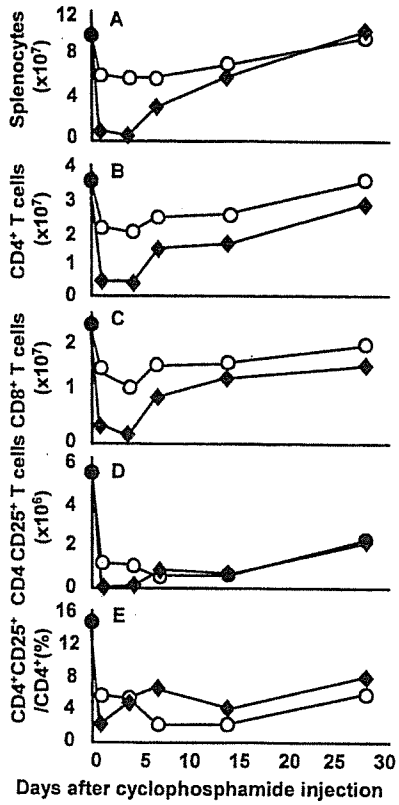

Figure 1. Alterations in T cell subsets following intraperitoneal injection of low- or high-dose cyclophosphamide. After 1,4,7,14 and 28 days after intraperitoneal injection of 20 or 200 mg/kg cyclophosphamide, numbers of splenocytes, $CD4^+$, $CD8^+$ and $CD4^+CD25^+$ T cells were determined as described in the Materials and methods. The data are means of two mice in each group. ○ and ♦, 20 and 200 mg/kg cyclophosphamide, respectively. The same experiments were repeated at least twice with similar results.

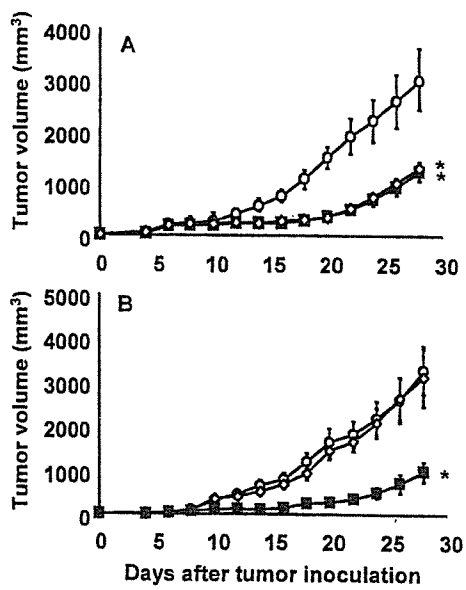

Figure 2. Anti-tumor effects of low- and high-dose cyclophosphamide in MH129 tumor-bearing C3H/HeN and nude mice. Mice (A, C3H/HeN; B, BALB/c nu/nu) were inoculated with $5x10^5$ MH129 cells on day 0. Groups of mice were also treated with 20 or 200 mg/kg cyclophosphamide on day 7, and injected with $4x10^6$ $CD4^+CD25^+$ T cells on day 8. Tumor sizes were monitored for 4 weeks. ○, control mice; ◇, mice treated with 20 mg/kg cyclophosphamide; ■, mice treated with 200 mg/kg cyclophosphamide; ▲, mice treated with 20 mg/kg cyclophosphamide and $4x10^6$ $CD4^+CD25^+$ T cells. The data are means ± SEM (n=8). *p<0.05, versus control mice (t-test). The same experiments were repeated at least twice with similar results.

depletion, *in vivo* growth of pre-existing murine hepatoma MH129 tumor. These findings appear to be highly crucial in a clinical setting of combined chemotherapy and immunotherapy for cancer treatment.

Materials and methods

Cell lines and mice used. MH129 cells, a mouse hepatoma cell line (13), were maintained in RPMI-1640 medium with 10% fetal calf serum and antibiotics. Six-week-old female C3H/HeN and BALB/c nu/nu mice were purchased from Charles River Japan (Tokyo, Japan) and kept in a specific pathogen-free facility. All experiments were conducted in accordance with the principles and procedures outlined in the Guideline for the Care and Use of Laboratory Animals of Nagasaki University.

Studies on the effect of cyclophosphamide on T cell subsets in the spleen. C3H/HeN mice were intraperitoneally injected with cyclophosphamide (20 or 200 mg/kg; Sigma, St. Louis, MI), and numbers of splenocytes, $CD4^+$, $CD8^+$ and $CD4^+CD25^+$ T cells were monitored for up to 4 weeks. The numbers of splenocytes were counted using a hemocytometer after lysis of red blood cells with ammonium chloride. The numbers of $CD4^+$, $CD8^+$ and $CD4^+CD25^+$ T cells were determined with FITC-conjugated anti-mouse CD4 (H129.19), PE-conjugated anti-CD8 (53-6.7) and PE-conjugated anti-CD25 (7D4) (PharMingen, San Diego, CA), respectively, on a FACScan flow cytometre using CellQuest software (BD Biosciences, Mountain View, CA).

Studies on the effect of low-dose cyclophosphamide on in vivo MH129 tumor growth. MH129 cells ($5x10^5$ cells/mouse) were subcutaneously injected into the flanks of mice. Tumor sizes were determined from caliper measurement using the standard formula (length x width^2/2). Groups of mice were treated by either intraperitoneal injection of cyclophosphamide (20 or 200 mg/kg) (Sigma) or 500 μg/mouse anti-CD25 antibody before or after tumor cell inoculation. A group of mice were also intraperitoneally injected with $4x10^6$ cells purified $CD4^+CD25^+$ T cells at the indicated time point. Anti-CD25 monoclonal antibody was purified from ascites of mice intraperitoneally injected with hybridoma PC61 using HiTrap™ protein G HP column (Amersham, Piscataway, NJ). $CD4^+CD25^+$ T cells (>90% pure) were isolated from splenocytes of naïve mice using a SpinSep Murine $CD4^+$ T-Cell kit (Veritas, Tokyo, Japan) and MACS CD25 MicroBead kit (Miltenyi Biotech, Bergisch Gladbach, Germany). Confirmation of $CD25^+$ cell depletion by PC61 was determined by staining splenocytes 4 days after antibody treatment with anti-CD25 antibody that recognize a different epitope of CD25 (7D4).

Histology. Tumor histology was examined on formalin-fixed tissue sections stained with hematoxylin and eosin (H&E).

