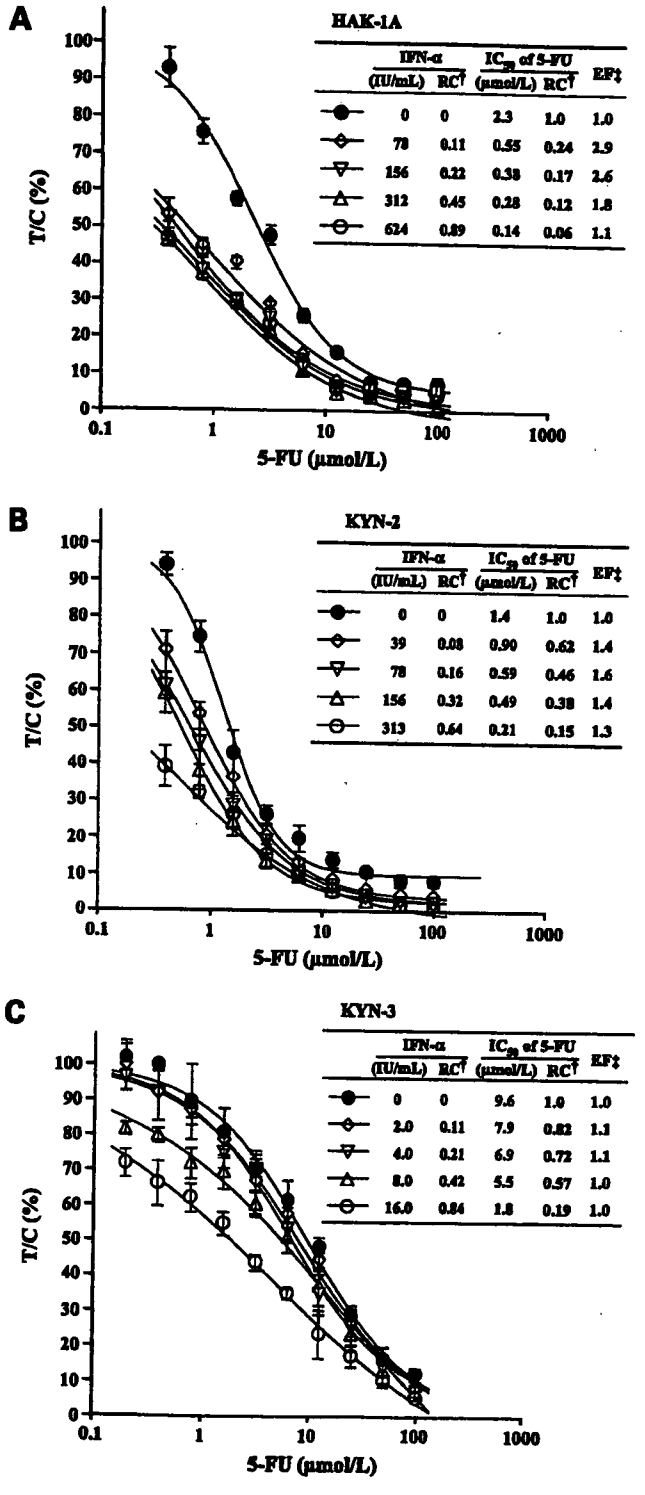

Figure 1. Antiproliferative effects of 5-FU and IFN-α on HAK-1A (A), KYN-2 (B), and KYN-3 (C) cells. Assays were carried out independently in quadruplicate. *Points*, mean; *bars*, SD. †, RC = (concentration) / (IC_{50} value). In this experiment, IC_{50} value of IFN-α against HAK-1A, KYN-2, and KYN-3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

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

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

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

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

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

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

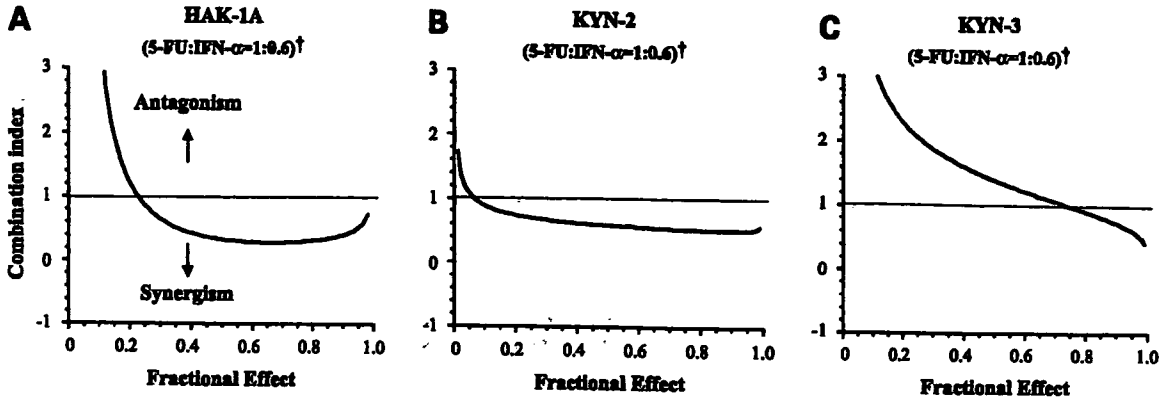

Figure 2. Quantitative analysis of synergy between 5-FU and IFN-α against HAK-1A (A), KYN-2 (B), and KYN-3 (C) cells using the CI method. CI > 1, antagonism; CI = 1, additive; CI < 1, synergistic. †, ratio of RC = (concentration of 5-FU) / (IC_{50} of 5-FU):(concentration of IFN-α) / (IC_{50} of IFN-α).

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

Discussion

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

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

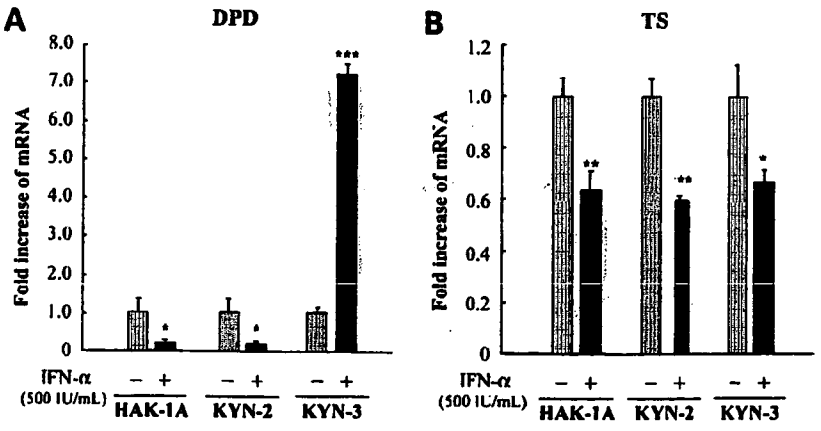

Figure 3. Effects of 500 IU/mL IFN-α on DPD (A) and TS (B) mRNA and protein expression levels in HAK-1A, KYN-2, and KYN-3 cells. Expression levels were measured by quantitative real-time reverse transcription-PCR and normalized to glyceraldehyde-3-phosphate dehydrogenase. The fold increases are shown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

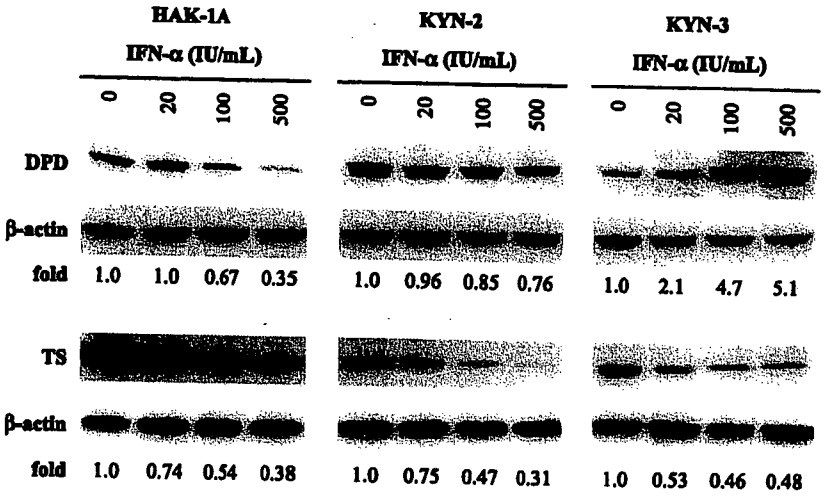

Figure 4. Protein expression levels of DPD and TS in HAK-1A, KYN-2, and KYN-3 cells treated with IFN-α. Protein expressions in HCC cells treated with IFN-α for 48 h were measured by Western blotting. Protein extracts of 10 μg/lane were loaded for KYN-3 cells, and 50 μg/lane were loaded for both HAK-1A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

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

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

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

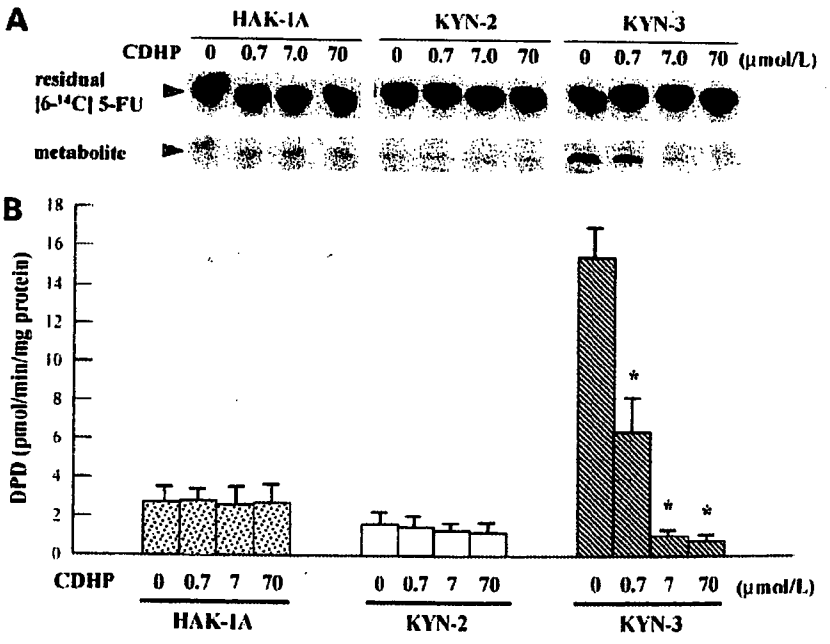

Figure 5. Inhibitory effect of CDHP on DPD activity. DPD activity was measured using [6-^{14}C]5-FU as a substrate. Residual [6-^{14}C]5-FU and metabolites were separated by TLC and visualized with an imaging analyzer (**A**). Each sample was developed on separated and independent TLC plate. Assays were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

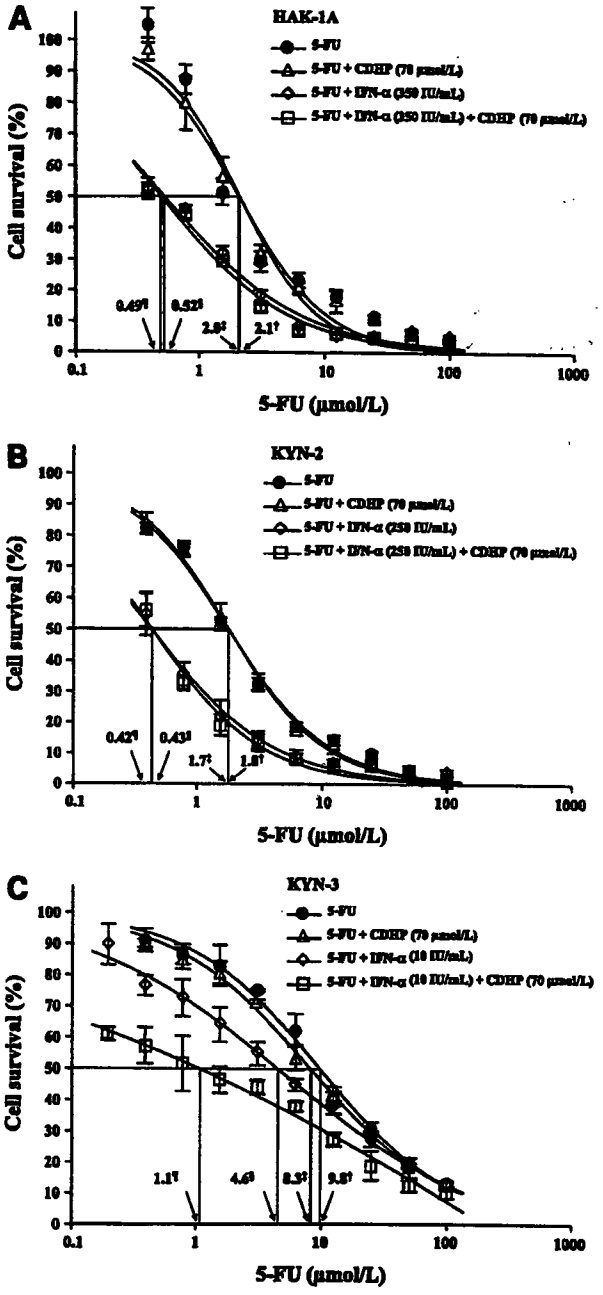

Figure 6. Effect of CDHP on combined antiproliferative effect of IFN-α and 5-FU in HAK-1A (A), KYN-2 (B), and KYN-3 (C) cells. Assays were carried out in quadruplicate. Experiments were repeated twice with similar results. IC_{50} values of 5-FU against HCC cells treated with 5-FU alone (†), 5-FU/CDHP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

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

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

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

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

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

Acknowledgments

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

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ABSTRACT

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

INTRODUCTION

INTERFERON-α (IFN-α) IS A MULTIFUNCTIONAL cytokine that possesses antiviral activity, antiproliferative activity, various immunoregulatory activities, antitelomerase activity, and antiangiogenesis activity.[1–3] The antiviral activity of IFN-α has attracted much attention, and IFN-α preparations have been used in the treatment of hepatitis B virus (HBV)-related and hepatitis C virus (HCV)-related chronic hepatitis in many countries.[4] IFN-α has been shown to possess highly suppressive effects on hepatocellular carcinogenesis in patients with virus-related chronic hepatitis,[5–7] but the mechanisms of action have not yet been clarified.

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

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

MATERIALS AND METHODS

Cell lines and cell culture

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

The cells were grown in Dulbecco's modified Eagle medium (DMEM) (Nissui Seiyaku, Co., Ltd., Tokyo, Japan) supplemented with 2.5% heat-inactivated (56°C, 30 min) fetal bovine serum (FBS) (Bioserum, Victoria, Australia), 100 U/mL penicillin, 100 μg/mL streptomycin (GIBCO-BRL/Life Technologies, Inc., Gaithersburg, MD), and 12 mmol/L sodium bicarbonate in a humidified atmosphere of 5% CO_2 in air at 37°C. HCC cells (2.0–5.0 $\times$ 10^5 cells per dish) were seeded on 100-mm tissue culture dishes (Asahi Techno Glass Corporation, Tokyo, Japan) and cultured with medium alone for 24 h to allow the cells to enter their logarithmic growth phase. Culture medium was not changed if not indicated.

IFN and reagents

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

Effects of IFN-α on AFP expression in HCC cells

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

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

Cell and culture medium preparation for AFP protein measurement

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

cDNA preparation and real-time quantitative RT-PCR

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

Cell cycle analysis

Cell lines were cultured with or without PEG-IFN-α2b (250 or 1000 IU/mL) for 24, 48, or 72 h, and cell cycle analysis was performed with a technique described elsewhere.[19] Briefly, cells were labeled with 10 mM BrdU (Sigma Chemical Co., St. Louis, MO) for 30 min, harvested, fixed in 70% cold ethanol at 4°C overnight, stained with anti-BrdU and propidium iodide (Sigma Chemical Co.), and then analyzed using a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA). The percentage of cells in the G_1, S or G_2/M phase was calcu-

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

RESULTS

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

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

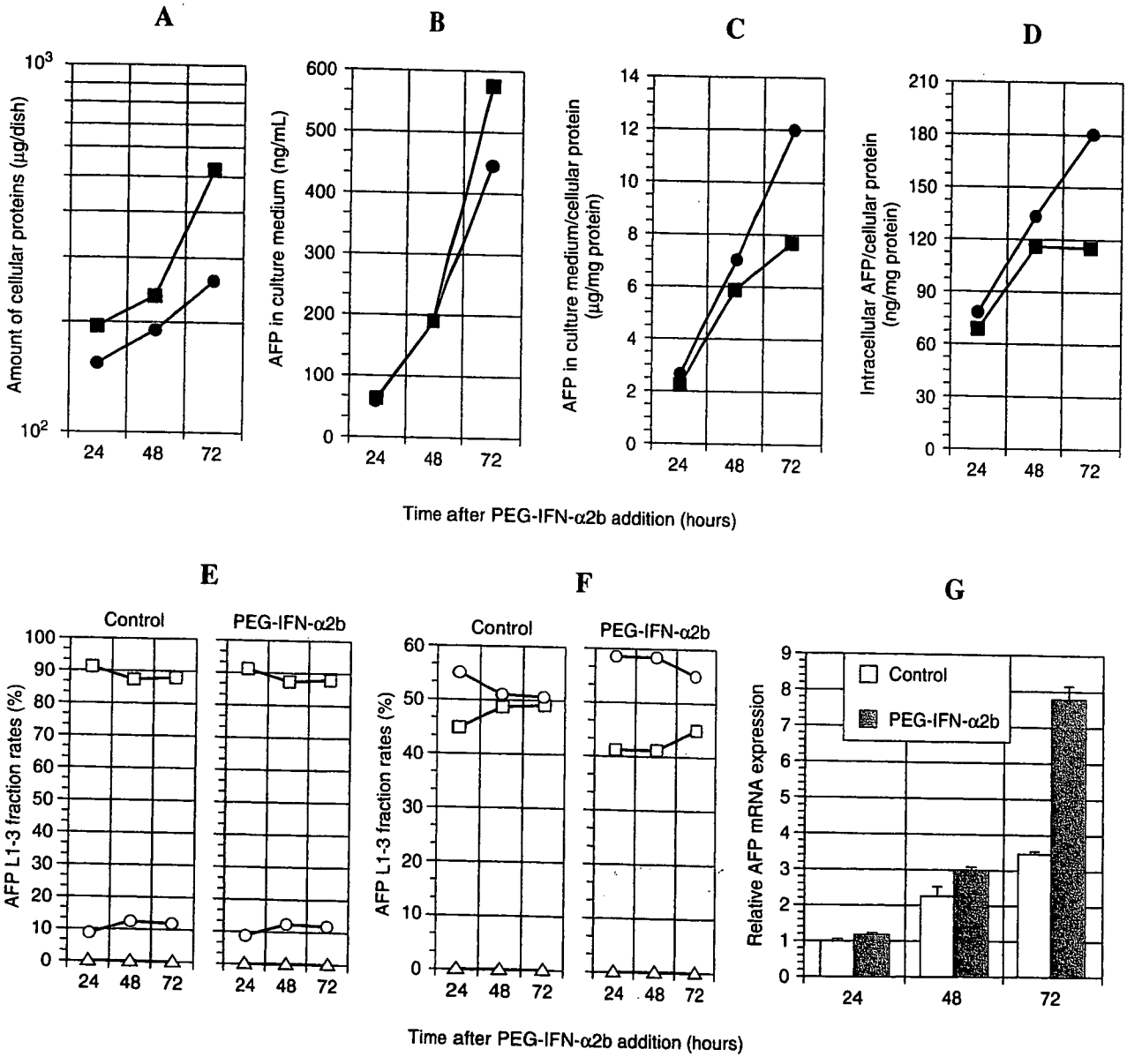

FIG. 1. Effects of PEG-IFN-α2b on the growth, expressions of AFP protein and mRNA, and AFP-L1, AFP-L2, and AFP-L3 rates in KIM-1 cells cultured for 24, 48, or 72 h. (**A**) Growth curve, (**B**) concentration of AFP in the culture supernatant, (**C**) amount of AFP in the culture supernatant per unit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

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

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

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

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

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

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

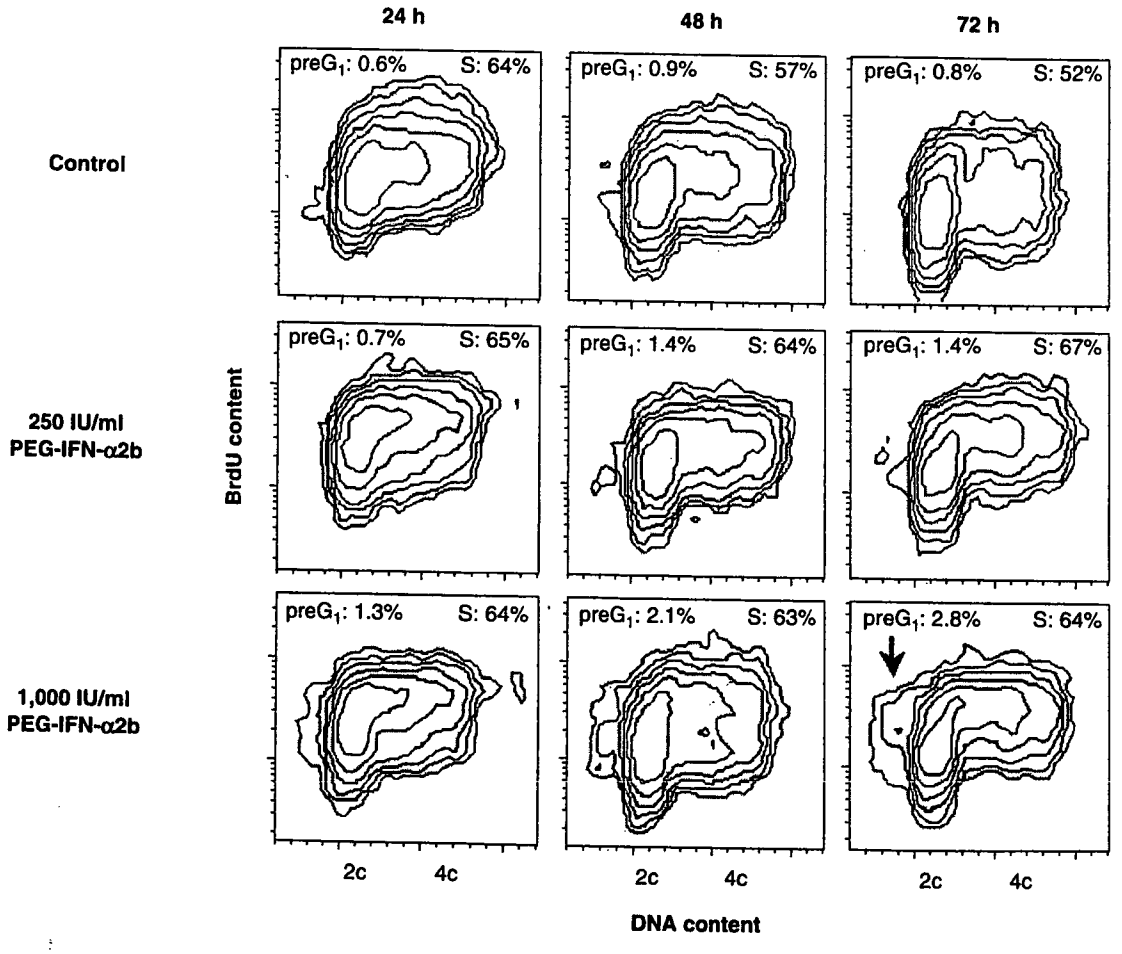

FIG. 2. Cell cycle analysis. KIM-1 cells were cultured with 250 or 1000 IU/mL PEG-IFN-α2b or medium alone (Control) for 24, 48, or 72 h. The cells were labeled with 10 mM BrdU for 30 min, fixed, then stained with anti-BrdU and propidium iodide and analyzed using a FACScan. The contour plots are shown.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

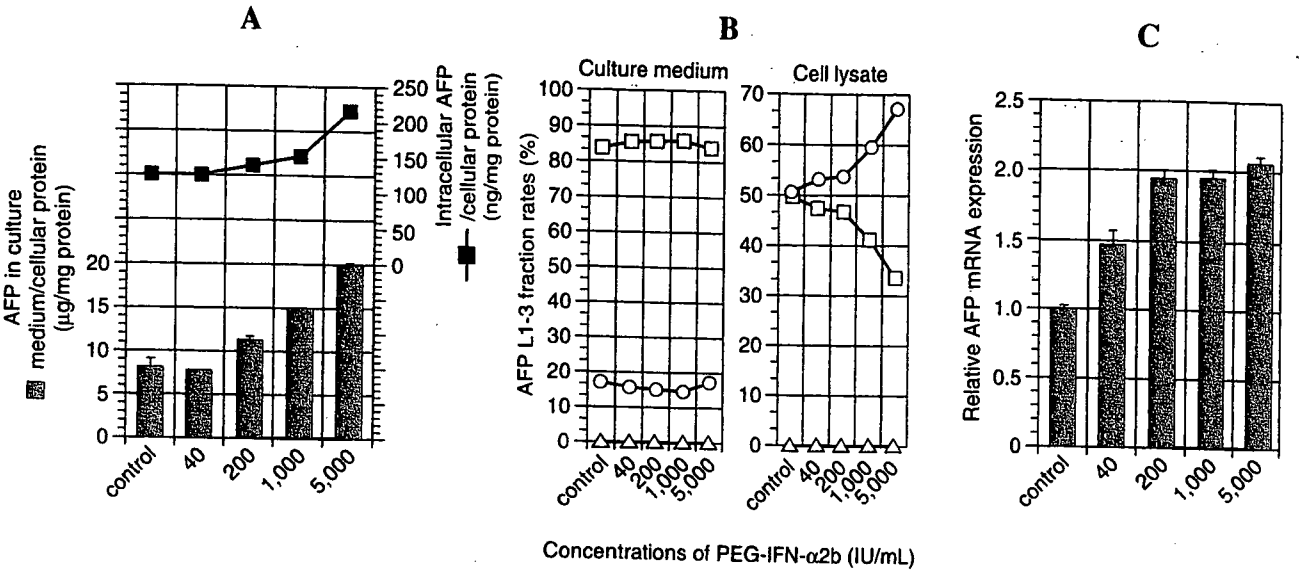

FIG. 3. Dose-dependent effects of PEG-IFN-α2b on the expressions of AFP protein and mRNA and on AFP-L1, AFP-L2, and AFP-L3 rates in KIM-1 cells cultured for 72 h. (**A**) Amount of AFP in the culture supernatant per unit volume of cellular protein (bottom, $n = 2$, mean ± SD) and amount of AFP in cell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

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

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

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

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

DISCUSSION

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

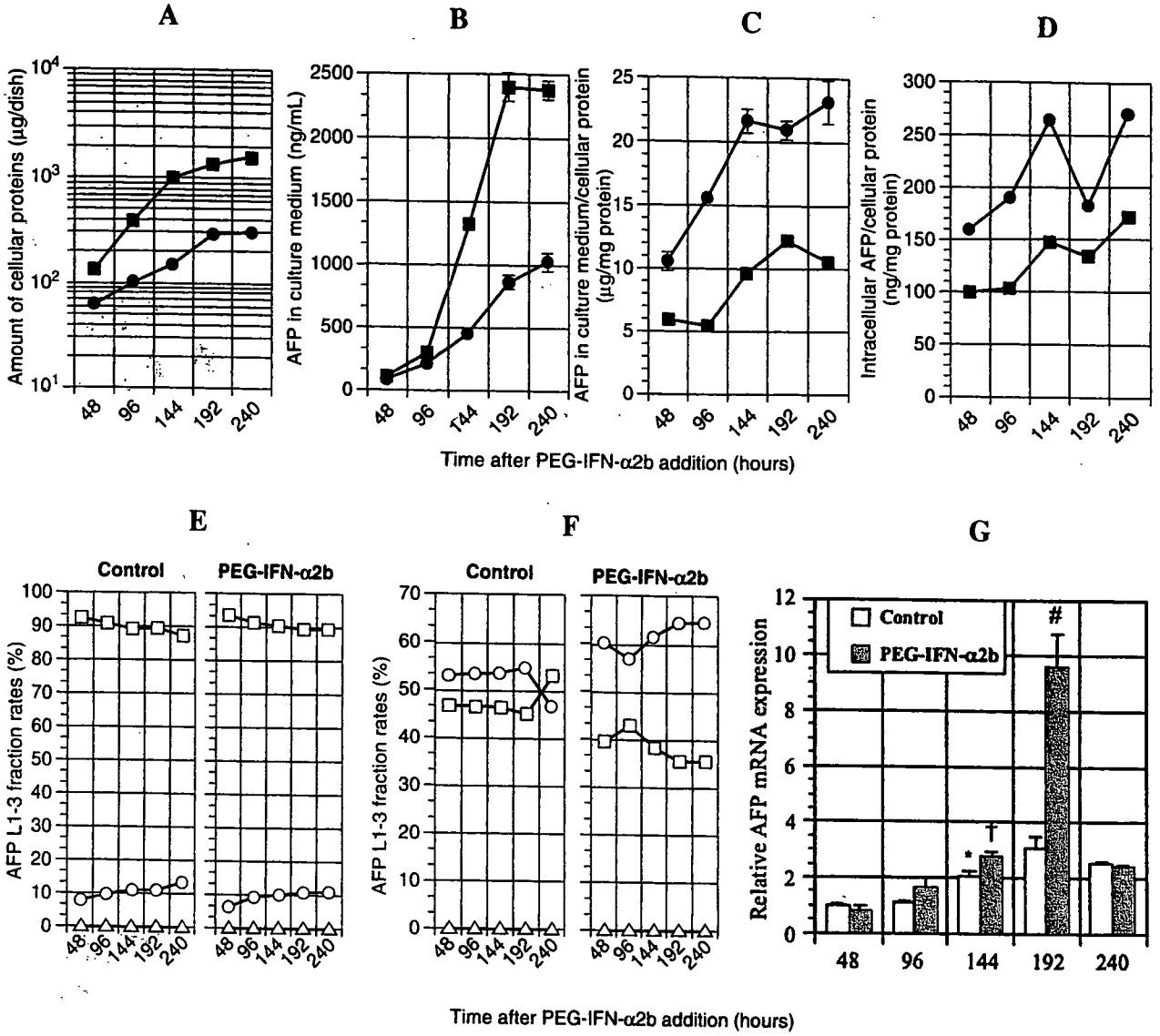

FIG. 4. Effects of PEG-IFN-α2b on the growth, expression of AFP protein and mRNA, and AFP-L1, AFP-L2, and AFP-L3 rates in KIM-1 cells cultured for 48, 96, 144, 192, or 240 h. (**A**) Growth curve, (**B**) concentration of AFP in the culture supernatant (mean ± SD, $n = 2$), (**C**) amount of AFP in the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

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

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

death of HCC cells that do not possess AFP production capability, whereas other HCC cells with AFP production capability are not affected, resulting in the upregulation of AFP expression. If this hypothesis is correct, AFP expression would be high or upregulated with time after IFN treatment. In our long-term experiment, AFP mRNA was downregulated at 240 h with PEG-IFN-α2b treatment, and this poses a question as to the correctness of this hypothesis.

There has been much progress in the characterization of cis-acting and trans-acting elements regulating human AFP gene expression. