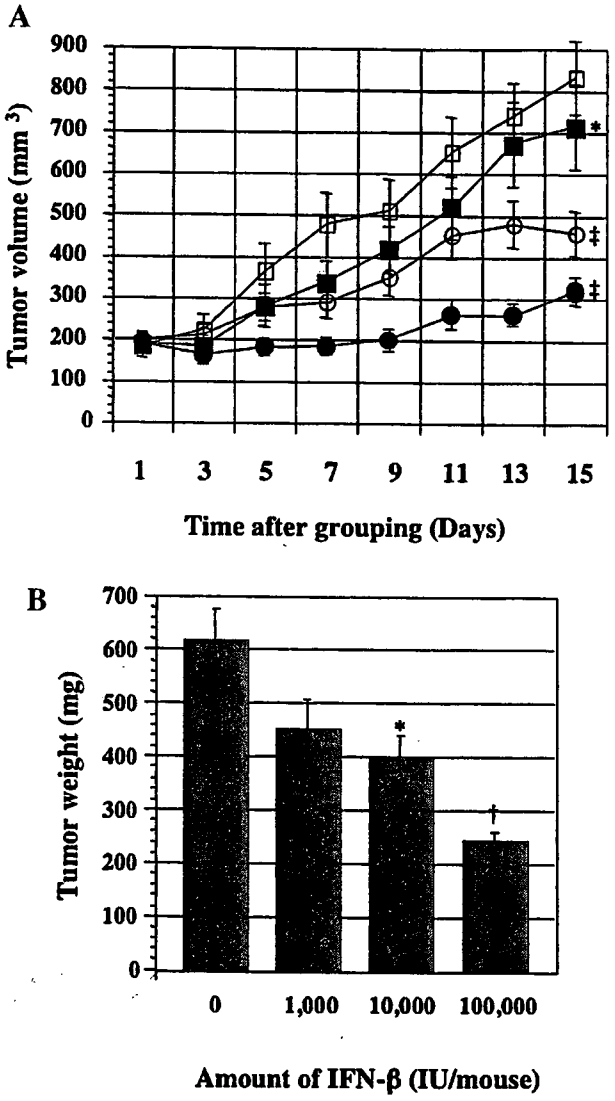

FIG. 3. Time course change in estimated tumor volume of s.c. transplanted HAK-1B in nude mice. (A) Mice received an s.c. injection of 1,000 (closed squares), 10,000 (open circles), or 100,000 (closed circles) IU IFN-β or PBS (control, open squares). (B) All mice were killed on day 15, and tumor weight was measured. Figures represent the average ± SE. The experiments were repeated twice, and almost identical results were obtained. *$p < 0.05$ vs. control; †$p < 0.001$ vs. control; ‡$p < 0.0001$ vs. control.

TABLE 2. ELISA, ARTERYLIKE BLOOD VESSELS, AND NUMBER OF APOPTOTIC CELLS IN HUMAN HCC (HAK-1B) TUMOR THAT WAS SUBCUTANEOUSLY TRANSPLANTED IN NUDE MICE

Treatment group[a]	*Control*	*IFN-β 1,000 IU*	*IFN-β 10,000 IU*	*IFN-β 100,000 IU*
VEGF[b] in tumor	20.0 ± 3.0[c]	27.7 ± 4.8	30.1 ± 3.2*	22.6 ± 3.9
bFGF[b] in tumor	46.8 ± 2.9	38.4 ± 4.6*	39.1 ± 1.7	48.6 ± 2.6
in serum	16.0 ± 4.4	6.2 ± 1.4*	7.8 ± 1.6	3.6 ± 0.4*
IL-8[b] in tumor	35.8 ± 3.2	37.3 ± 3.0	39.6 ± 3.5	36.7 ± 2.5
in serum	27.0 ± 2.2	11.0 ± 2.4**	10.9 ± 1.7***	8.1 ± 1.4***
Arterylike blood vessel[d]				
inside tumor	0.381 ± 0.074	0.318 ± 0.053	0.390 ± 0.075	0.413 ± 0.057
tumor margin	0.621 ± 0.081	0.646 ± 0.069	0.770 ± 0.128	0.701 ± 0.077
Apoptotic cell[e]	67.2 ± 5.2	73.9 ± 3.8	113.4 ± 5.4***	155.1 ± 7.9***

[a]Cultured HAK-1B cells (1×10^7) were transplanted s.c. into nude mice. Eight days later, when the largest diameter of the tumor reached approximately 5–10 mm, mice in each group were treated every day with i.p. injections of IFN-β or saline (Control).

[b]ELISA data (VEGF, bFGF, and IL-8) are expressed as ng/100 pg total protein.

[c]Average ± SE.

[d]Arterylike blood vessels: number of blood vessels in the tumor and in the borderline area between the tumor nodule and surrounding tissues was counted in each specimen, and the average number per area in each group was obtained.

[e]Number of apoptotic cells was counted in eight 0.25-mm^2 areas in each section, and the average number per area in each group was obtained.

*$p < 0.05$ vs. Control; **$p < 0.005$ vs. Control; ***$p < 0.001$ vs. Control.

that the difference in such effects as antiproliferation and potency between IFN-α and IFN-β may be largely related to the different interaction with type I IFN receptor. We recently examined chronologic changes in IFNAR-2 expression in HAK-1B cells cultured with PEG-IFN-α2b and found that the expression of IFNAR-2 was significantly downregulated at 3 h compared with the control, then significantly upregulated at 48 h, and returned to the control level at 72 h.[21] In the current study, we also examined chronologic changes of IFNAR-2 expression with IFN-β by using the same HCC cell line, HAK-1B, for up to 72 h of culture and compared the current results with the previous results on PEG-IFN-α2b. As a result, IFNAR-2 expression after IFN-α or IFN-β exposure was not different, and we could not find a difference at this point.

IFN-β *in vivo* dose-dependently suppressed the growth of human HCC that was transplanted s.c. to nude mice, and growth suppression occurred even at 1/2.4 of the clinical dose for chronic hepatitis C patients. By considering the IC_{50} of *in vitro* IFN-β that was 1/43 of the consensus IFN-α[24] and by comparing to our previous *in vivo* findings of consensus IFN-α,[24] IFN-β is expected to have much stronger antiproliferative effects than those found in the current results. There was no difference in the antiproliferative effects between the i.p. and i.v. administrations of IFN-β. The reason for this lower level of antiproliferative effects has not yet been described fully, but pharmacokinetic studies indicate that IFN-β exhibits an extremely short half-life in the blood system after i.m. or i.v. protein administration.[48,49] Therefore, the short half-life of IFN-β in serum may be related to the *in vivo* findings.

In our recent study on PEG-IFN-α2b and IFN-α2b, which are equal in their antivirus activity level,[21] their *in vivo* antiproliferative effects on HCC were comparatively examined. The antiproliferative effect was significantly higher in PEG-IFN-α2b, with a longer serum half-life than IFN-α2b. This suggested that the antitumor effect is higher when IFN is present in the serum for a longer time. Similar findings were reported for IFN-β. In clinical practice, the clearance rate of serum HCV RNA and the ratio of 2′,5′-oligoadenylate synthetase (2′,5′-OAS) activity were significantly higher in chronic hepatitis patients who received 3 MU IFN-β i.v. twice a day than those who received 6 MU IFN-β daily.[50,51] In animal experiments, Sung et al.[52] examined the pharmacokinetic properties of albuferon in rhesus monkeys. Albuferon is a novel recombinant protein derived from the gene fusion of IFN-β and human serum albumin, and its half-life is longer than that of IFN-β. They found an enhanced pharmacodynamic response, with increases in both neopterin and 2′,5′-OAS expression levels. Therefore, it is necessary to conduct further *in vivo* studies that use a more frequent administration of IFN-β or IFN-β with a longer half-life.

Our current study examined the mechanism of *in vivo* antiproliferative effects by monitoring apoptosis, angiogenesis, and the expression of angiogenesis factors. As a result, the number of apoptotic cells increased with the increase in the IFN-β dose, and this showed the occurrence of antiproliferation due to apoptosis induction, as was found for IFN-α.[22,24] On the other hand, angiogenesis inhibition was not observed as well as for PEG-IFN-α2b[21]; that is, the number of blood vessels in and around the tumor did not decrease significantly. Several studies reported that IFN-β inhibits angiogenesis by suppressing the expression of such angiogenesis factors as bFGF[53,54] and IL-8.[55,56] However, in the current study, decreased expression of bFGF and IL-8 in the serum of IFN-β groups, in particular the 100,000 IU group, was thought to be the result of tumor size reduction. Regarding the expressions in nude mouse tumors, IL-8 expression was not significantly different among the groups, and VEGF expression was significantly increased in the 10,000 IU IFN-β group. In the 1,000 IU group,

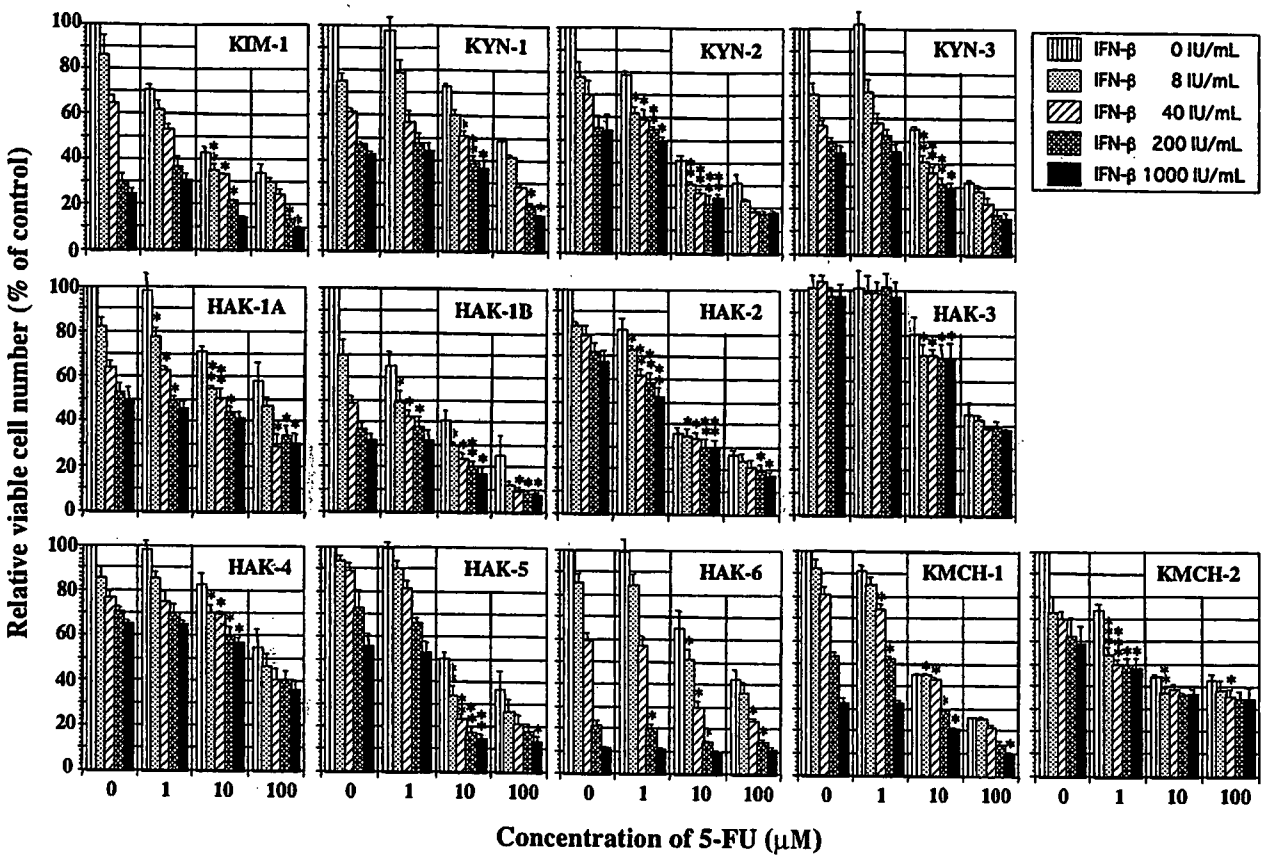

FIG. 4. Antiproliferative effect of IFN-β in combination with various doses of 5-FU. Cells were incubated with IFN-β in the presence of various concentrations of 5-FU, and a relative viable cell number (% of control) was determined after 72 h of culture. Eight samples were used in each experiment, which was repeated at least three times for each cell line. Figures represent the average ± SE. *Synergistic effect with the range of the combination index (CI) of 0.3–0.7; **strong synergistic effect with the range of the CI of 0.1–0.3; ***very strong synergistic effect with the range of CI of <0.1. Synergistic effects and the CI were assessed by median-effect principle analysis.

bFGF expression decreased significantly from the level of the control, and the number of blood vessels decreased inside the tumor. Therefore, bFGF and blood vessels might be related, and in the 1,000 IU group, IFN could suppress the expression of angiogenesis factors at a biologically optimal concentration as Tedjarati et al.[57] reported, even though there was no specific decrease in tumor size. VEGF expression in HAK-1B cells *in vitro* was upregulated in the same manner as for IFN-α.[58]

The clinical efficacy of chemotherapy in combination with IFN-α has already been reported in HCC.[17–19] As *in vitro* data suggest that the antiproliferative potency of IFN-β is greater than that of IFN-α,[41–43] IFN-β in combination with chemotherapeutic drugs would be a more promising therapeutic approach in the treatment of HCC. Makower and Wadler[59] described that IFN-β is a more potent modulator of 5-FU cytotoxicity *in vitro* than IFN-α. In the current study, we examined the cooperative effects of IFN-β with 5-FU on 13 human liver cancer cell lines using growth inhibitory assays and median-effect principle analyses and found synergistic antiproliferative effects in all cell lines at various degrees. The possible reported mechanisms of these synergistic effects are as follows[59–61]: (1) IFN-β may increase the amount of active 5-FU metabolite and inhibit the activity of thymidylate synthase, (2) IFN-β may alter the expression of enzymes that affect 5-FU metabolism (e.g., thymidine phosphorylase, dihydropyrimidine dehydrogenase, uridine phosphorylase, and thymidine kinase), and (3) 5-FU may upregulate the expression levels of type I IFN receptor subunits. Oie et al.[61] examined the mechanisms of the antiproliferative effect of combination treatment with IFN-α and 5-FU by using 6 HCC cell lines and confirmed that the upregulation of type I IFN receptor by 5-FU is the most important mechanism of synergistic effects.

Our findings showed that IFN-β even at a relatively low dose has a potent antiproliferative effect on HCC *in vitro*, and the *in vivo* antitumor effect was expressed at the clinical dose. These data suggest the potential clinical application of IFN-β in the prevention and treatment of HCC. Strong growth suppression on HCC could be expected when a relatively low dose of IFN-β with or without 5-FU is continuously or frequently administered to tumor-feeding arteries of HCC.

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Alteration of dihydropyrimidine dehydrogenase expression by IFN-α affects the antiproliferative effects of 5-fluorouracil in human hepatocellular carcinoma cells

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Abstract

Dihydropyrimidine dehydrogenase (DPD) is the rate-limiting enzyme in the catabolism of 5-fluorouracil (5-FU) and its activity is closely associated with cellular sensitivity to 5-FU. This study examines the role of DPD in the antiproliferative effects of 5-FU combined with IFN-α on hepatocellular carcinoma (HCC) cells in culture and asks whether IFN-α could affect DPD expression. The combined action of IFN-α and 5-FU on three HCC lines was quantified by a combination index method. Coadministration of IFN-α and 5-FU showed synergistic effects against HAK-1A and KYN-2 but antagonistic effects against KYN-3. The cellular expression levels of DPD mRNA and protein were markedly up-regulated in KYN-3 cells by IFN-α but were down-regulated in HAK-1A and KYN-2. The expression of thymidylate synthase mRNA and protein was down-regulated by IFN-α in all three cell lines. Coadministration of a selective DPD inhibitor, 5-chloro-2,4-dihydroxypyridine (CDHP), enhanced the antiproliferative effect of 5-FU and IFN-α on KYN-3 ~4-fold. However, the synergistic effects of 5-FU and IFN-α on HAK-1A and KYN-2 were not affected by CDHP. The antiproliferative effect of 5-FU could thus be modulated by IFN-α, possibly through DPD expression, in HCC cells. Inhibition of DPD activity by CDHP may enhance the efficacy of IFN-α and 5-FU combination therapy in patients with HCC showing resistance to this therapy. [Mol Cancer Ther 2007;6(8):2310–8]

Introduction

5-Fluorouracil (5-FU) is widely used in the treatment of various gastrointestinal cancers and other types of tumor. It is converted to the active metabolite 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP) and inhibits thymidylate synthase (TS) activity competitively through the formation of a ternary complex of FdUMP, TS, and 5,10-methylenetetrahydrofolate. Cancer cells with high levels of FdUMP and low levels of TS are thus known to be sensitive to 5-FU (1).

Dihydropyrimidine dehydrogenase (DPD) is a rate-limiting enzyme involved in the degradation of pyrimidine bases and pyrimidine-based antimetabolites, such as 5-FU, and so diminishes the antitumor activity of 5-FU. This catabolism occurs mainly in the liver. DPD activity shows wide variation in both cancer patients and the healthy population (2).

The human DPD gene (*DPYD*) is located on chromosome 1p22. It is a single copy 950-kb gene comprising 23 exons (3), in which 39 mutations and polymorphisms have been identified (4–6). Abnormalities of *DPYD* that decrease DPD activity are observed in 3% to 5% of the total population (7), and several patients with congenital DPD deficiency were reported as suffering from severe toxicity after the administration of 5-FU (8). DPD activity in tumor cells is critical to the antitumor effects of 5-FU (9), and its inhibition is expected to enhance these effects.

Hepatocellular carcinoma (HCC) is the fifth most common malignancy in the world. The most effective treatment for patients with HCC is the surgical resection of hepatic lesions. Local therapeutic approaches, such as transcatheter arterial embolization (10), percutaneous transhepatic ethanol injection (11), microwave coagulation (12), and radiofrequency ablation (13), are also effective. However, these therapies are not sufficient for patients with advanced HCC, for whom surgery is often not suitable and whose 5-year survival rate is extremely low (14). For patients with advanced HCC, the clinical response of

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almost every anticancer drug is insufficient, and several combination chemotherapies have been tried. Combined chemotherapy with 5-FU and IFN-α has been used previously for patients with advanced HCC, with improved therapeutic effects (15–17). However, these reports of positive effects are contradicted by a previously observed lack of antitumor activity accompanied by increased toxicity (18). To improve the therapeutic index of 5-FU and IFN-α combination therapy, it is therefore important to establish the cause of this occasional decrease in efficacy and increase in side effects.

Plausible mechanisms, such as an increase in FdUMP, TS inhibition rate, and thymidine phosphorylase activity, a decrease in TS levels, and altered 5-FU pharmacokinetics, have been suggested to explain the improved therapeutic effects of IFN-α and 5-FU (19–23). Takaoka et al. (24) showed that transcription of the *p53* gene is induced by IFN-α/IFN-β, accompanied by an increase in p53 protein level, and that the apoptotic response by IFN-β combined with 5-FU was enhanced. Milano et al. (25) reported that IFN-α inhibits DPD activity in human tumor cells, suggesting that inhibition of DPD activity could be involved in 5-FU–induced antiproliferative activity. Consensus IFN was shown to enhance the antiproliferative effect of 5-FU against hepatoma cells through down-regulation of DPD expression (23). By contrast, increased expression of DPD protein by IFN-γ was reportedly observed at a concentration equivalent to that in the sera of patients (26).

According to the presence or absence of synergism by the combination of 5-FU and IFN-α, we classified six human HCC cell lines into two groups: the S-group containing three cell lines, which showed a synergistic effect, and the A-group containing the remaining three cell lines, which showed additive effects (27). The expression levels of type I IFN receptor subunits were specifically up-regulated by 5-FU in all three cell lines of the S-group but not in those of the A-group (27). In this study, we asked whether DPD could limit the antiproliferative effect of 5-FU against HCC cells in culture when 5-FU was applied in combination with IFN-α. This work also shows that inhibiting DPD activity in HCC cells with high DPD levels improves the efficacy of 5-FU combined with IFN-α, following up-regulation by IFN-α.

Materials and Methods

Drugs

5-FU was purchased from Kyowa Hakko Kogyo Co. Ltd. (5-FU Injection 250 Kyowa) and natural human IFN-α was purchased from Otsuka Pharmaceutical Co. Ltd. (OIF). 5-Chloro-2,4-dihydroxypyridine (CDHP) was a gift from Taiho Pharmaceutical Co. Ltd.

Cell Lines

HCC cell lines, KYN-2, KYN-3, and HAK-1A (28, 29), were grown in DMEM (Nissui Seiyaku Co.) with 10% fetal bovine serum (FetalClone III, Hyclone) in a humidified atmosphere of 5% CO_2 at 37°C. We confirmed the expression of type I IFN receptor subunits 1 and 2 in these three HCC cell lines (27).

Cytotoxicity Tests

Cells were seeded into 96-well plates at 1,000 cells/100 μL/well and incubated overnight. On the following day, 100-μL aliquots containing IFN-α and/or 5-FU with or without CDHP were added to each well and cells were cultured for a further 5 days. The number of viable cells was estimated by assaying the activity of cellular succinate dehydrogenases using WST-8 reagent (Cell Counting Kit-8, Dojindo; ref. 30). We confirmed that untreated groups of KYN-2, KYN-3, and HAK-1A cells grew exponentially for 6 days under these experimental conditions (data not shown).

Combination Index Analysis

The combined effects of 5-FU and IFN-α were quantified using a combination index (CI) method developed by Chou and Talalay (31). This method involves plotting dose-effect curves, for each agent and their combination, using the median-effect equation: $fa / fu = (D / Dm)^m$, where D is the dose of the drug, Dm is the dose required for a 50% effect (equivalent to IC_{50}), fa and fu are the affected and unaffected fractions, respectively ($fa = 1 - fu$), and m is the exponent signifying the sigmoidicity of the dose-effect curve.

In this study, relative concentrations (RC) of IFN-α and 5-FU, determined as (concentration) / (IC_{50} value), were used for analysis. The computer software Xlfit version 2.0.6 (ID Business Solutions Ltd.) was used to calculate the values of Dm and m. The CI used for the analysis of the drug combinations was determined by the isobologram equation for mutually nonexclusive drugs that have different modes of action: $CI = (D)_1 / (Dx)_1 + (D)_2 / (Dx)_2 + (D)_1(D)_2 / (Dx)_1(Dx)_2$, where $(D)_1$ and $(D)_2$ are RCs of drugs 1 and 2 and x is the percentage of inhibition. Combination indices $CI < 1$, $CI = 1$, and $CI > 1$ indicate synergism, additive effects, and antagonism, respectively.

Quantitative Real-time Reverse Transcription-PCR

Total RNA was extracted using Isogen (Nippon Gene Co., Ltd.) and reverse transcribed using a reverse-transcription system (Promega Corp.) according to the manufacturer's instructions. Quantitative real-time reverse transcription-PCR was done with an ABI Prism 7300 (PE Applied Biosystems). The primers used were as follows: TS 5′-GAATCACATCGAGCCACTGAAA-3′ (forward primer), 5′-CAGCCCAACCCCTAAAGACTGA-3′ (reverse primer), and 5′-(FAM)TTCAGCTTCAGCGAGAACCCAGA(TAMRA)-3′ (probe) and DPD 5′-AATGATTCGAAGAGCTTTTGAAGC-3′ (forward primer), 5′-GTTCCCCGGATGATTCTGG-3′ (reverse primer), and 5′-(FAM)TGCCCTCACCAAAACTTTCTCTCTTGATAAGGA(TAMRA)-3′ (probe). Primers and Taqman probes for glyceraldehyde-3-phosphate dehydrogenase were prepared by Assay-on-Demand Gene Expression Products (PE Applied Biosystems).

Western Blotting

HCC cells were cultured for 48 h with 0, 20, 100, or 500 IU/mL IFN-α. Total protein was extracted using a protein extraction reagent (M-PER, Pierce) supplemented with protease inhibitors (Halt Protease Inhibitor

Cocktail kit, Pierce). Cell lysates were loaded into 7.5% SDS-polyacrylamide gels. After electrophoresis, the separated proteins were electrotransblotted onto polyvinylidene difluoride membranes (Immobilon-P membrane, Millipore). After blocking, membranes were probed with antihuman DPD polyclonal rabbit antibody and antihuman TS monoclonal mouse antibody (gifts from Taiho Pharmaceutical). The proteins were visualized using horseradish peroxidase–conjugated antibodies (Pierce) followed by enhanced chemiluminescence (Pierce). The intensity of luminescence was quantified using an image analysis system (LAS-1000, Fuji Film).

Enzyme Assay for DPD Activity

DPD activity was measured using [6-^{14}C]5-FU as a substrate (32, 33). Cells were homogenized and centrifuged at 105,000 × *g* for 60 min at 4°C, and the supernatant was used for assay. A reaction mixture containing 10 mmol/L potassium phosphate (pH 8.0), 0.5 mmol/L EDTA, 0.5 mmol/L 2-mercaptoethanol, 2 mmol/L DTT, 5 mmol/L $MgCl_2$, 20 μmol/L [6-^{14}C]5-FU, 0.1 mmol/L NADPH, and 25 μL of cell extract in a total volume of 50 μL was incubated at 37°C for 30 min. After chemical hydrolyzation and neutralization using KOH and $HClO_4$, a 5-μL aliquot was applied to a TLC plate (2.5 × 20 cm, silica gel 60 F_{254} plate, Merck) and developed with a mixture of ethanol and 1 mol/L ammonium acetate (5:1, v/v) and diethylether, acetone, chloroform, and water (50:50:40:1, v/v). DPD activity was determined as the sum of the products converted from 5-FU (i.e., dihydrouracil, 2-fluoro-β-ureidopropionic acid, and 2-fluoro-β-alanine) that were visualized and quantified with an imaging analyzer (BAS-2000, Fujix).

Results

Comparison of Drug Sensitivity and mRNA Levels of DPD and TS in Three HCC Cell Lines

The sensitivities of three HCC lines, HAK-1A, KYN-2, and KYN-3, to separately administered 5-FU and IFN-α were determined as IC_{50} values. KYN-3 was the most resistant to 5-FU of the three HCC lines. The IC_{50} values of IFN-α in HAK-1A, KYN-2, and KYN-3 cells were 720, 510, and 24 IU/mL, respectively. KYN-3 cellular sensitivity to IFN-α was therefore approximately 30-fold and 20-fold higher than in HAK-1A and KYN-2 cell lines, respectively. The IC_{50} value of IFN-α in HepG2 cells established from human hepatoblastoma and widely used in experiments was found to be over 10,000 IU/mL (data not shown).

Sensitivity was then compared with mRNA levels of DPD and TS, and DPD activity (as conversion rate from [6-^{14}C]5-FU to its metabolites) in the three cell lines, which were shown to be broadly comparable (Table 1). DPD mRNA levels relative to those of KYN-3 cells, taken as 100%, and those of TS relative to HAK-1A, taken as 100%, are shown in Table 1. The cellular level of TS mRNA in KYN-2 cells was much lower than in the other two lines. Basal DPD activity in KYN-3 cells was approximately 5.5-fold and 9.1-fold higher than in HAK-1A and KYN-2 cell lines, respectively.

Quantitative Analysis of the Combination Effects of 5-FU and IFN-α

Dose-response curves of 5-FU alone and in combination with various concentrations of IFN-α are shown in Fig. 1A to C. Because sensitivities to IFN-α and 5-FU alone were different for each HCC cell line, RCs to the IC_{50} value were used. As the concentration of combined IFN-α was increased, the dose-response curves of 5-FU were shifted down in an IFN-α concentration-dependent manner in all three HCC cell lines. For instance, following cotreatment with 312 IU/mL (RC = 0.45) IFN-α,the dose-response curve of HAK-1A to 5-FU was significantly shifted down and the 5-FU IC_{50} value of 2.3 μmol/L was reduced to 0.28 μmol/L. However, there were clear differences between the three HCC cell lines. For KYN-3 cells, after treatment with the equivalent RC, 0.42 (8.0 IU/mL) IFN-α,the dose-response curve of 5-FU was significantly shifted down, but the 5-FU IC_{50} value of 9.6 μmol/L was only reduced to 5.5 μmol/L.

An enhancement factor (EF) was defined to evaluate synergism between 5-FU and IFN-α, based on a 50% antiproliferative effect, as EF = 1 / [(RC of IFN-α) + (RC of 5-FU)]. When EF is 1, this combined effect is additive; values >1 or <1 imply synergistic or antagonistic effects, respectively. EF values of HAK-1A and KYN-2 were >1 at almost all combined doses, but EF values of KYN-3 at all tested combined doses were closer to 1. The combined effect of 5-FU and IFN-α on HAK-1A and KYN-2 cells was thus judged to be synergistic and that on KYN-3 to be additive, consistent with our previous study (27).

Table 1. HCC cell sensitivities to 5-FU and IFN-α, DPD, and TS mRNA expression levels and DPD activities

Cell line	IC_{50}*		Relative mRNA levels†		DPD activity‡ (pmol/min/mg protein)
	5-FU (μmol/L)	IFN-α (IU/mL)	DPD	TS	
HAK-1A	2.1	720	15	100	2.8 ± 0.9
KYN-2	1.8	510	23	14	1.7 ± 0.7
KYN-3	9.8	24	100	46	15.5 ± 1.6

*The IC_{50} value that caused 50% growth inhibition was calculated from the log-logit regression line. The assays were carried out in quadruplicate. Experiments were repeated twice with essentially similar results.

†DPD mRNA levels are shown relative to those of KYN-3 cells, taken as 100%, and TS mRNA levels are shown relative to those of HAK-1A cells, taken as 100%.

‡Determinations were carried out in triplicate and data represent mean ± SD.

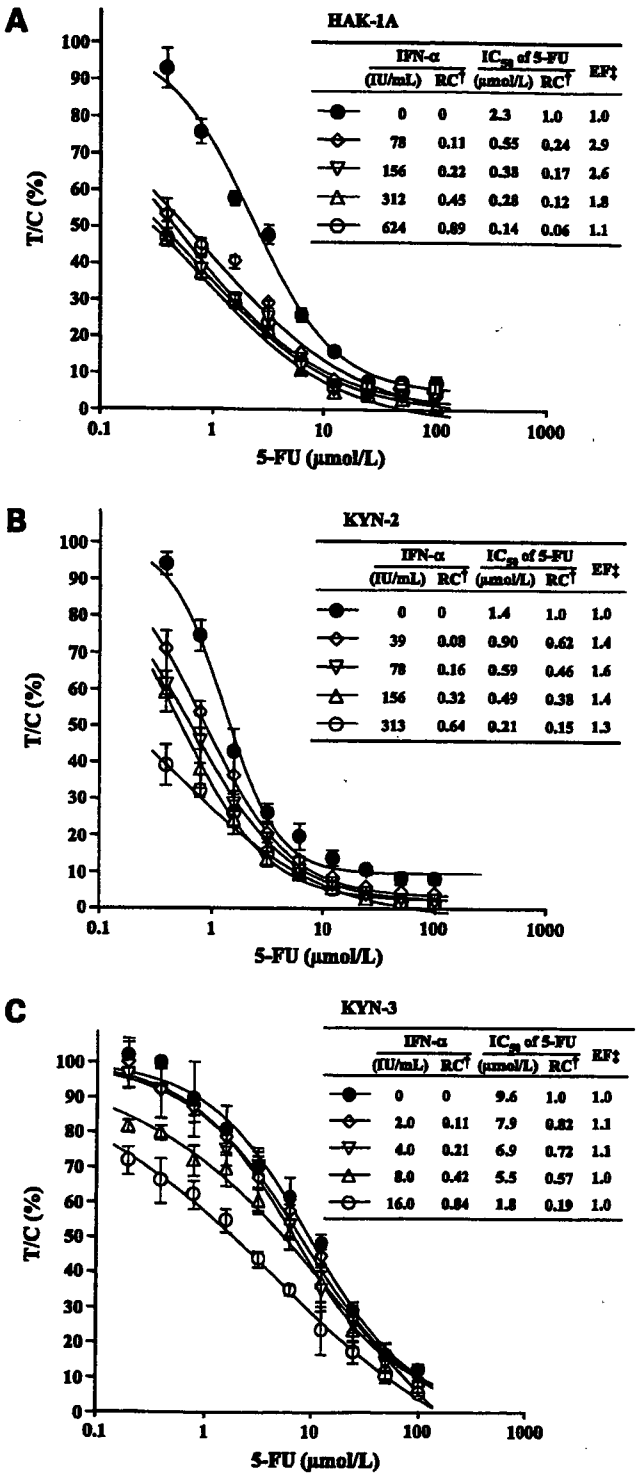

Figure 1. Antiproliferative effects of 5-FU and IFN-α on HAK-1A (**A**), KYN-2 (**B**), and KYN-3 (**C**) cells. Assays were carried out independently in quadruplicate. *Points*, mean; *bars*, SD. †, RC = (concentration) / (IC_{50} value). In this experiment, IC_{50} value of IFN-α against HAK-1A, KYN-2, and KYN-3 was 700, 490, and 20 IU/mL, respectively. ‡, EF = 1 / [(RC of IFN-α) + (RC of 5-FU)]. EF > 1, synergistic; EF = 1, additive; EF < 1, antagonistic. Experiments were repeated twice with similar results.

The synergism of IFN-α and 5-FU at various fractional efficacies was then quantified using the CI methods of Chou and Talalay (31). The antiproliferative effects of 5-FU on HAK-1A and KYN-2 cells were synergistically enhanced by IFN-α, indicated by CI values of <1 at most fractional effects (Fig. 2A and B). By contrast, the CI value in KYN-3 cells was >1 for fractional effects of 0.7 and under (Fig. 2C). The combined effect of IFN-α and 5-FU against KYN-3 thus seems to be antagonistic, except at very high fractional effect levels. These data show that the degree of synergism between IFN-α and 5-FU against KYN-3 cells (with a higher CI value) is weaker than against HAK-1A and KYN-2 cells over a large range of fractional effects.

Altered Expression of mRNA and Protein of TS and DPD by IFN-α

Cellular mRNA levels of DPD and TS were examined following treatment with or without 500 IU/mL IFN-α for 24 h (Fig. 3A and B). IFN-α treatment resulted in an ~7.5-fold increase in DPD mRNA levels in KYN-3 cells while reducing DPD mRNA levels in HAK-1A and KYN-2 cells to approximately one quarter and one third of that in IFN-α untreated cells, respectively (Fig. 3A). TS mRNA levels in all three HCC cell lines decreased after treatment with IFN-α to ~60% of those in untreated cells (Fig. 3B).

Cellular protein levels of DPD and TS were also determined by Western blot analysis. The intensities of blotted bands were normalized by that of β-actin, and fold increase was measured as the relative intensity to that of untreated cells, taken as 1.0. Protein extracts of 10 μg/lane were loaded for KYN-3 cells, and 50 μg/lane were loaded for both HAK-1A and KYN-2 cells (Fig. 4). DPD protein levels were roughly comparable with mRNA levels and DPD activity in all three cell lines. Expression of DPD protein, molecular weight of 110,000, was down-regulated in HAK-1A and KYN-2 cells when treated with over 100 IU/mL IFN-α for 48 h. Conversely, that in KYN-3 cells was up-regulated after treatment with over 20 IU/mL IFN-α for 48 h in a concentration-dependent manner (Fig. 4). Expression of TS protein, molecular weight of 36,000, was down-regulated to a similar extent in a concentration-dependent manner in all three HCC cell lines when treated with IFN-α.

Effect of a DPD Selective Inhibitor on the Antiproliferative Effect of 5-FU and IFN-α

The combined antiproliferative effects of 5-FU and IFN-α can be modulated by treatment with a DPD competitive inhibitor, CDHP. We first examined the effect of CDHP on DPD activity in three HCC cell lines. KYN-3 cell extracts showed relatively high DPD activity of ~15 pmol/min/mg protein (consistent with Table 1 findings). By contrast, DPD activity in HAK-1A and KYN-2 cells was very low with a marginal limit of evaluation for enzyme activity. DPD activity in KYN-3 cells was reduced to 40%, 7%, and 5% of basal activity after treatment with 0.7, 7.0, and 70 μmol/L CDHP, respectively (Fig. 5). There seemed to be almost no marked inhibition of DPD activity by CDHP in the other two cell lines, possibly due to their low enzyme activities.

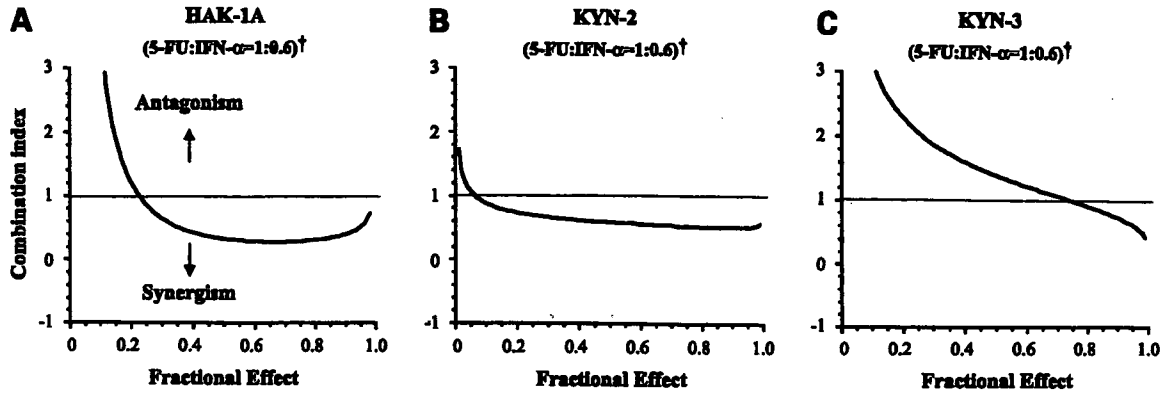

Figure 2. Quantitative analysis of synergy between 5-FU and IFN-α against HAK-1A (A), KYN-2 (B), and KYN-3 (C) cells using the CI method. CI > 1, antagonism; CI = 1, additive; CI < 1, synergistic. †, ratio of RC = (concentration of 5-FU) / (IC_{50} of 5-FU):(concentration of IFN-α) / (IC_{50} of IFN-α).

We next examined whether cotreatment of CDHP could modulate the antiproliferative effect of 5-FU alone and in combination with IFN-α in three HCC cell lines. As seen in Fig. 6A to C, the dose-response curves of 5-FU alone in HAK-1A, KYN-2, and KYN-3 cells and those of 5-FU combined with IFN-α in HAK-1A and KYN-2 cells were not shifted by cotreatment with CDHP. None of these differences were significant at all data points. By contrast, there seemed a marked and significant shift of the 5-FU and IFN-α combined dose-response curve in KYN-3 cells to CDHP: the 5-FU IC_{50} value of 4.6 μmol/L was reduced to 1.1 μmol/L by cotreatment with CDHP in the presence of IFN-α (Fig. 6C).

Discussion

Consistent with the findings of our recent study (27), the combination of 5-FU and IFN-α showed a synergistic antiproliferation effect on two HCC cell lines, HAK-1A and KYN-2, and an additive or antagonistic antiproliferation effect on KYN-3 cells (Figs. 1 and 2). Expression of type 1 IFN receptor was specifically up-regulated by exposure to 5-FU in both HAK-1A and KYN-2, but not KYN-3 cells, suggesting that the modulation of IFN receptor expression by 5-FU could play a pivotal role in therapeutic efficacy (27). In this study, we further examined the molecular events underlying the antiproliferative effects of 5-FU and IFN-α. One of the major mechanisms of antiproliferative activity of 5-FU is the inhibition of TS activity with formation of the ternary complex of FdUMP, TS, and 5,10-methylenetetrahydrofolate. However, we observed a marked IFN-α–induced decrease in TS expression at similar levels in all three cell lines, suggesting that modulation of TS expression itself might not be directly involved in the absence or presence of synergism by the combination of 5-FU and IFN-α.

DPD is a key enzyme involved in 5-FU inactivation, which modulates FdUMP levels and controls formation of

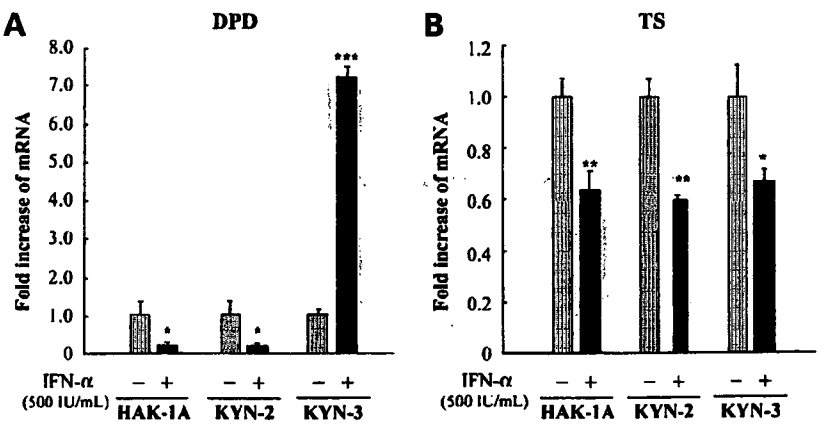

Figure 3. Effects of 500 IU/mL IFN-α on DPD (A) and TS (B) mRNA and protein expression levels in HAK-1A, KYN-2, and KYN-3 cells. Expression levels were measured by quantitative real-time reverse transcription-PCR and normalized to glyceraldehyde-3-phosphate dehydrogenase. The fold increases are shown relative to the initial level, taken as 1.0. Determinations were carried out in triplicate. *Dotted and black columns,* mean value of relative mRNA levels in HCC cells untreated and treated with IFN-α, respectively; *bars,* SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, differences are statistically significant by Welch's test, compared with untreated groups.

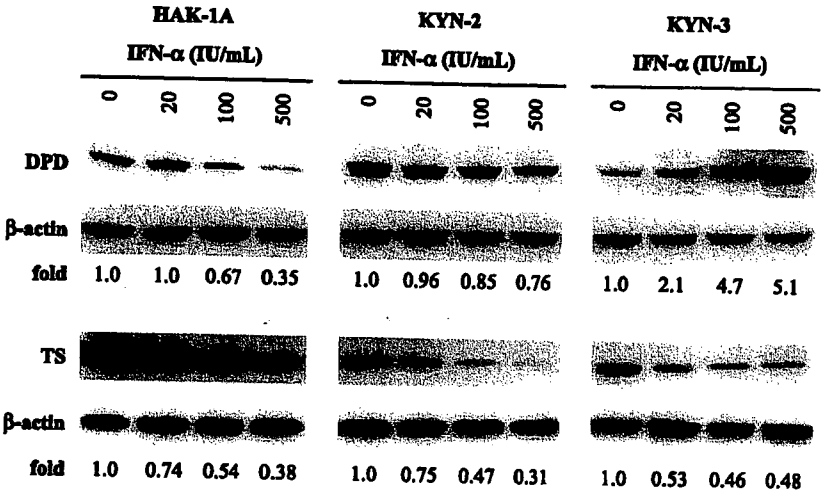

Figure 4. Protein expression levels of DPD and TS in HAK-1A, KYN-2, and KYN-3 cells treated with IFN-α. Protein expressions in HCC cells treated with IFN-α for 48 h were measured by Western blotting. Protein extracts of 10 μg/lane were loaded for KYN-3 cells, and 50 μg/lane were loaded for both HAK-1A and KYN-2 cells. Experiments were repeated twice with similar results. The intensities of immunoblotted bands were quantified by image analyzing methods. The fold increases relative to the initial level, taken as 1.0, are shown under the bands.

the ternary complex. Several clinical studies have shown that the intratumoral expression level of DPD is closely associated with clinical responses to 5-FU in patients with colorectal cancer (1), gastric cancer (34), and non–small cell lung cancer (35). In our present study, expression of DPD protein and mRNA levels in KYN-3 cells were specifically increased 5-fold or more over the basal level after exposure to IFN-α (Figs. 3 and 4). By contrast, down-regulation of DPD by IFN-α was observed in both HAK-1A and KYN-2.

In these two HCC cell lines, we previously proposed that 5-FU–induced up-regulation of the IFN receptor was the main mechanism underlying the synergistic antiproliferative effect of 5-FU and IFN-α (27). Moreover, down-regulation of DPD by IFN-α in these two cell lines might be involved in the synergistic effect. By contrast, up-regulation of DPD by IFN-α might account for the antagonism between IFN-α and 5-FU in KYN-3 cells.

Shestopal et al. (36) reported that 5′ flanking region of *DPYD* gene lacks the canonical TATA and CCAAT boxes, however, contains several *cis*-acting regulatory elements including binding sites for activator protein-2, nuclear factor-κB, Sp1, and Egr families. About the regulatory mechanism for IFN-α modulation of *DPYD* expression, we

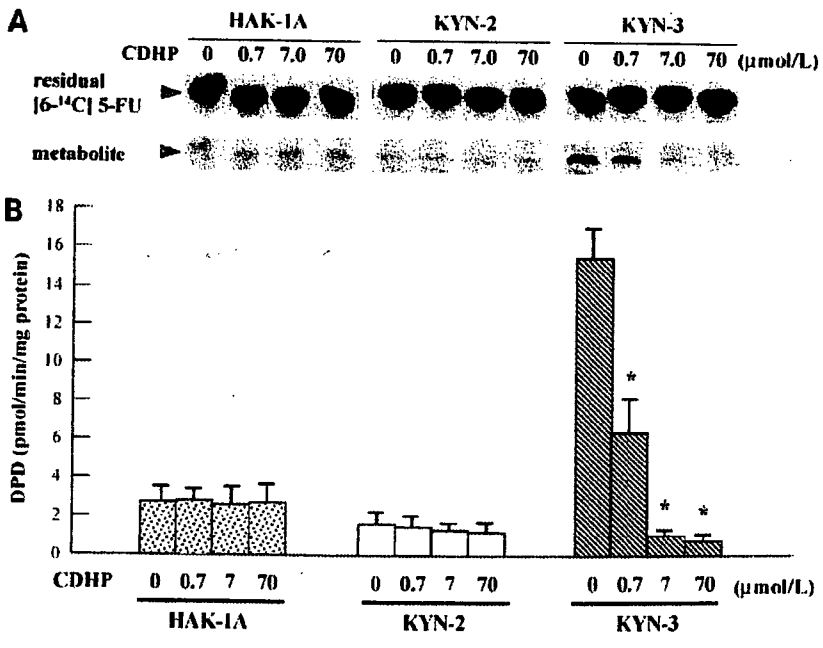

Figure 5. Inhibitory effect of CDHP on DPD activity. DPD activity was measured using [6-^{14}C]5-FU as a substrate. Residual [6-^{14}C]5-FU and metabolites were separated by TLC and visualized with an imaging analyzer (**A**). Each sample was developed on separated and independent TLC plate. Assays were carried out in triplicate. *Columns,* DPD; *bars,* SD (**B**). *, $P < 0.001$, differences are statistically significant by Welch's test, compared with CDHP untreated group.

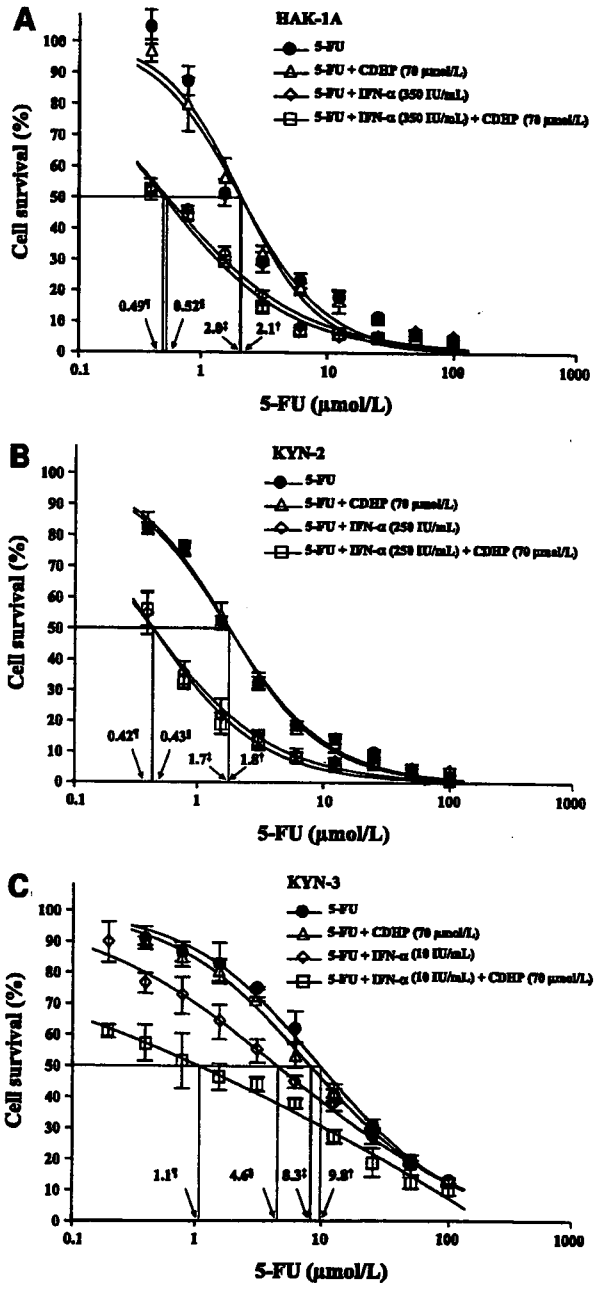

Figure 6. Effect of CDHP on combined antiproliferative effect of IFN-α and 5-FU in HAK-1A (**A**), KYN-2 (**B**), and KYN-3 (**C**) cells. Assays were carried out in quadruplicate. Experiments were repeated twice with similar results. IC_{50} values of 5-FU against HCC cells treated with 5-FU alone (†), 5-FU/CDHP (‡), 5-FU/IFN-α (§), and 5-FU/IFN-α/CDHP (¶).

analyzed the sequence of 1.2 kb of 5′ flanking region of *DPYD* and found that this region contains two putative consensus binding sites for signal transducer and activator of transcription families (data not shown), suggesting that gene expression of *DPYD* is highly susceptible to IFN-α, which strongly activates signal transducer and activator of transcription 1 and signal transducer and activator of transcription 2 through the Janus-activated kinase-signal transducer and activator of transcription pathway. However, it remains unclear why *DPYD* expression is differentially controlled by IFN-α in HCC cell lines. Further elucidation of this differential regulatory mechanism at the molecular basis is required.

Our present study showed that cotreatment with the DPD inhibitor CDHP further synergistically enhanced the antiproliferative effect of 5-FU and IFN-α in KYN-3 cells only and not in HAK-1A and KYN-2 cells (Fig. 6). The antiproliferative effect of 5-FU alone was only slightly altered by CDHP cotreatment in KYN-3 cells, if any, and not at all in the other two cell lines (Fig. 6). This IC_{50} reduction, however, was not statistically significant. Basal DPD activity and expression levels were much higher in KYN-3 cells than in HAK-1A and KYN-2 cells. Moreover, cellular DPD levels were specifically up-regulated >5-fold in KYN-3 cells by IFN-α, but this was not observed in HAK-1A and KYN-2 cells. It was presumed that 15 pmol/min/mg protein of basal DPD activity in KYN-3 cells was up-regulated to 80 to 110 pmol/min/mg protein. Taken together, this suggests that a relatively high DPD activity might be more susceptible to inhibition by CDHP, resulting in an apparent synergistic effect of CDHP on the antiproliferative effect by 5-FU and IFN-α.

Certain levels of DPD in cancer cells could be sensitive to CDHP-induced inhibition. A relevant study by Takechi et al. (37) showed that the antiproliferative activity of 5-FU could be markedly enhanced by cotreatment with 69 μmol/L CDHP in two human tumor cell lines with relatively high DPD activities, approximately 101 and 153 pmol/min/mg protein, respectively, but not those with low enzyme activity, 33 pmol/min/mg protein. However, this plausible mechanism why CDHP did induce synergism of 5-FU and IFN-α against only KYN-3 cells requires further study to validate these findings.

CDHP has been applied as a modulator in the newly developed antimetabolite TS-1 (Taiho Pharmaceutical). TS-1 consists of tegafur, CDHP, and potassium oxonate in a molar ratio of 1:0.4:1 (38). Potassium oxonate is a competitive inhibitor for orotate phosphoribosyltransferase that activates 5-FU. Potassium oxonate is mainly distributed in the gastrointestinal tract after p.o. administration and prevents gastrointestinal toxicity induced by 5-FU without reducing 5-FU activity in tumor (38, 39). In Japan, TS-1 has been used to treat patients with gastric, head and neck, and pancreatic cancers and shows potent therapeutic efficacy against gastric tumors, with a response rate of 46.5% (40–42). Nakamura et al. (43) applied a new combination regimen of TS-1 and IFN-α to advanced HCC patients with portal vein thrombus and multiple pulmonary metastases and observed some improvement in therapeutic efficacy. The combination of TS-1 and IFN-α could therefore be effective against patients with advanced HCC. However, the side effects of these combination therapies should be seriously considered before their implementation. Further studies

are required to determine how DPD could be differentially controlled between normal cells including normal hepatic cells and malignant hepatic cells in patients with HCC when DPD inhibitory drugs are introduced.

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Effects of IFN-α on α-Fetoprotein Expressions in Hepatocellular Carcinoma Cells

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ABSTRACT

We investigated the effects of pegylated (PEG)-IFN-α2b on α-fetoprotein (AFP) expression as demonstrated by protein and mRNA levels in six human hepatocellular carcinoma (HCC) cell lines. The number of KIM-1 cells in culture with PEG-IFN-α2b decreased between 24 amd 240 h, whereas the levels of intracellular and secreted AFP per cellular protein increased (except at 192 h), with levels 1.9-fold and 2.9-fold higher at maximum, respectively, than cells without PEG-IFN-α2b (control). The mRNA level increased between 72 and 192 h, when the level was 3-fold higher than that of the control. In the 72-h culture with 40–5000 IU/mL PEG-IFN-α2b, there were dose-dependent increases in AFP protein and mRNA expression and dose-dependent decrease in cell number resulting from apoptosis and blockage of the cell cycle at the S-phase. The rate of fucosylated AFP in the cell lysate decreased in a dose-dependent and time-dependent manner. In the PEG-IFN-α2b culture of the other five HCC cell lines, cell proliferation was suppressed, but the expressions of AFP protein and mRNA increased in only two cell lines, and suppression of cell proliferation was not related to the increase in AFP expressions. Our findings demonstrated that PEG-IFN-α2b induces an increase in AFP expression at both the protein and mRNA levels.

INTRODUCTION

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 (HBV)-related and hepatitis C virus (HCV)-related chronic hepatitis in many countries.[4] IFN-α has been shown to possess highly suppressive effects on hepatocellular carcinogenesis in patients with virus-related chronic hepatitis,[5–7] but the mechanisms of action have not yet been clarified.

Determination of the serum α-fetoprotein (AFP) level is used in the diagnosis and monitoring of hepatocellular carcinoma (HCC) patients. The lens culinaris agglutinin (LCA)-reactive fraction of AFP (AFP-L3), that is, the fucosylated variant of AFP, is sensitive and specifically shows the localization of HCC.[8,9] It is also important in the prediction of poor prognosis of HCC.[10] Some chronic hepatitis C patients have consistently high AFP values without there being any evidence of HCC, and some researchers consider such patients to be at high risk for HCC.[11,12] Murashima et al.[13] reported that IFN-α administration significantly reduced serum AFP levels in patients with chronic hepatitis C and high levels of AFP, and the effects were observed in both responders and nonresponders. IFN-α administration may thereby diminish the risk of hepatocarcinogenesis, but the mechanisms by which IFN-α decreases serum AFP levels have not been clarified. Chronic hepatitis C patients with a high level of AFP may have very early stage, clinically undetectable HCC cells, and IFN-α may directly induce apoptosis in AFP-producing cells or may act on HCC cells and downregulate AFP gene expression and secretion. As the effects of IFN-α on AFP expression in terms of mRNA and

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protein levels have not been studied in depth, we examined this aspect by using pegylated IFN-α2b (PEG-IFN-α2b) and HCC cell lines.

MATERIALS AND METHODS

Cell lines and cell culture

We used six HCC cell lines (KIM-1, KYN-1, KYN-2, KYN-3, HAK-1A, and HAK-1B) that were originally established and characterized in our laboratory. KIM-1 produces a relatively large amount of AFP, and the other cell lines produce little or none. These six cell lines were previously confirmed to retain morphologic and functional characteristics of the original tumor.[14–19]

The cells were grown in Dulbecco's modified Eagle medium (DMEM) (Nissui Seiyaku, Co., Ltd., Tokyo, Japan) supplemented with 2.5% heat-inactivated (56°C, 30 min) fetal bovine serum (FBS) (Bioserum, Victoria, 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. HCC cells (2.0–5.0 $\times$ 10^5 cells per dish) were seeded on 100-mm tissue culture dishes (Asahi Techno Glass Corporation, Tokyo, Japan) and cultured with medium alone for 24 h to allow the cells to enter their logarithmic growth phase. Culture medium was not changed if not indicated.

IFN and reagents

PEG-IFN-α2b (PEG Intron) was provided by Schering-Plough K.K. (Osaka, Japan). The specific activity of PEG-IFN-α2b was 6.4 $\times$ 10^7 IU/mg protein. Antibromodeoxyuridine (BrdU) antibody was purchased from BD Biosciences (San Jose, CA), and control normal mouse IgG1 was from DAKO (Glostrup, Denmark).

Effects of IFN-α on AFP expression in HCC cells

In a short-term time course experiment, KIM-1 cells were cultured with fresh medium alone (control) or medium containing 1000 IU/mL PEG-IFN-α2b for 24, 48, or 72 h. We estimated the amount of AFP protein, rate (%) of AFP-L1, AFP-L2, and AFP-L3 in the cultured cells and culture medium, the amount of total cellular protein, and the level of AFP mRNA expression in the cultured cells. The amount of AFP in the cultured cells and spent medium was divided by the amount of total cellular protein of the cultured cells, and the levels of AFP per unit volume of cellular protein were compared between the cells treated with PEG-IFN-α2b and the control. The number of cells was estimated by the amount of the total cellular protein. In a multidose experiment, KIM-1 cells were cultured with fresh medium alone (control) or medium containing 40, 200, 1000, or 5000 IU/mL PEG-IFN-α2b for 72 h, and AFP expression was estimated as described. In a long-term time course experiment, the medium was changed and AFP levels were estimated every 48 h until 240 h.

The effect of PEG-IFN-α2b on AFP expression in HCC cells was also investigated in the other five HCC cell lines. The cells were cultured with fresh medium alone (control) or medium containing 1000 IU/mL PEG-IFN-α2b for 72 h, and AFP expressions as demonstrated by protein and mRNA levels were measured.

Cell and culture medium preparation for AFP protein measurement

Cultured cells were dispersed with trypsin-EDTA, collected, and resuspended in culture medium, centrifuged for 5 min (300g, 4°C), resuspended in an appropriate amount of ice-cold, Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (PBS) containing 100 μg/mL phenylmethylsulfonyl fluoride, sonicated for 10 sec at maximal power of the Ultrasonic Generator (model US-50, Nihonseiki Kaisha Ltd., Tokyo, Japan), and centrifuged for 10 min (12,000g, 4°C). The supernatant was saved as cell lysate for the measurement of intracellular AFP levels. Culture media were collected from the dishes and centrifuged for 10 min (1000 g, 4°C), and the supernatant was saved for measurement of AFP level in spent media. The total amounts of AFP in the cell lysates and spent media were measured by chemiluminescence immunoassay, using ARCHITECT AFP kits (Abbott Japan Co., Ltd, Tokyo, Japan). The rates (%) of AFP-L1, AFP-L2, and AFP-L3 in the samples were measured by lectin affinity electrophoresis coupled with antibody affinity blotting, using AFP differentiation kit L (Wako Pure Chemicals Inc., Osaka, Japan). The amount of total cellular proteins was determined using the BCA protein assay reagent (Pierce, Rockford, IL).

cDNA preparation and real-time quantitative RT-PCR

AFP mRNA expression levels in HCC cells were assessed using a quantitative real-time RT-PCR method. Total RNA was obtained with RNeasy Mini Kits (Qiagen K.K., Tokyo, Japan) and reverse transcribed with a reverse transcription system (Promega Corporation, Madison, WI) according to the manufacturer's instructions. The reaction for converting to cDNA was performed under the following conditions: 10 min at 70°C, 10 min at ambient temperature, 30–40 min at 42°C, 5 min at 95°C, and 5 min at 4°C. cDNA was stored at −20°C until use. The two-step TaqMan real-time quantitative RT-PCR was performed on the ABI Prism 7300 Sequence Detection System (PE Applied Biosystems, Foster City, CA). The reactions were performed for 2 min at 50°C, then 10 min at 95°C, followed by 40 cycles of 95°C for 15 sec and 60°C for 1min. The results were normalized for GAPDH expression and used as an internal standard. Primers and TaqMan probes of AFP and GAPDH were prepared by Assay-on-Demand Gene Expression Products (PE Applied Biosystems).

Cell cycle analysis

Cell lines were cultured with or without PEG-IFN-α2b (250 or 1000 IU/mL) for 24, 48, or 72 h, and cell cycle analysis was performed with a technique described elsewhere.[19] 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 then analyzed using a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA). The percentage of cells in the G_1, S or G_2/M phase was calcu-

lated from a dot or contour plot obtained for the flow cytometric analyses of double-stained cells.

RESULTS

Effects of PEG-IFN-α2b on growth and AFP expressions in KIM-1 cells

In the short-term experiment, the cellular protein level (an indicator of cell number) of KIM-1 cells in the PEG-IFN-α2b culture started to decrease at 24 h, decreasing to approximately 50% of the control at 72 h (Fig. 1A). AFP levels in the culture medium at 24 and 48 h were almost the same in the control and the PEG-IFN-α2b culture cells, but the level in the PEG-IFN-α2b culture cells decreased to 78% of the control at 72 h (Fig. 1B). In the comparison of AFP levels in culture medium and cell lysate per unit volume of cellular protein, the levels tended to increase with time in the PEG-IFN-α2b culture cells compared with the control (Fig. 1 C, D). AFP-L3 fraction rates (%) in culture supernatant were similar in the control and the PEG-IFN-α2b culture cells (Fig. 1E). The rate of AFP-L3 in cell lysate tended to increase slightly with time in both the control and the PEG-IFN-α2b culture cells, but the rates were lower in the PEG-IFN-α2b culture cells (Fig. 1F). AFP-L3 fraction rates

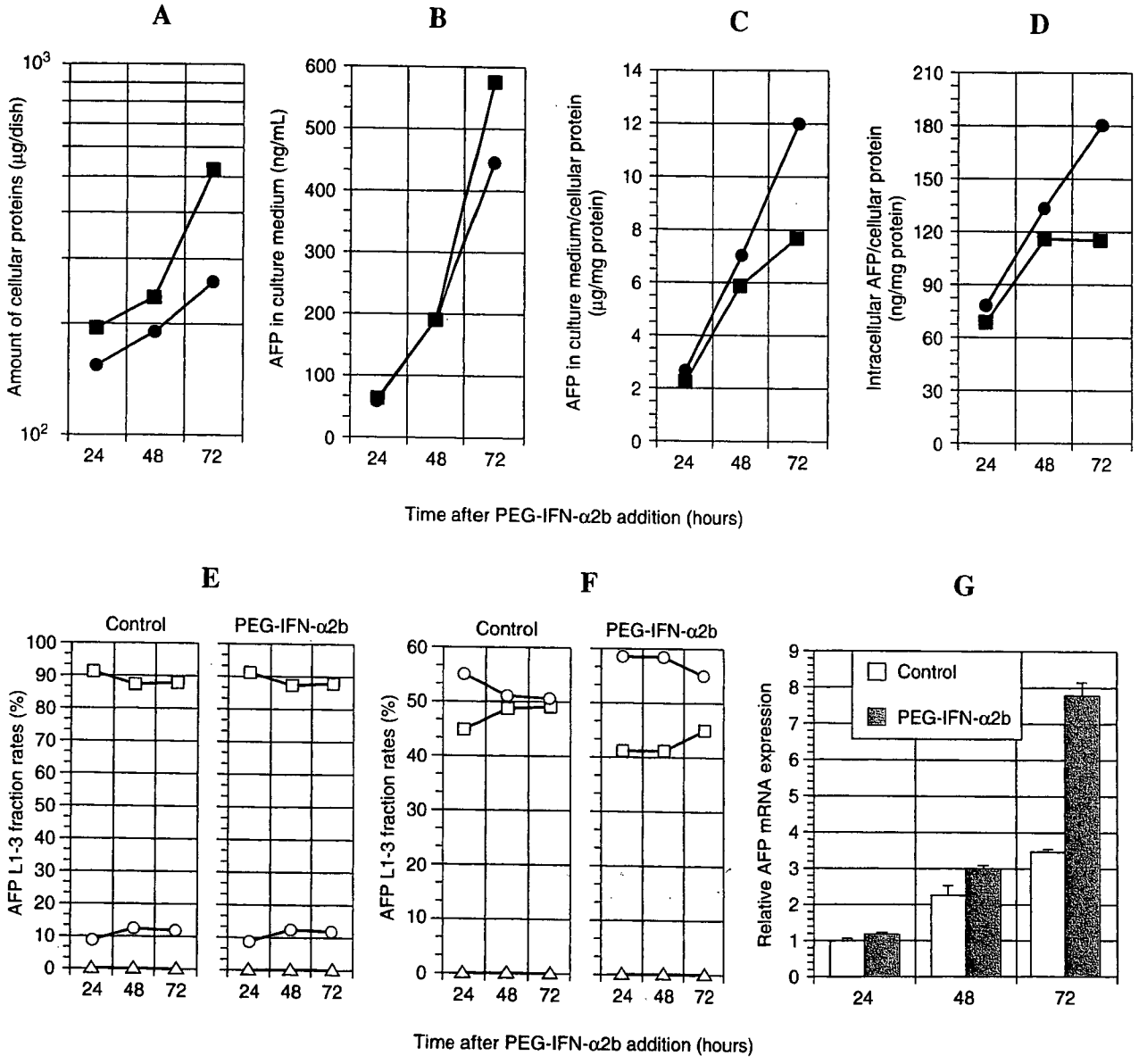

FIG. 1. Effects of PEG-IFN-α2b on the growth, expressions of AFP protein and mRNA, and AFP-L1, AFP-L2, and AFP-L3 rates in KIM-1 cells cultured for 24, 48, or 72 h. (**A**) Growth curve, (**B**) concentration of AFP in the culture supernatant, (**C**) amount of AFP in the culture supernatant per unit volume of cellular protein, and (**D**) amount of AFP in cell lysate per unit volume of cellular protein. KIM-1 cells were cultured with (filled circles) or without (filled squares) 1000 IU/mL PEG-IFN-α2b. (**E**) Rates (%) of AFP-L1 (open circles), AFP-L2 (open triangles), and AFP-L3 (open squares) in the supernatants of culture medium, and (**F**) those rates in cell lysate. (**G**) AFP mRNA expression (n = 2, mean ± SD).

(%) were lower in cell lysate than in culture supernatant. Expression of AFP mRNA in the PEG-IFN-α2b culture cells was nearly twice that of the control at 72 h (Fig. 1G).

Cell cycle analysis showed that the number of KIM-1 cells in the S-phase increased in the culture with 250 or 1000 IU/mL PEG-IFN-α2b for 48 or 72 h, indicating that PEG-IFN-α2b induced S-phase arrest (Fig. 2). In addition, the number of cells at the preG$_1$ phase increased with contact with PEG-IFN-α2b in a dose-dependent and time-dependent manner, indicating the induction of apoptosis (Fig. 2).

Dose-dependent effects of PEG-IFN-α2b on growth and AFP expressions in KIM-1 cells

The number of KIM-1 cells decreased dose dependently to approximately 20% of the control in the culture with 5000 IU/mL PEG-IFN-α2b for 72 h. AFP levels in the culture medium or cell lysate per unit volume of cellular protein tended to increase dose dependently (Fig. 3A). The AFP-L3 rate in culture supernatant was not related to the PEG-IFN-α2b level, but the AFP-L3 rate in cell lysate decreased with increase of PEG-IFN-α2b levels (Fig. 3B). AFP-L3 fraction rates (%) were lower in cell lysate than in culture supernatant. Expression of AFP mRNA increased with contact with PEG-IFN-α2b, and expression was approximately two times higher in the cells with 200–5000 IU/mL PEG-IFN-α2b in the culture medium than in the control (Fig. 3C).

Long-term time-dependent effects of PEG-IFN-α2b on growth and AFP expression in KIM-1 cells

From 48 h after the addition of PEG-IFN-α2b to the cultures of KIM-1 cells, the cellular protein level (an indicator of cell number) started to decrease compared with the control and was approximately 20% of the control after 96 h (Fig. 4A). AFP in culture supernatant was <50% that in the control after 144 h (Fig. 4B). AFP level per unit volume of cellular protein was 1.7–2.9-fold higher in the PEG-IFN-α2b cultures compared with the control at all measurement time points, and the levels in both groups tended to increase with time (Fig. 4C). AFP level in cell lysate per unit volume of cellular protein was also 1.4–1.9-fold higher in the PEG-IFN-α2b culture compared with the control and tended to increase with time except at 192 h (Fig. 4D). AFP-L3 rates (%) in culture supernatant were almost

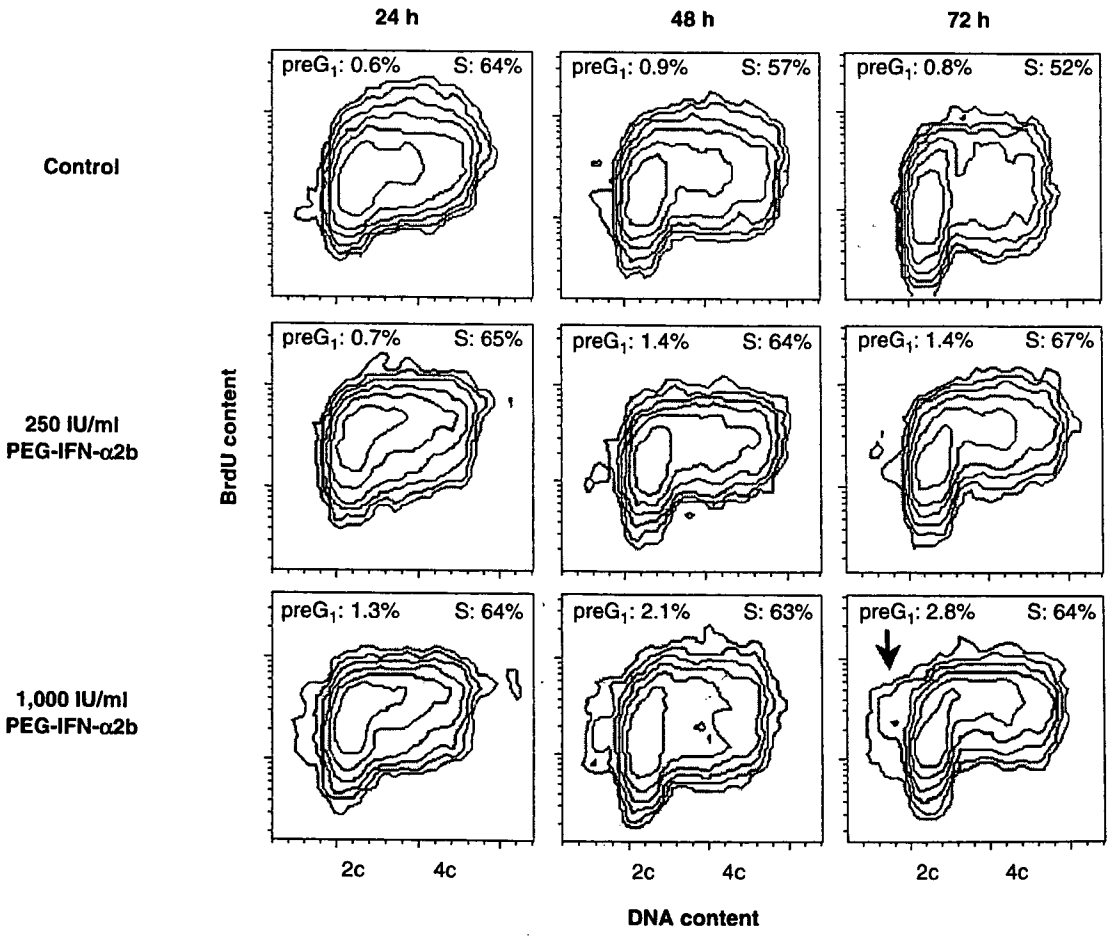

FIG. 2. Cell cycle analysis. KIM-1 cells were cultured with 250 or 1000 IU/mL PEG-IFN-α2b or medium alone (Control) for 24, 48, or 72 h. The cells were labeled with 10 mM BrdU for 30 min, fixed, then stained with anti-BrdU and propidium iodide and analyzed using a FACScan. The contour plots are shown. The arrow shows the area of the preG$_1$ phase.

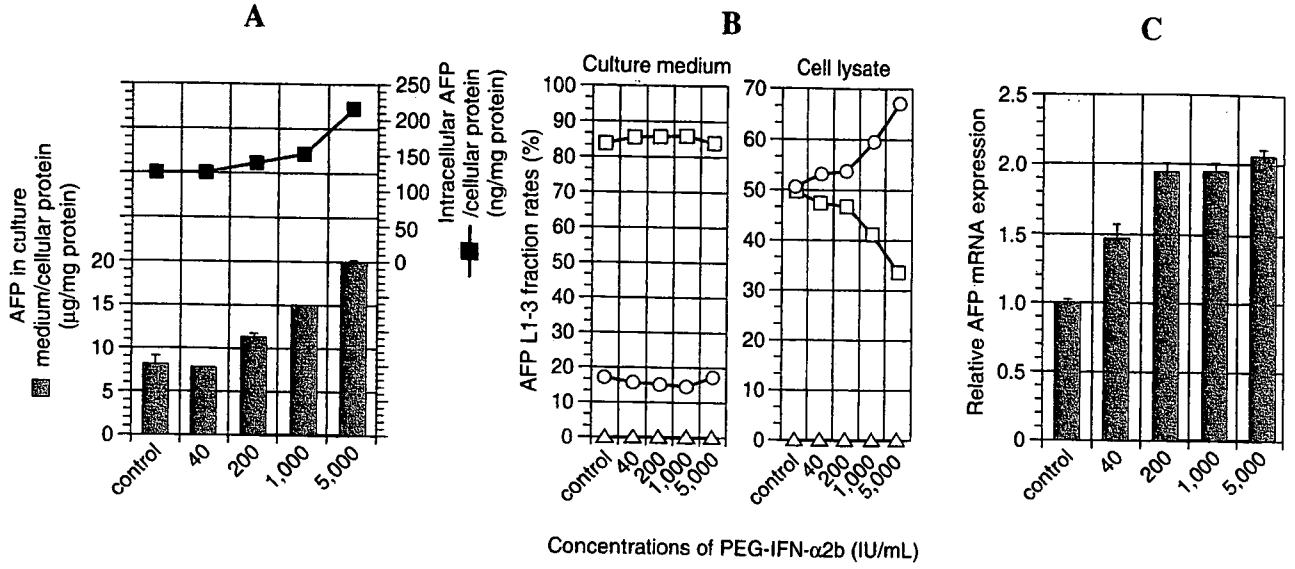

FIG. 3. Dose-dependent effects of PEG-IFN-α2b on the expressions of AFP protein and mRNA and on AFP-L1, AFP-L2, and AFP-L3 rates in KIM-1 cells cultured for 72 h. (**A**) Amount of AFP in the culture supernatant per unit volume of cellular protein (bottom, $n = 2$, mean ± SD) and amount of AFP in cell lysate per unit volume of cellular protein (top). (**B**) Rates (%) of AFP-L1 (circles), AFP-L2 (triangles), and AFP-L3 (squares) in culture supernatants (left) and cell lysate (right). (**C**) AFP mRNA expression ($n = 2$, mean ± SD).

the same between the PEG-IFN-α2b culture cells and the control (Fig. 4E); however, the AFP-L3 rate in cell lysate was lower in the culture cells than in the control and tended to decrease slightly with time after 96 h (Fig. 4F). AFP-L3 fraction rates (%) were lower in cell lysate than in culture supernatant. Expression of AFP mRNA increased in both groups up to 192 h. In the PEG-IFN-α2b culture cells, the mRNA level was significantly higher or tended to be high at 96, 144, and 192 h compared with the control, and the level in the culture cells increased approximately 3-fold at 192 h (Fig. 4G).

Effects of PEG-IFN-α2b on growth and AFP expressions in the other five HCC cell lines

PEG-IFN-α2b suppressed the growth of the other five HCC cell lines to 40%–75% of the control. AFP in cell lysate was detected at a very low volume in KYN-1 and KYN-2 cells with PEG-IFN-α2b at 1.5 and 1.3 ng/mg cellular protein, respectively. In the culture supernatant, AFP was expressed at a very low level in KYN-2, and the level in the cells with PEG-IFN-α2b increased approximately 2.5-fold over that of cells that had no contact with PEG-IFN-α2b (0.05 and 0.02 μg/mg protein, respectively). Expression of AFP mRNA was also detected only in the KYN-1 and KYN-2 cells, and the level of KYN-2 without PEG-IFN-α2b was approximately 4-fold that of KYN-1 without PEG-IFN-α2b. The level of both cell lines increased 2–4-fold with PEG-IFN-α2b treatment.

Intraassay and interassay variations of the total AFP protein levels and AFP-L3 rates in the supernatant of culture medium and cell lysate in the present experiments were estimated by the coefficient of variation (%)(CV) values. The CV values of intraassay and interassay variation of the total AFP protein levels were <16.6% and <28.3%, respectively, and those of AFP-L3 rates were 1.4% and 7.1%, respectively.

DISCUSSION

Only Nakamura et al.[20] have studied *in vitro* effects of IFN on AFP expression in HCC cells, and they reported that IFN-α2a increased AFP protein expression on the surface of NuE, an hepatoma cell line. In our current study, we used KIM-1, an HCC cell line that produces AFP, and examined the effects of PEG-IFN-α2b on cell growth and expression of AFP protein and mRNA. In our short-term and long-term experiments, the addition of PEG-IFN-α2b resulted in a chronologic decrease of KIM-1 cells and of the AFP level in culture supernatant. However, in the comparison of AFP protein production per unit volume of cellular protein, both the intracellular level and the level in culture supernatant were observed to increase with time (except at 192 h), and the AFP mRNA level also increased up to 192 h. In addition, upregulation of AFP protein and mRNA expressions by PEG-IFN-α2b was dose dependent. These results, as well as the results with KYN-1 and KYN-2, suggested that PEG-IFN-α2b affects human HCC cells at the transcriptional level and then upregulates AFP expressions. Therefore, if the serum AFP level in HCC patients or HCC-suspected patients increases after IFN administration, IFN would affect HCC and upregulate AFP production, or the number of AFP-producing cells would be increased. A lowering of the serum AFP levels indicates a decrease in AFP-producing cells because functional decrease of AFP production does not occur as a result of treatment. In the current experiments, we did not examine the ef-

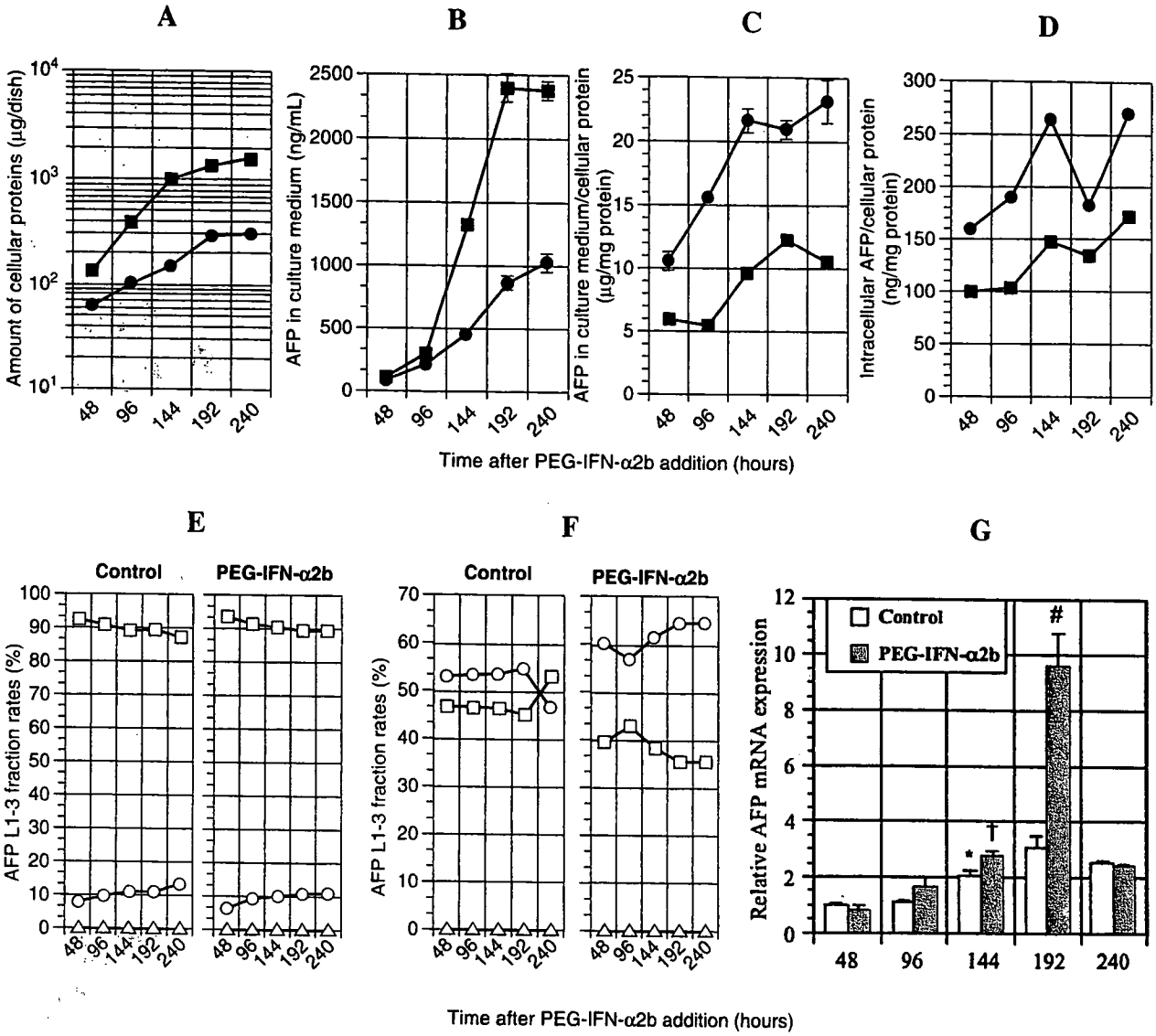

FIG. 4. Effects of PEG-IFN-α2b on the growth, expression of AFP protein and mRNA, and AFP-L1, AFP-L2, and AFP-L3 rates in KIM-1 cells cultured for 48, 96, 144, 192, or 240 h. (**A**) Growth curve, (**B**) concentration of AFP in the culture supernatant (mean ± SD, $n = 2$), (**C**) amount of AFP in the culture supernatant per unit volume of cellular protein (mean ± SD, $n = 2$), and (**D**) amount of AFP in cell lysate per unit volume of cellular protein of KIM-1 cells cultured with (filled circles) or without (filled squares) 1000 IU/mL PEG-IFN-α2b. (**E**) Rates of AFP-L1 (open circles), AFP-L2 (open triangles), and AFP-L3 (open squares) in the culture supernatants, and (**F**) those in cell lysate. (**G**) AFP mRNA expression ($n = 5$, mean ± SE). The data are the mean of two independent experiments. $^{*}p < 0.05$–0.0003 vs. control values at 48, 96, or 240 h, $^{\dagger}p < 0.03$–0.0001 vs. PEG-IFN-α2b values at 48, 96, or 192 h, or control value at 144 h; $^{\#}p < 0.001$–0.0001 vs. all the other data.

fects of non-PEG-IFN-α2b on AFP expression. However, we can expect effects with non-PEG-IFN-α2b comparable to those of PEG-IFN-α2b because previous reports, including our study,[21] demonstrated that the potency of PEG-IFN-α2b, defined as bioactivity independent of protein concentration, was comparable to that of IFN-α2b at both the molecular and cellular levels in a battery of *in vitro* assays.[22]

As shown in a previous cell cycle study using BALL-1 IFN-α,[19] our current results confirmed that PEG-IFN-α2b induces apoptosis and cell cycle blockage at the S-phase in KIM-1 cells. PEG-IFN-α2b was shown previously to suppress cell growth in the HAK-1B cell line via a similar growth suppression mechanism,[21] but AFP expression in HAK-1B was not upregulated. These findings may suggest that the growth suppression mechanisms, such as induction of apoptosis and cell cycle blockage at the S-phase, are not related to AFP upregulation. Nakabayashi et al.[23] investigated the relationship between AFP secretion and growth of human hepatoma cell lines after treatment with such hormones as insulin and dexamethasone and also indicated that AFP secretion was unrelated to the change in growth rate. These current findings indicated, however, the possibility that PEG-IFN-α2b treatment may cause selective