Table I. Probe and primer pair sequences for six factors regulating 5-FU sensitivity.

Gene		Sequences
TS[a]	Probe:	5'-(FAM)TTCAGCTTCAGCGAGAACCCAGA(TAMRA)-3'
	Forward primer:	5'-GAATCACATCGAGCCACTGAAA-3'
	Reverse primer:	5'-CAGCCCAACCCCTAAAGACTGA-3'
DPD[b]	Probe:	5'-(FAM)TGCCCTCACCAAAACTTTCTCTCTTGATAAGGA(TAMRA)-3'
	Forward primer:	5'-AATGATTCGAAGAGCTTTTGAAGC-3'
	Reverse primer:	5'-GTTCCCCGGATGATTCTGG-3'
OPRT[c]	Probe:	5' (FAM)CTCCTTATTGCGGAAATGAGCTCCACC(TAMRA)-3'
	Forward primer:	5'-TCCTGGGCAGATCTAGTAAATGC-3'
	Reverse primer:	5'-TGCTCCTCAGCCATTCTAACC-3'
TP[d]	Probe:	5'-(FAM)CAGCCAGAGATGTGACAGCCACCGT(TAMRA)-3'
	Forward primer:	5'-CCTGCGGACGGAATCCT-3'
	Reverse primer:	5'-GCTGTGATGAGTGGCAGGCT-3'
UP[e]	Probe:	5'-(FAM)TGCTCCAACGTCACTATCATCCGCAT(TAMRA)-3'
	Forward primer:	5'-TGACTGCCCAGGTAGAGACTATCC-3'
	Reverse primer:	5'-AGACCTATCCCACCAGAAGTGC-3'
TK[f]	Probe:	5'-(FAM)TGGCCTGGATTCACGCCCTCTTG(TAMRA)-3'
	Forward primer:	5'-AGCCTTGGCCCACACTGA-3'
	Reverse primer:	5'-CCAGAGGTAGGAAGGGCTTTG-3'

[a]Thymidylate synthase; [b]dihydropyrimidine dehydrogenase; [c]orotate phosphoribosyl transferase; [d]thymidine phosphorylase; [e]uridine phosphorylase; and [f]thymidine kinase.

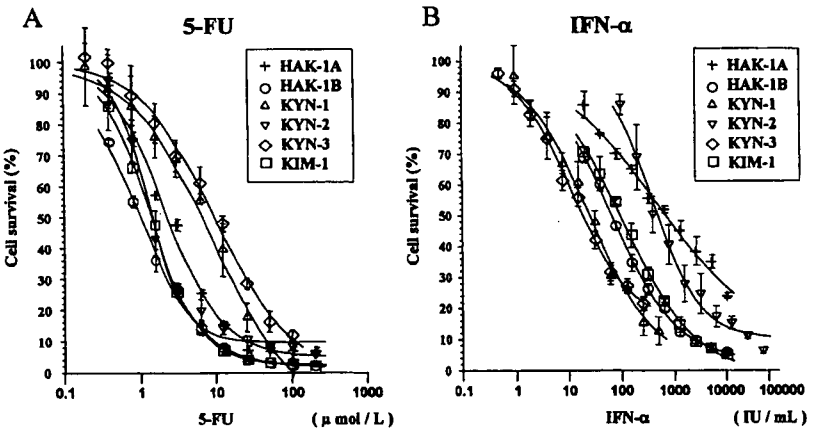

Figure 1. Antiproliferative effects of IFN-α and 5-FU in six HCC cell lines, HAK-1A, HAK-1B, KYN-1, KYN-2, KYN-3, and KIM-1. Cells were exposed to drugs for 5 days. Curves show the fitted dose-response curves for 5-FU (A) and IFN-α (B). Assays were carried out in quadruplicate. Data points represent the mean ± SD of the cell survival ratio relative to that of untreated cells, taken as 100%. Experiments were repeated twice with essentially similar results.

to knock down IFNAR1 (j03171_stealth_189) and IFNAR2 (x89772_stealth_1054) expression. Sub-confluent KYN-1 cells were cultured overnight in Opti-MEM I medium, then 40 nmol/l siRNA and Lipofectamine™ 2000 (Invitrogen™ Life Technologies) were applied according to the manufacturer's instructions. After 4 h, cells were harvested from the culture plates and seeded into 96-well plates. After a further 8-h incubation, IFN-α was applied and cells were cultured for 5 more days. The numbers of cells were estimated by WST-8 method (31).

Results

Antiproliferative effects of IFN-α and 5-FU in six HCC cell lines. When the antiproliferative effects of IFN-α and 5-FU on six HCC cell lines were examined, the growth inhibition seen

Table II. Relative mRNA expression levels of type I IFN receptor subunits and six factors regulating sensitivity to 5-FU in six HCC cell lines.

Cell line	Relative mRNA levels [a]								IC_{50}[j]	
	IFNAR1[b]	IFNAR2[c]	TS[d]	DPD[e]	OPRT[f]	TP[g]	UP[h]	TK[i]	IFN-α (IU/ml)	5-FU (μmol/l)
HAK-1A	23	5	100	1	100	6	16	91	700	2.3
HAK-1B	100	100	15	59	84	100	100	43	66	1.0
KYN-1	99	54	67	100	71	15	94	99	25	7.2
KYN-2	5	13	9	4	38	34	2	92	490	1.4
KYN-3	27	14	66	13	55	31	36	92	19	9.6
KIM-1	38	27	20	87	42	55	19	100	94	1.4

[a]The mRNA levels were examined by quantitative real-time RT-PCR and normalized with GAPDH. Relative mRNA level shows the average of the ratio relative to the highest level in six HCC cell lines of 100 in respective factor in triplicate determinations. [b]Type I interferon receptor subunit 1; [c]type I interferon receptor subunit 2; [d]thymidylate synthase; [e]dihydropyrimidine dehydrogenase; [f]orotate phosphoribosyl transferance; [g]thymidine phosphorylase; [h]uridine phosphorylase, and [i] thymidine kinase. [j]IC_{50}, drug concentration reducing the cell growth to 50% of that of non-treated cells. Cytotoxicity tests were carried out in quadruplicate.

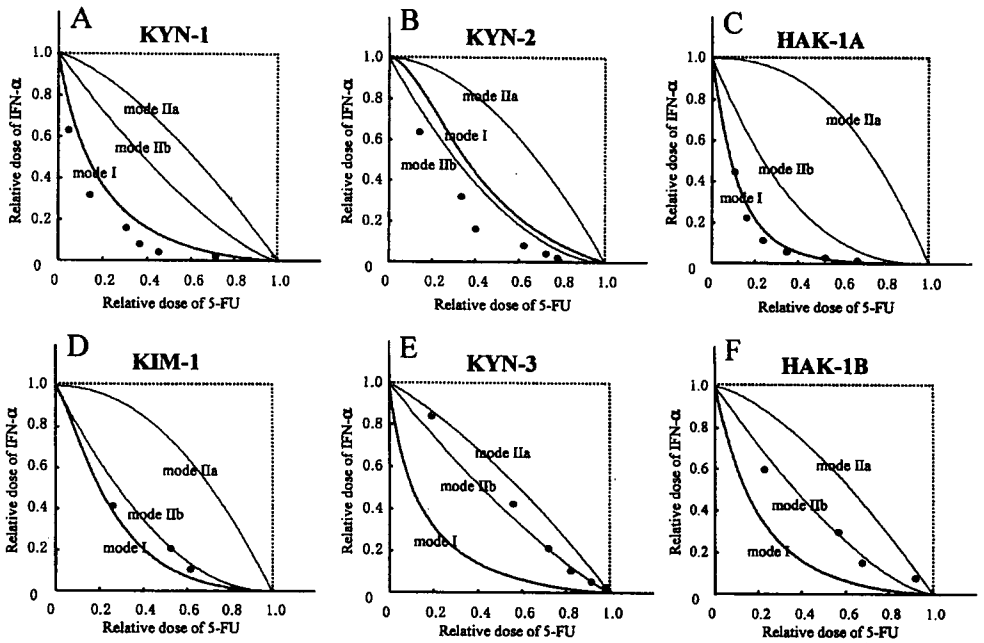

Figure 2. Combination effects of IFN-α and 5-FU. Isobologram analysis of IFN-α and 5-FU against six HCC cell lines. Cells were exposed to IFN-α and 5-FU for 5 days simultaneously. The area surrounded by the outer 2 curves of Mode I, IIa and IIb curves is the envelope of additivity. Relative doses of the drugs were calculated as concentration of 5-FU/IC_{50} of 5-FU and concentration of IFN-α/IC_{50} of IFN-α. Closed circles represent combined concentrations of 5-FU and IFN-α that inhibited cell growth by 50%. Assays were carried out in quadruplicate. Experiments were repeated twice with essentially similar results.

in all HCC cell lines was dose-dependent (Fig. 1). The HCC cell lines showed varied sensitivities to IFN-α, with IC_{50} values over a 25-fold range, from 19 to 700 IU/ml (Table II). KIM-1, KYN-1, KYN-3, and HAK-1B were highly sensitive to IFN-α with IC_{50} values below 100 IU/ml, while HAK-1A and KYN-2 appeared to be more resistant with IC_{50} values of 700 and 490 IU/ml, respectively. KYN-1 and KYN-3 responded weakly to 5-FU with IC_{50} values of 7.2 and 9.6 μmol/l, respectively, while the remaining four HCC cell lines were approximately three times more sensitive to 5-FU.

Expression of type I IFN receptor and factors regulating 5-FU sensitivity in six HCC cell lines. Table II shows the relative levels of basal mRNAs for IFNAR1, IFNAR2 and factors regulating sensitivity to 5-FU, with an arbitrary maximum value of 100. The mRNA levels of IFNAR1 were highest in

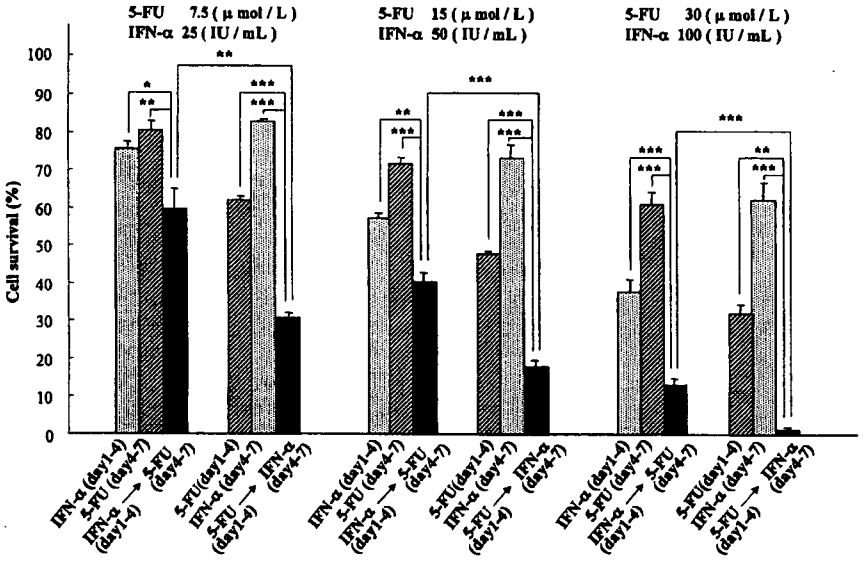

Figure 3. Schedule dependency of synergism of IFN-α and 5-FU. KYN-1 cells were seeded on day 0 and exposed to IFN-α or 5-FU for 3 days (day 1-4). On day 4, culture medium was removed and washed with PBS three times and fresh medium containing another drug was added. Cells were cultured for a further 3 days (day 4-7). The number of viable cells was estimated using the WST-8 assay. Experiments were carried out in triplicate independently. The columns show the mean value and bars represent SDs. Dotted column, IFN-α alone; striped column, 5-FU alone; solid column, sequential treatment of 5-FU and IFN-α. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$; statistically significant difference between the indicated data points by Welch's test.

the IFN-α-sensitive cell lines HAK-1B and KYN-1, while those in the other four HCC lines, which included both IFN-α-sensitive and resistant lines, were only 5-40% of their levels in HAK-1B. As for IFNAR-1, the levels of IFNAR-2 mRNA were relatively higher in HAK-1B and KYN-1. Moreover, both IFN-α-resistant cell lines, HAK-1A and KYN-2, had relatively lower levels of IFNAR-1 and IFNAR-2 mRNAs, while the other IFN-α-sensitive cell lines had relatively higher levels of mRNA for both subunits.

Of the six factors regulating sensitivity to 5-FU, low levels of TS and DPD and high levels of OPRT, TP, UP and TK render cancer cells sensitive to 5-FU in *in vitro* assays. Cellular TS mRNA levels were relatively high in two 5-FU-resistant lines, KYN-1 and KYN-3, while DPD mRNA levels were high only in KYN-1 cells. Cellular TP mRNA levels were relatively low in the 5-FU-resistant KYN-1, but high in the 5-FU-sensitive HAK-1B cells. However, overall there was no clear association between the cellular mRNA levels of each factor and the 5-FU sensitivity of the six HCC lines.

Combined effect of IFN-α and 5-FU on six HCC cell lines. The isobologram method yielded three curves, Mode I, Mode IIa and Mode IIb. All of the data points for combined treatments against KYN-1 and KYN-2 cell lines, and three out of six data points for HAK-1A, fell in the area suggesting a synergistic effect and the other three points for HAK-1A cells were almost on the mode I curve (Fig. 2A and C). Therefore the combined effect of IFN-α and 5-FU on these three cell lines was judged to be synergistic. By contrast, all of the data points for combined treatments of HAK-1B, KYN-3, and KIM-1 cells fell within the envelope of additivity, and the combined effects on these cell lines were judged to be additive (Fig. 2D and F). Based on these results, we separated the six cell lines into two groups: the S-group, showing synergistic responses, and consisting of KYN-1, KYN-2, and HAK-1A, and the A-group, characterized by additive responses, and consisting of HAK-1B, KYN-3, and KIM-1.

As shown in Fig. 3, schedule-dependent interactions between IFN-α and 5-FU were examined using KYN-1 cells showing synergistic effect with simultaneous treatment of IFN-α and 5-FU. Sequential exposure to 5-FU followed by IFN-α showed much stronger antiproliferative effect than the reverse sequence at all tested concentration sets of 5-FU and IFN-α.

Effect of IFN-α on protein expression of factors regulating 5-FU sensitivity. The metabolism of 5-FU is shown in Fig. 4A. The antitumor effects of 5-FU primarily depend on levels of its metabolic enzymes in *in vitro* study. We selected six enzymes, TS, DPD, OPRT, TP, UP and TK, that were reported to be closely associated with sensitivity and/or resistance to 5-FU. We examined their protein levels in these six cell lines when treated with IFN-α at 500 IU/ml for 48 h (Fig. 4B). The expression levels of TS, OPRT and TK were down-regulated after treatment with IFN-α for 48 h, not only cell lines in the S-group, but also in the A-group. By contrast, treatment with IFN-α resulted in a 3-fold increase in DPD protein level in KYN-3 cells. We also observed an up-regulation of TP in IFN-α treated KYN-1 and HAK-1A cells, both in the S-group, by 6.9- and 2.8-fold respectively. However, none of the six factors regulating sensitivity to 5-FU was consistently modulated in response to IFN-α in all of the S-group or all of the A-group cell lines.

Effect of 5-FU on expression of IFNAR1 and IFNAR2 in six HCC cell lines. The relative mRNA levels of IFNAR1 and

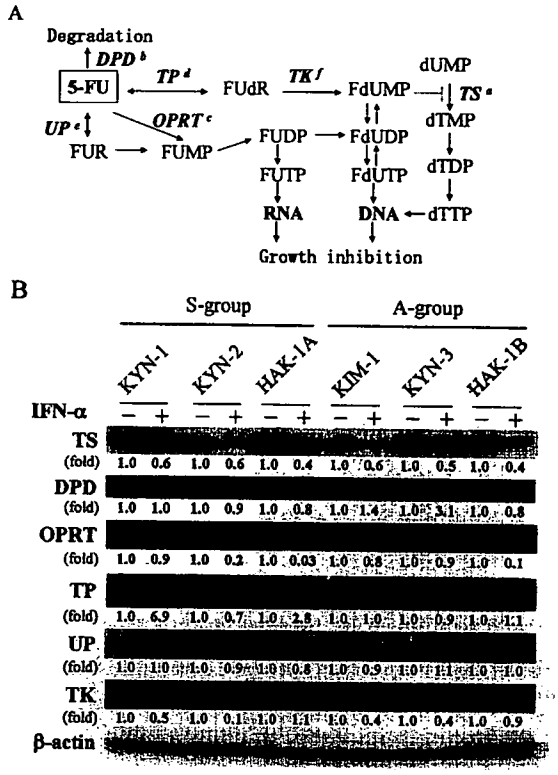

Figure 4. Effect of IFN-α on factors regulating sensitivity to 5-FU. (A) Metabolism of 5-FU. [a]TS, thymidylate synthase; [b]DPD, dihydropyrimidine dehydrogenase; [c]OPRT, orotate phosphoribosyl transferase; [d]TP,thymidine phosphorylase; [e]UP, uridine phosphorylase; [f]TK,thymidine kinase; FUdR, 5-fluoro-2'-deoxyuridine; FdUMP, 5-fluoro-2'-deoxyuridine-5'-monophosphate; FdUDP, 5-fluoro-2'-deoxyuridine-5'-diphosphate; FdUTP, 5-fluoro-2'-deoxyuridine-5'-triphosphate; FUR, 5-fluorouridine; FUMP, 5-fluorouridine-5'-monophosphate; FUDP, 5-fluorouridine-5'-diphosphate; FUTP, 5-fluorouridine-5'-triphosphate. TS is a target enzyme of active metabolite FdUMP from 5-FU, and DPD inactivates 5-FU. OPRT, TP, UP and TK were concerned with conversion of 5-FU to its active form. Low levels of TS and DPD and high levels of OPRT, TP, UP and TK render cancer cells sensitive to 5-FU in *in vitro* assays. (B) Protein expression of six enzymes after 48-h treatment with 500 IU/ml IFN-α were determined by Western blot analysis. Values underneath the bands represent the relative density to that of drug untreated cells, taken as 1.0. Cell lines in the S-group showed synergistic effect, and those in the A-group showed additive effect with combination of IFN-α and 5-FU.

IFNAR2 in 5-FU-treated HCC cells, compared to untreated cells, are shown in Fig. 5. In HAK-1A, KYN-1 and KYN-2 in the S-group, treatment with 5-FU induced an approximately 4-fold increase in IFNAR-1 mRNA levels when compared to untreated cells (Fig. 5A). By contrast, there appeared only a slight or no increase in IFNAR-1 mRNA in KIM-1, KYN-3, and HAK-1B in the A-group. We observed a 2.5- to 3-fold increase in IFNAR2 mRNA in KYN-1 and HAK-1A, but not in KYN-2 in the S-group by 5-FU. By contrast, there appeared no increase of IFNAR2 mRNA by 5-FU in KIM-1, KYN-3, and HAK-1B cells in the A-group (Fig. 5B). Time-dependent kinetics for the expression of both type I IFN receptor subunits showed a marked increase in IFNAR1 and IFNAR2 mRNA levels at 3 h in KYN-1 cells, but not in KYN-3 cells when treated with 5-FU (Fig. 6A and B). In KYN-1 cells, Western blot analysis also showed an approximate 4-fold increase in protein levels of IFNAR1, 24 h after exposure to 5-FU (Fig. 6C). Treatment of KYN-1 cells with 5-FU induced an approximate 7-fold increase in IFNAR2 protein. In contrast, in KYN-3 cells in the A-group, the levels of the IFNAR2-related molecules R2 were not increased by 5-FU treatment (Fig. 6D).

Effect of knockdown of IFNAR1 and IFNAR2 by siRNA on antiproliferative effect of IFN-α. We next examined whether cellular levels of IFNAR1 and/or IFNAR2 were closely associated with IFN-α-induced antiproliferative effect in KYN-1 and KYN-3. Cellular levels of IFNAR1 and IFNAR2 proteins were markedly reduced by the relevant siRNAs, but not by scrambled RNAs (Fig. 7A). Moreover, the antiproliferative effect of IFN-α on both KYN-1 and KYN-3 cells was abrogated by knockdown of either IFNAR1 or IFNAR2, while the scrambled RNAs had no effect (Fig. 7B). Quantitative analysis of the cell survival curves of KYN-1 revealed that the knockdown of IFNAR1 and IFNAR2 increased the IC_{50} values for IFN-α, by 7-fold (231 IU/ml) and 5-fold (158 IU/ml) respectively, compared to the IC_{50} values (31 IU/ml) for cells untreated with siRNAs and treated with scramble RNAs (Fig. 7C and Table III). In KYN-3 cells, the knockdown of IFNAR1 and IFNAR2 increased the IC_{50} values for IFN-α from 21 IU/ml for untreated control cells to over 1000 IU/ml respectively. The antiproliferative activity of IFN-α

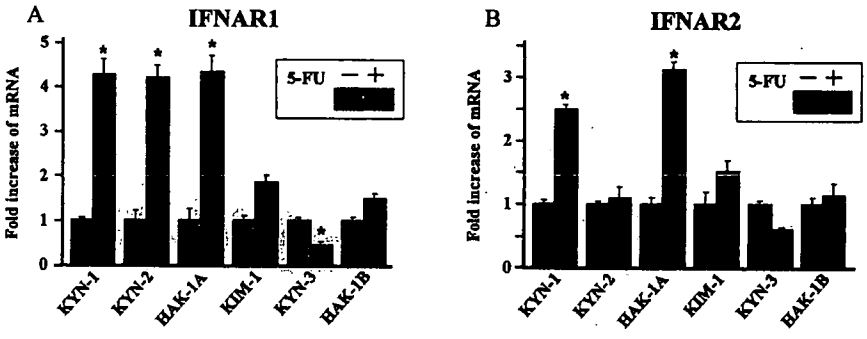

Figure 5. Expression of IFNAR1 and IFNAR2 mRNA, in HCC cells treated with 5.0 μmol/l 5-FU for 3 h. The increases in mRNA are shown relative to the initial level, taken as 1.0. Dotted and black columns show the mean mRNA levels in drug-untreated and 5-FU-treated cells, respectively. (A) IFNAR1, (B) IFNAR2. Determinations were carried out in triplicate, and bars represent the SDs. Experiments were repeated twice with essentially similar results. *Difference is >2-fold and statistically significant by Welch's test ($P<0.05$) as compared with untreated cells.

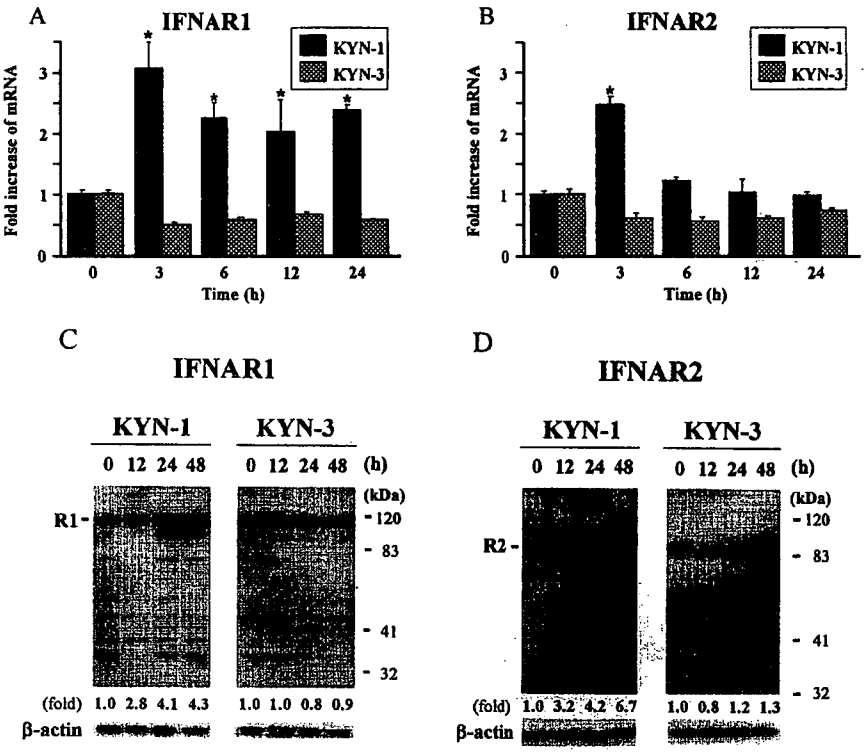

Figure 6. Time course of IFNAR1 and IFNAR2 mRNA and protein expression in KYN-1 and KYN-3 cells treated with 5 μmol/l of 5-FU. Increases in mRNA expression levels for IFNAR1 (A) and IFNAR2 (B) are shown relative to the initial level, taken as 1.0, in KYN-1 and KYN-3 cells. Black columns represent the mean increase of mRNA levels in KYN-1 cells and meshed columns show increases in KYN-3 cells. Determinations were carried out in triplicate, and bars indicate the SD. *Difference is >2-fold and statistically significant by Welch's test ($P<0.05$), compared with initial level. Experiments were repeated twice with essentially similar results. (C) IFNAR1 protein expression in cells treated with 5-FU. R1 is an ~110-kDa band detected by Western blotting. (D) IFNAR2 protein expression in cells treated with 5-FU. R2 is an ~100-kDa band that may be the long form of IFNAR2. Experiments were repeated three times with essentially similar results.

in KYN-1 and KYN-3 cells was thus confirmed to be dependent on the expression levels of both IFNAR1 and IFNAR2.

Discussion

In this study, six independently established HCC cell lines were subjected to simultaneous treatment with 5-FU and IFN-α and classified into two groups on the basis of their antiproliferative activity according to isobologram analysis: KYN-1, KYN-2 and HAK-1A, in which the two drugs had a synergistic effect, were in the S-group, and HAK-1B, KYN-3 and KIM-1, in which the two drugs had an additive effect, were in the A-group. We further asked whether treatment with either 5-FU or IFN-α could modulate the expression of factors likely to be involved in the classification of the HCC cell lines into the S- or A-group.

We first determined cellular levels of mRNA and protein of TS, DPD, OPRT, TP, UP, and TK genes that are known to be involved in sensitivity to 5-FU (Table II and Fig. 4B). Basal levels of these cellular proteins in six cell lines used in this study were found to be comparable to their mRNA levels of these six enzymes. We examined whether exposure to IFN-α could modulate the protein expression levels of these six genes. Cellular expression levels of TS were not significantly different in cell lines of the S- and A-group, suggesting that TS might not be a critical factor determining whether the combination effect is synergic or additive. None

Table III. IC_{50} values of IFN-α against IFNARs reduced KYN-1 and KYN-3 cell lines.

Treatment	IC_{50} (IU/ml) KYN-1	KYN-3
None	31	21
IFNAR1 knock down	231	>1000
scramble of IFNAR1 siRNA	34	19
IFNAR2 knock down	158	>1000
scramble of IFNAR2 siRNA	28	20

The IFNARs genes were silenced by Stealth™ RNAi (Invitrogen™ Life Technologies). KYN-1 and KYN-3 cells were treated with siRNAs and exposed to IFN-α for 5 days. IC_{50} values were calculated from dose-response curves shown in Fig. 7C.

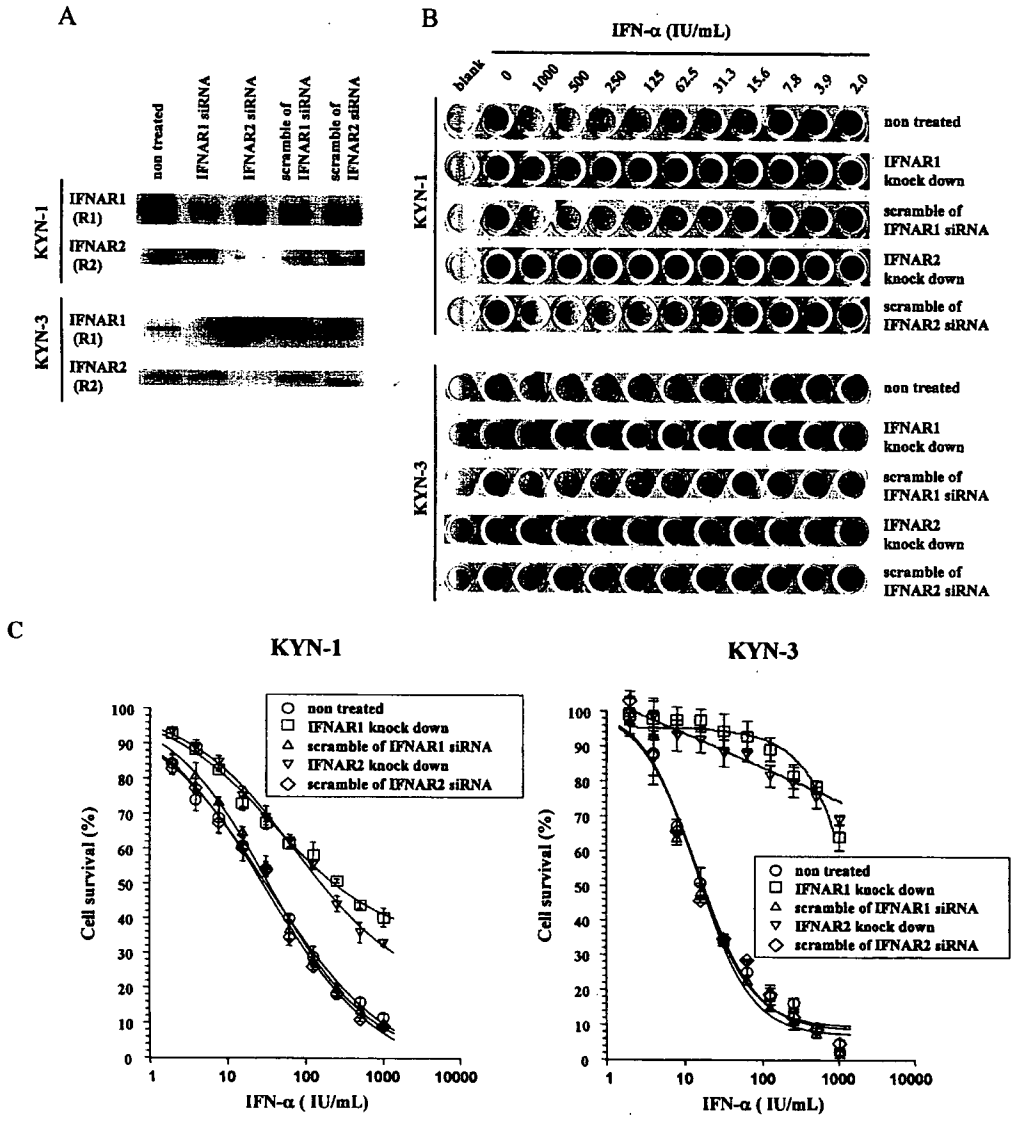

Figure 7. Dependency of antiproliferative activity of IFN-α on IFNAR1 and IFNAR2 expression. (A) Silencing of the IFNARs genes by siRNAs was confirmed by Western blotting. (B) KYN-1 and KYN-3 cells were treated with siRNAs and exposed to IFN-α for 5 days. Cells were stained by crystal violet. Assays were carried out in quadruplicate and one line of wells per condition of treatment is shown respectively. (C) Growth inhibitory effects of IFN-α on KYN-1 and KYN-3 cells pre-treated with siRNAs. Lines show the fitted curves of dose-response. Cell viability data is shown as means ± SD (n=4). The differences between IFNARs siRNA pre-treated group and their respective scrambled control are statistically significant at all concentrations tested, by Welch's t-test ($P<0.01$). Experiments were repeated twice with essentially similar results. IC_{50} values are shown in Table III.

of the other genes were specifically down- or up-regulated in a similar manner in the 3 cell lines of the S- or A-group.

We next examined whether exposure to 5-FU could modulate expression levels of both mRNA and protein of IFNAR1 and IFNAR2. Knock down of IFNAR1 or IFNAR2 gene by its specific siRNA markedly desensitized the sensitivity of hepatic cancer cells to IFN-α. This suggested that both IFNAR1 and IFNAR2 are closely associated with antiproliferative effect by IFN-α. We further observed an approximate 4-fold increase in mRNA of IFNAR1 in the three cell lines in the S group, but no increase in the three cell lines in the A-group, when treated with 5-FU. Western blot analysis showed an approximate 4-fold increase in IFNAR1 in a time-dependent manner and an approximate 7-fold increase in IFNAR2 in 5-FU-treated KYN-1 cells, but not in KYN-3. Treatment with 5-FU thus could induce specific expression of both IFNAR1 and IFNAR2 in HCC cell lines in the S-group, but not in the A-group. A relevant study by Kondo has reported that a combination of IFN-α and 5-FU strongly induced apoptosis in HCC cells in association with Bcl-2 family members by activation of IFNAR2 signal (33). Ota and colleagues have recently demonstrated a significant correlation of IFNAR2 expression and response rates in patients with HCC when treated with a combination of IFN-α and 5-FU (6). Collectively,

up-regulation of IFNAR1 and IFNAR2 by 5-FU might play a pivotal role in synergism of IFN-α combined with 5-FU against HCC.

In conclusion, we present a novel finding that synergy between 5-FU and IFN-α in HCC cells is mediated through the 5-FU-induced up-regulation of the type I IFN receptor. The translation of this *in vitro* result into clinical application, particularly the identification of whether or not 5-FU up-regulates the type I IFN receptor in a specific patient, would have a tremendous impact on the selection of the best treatment modality. Practical methods for determining the up-regulation of the type I IFN receptor, for selecting responders to combined IFN-α and 5-FU therapy should be the subject of further research. Moreover, our assay system using HCC cell lines could provide novel insights of practical significance, about other anticancer agents besides 5-FU, which could efficiently up-regulate the expression of the type I IFN receptor.

Acknowledgements

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HEPATOLOGY

Growth inhibitory effects of interferon-α subtypes vary according to human liver cancer cell lines

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Abstract

Background: Interferon (IFN)-α preparations used in the treatment of viral and neoplastic disease consist of single or multiple IFN-α subtypes that may possess different biological activity, but there are no data on liver cancer cells.

Methods: Antiproliferative effects and the mechanisms of growth inhibition of five IFN-α subtypes (α1, α2, α5, α8 and α10) were examined *in vitro* using 13 human liver cancer cell lines.

Results: The antiproliferative effect of each IFN-α subtype was different in each cell line. The 50% growth inhibitory concentration (IC50) on an antiviral unit basis showed that α5 presented the most potent antiproliferative effects in 11 of the 13 cell lines, and α8 in two cell lines. On average, the antiproliferative effects were strong in descending order from α5, α8, α10, α2 to α1. On weight basis, the most potent antiproliferative effect was shown by α8 in nine of the 13 cell lines, α5 in four cell lines, and the potency of the effects on average in descending order was α8, α5, α10, α2 and α1. No significant difference was observed between natural and recombinant α2. The mechanism of growth inhibition of each subtype in HAK-1B and KMCH-1 cell lines were apoptosis and S-phase arrest, and their induction levels were related to a certain degree to the antiproliferative effects.

Conclusions: Our findings show that the antiproliferative effect of each IFN-α subtype varies according to the cell line, but that the cells are relatively or absolutely responsive to α5 and α8 subtypes.

Introduction

Interferon (IFN)-α is a multifunctional cytokine that possesses antiviral activity, antiproliferative activity and various immunoregulatory activities.[1] The antiviral activity of IFN-α has attracted a great deal of attention, and IFN-α preparations have been used in the treatment of hepatitis B virus and hepatitis C virus related chronic hepatitis in many countries.[2] Recently, IFN-α has been shown to possess highly suppressive effects on hepatocellular carcinogenesis in patients with virus-related chronic hepatitis,[3–5] but the mechanisms of its action have not yet been clarified. We previously reported that the human lymphoblastoid IFN-α derived from Sendai virus-induced BALL-1 cells (BALL-1 IFN-α) directly suppressed cell-proliferation in 13 liver cancer cell lines to various degrees by inhibiting cell cycle progression with or without apoptosis *in vitro*.[6] This suggests that the direct antiproliferative action of IFN-α may be involved in IFN-α's suppressive mechanisms on hepatocellular carcinogenesis. In clinical practice, IFN-α preparation alone or in combination with other anticancer medicine such as 5-fluorouracil has been applied in the treatment of malignant diseases including leukemia, renal cancer,[2,7] and advanced hepatocellular carcinoma (HCC).[8]

Human IFN-α comprises a family of structurally and functionally related genes of at least 14 subtypes.[9,10] Natural IFN-α preparations, such as human lymphoblastoid IFN-α, consist of a mixture of distinct IFN-α subtypes. Several studies suggest that IFN-α subtypes display significant differences in specific activities such as antiviral activity and antiproliferative activity,[11–18] as well as in binding affinities to the type I IFN receptor.[13,19] The activity levels also varied greatly depending on the target cells. For the prevention and treatment of HCC by IFN-α, it is important to know the potency of the antiproliferative effect of each IFN-α subtype. However, there have been no in-depth studies on HCC cells.

Our current study examined the antiproliferative effects of five IFN-α subtypes on 13 liver cancer cell lines whose type I IFN receptor expression and growth inhibition by BALL-1 IFN-α had been previously investigated.[6] The present study showed that each IFN-α subtype presents very different antiproliferative activities in different cell lines.

Methods

Cell lines and cell culture

This study used 11 human HCC cell lines (KIM-1,[20] KYN-1,[21] KYN-2,[22] KYN-3,[23] HAK-1A,[24] HAK-1B,[24] HAK-2,[25] HAK-3, HAK-4, HAK-5 and HAK-6)[6] and two combined hepatocellular and cholangiocarcinoma cell lines (KMCH-1[26] and KMCH-2).[27] The cell lines were originally established and characterized in our laboratory and were previously confirmed to retain the morphological and functional characteristics of the original tumor. KIM-1, KYN-1, HAK-2 and HAK-3 were established from moderately differentiated HCC; KYN-2, KYN-3 and HAK-6 from moderately to poorly differentiated HCC; HAK-4 from poorly differentiated HCC; and HAK-5 from sarcomatous HCC. HAK-1 A and HAK-1B were established from a single HCC nodule showing a three-layered structure with a different histological grade in each layer. HAK-1 A resembles well-differentiated HCC cells in the outer layer of the original tumor, and HAK-1B resembles poorly differentiated cells in the inner layer.

The cells were grown in a culture medium consisting of Dulbecco's modified Eagle medium (Nissui Seiyaku, Tokyo, Japan) supplemented with 2.5% heat-inactivated (56°C, 30 min) fetal bovine serum (Bioserum, Melbourne, Australia), 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco BRL/Life Technologies, Gaithersburg, MD, USA) and 12 mM sodium bicarbonate, in a humidified atmosphere of 5% CO_2 at 37°C. Cells were cultured for 24 h prior to the experiments to allow the cells to enter their logarithmic growth phase. In colorimetric cell growth assay and cell cycle analysis experiments, the medium with or without IFN-α was renewed 48 h after the initial treatment.

Cloning and expression of recombinant human interferon-α

Recombinant human IFN-α subtypes (α1, α2, α5, α8 and α10) were expressed via the pET-3a-BL21 system. Briefly, total RNA was purified from BALL-1 cells using the guanidine method. Reverse transcription-polymerase chain reaction (RT-PCR) was performed and the amplified products were cloned into pCR-Script (+) and sequenced. The amplified products of human IFN-α cDNA were then subcloned into the expression vector pET-3a. For expression, the constructs were transformed into BL-21 and the transformants were cultured in L-broth and inducted by 0.2 mM IPTG for 3 h. Cells were harvested and sonicated in phosphate-buffered saline (PBS). Each subtype was purified from supernatants in three steps; hydrophobic chromatography, ion-exchange chromatography, and gel filtration. Specific activity was measured in a cytopathic effect (CPE) reduction assay with the Sindbis virus in FL cells: 2.54×10^6 IU/mg for subtype α1; 7.17×10^7 IU/mg for α2; 3.98×10^7 IU/mg for α5; 2.98×10^8 IU/mg for α8; and 4.87×10^7 IU/mg for α10.

Preparation of natural human interferon-α2

Natural human IFN-α2 (nIFN-α2) was separated from human lymphoblastoid IFN-α (OIF) with anti-IFN-α antibody NK-2 Sepharose (RESELUTE NK-2, LONZA, Slough, UK) and eluted with 0.1 M citric acid (pH 2.0) containing 0.3 M NaCl. Separated nIFN-α2 was collected and dialyzed at 0.05 M Tris-HCl buffer (pH 8.3) for 20 h at 4°C. IFN-α2 was then prepared from nIFN-α by ion-exchange high-performance liquid chromatography (HPLC) on a DEAE-5PW Column (Tosho, Tokyo, Japan). The protein concentration was determined in terms of human serum albumin using the Bradford method. The specific activity of natural IFN-α2 was estimated at 1.2×10^8 IU/mg protein measured in a CPE reduction assay with the Sindbis virus in FL cells.

Effect of interferon-α subtypes on the proliferation of hepatocellular carcinoma and combined hepatocellular and cholangiocarcinoma cell lines

The effect of each IFN-α subtype on cultured cell proliferation was investigated using colorimetric assays with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide cell growth assay kits (Chemicon, Temecula, CA, USA) as previously described.[6] Briefly, cultured cells ($1.5–8 \times 10^3$ cells per well) were seeded on 96-well plates (Falcon, Becton Dickinson Labware, Tokyo, Japan), cultured for 24 h and the medium was then replaced with a fresh 100-μL medium with or without one of the IFN-α subtypes (1–1024 IU/mL). After culture for 24, 48, 72 or 96 h, the number of viable cells was examined. Six to eight samples were used in each experiment, and each experiment was repeated at least twice in order to confirm the reproducibility of the test results. The 50% inhibitory concentration (IC50) of each cell line was estimated at 96 h of culture with each IFN-α subtype on an antiviral unit basis. The IC50 of the average of 13 cell lines was estimated on an antiviral unit basis as well as on a weight basis.

Cell cycle analysis

Cells were cultured with or without IFN-α (1000 U/mL) for 96 h, harvested, fixed in 70% cold ethanol at 4°C for 3 h, and then stained with 40 μg/mL propidium iodide (Sigma Chemical, St. Louis, MO, USA) for 10 min. Cell cycle and apoptosis were analyzed with an EPICS XL flow cytometer and WINCYCLE software (Beckman Coulter, Fullerton, CA, USA).

Statistics

The significance of differences was estimated using the unpaired and/or paired Student's *t*-test (two-tailed).

Results

Effects of interferon-α subtypes on the growth of 13 liver cancer cell lines

Figure 1 shows IC50 of the five IFN-α subtypes on an antiviral unit basis in the 13 liver cancer cell lines. Each subtype presented different antiproliferative effects in each cell line. Among the 13

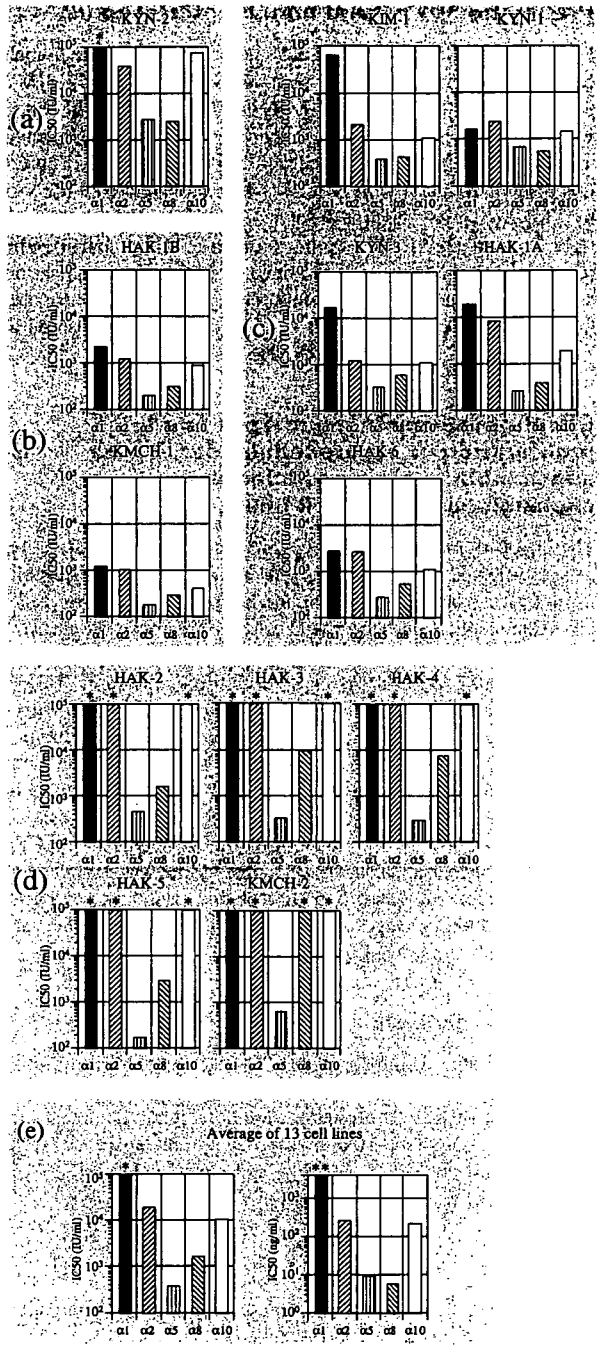

Figure 1 Antiproliferative effects of five interferon (IFN)-α subtypes in 13 liver cancer cell lines. Fifty percent inhibitory concentration (IC50) values (shown as the bars) were obtained from cells cultured with various concentrations of the five IFN-α subtypes for 96 h. (a) KYN-2 showed a weak response to every subtype. (b) HAK-1B and KMCH-1 showed a relatively good response to every subtype. (c) KIM-1, KYN-1, KYN-3, HAK-1 A and HAK-6 responded highly to α5 and α8, and also to α10. (d) HAK-2, HAK-3, HAK-4, HAK-5 and KMCH-2 responded very weakly to α1, α2 and α10, while the response of the cells was high to α5 and varied to α8. (e, left) On average, of the 13 cell lines, IC50 on an antiviral unit basis to α5 was the least (383.1 IU/mL) followed by α8 (1691.5 IU/mL), α10 (10 926 IU/mL), α2 (19 627.8 IU/mL) and α1 (higher than 100 000 IU/mL) (e, right). On a weight basis (ng/mL), the average IC50 to α8 (5.7 ng/mL) became less than that to α5 (9.6 ng/mL). *IC50 value of more than 1.0×10^5 IU/mL; **IC50 value of more than 4.0×10^3 ng/mL.

cell lines, KYN-2 (Fig. 1a) responded weakly to every subtype (IC50 > 1000 IU/mL), while the remaining 12 cell lines responded to at least one subtype (IC50 < 1000 IU/mL). The seven cell lines (KIM-1, KYN-1, KYN-3, HAK-1 A, HAK-1B, HAK-6 and KMCH-1) (Fig. 1b,c) were highly responsive to α5 and α8, and α10 was the next most potent subtype. In six of the seven cell lines (those except KYN-1), IC50 to α5 was the least among the five subtypes. The response to α1 and α2 varied among the cell lines. HAK-1B and KMCH-1 (Fig. 1b) were also relatively responsive to α1 and α2. Among the seven cell lines, the difference in IC50 to α5 and to α8 was less than two times. The remaining five cell lines (HAK-2, HAK-3, HAK-4, HAK-5 and KMCH-2) responded very weakly to α1, α2 and α10 (Fig. 1d), while the response of the cells was strong to α5 and varied to α8. The difference in IC50 to α5 and α8 in the five cell lines except HAK-2 (i.e. HAK-3, HAK-4, HAK-5 and KMCH-2) was greater than 15 times. On average, of the 13 cell lines, IC50 to α5 was the least (383.1 IU/mL) followed by α8 (1691.5 IU/mL), α10 (10 926 IU/mL), α2 (19 627.8 IU/mL) and α1 (higher than 100 000 IU/mL) (Fig. 1e, left). On a weight basis (ng/mL), the specific activity of α8 was approximately 7.5 times higher than α5, that is, the average IC50 to α8 (5.7 ng/mL) became less than that to α5 (9.6 ng/mL). The order of the remaining three subtypes was similar to the data obtained on an antiviral unit basis (Fig. 1e, right). In our current study, weight based analysis of each cell line showed less IC50 to α5 than to α8 in nine cell lines (KYN-1, KYN-2, KYN-3, HAK-1 A, HAK-1B, HAK-2, HAK-6, KIM-1 and KMCH-1), while both IC50 on an antiviral unit basis and a weight basis was the least to α5 in the remaining four cell lines (HAK-3, HAK-4, HAK-5 and KMCH-2).

When chronological changes in the ratio of viable cell numbers in the cultures with 1024 IU/mL of each IFN-α subtype to the number in the cultures with medium only were monitored, with α5 the ratio in the 11 cell lines (except KYN-1 and KMCH-2) started to decrease with time from 24 h after the addition. The addition of the other subtypes induced a slight decrease in the ratio only in one or two cell lines, but induced a slight increase in nine cell lines (except KYN-3, HAK-1A, HAK-1B, and KMCH-1) at 24 h, but the number started to decrease after 24 h. A time-dependent antiproliferative effect was observed up to 96 h in 11 cell lines (except HAK-3 and HAK-4), whereas in HAK-3 and HAK-4 cell lines the time-dependent effects of the subtypes except α5 became unobservable after 72 h. Sensitivity to the growth-suppressive effect of each IFN-α subtype was not related to the histological grade of the original tumors of each cell line.

Effects of natural and recombinant interferon-α2 on the growth of 10 liver cancer cell lines

Natural IFN-α2 is thought to possess a certain number of glycosylation sites, while recombinant IFN-α2 (rIFN-α2) does not. In the

three liver cancer cell lines (KYN-1, KYN-3 and HAK-2), relative viable cell number 96 h after adding 1024 IU/mL of nIFN-α2 was lower by 10% or more than that after the addition of rIFN-α2, whereas the number was higher by 10% or more in one cell line (KYN-2). In the other cell lines, the numbers showed changes within the 10% range. On average, in the 10 cell lines no significant differences were obtained between natural and recombinant IFN-α2 (Fig. 2).

Growth inhibitory mechanisms of five interferon-α subtypes in two representative liver cancer cell lines

The cell cycle was analyzed in HAK-1B and KMCH-1 by adding 1000 IU/mL of each IFN-α subtype. Every subtype induced apoptosis and S-phase arrest, and the effect was the most remarkable with α5. In addition, the induction levels of the subtypes were related to a certain degree to the antiproliferative effects (Fig. 3).

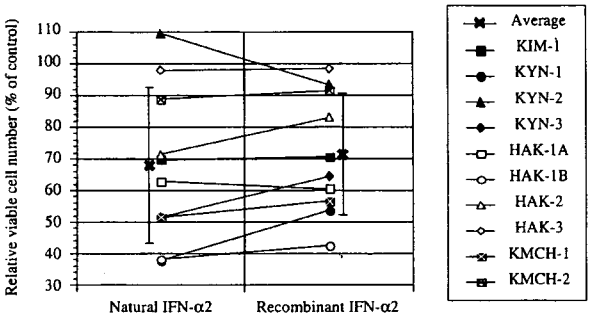

Figure 2 Antiproliferative effects of natural and recombinant interferon (IFN)-α2 on 10 liver cancer cell lines. Relative viable cell numbers (% of control) were obtained from cells cultured with 1024 IU/mL of natural or recombinant IFN-α2 for 96 h. There were no significant differences between natural and recombinant IFN-α2.

Discussion

We investigated the antiproliferative effects of five IFN-α subtypes (α1, α2, α5, α8 and α10) against 13 liver cancer cell lines at different histological grades. The antiproliferative activities of IFN-α subtypes were studied on various neoplastic cells such as renal cell carcinoma and leukemia.[11–14,18] However, there are no studies that have examined a large number of liver cancer cell lines that are at different histological grades and possess different biological activities. Because the morphological and biological features of liver cancer cells are diverse, examination of many cell lines should be conducted to provide more precise information. In the current study, each of the 13 cell lines presented different sensitivity to the antiproliferative effect of each IFN-α subtype, and there were roughly four patterns (as shown in Fig. 1a–d). This showed that each cell line responds differently to each subtype. We compared the antiproliferative effects of the subtypes on both an antiviral unit basis[18] and a weight basis.[13,14] An antivirus basis was used because most of the IFN-α preparations are administered on an antiviral unit basis in the clinical treatment of chronic hepatitis, HCC and renal cell carcinoma (RCC). Comparison between these two bases showed: (i) on an antiviral unit (IU) basis the most potent antiproliferative effect was presented by α5 in 11 cell lines, α8 in two cell lines, and on an average of the 13 cell lines, potency was in the order from α5, α8, α10, α2 to α1; and (ii) on a weight (ng) basis, the most potent antiproliferative effect was presented by α8 in nine cell lines, α5 in four cell lines, and on average, potency was in the order from α8, α5, α10, α2 to α1. In previous studies, IFN-α subtypes displayed significant differences in their antiproliferative activity, and the activity also varied greatly depending on the target cells. Some studies reported that α8 was the most potent inhibitor in the RCC cell line and in cells derived from chronic myelogenous leukemia.[14,18] Other studies reported that α10 was the most potent in RCC cell lines and Burkitt's lymphoma cell line on a weight basis.[12,13] In our current study, the most potent subtype on a weight basis was α8 in the majority of the cell lines, but in four cell lines α5 was the most potent. On average, of the 13 cell lines, α5 was next to α8, and this has not been reported on other cell types. Therefore, this was thought to be a characteristic feature

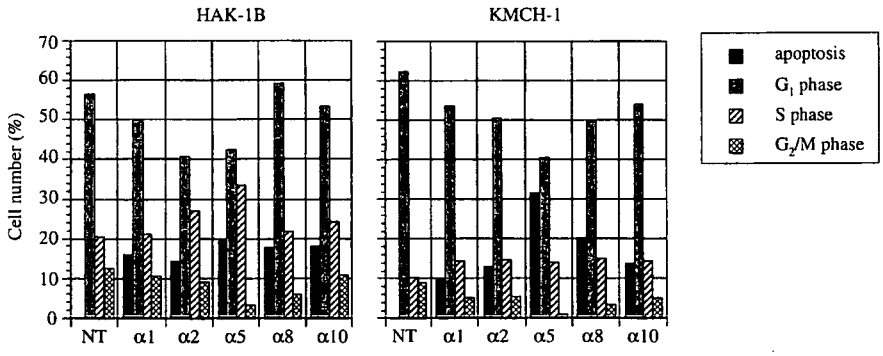

Figure 3 Analysis of apoptosis and cell cycle in two liver cancer cell lines cultured with or without (NT) 1000 IU/mL of one of the five interferon (IFN)-α subtypes for 96 h. HAK-1B and KMCH-1 cells were stained with propidium iodide (PI), and analyzed with an EPICS XL. The percentages of apoptotic cells and of cells in the G_1, S and G_2/M phases were analyzed using WINCYCLE software (Beckman Coulter, Fullerton, CA, USA). Every subtype induced apoptosis and S-phase arrest, and their induction levels were related to a certain degree to the antiproliferative effects.

of HCC. Castelruiz *et al.*[28] found that IFN-α5 was the sole IFN-α subtype expressed in normal liver tissue, and the volume of IFN-α decreased in the liver of patients with chronic hepatitis C infection. Our findings indicate that a decrease in α5 that possesses potent antiproliferative effects in the liver of chronic hepatitis C patients would assist carcinogenesis or the growth of cancer cells into the surrounding tissues. The administration of IFN-α5 to chronic hepatitis C patients or the normalization of its expression in the liver tissue after reducing chronic hepatic inflammation is important in preventing hepatocarcinogenesis.

Yamamoto *et al.*[15] compared the antiviral activity of the five IFN-α subtypes on a weight basis using eight of the 13 liver cancer cell lines (KIM-1, KYN-1, KYN-2, KYN-3, HAK-1 A, HAK-1B, HAK-2, HAK-3), and reported that α8 was the most potent subtype in seven cell lines (except HAK-3). Their findings regarding α8 agreed with our findings on antiproliferative effects; however, in our study α5 was the next most potent subtype, which differs from their results. This difference indicates that the antiproliferative effects and antiviral effects of IFN-α subtypes are induced by different mechanisms. Arora *et al.*[29] reported that these two effects are mediated through different signaling pathways. Other studies showed that there are type I IFN-specific[17] or IFN-α subtype-specific[16] intracellular signaling pathways that produce different biological responses. Further studies are necessary to explore the relationship between the signaling pathway of α5 and the activation of IFN-stimulated genes related to antiviral effects and antiproliferation effects.

We previously studied the expression of type I IFN receptors, particularly the IFNAR-2 subunit protein, and the response to the antiproliferative effects of BALL-1 IFN-α as well as the mechanism of growth suppression by IFN-α in 13 liver cancer cell lines.[6] In the present study, the relationship between the antiproliferative effects of the IFN-α subtype and the expression of the IFNAR-2 subunit on the cell surface was not significant, and the sensitivity of cells such as HAK-3, HAK-4 and KMCH-2 that have low cell surface IFNAR-2 expression (positive cell rates: 1.0%~2.3%)[6] to the antiproliferative effects of the subtypes tended to be low, whereas a relatively good response was obtained to every subtype in HAK-1B and KMCH-1 that have relatively high IFNAR-2 expression (positive cell rates: 20.8% and 13.2%, respectively).[6] With the other cell lines where the IFNAR-2 expression level is in the range between the above-mentioned two groups (positive cell rates: 3.0–9.0%),[6] no clear relationship was obtained. Therefore, only those cell lines that have either high or low IFNAR-2 expression could possess a certain relationship between the subtypes and the antiproliferative effects.

BALL-1 IFN-α consists of the α2 subtype (approximately 75%) and the α8 (25%) subtype. All cell lines, except KYN-2, KYN-3 and KMCH-2, indicated a relationship between their response to the antiproliferative effects of BALL-1 IFN-α and their sensitivity to IFN-α8. Different from recombinant IFN-α2, BALL-1 IFN-α-derived natural IFN-α2 receives certain glycosylation, but in our current results there were no specific effects of the presence or absence of α2 glycosylation on the proliferation of liver cancer cells, but the effects of α2 glycosylation were indicated to be dependent on the target cells. This finding agreed with that of Yanai *et al.*[14]

The mechanisms of the antiproliferative effects of IFN-α subtypes were examined in HAK-1B and KMCH-1 which showed a relatively good response to every subtype. The results were the same as those found with BALL-1 IFN-α, that is, induction of apoptosis and S-phase arrest,[6] and their induction levels were related to the antiproliferative effects to a certain degree.

In conclusion, each liver cancer cell line shows different levels of sensitivity to the antiproliferative effects of IFN-α subtypes, and every cell line is relatively or absolutely responsive to at least α5 and α8, although the reasons are not yet clear. It has been suggested that differences in the binding affinities of the IFN-α subtypes to the receptors produce a difference in biological potency.[13,30] This agrees with our recent findings[31] that consensus IFN (IFN-αCon1) that has higher affinity to the receptors[32] expressed more potent antiproliferative effects on an antiviral unit basis in most of the 13 liver cancer cell lines than did BALL-1 IFN-α. Further studies on the relationship among the antiproliferative effects, binding affinities to the receptors, and intracellular signaling pathway activation of IFN-α subtypes are necessary to clarify the difference in the antiproliferative effects among IFN-α subtypes.

Finally, our data indicate that IFN-α subtypes have different antiproliferative effects *in vitro*, and such *in vitro* differences among IFN-α subtypes should be appreciated in clinical application. For instance, the administration of IFN-α preparations containing high proportions of IFN-α5 or IFN-α8 would be more efficient in terms of the prevention and treatment of HCC.

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Clinical Studies

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

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

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

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

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

Materials and methods

Cell lines and cell culture

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

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

IFN and reagents

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

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

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

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

Morphological observation

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

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

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

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

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

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

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

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

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

Morphological examination of the subcutaneous tumors of nude mice

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

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

ELISA

The tumors were cut into pieces, and an appropriate amount was homogenized in 500 µl of ice-cold Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline containing 100 µg/ml phenymethylsulfonyl fluoride using a pellet pestle. The mixture was centrifuged for 10 min (12 000 g, 4 °C), and the supernatant was stored at −20 °C until use. The amount of the IFNAR-2 subunit in the supernatant was measured using ELISA kits (Otsuka Pharmaceutical Co. Ltd., Tokyo, Japan). The amount of tissue protein was determined using a BCA protein assay reagent (Pierce, Rockford, IL).

Statistics

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

Results

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

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

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

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

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

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

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

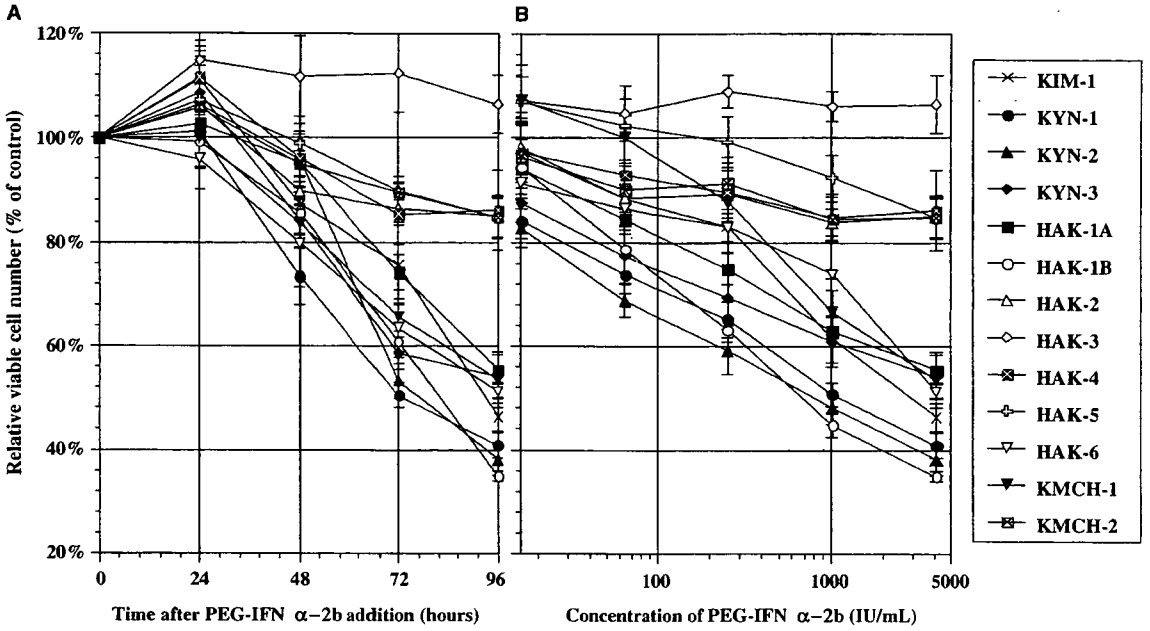

Fig. 1. Antiproliferative effect of pegylated IFN-α2b (PEG-IFN-α2b). (A) Chronological changes in relative viable cell number (% of the control) after adding 4096 IU/ml of PEG-IFN-α2b. Growth was suppressed with time in 10 cell lines. (B) 96 h after adding 16, 64, 256, 1024, or 4096 IU/ml of PEG-IFN-α2b.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

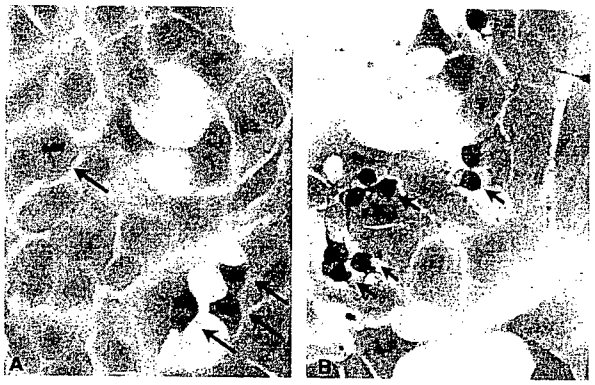

Fig. 2. Photomicrograph of HAK-1B cells cultured for 72 h on a Lab-Tek Chamber slide. (A) Without pegylated IFN-α2b (PEG-IFN-α2b) in culture medium. Some mitotic figures (long arrows) were noted. (B) With 4096 IU/ml of PEG-IFN-α2b in culture medium. Apoptotic cells (short arrows) characterized by cytoplasmic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

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

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

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

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

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

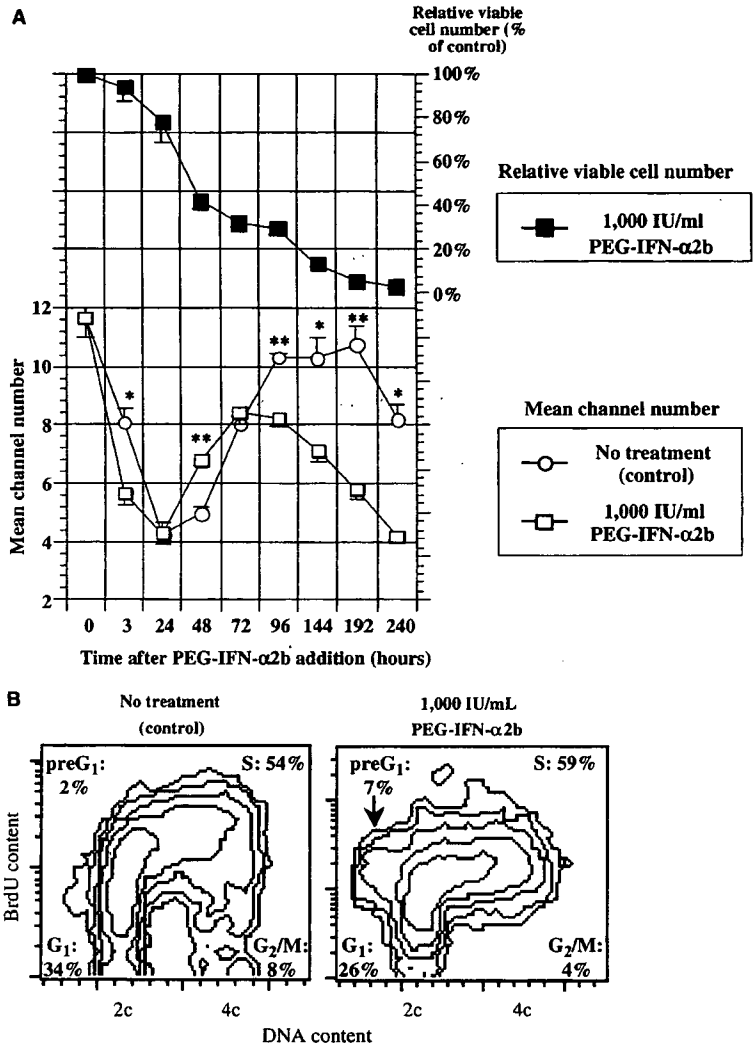

Fig. 3. Effects of 1000 IU/ml of pegylated IFN-α2b (PEG-IFN-α2b) on growth and IFN-α receptor-2 (IFNAR-2) expression in HAK-1B cells. (A) Time-course changes in relative viable cell number (% of Control) and IFNAR-2 expression before and after 1000 IU/ml of PEG-IFN-α2b addition. Cells reacted with anti-IFNAR-2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

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

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