Results

Low-, but not high-dose cyclophosphamide selectively depletes $CD4^+CD25^+$ T cells. We first examined the effects of low- and high-dose cyclophosphamide on the kinetics of T

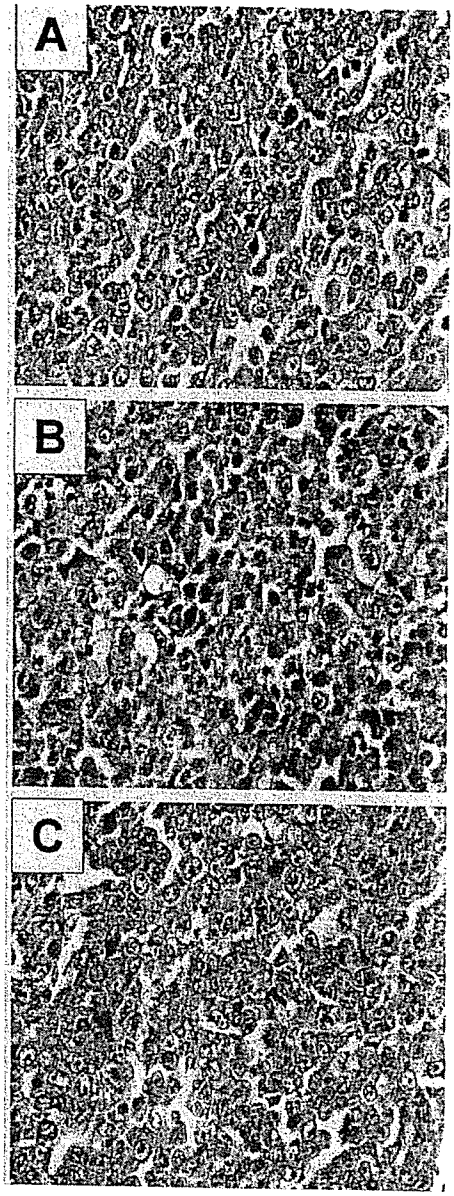

Figure 3. H&E staining of MH129 tumors from control mice (A) and mice treated with 20 or 200 mg/kg cyclophosphamide (B and C). Tumors were removed on day 22 from mice. Magnification of x400.

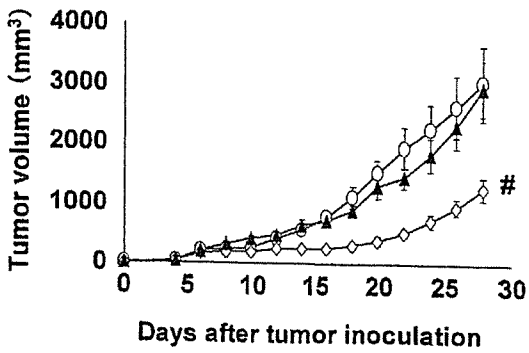

Figure 4. Anti-tumor effects of low-dose cyclophosphamide with/without repletion of CD4+CD25+ T cells in MH129 tumor-bearing C3H/HeN mice. Mice were inoculated with $5x10^5$ MH129 cells on day 0. Groups of mice were also treated with 20 mg/kg cyclophosphamide on day 7, and injected with $4x10^6$ CD4+CD25+ T cells on day 8. Tumor sizes were monitored for 4 weeks. ○, control mice; ◇, mice treated with 20 mg/kg cyclophosphamide; ▲, mice treated with 20 mg/kg cyclophosphamide and $4x10^6$ CD4+CD25+ T cells. The data are means ± SEM (n=8). *p<0.05, versus control mice (t-test). The same experiments were repeated at least twice with similar results.

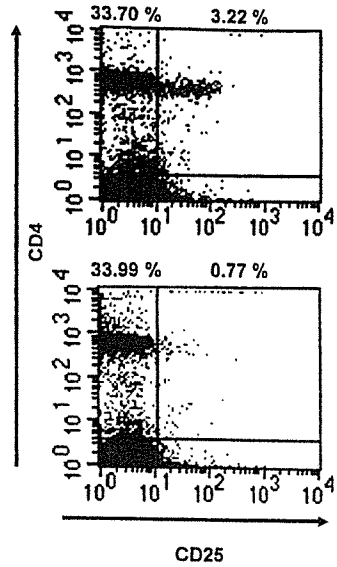

Figure 5. Flow cytometric analysis of CD4 and CD25 expression on splenocytes in mice untreated (A) or treated with 0.5 mg anti-CD25 antibody (B). Four days after antibody treatment, splenocytes were analyzed for CD4 and CD25 expressions by FACScan as described in Materials and methods.

lymphocyte subsets in the spleens of C3H/HeN mice. Flow cytometric analysis of splenocytes was performed 1, 4, 7, 14 and 28 days after intraperitoneally injecting 20 or 200 mg/kg cyclophosphamide (day 0). Fig. 1 shows that 20 mg/kg cyclophosphamide decreased the numbers of splenocytes, CD4+ and CD8+ T cells by ~50% from day 1. The decrease peaked on day 4 and continued for at least 2 weeks. However, a decline in CD4+CD25+ T cell number was more profound (~85% decrease) and recovered more slowly than CD4+ cells, thus leading to the remarkably lower ratios of CD4+CD25+ T cells to CD4+ T cells throughout the experimental period. In contrast, 200 mg/kg cyclophosphamide severely decreased the numbers of all the T cell subsets examined by >90% although the decreased ratios of CD4+CD25+ T cells to CD4+ T cells were still observed. These data clearly indicate the selective suppression of CD4+CD25+ regulatory T cells by low-dose, but not high-dose, cyclophosphamide.

Anti-tumor effect of low-dose cyclophosphamide is immune-mediated, while that of high-dose cyclophosphamide is attributed solely to direct cytotoxic effect. To see how critically the aforementioned phenomenon plays a role in tumor immunity, anti-tumor effects of low- and high-dose cyclophosphamide were compared in an *in vivo* tumor model with a mouse hepatoma cell line, MH129, and syngeneic immuno-competent C3H/HeN and immuno-incompetent nude mice. Surprisingly, both 20 and 200 mg/kg cyclophosphamide injected 7 days after tumor cell inoculation significantly suppressed the growth of MH129 tumors in C3H/HeN mice (Fig. 2A). However, the suppressive effect of low-dose cyclophosphamide was no longer observed in nude mice (Fig. 2B), indicating that the anti-tumor effect of low-dose cyclophosphamide observed in immuno-competent mice appears to be attributed to anti-tumor immunity. In contrast, high-dose cyclophosphamide was equally effective in both mice, indicating that the effect is solely due to direct cytotoxicity.

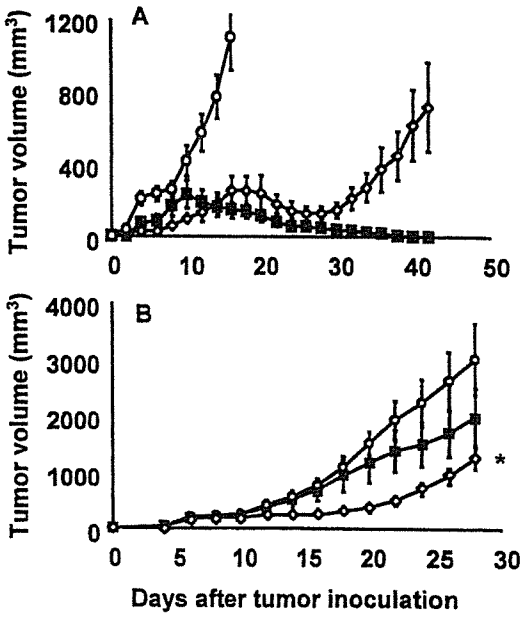

Figure 6. Comparison of anti-tumor effects of low-dose cyclophosphamide and anti-CD25 antibody administered before and after tumor cell inoculation in C3H/HeN mice. C3H/HeN mice were inoculated with $5x10^5$ MH129 cells on day 0. Groups of mice were also treated with 500 μg/mouse anti-CD25 antibody (A) or 20 mg/kg cyclophosphamide (B) on day -4 or +4. Tumor sizes were then monitored for 4 or 7 weeks. ○, control mice; ◇, mice treated with cyclophosphamide or anti-CD25 antibody after tumor cell inoculation; ■, mice treated with cyclophosphamide or anti-CD25 antibody before tumor cells inoculation. The data are means ± SEM (n=8). *$p<0.05$, versus control mice. The same experiments were repeated at least twice with similar results.

In histological examinations (Fig. 3), tumors from mice treated with low-dose cyclophosphamide showed higher intratumoral lymphocyte infiltrations as compared to those in control and high-dose cyclophosphamide-treated mice, data compatible with immune and non-immune mediated anti-tumor effects of low- and high-dose cyclophosphamide, respectively.

Anti-tumor effect of low-dose cyclophosphamide is mediated by depletion of CD4+CD25+ T cells. The next study was performed to evaluate whether anti-tumor immunity induced by low-dose cyclophosphamide resulted from the selective CD4+CD25+ T cell depletion. For this purpose, following MH129 inoculation on day 0 and low-dose cyclophosphamide injection on day 7, CD4+CD25+ T cells ($4x10^6$ cells/mouse) purified from naïve mice were injected on day 8. Fig. 4 shows that repletion of CD4+CD25+ T cells completely abolished the anti-tumor effect of low-dose cyclophosphamide. These data clearly implicate the anti-tumor effect of low-dose cyclophosphamide in its selective depletion of CD4+CD25+ T cells.

Timing of administration to obtain the optimal anti-tumor immunity is different between low-dose cyclophosphamide and anti-CD25 antibody. Because monoclonal anti-CD25 antibody (PC61) has been widely used to deplete CD4+CD25+ T cells in previous studies (14-16), the consequence of antibody-mediated CD4+CD25+ T cell depletion was compared to that of cyclophosphamide-mediated CD4+CD25+ T cell depletion in the MH129 tumor model. In our preliminary dose-escalating experiment, intraperitoneal injection of 0.5 mg anti-CD25 antibody (PC61) maximally depleted CD4+CD25+ T cells (Fig. 5). Fig. 6 shows that four day-prior injection of anti-CD25 antibody completely eradicated MH129 tumors but, 4 days later, the injection only transiently inhibited tumor growth, which is consistent with data previously reported with several cell lines including Meth A, MOPC-70A and RL Male1 cells (15,16). However, the results were opposite in the case of cyclophosphamide; injection of cyclophosphamide on day -4 of tumor cell inoculation was less effective than that on day +4.

Discussion

Although cyclophosphamide-induced suppression of CD4+CD25+ regulatory T cells has previously been demonstrated, attention has not been focused on the concentrations of cyclophosphamide used. Thus, the amounts used varied from 30 to 200 mg/kg in mice and rats (8-11). Furthermore, an immuno-enhancing effect has also been described with doses of cyclophosphamide ranging from 10 to 300 mg/kg (2). Therefore, we first compared the outcomes of high-dose (200 mg/kg) and low-dose (20 mg/kg) cyclophosphamide administration on T cell subsets in mice. Our data clearly demonstrate that, although both high- and low-dose cyclophosphamide markedly suppressed the number of CD4+CD25+ T cells and the ratios of CD4+CD25+ T cells to CD4+ T cells, their effects on the numbers of CD4+ T and CD8+ T cells were quite different. Thus, decreases in absolute numbers of CD4+ T and CD8+ T cells by high-dose cyclophosphamide were much more evident than those by low-dose cyclophosphamide (>90% versus ~50% decreases). These observations indicate that CD4+CD25+ regulatory T cells are selectively suppressed, but conventional, effector T cells appear to be spared by low-dose cyclophosphamide. In contrast, high-dose cyclophosphamide induces quantitatively substantial decreases in all T cell subsets, presumably eradicating both regulatory and effector T cells.

Regarding the mechanism(s) for this selective depletion of CD4+CD25+ T cells with cyclophosphamide, Lutsiak *et al* (10) showed the increased sensitivity of CD4+CD25+ T cells to apoptosis in 100 mg/kg cyclophosphamide-treated mice, leading to the impaired homeostatic proliferation of this T cell subpopulation. Ercolini *et al* (11) also demonstrated selective depletion of the cycling population of CD4+CD25+ T cells with 100 mg/kg cyclophosphamide. In addition, Ikezawa *et al* (9) and Lutsiak *et al* (10) found that cyclophosphamide suppressed not only number but also functional property of CD4+CD25+ T cells.

The long-lasting depletion of CD4+CD25+ T cells by low-dose cyclophosphamide enabled us to observe the effect of cyclophosphamide on tumor immunity. Strong growth inhibition of MH129 tumors by low-dose cyclophosphamide in the syngeneic immuno-competent but not immuno-incompetent nude mice, extensive intratumoral lymphocyte infiltration and disappearance of this anti-tumor effect by CD4+CD25+ T cell repletion collectively indicate that the anti-tumor effect of low-dose cyclophosphamide is mediated by its immuno-enhancing potential induced by CD4+CD25+ T cell depletion,

rather than its direct cytotoxic effect as an alkylating agent. In contrast, the similar anti-tumor effects of high-dose cyclophosphamide in mice and sparse intratumoral lymphocyte infiltration indicates that the anti-tumor effect of high-dose cyclophosphamide is solely comprised of the direct cytotoxic effect. Thus, we definitely demonstrate the difference in the effects of low- and high-doses of cyclophosphamide on anti-tumor immunity. Thus, low-dose cyclophosphamide enhances anti-tumor immunity by selectively depleting $CD4^+CD25^+$ regulatory T cells, while high-dose cyclophosphamide does not because of elimination of both effector and regulatory T cells. Our data suggest that 50% decreases in conventional, effector $CD4^+$ T and $CD8^+$ T cell numbers by low-dose cyclophosphamide are unlikely to affect overall immune reaction, but the >90% decreases seem to be critical.

Although the timing of cyclophosphamide injection to obtain the optimal result is controversial in other literatures (17,18), administration of cyclophosphamide after tumor inoculation is more efficacious than prior injection in this study. These data are similar to those in the recent report obtained with the agonistic anti-glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR) antibody (DTA-1) (19). In contrast, the anti-tumor effect of anti-CD25 antibody-mediated $CD4^+CD25^+$ T cell depletion is higher when antibody is given before tumor cell inoculation than after tumor cell injection in the present and previous studies (15,16). This is at least partly because CD25 is also expressed on activated T cells (14). Thus, anti-CD25 antibody can eliminate not only regulatory cells but also activated effector cells (both $CD4^+CD25^+$) when administered after tumor cell inoculation. On the contrary, low-dose cyclophosphamide does not seem to affect activated $CD4^+CD25^+$ effector T cells. Anti-GITR antibody stimulates the activated $CD4^+CD25^+$ effector T cells (20). In this regard, use of cyclophosphamide or agonistic anti-GITR antibody appears to be more practical in a clinical setting.

Since recent studies show an increase in $CD4^+CD25^+$ T cells in patients with various cancers (21-23), suppression of number and/or function of $CD4^+CD25^+$ T cells may be critical for successful anti-cancer immunotherapy. It may be worthy noting here that selective suppression of $CD4^+CD25^+$ T cells has also been recently described in methotrexate (8) and fludarabine (24).

It should be emphasized here that the low-dose cyclophosphamide we used in this study (20 mg/kg) approximately corresponds to a dose of cyclophosphamide commonly used in cancer chemotherapy in humans (1000 mg/m^2) (25). Furthermore, 200-300 mg/m^2 cyclophosphamide has widely been used as an immuno-potentiating agent in cancer immunotherapy (2). Therefore, it may be particularly important to scrutinize the effect of lower-dose cyclophosphamide (20 mg/kg or less) on immune function including number and function of $CD4^+CD25^+$ regulatory T cells in humans.

In conclusion, we here report that low-dose (20 mg/kg) cyclophosphamide selectively depletes $CD4^+CD25^+$ T cells, thereby enhancing anti-tumor immunity. These findings appear to be highly critical in terms of combined chemotherapy and immunotherapy for cancer treatment in humans. Further studies on elucidating the molecular mechanisms for cyclophosphamide-mediated suppression of regulatory T cells will help us better understand regulatory T cell physiology and develop novel strategies for cancer treatment.

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The up-regulation of type I interferon receptor gene plays a key role in hepatocellular carcinoma cells in the synergistic antiproliferative effect by 5-fluorouracil and interferon-α

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Abstract. Combination therapy with interferon (IFN)-α and 5-fluorouracil (5-FU) has been reported to show an improved therapeutic efficacy in patients with advanced hepatocellular carcinoma (HCC) but the mechanism behind this has not been completely elucidated. We examined the molecular events underlying the antiproliferative effects of IFN-α and 5-FU in combination using six human HCC cell lines. When the antiproliferative effects of administering IFN-α and 5-FU together were analyzed using isobolograms, we found that the cell lines could be divided into two groups: the S-group containing three cell lines, which showed synergistic effects, and the A-group, containing the remaining three cell lines, which showed additive effects. Real-time RT-PCR and Western blot analyses revealed that the expression levels of type I IFN receptor subunits, IFNAR1 and IFNAR2, were specifically up-regulated by 5-FU in all three cell lines of the S-group with the exception of IFNAR2 in one cell line, but not in those of the A-group. IFN-α modulated the protein expression levels of six enzymes regulating sensitivity to 5-FU, but none of them were down- or up-regulated in the same way in all members of the S- or A-group. In conclusion, the 5-FU-induced modulation of IFN receptor expression could play a pivotal role in the therapeutic efficacy of IFN-α combined with 5-FU. Measuring the expression levels of IFN receptors, and their ability to be up-regulated, may be a promising method for selecting HCC patients for this type of combination therapy.

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Abbreviations: IFN-α, interferon α; 5-FU, 5-fluorouracil; HCC, hepatocellular carcinoma; IFNAR1, type I IFN receptor subunit 1; IFNAR2, type I IFN receptor subunit 2; FdUMP, 5-fluoro-2'deoxyuridine-5'-monophosphate; TS, thymidylate synthase; TP, thymidine phosphorylase; DPD, dihydropyrimidine dehydrogenase; OPRT, orotate phosphoribosyl transferase; UP, uridine phosphorylase; TK, thymidine kinase; RT-PCR, reverse transcription-polymerase chain reaction

Key words: interferon-α, 5-fluorouracil, combination therapy, synergism, interferon receptor, hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC), the most common primary liver cancer, is one of the most frequent and aggressive malignant tumors, and its incidence is increasing. The surgical resection of hepatic lesions is the most effective treatment for patients with HCC, and local therapeutic approaches, such as transcatheter arterial embolization (1), percutaneous transhepatic ethanol injection (2), microwave coagulation (3), and radiofrequency ablation (4) have also been reported to be effective. However, these therapies are still not effective for patients with advanced HCC, who are often not suitable for surgery and whose 5-year survival rate is extremely low (5). One chemotherapeutic strategy is combined chemotherapy with 5-fluorouracil (5-FU) and interferon (IFN)-α. Monden and colleagues have reported a beneficial therapeutic effect in a patient with recurrent HCC and multiple lung and bone metastases (6-8). However, this combined treatment was accompanied by increased toxicity, as manifested by an elevated incidence of mucositis and neurological and hematological side effects (9). It is therefore important to understand

the exact mechanism of this combination therapy so that patients likely to respond can be selected and unnecessary side effects can be avoided.

5-FU has two major antitumor mechanisms: one involves its active metabolite 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP), inhibiting the activity of thymidylate synthase (TS) and consequently DNA synthesis; the other is related to the incorporation of 5-FU metabolite into RNA and DNA, thereby disrupting normal RNA processing and function. The sensitivity of cancer cells to 5-FU is often influenced by the enzymes affecting 5-FU metabolism, including dihydropyrimidine dehydrogenase (DPD), orotate phosphoribosyl transferase (OPRT), thymidine phosphorylase (TP), uridine phosphorylase (UP), and thymidine kinase (TK). In contrast, IFNs are divided into type I and type II. The human type I IFN family is composed of at least 14 structurally related IFN-α subtypes and single IFN-ß and IFN-ω subtypes. Type I IFNs have various biological activities, including antiviral, anti-proliferative, immunomodulatory (10-12), and anti-angiogenic effects (13,14), mediated by the type I IFN receptor. This receptor is composed of two functional transmembrane subunits, type I IFN receptor subunit 1 (IFNAR1) and subunit 2 (IFNAR2), cooperating to form a high-affinity receptor for all type I IFNs (15,16). IFNAR2 is the major binding subunit and IFNAR1 is necessary for the tight binding. Of these receptor molecules, the expression levels of the type I IFN receptor were closely correlated with the response rates to IFN treatment in patients with chronic hepatitis C (17), and overexpression of the IFNAR2 markedly increased the anti-proliferative activity of IFNs and their capacity to induce apoptosis (18), suggesting that the type I IFN receptor is a key molecule for the antitumor activity of IFN-α.

Concerning possible mechanisms behind IFN-α and 5-FU showing improved therapeutic efficacy, increased FdUMP concentrations, decreased protein level of TS, an increase in TS inhibition rate and TP activity, and an alteration in 5-FU pharmacokinetics by combined IFN-α have been reported (19-24). Eguchi *et al* have reported that augmentation of the antitumor effect of 5-FU by IFN-α might in part be attributable to the up-regulation of $p27^{Kip1}$ blocking cell cycle progression (25). However, none of these theories provide a consistent mechanism for the exact rationale of this combination therapy. Furthermore, almost all studies have assumed that IFN-α plays a role in modulating the antitumor activity of 5-FU.

In this study, we provide evidence that the modulation of IFN receptor expression by 5-FU is specifically associated with the improved efficacy rather than the cellular modulation of the enzymes that regulate the sensitivity to 5-FU by IFN-α.

Materials and methods

Drugs. Natural human IFN-α was purchased from Otsuka Pharmaceutical Co., Ltd. (OIF, Tokyo, Japan) and 5-FU was purchased from Kyowa Hakko Kogyo Co., Ltd. (5-FU Injection 250 Kyowa, Tokyo, Japan).

Cell lines. HCC cell lines, KIM-1, KYN-1, KYN-2, KYN-3, HAK-1A and HAK-1B (26-30), were grown in Dulbecco's modified Eagle's medium (Nissui Seiyaku Co., Tokyo, Japan) with 10% fetal bovine serum (FBS) (FETALCLONE III, Hyclone, UT, USA) in a humidified atmosphere of 5% CO_2 at 37°C.

Antiproliferation test. Cells were seeded into 96-well plates at 1,000 cells/0.1 ml/well and incubated overnight. On the following day, 100 μl aliquot containing IFN-α and 5-FU was added and cultured for a further 5 days. In study of schedule-dependent synergy, HCC cells were seeded into 12-well plates at 5,000 cells/1 ml/well. On the following day, 1-ml aliquot containing drugs was added. After a further 3 days, culture medium was exchanged for fresh medium including another drug and cultured for a further 3 days. The number of viable cells was estimated by activity of cellular dehyrogenases using WST-8 reagent (Cell Counting Kit-8, DOJINDO, Kumamoto, Japan) (31).

Isobologram analysis. To analyze the mode of interaction between 5-FU and IFN-α, the combined doses that reduced cell growth by 50% were plotted as isobolograms, according to the method of Steel and Peckham (32). The envelope of additivity surrounded by mode I (heteroaddition), IIa and IIb (isoaddition) curves, was constructed based on the dose-response curves of IFN-α and 5-FU alone. Thus, when the data points for the combined drugs fell within this envelope, the combined effect was judged to be additive. When the points fell in the area under the envelope of additivity, the combined effect was judged as synergistic, because in this case, 50% inhibition was produced by a lower concentration than predicted on an additive basis.

cDNA preparation and quantitative real-time RT-PCR. Total RNA was extracted using Isogen (Nippon Gene Co., Ltd., Tokyo, Japan) and reverse transcribed using a reverse transcription system (Promega Corp., Madison, WI) according to the manufacturer's instructions. RT-PCR was performed with an ABI PRISM 7300 (PE Applied Biosystems, Foster City, CA). The sequences of the primers and probes are shown in Table I, and those for IFNAR1, IFNAR2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Applied Biosystems.

Western blotting. HCC cells were cultured with various concentrations of 5-FU or IFN-α. Total protein was extracted using protein extraction reagent (M-PER™, Pierce, Rockford, IL) supplemented with protease inhibitors (Halt™ protease inhibitor cocktail kit, Pierce). Cell lysates were subjected to SDS-PAGE and transferred to Immobilon-P transfer membranes (Millipore, Bedford, MA). After blocking, membranes were probed with anti-TS, -OPRT and -TP monoclonal and anti-DPD polyclonal antibodies (a gift from TAIHO Pharmaceutical Co., Ltd., Tokyo, Japan), monoclonal antibody against TK (abcam, Cambridge, UK), polyclonal antibodies against IFNAR1, IFNAR2 and UP (Santa Cruz Biotechnology, Santa Cruz, CA). The proteins were visualized using HRP-conjugated antibodies followed by enhanced chemiluminescence (Pierce). The intensity of luminescence was quantified using an image analysis system (LAS-1000, Fuji Film, Tokyo, Japan).

Silencing IFNAR1 and IFNAR2 genes. Stealth™ RNAis (Invitrogen™ Life Technologies, San Diego, CA) were used

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.

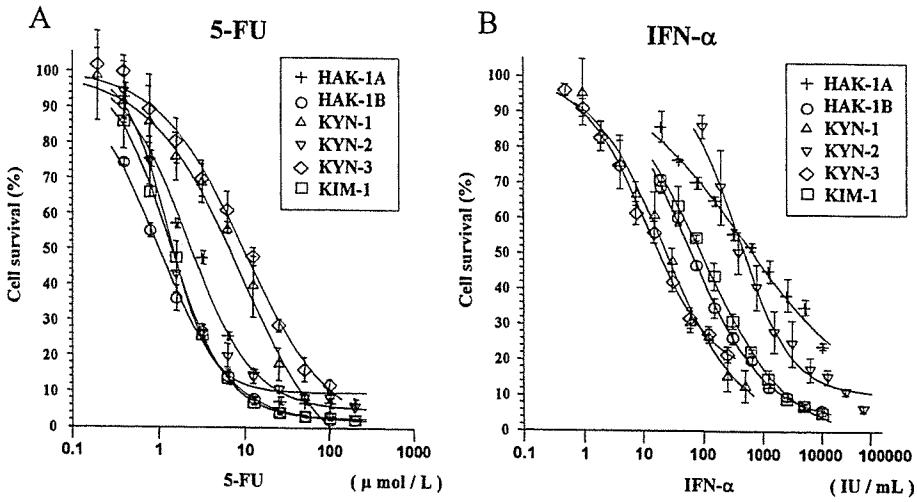

Figure 1. Antiproliferative effects of IFN-α and 5-FU in six HCC cell lines, HAK-1A, HAK-1B, KYN-1, KYN-2, KYN-3, and KIM-1. Cells were exposed to drugs for 5 days. Curves show the fitted dose-response curves for 5-FU (A) and IFN-α (B). Assays were carried out in quadruplicate. Data points represent 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.

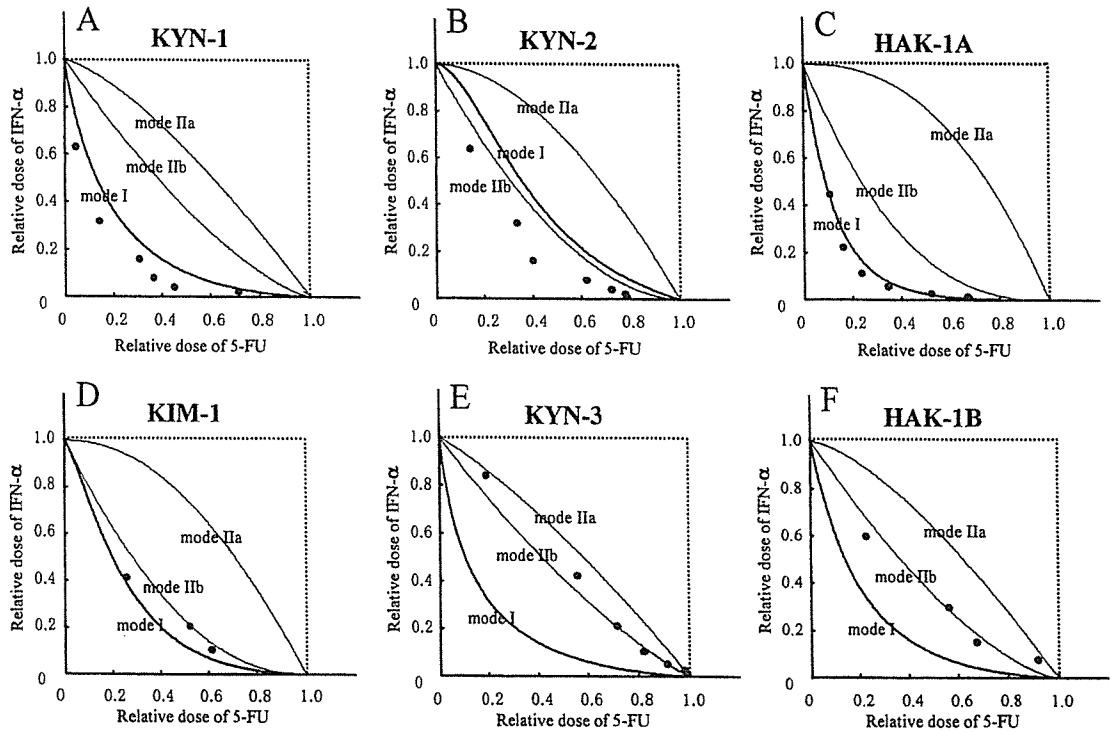

Figure 2. Combination effects of IFN-α and 5-FU. Isobologram analysis of IFN-α and 5-FU against six HCC cell lines. Cells were exposed to IFN-α and 5-FU for 5 days simultaneously. The area surrounded by the outer 2 curves of Mode I, IIa and IIb curves is the envelope of additivity. Relative doses of 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

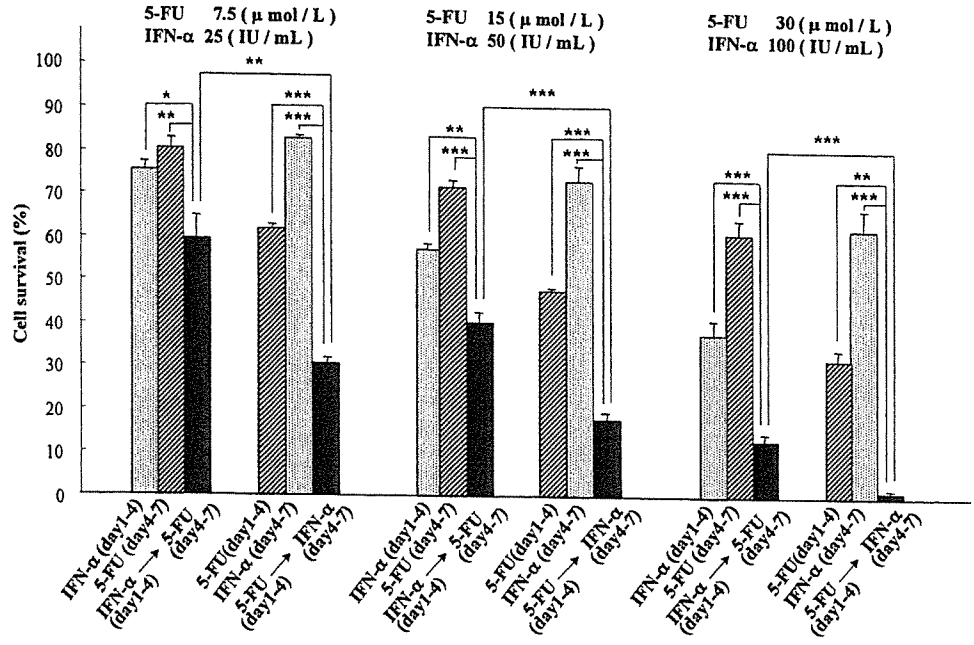

Figure 3. Schedule dependency of synergism of IFN-α and 5-FU. KYN-1 cells were seeded on day 0 and exposed to IFN-α or 5-FU for 3 days (day 1-4). On day 4, culture medium was removed and washed with PBS three times and fresh medium containing another drug was added. Cells were cultured for a further 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

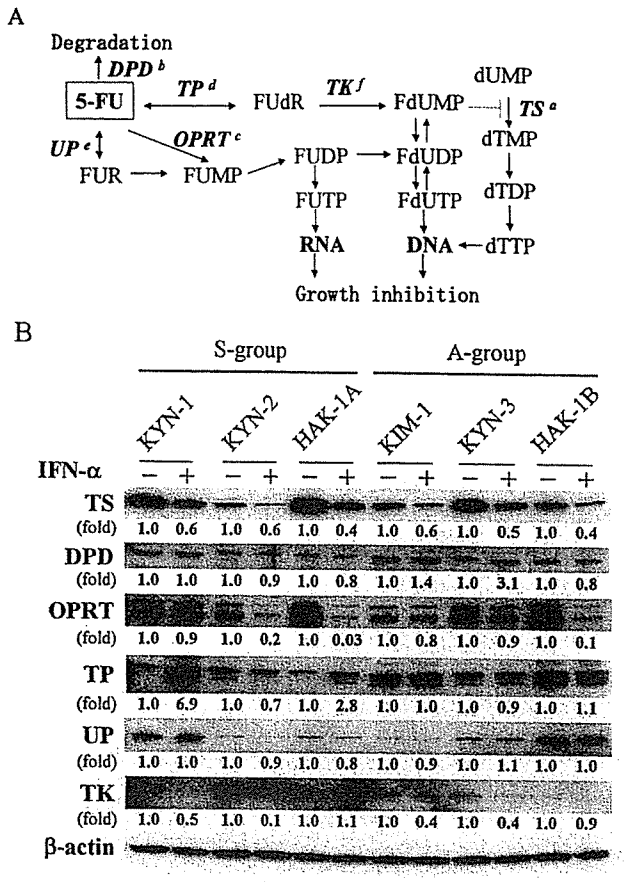

Figure 4. Effect of IFN-α on factors regulating sensitivity to 5-FU. (A) Metabolism of 5-FU. [a]TS, thymidylate synthase; [b]DPD, dihydropyrimidine dehydrogenase; [c]OPRT, orotate phosphoribosyl transferase; [d]TP,thymidine phosphorylase; [e]UP, uridine phosphorylase; [f]TK,thymidine kinase; FUdR, 5-fluoro-2'-deoxyuridine; 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

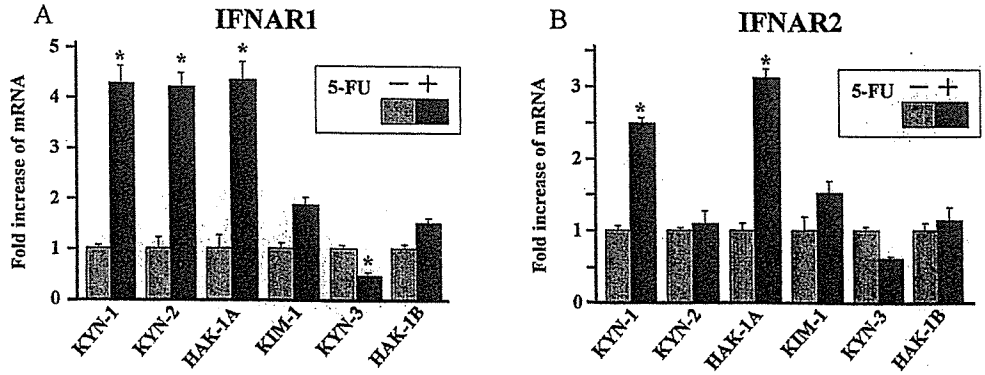

Figure 5. Expression of IFNAR1 and IFNAR2 mRNA, in HCC cells treated with 5.0 μmol/l 5-FU for 3 h. The increases in mRNA are shown relative to the initial level, taken as 1.0. Dotted and black columns show the mean mRNA levels in drug-untreated and 5-FU-treated cells, respectively. (A) IFNAR1, (B) IFNAR2. 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.

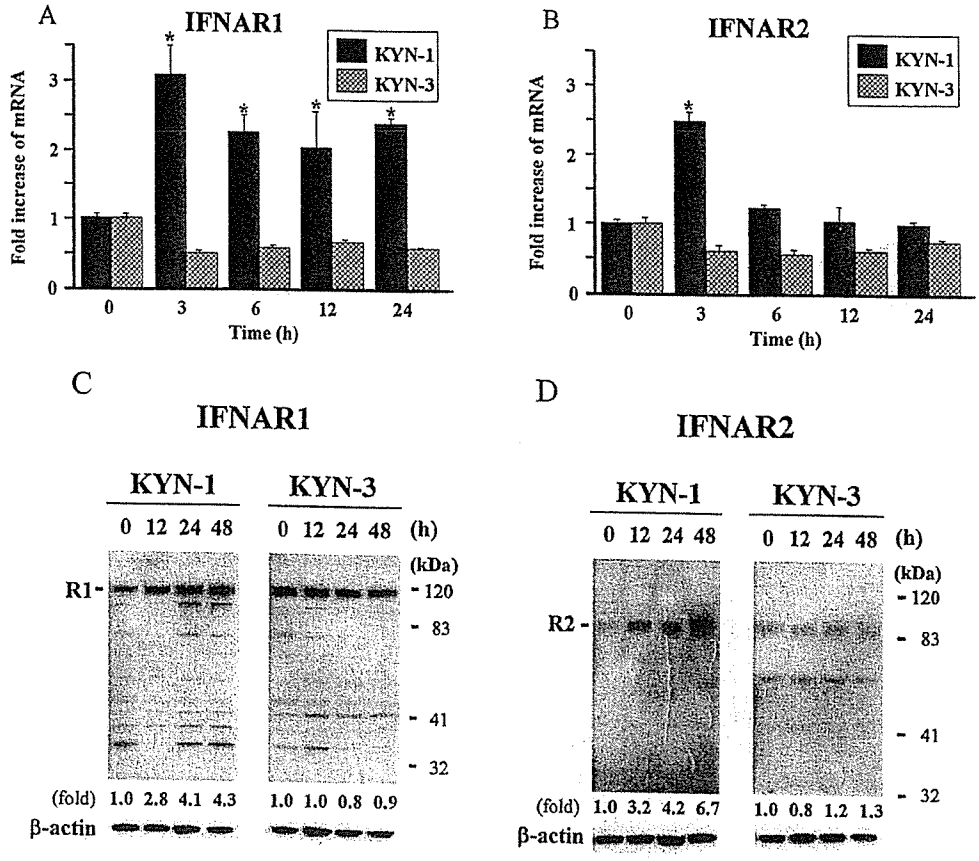

Figure 6. Time course of IFNAR1 and IFNAR2 mRNA and protein expression in KYN-1 and KYN-3 cells treated with 5 μmol/l of 5-FU. Increases in mRNA expression levels for IFNAR1 (A) and IFNAR2 (B) are shown relative to the initial level, taken as 1.0, in KYN-1 and KYN-3 cells. Black columns represent the 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.

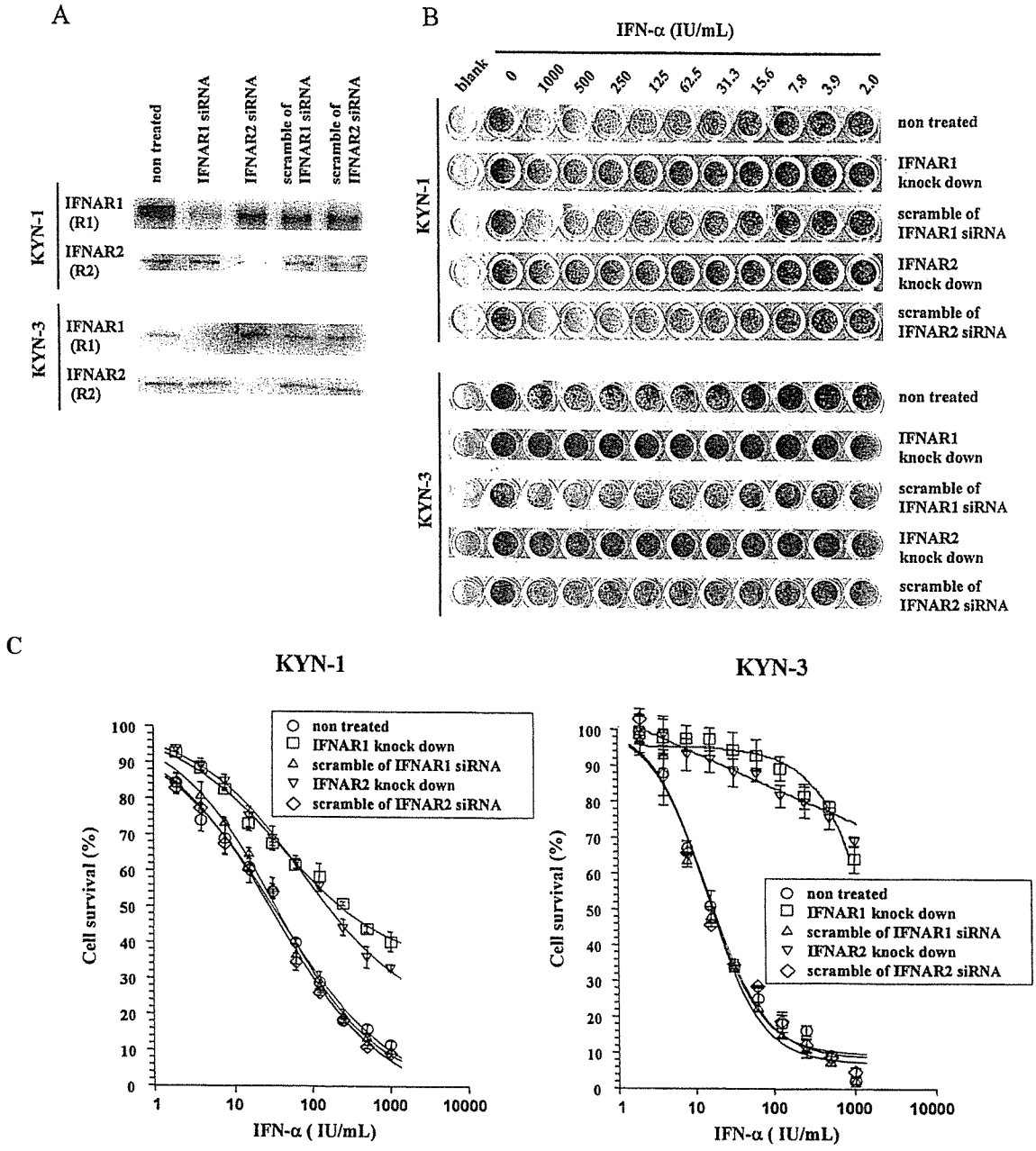

Figure 7. Dependency of antiproliferative activity of IFN-α on IFNAR1 and IFNAR2 expression. (A) Silencing of the IFNARs genes by siRNAs was confirmed by Western blotting. (B) KYN-1 and KYN-3 cells were treated with siRNAs and exposed to IFN-α for 5 days. Cells were stained by crystal violet. Assays 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.

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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

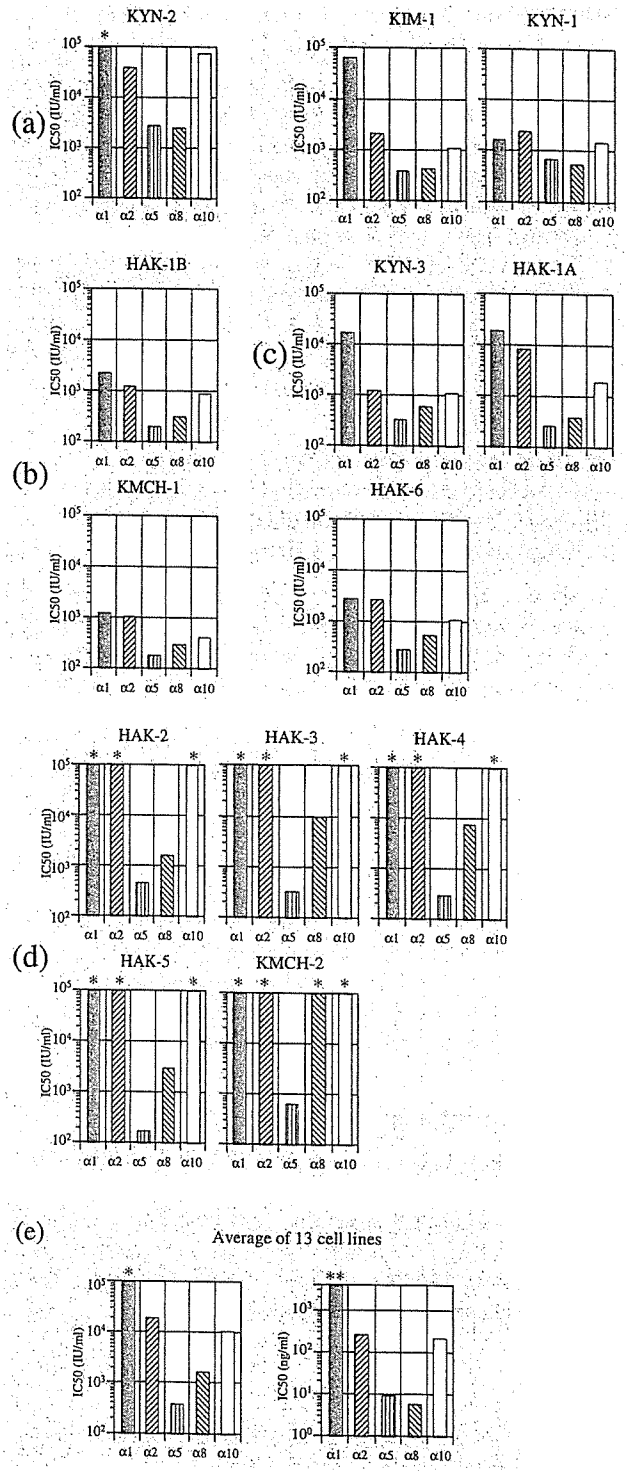

Figure 1 Antiproliferative effects of five interferon (IFN)-α subtypes in 13 liver cancer cell lines. Fifty percent inhibitory concentration (IC50) values (shown as the bars) were obtained from cells cultured with various concentrations of the five IFN-α subtypes for 96 h. (a) KYN-2 showed a weak response 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).

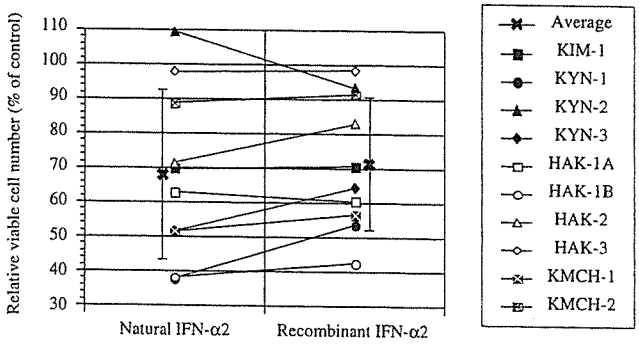

Figure 2 Antiproliferative effects of natural and recombinant interferon (IFN)-α2 on 10 liver cancer cell lines. Relative viable cell numbers (% of control) were obtained from cells cultured with 1024 IU/mL of natural or recombinant IFN-α2 for 96 h. There were no significant differences between natural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

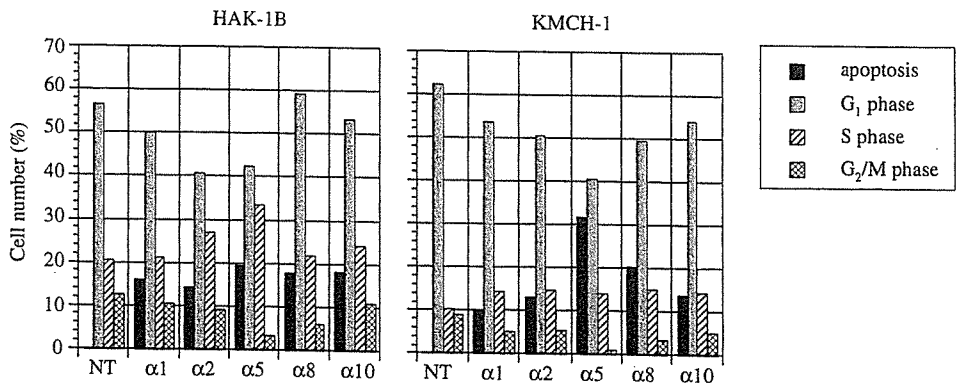

Figure 3 Analysis of apoptosis and cell cycle in two liver cancer cell lines cultured with or without (NT) 1000 IU/mL of one of the five interferon (IFN)-α subtypes for 96 h. HAK-1B and KMCH-1 cells were stained with propidium iodide (PI), and analyzed with an EPICS XL. The percentages of apoptotic cells 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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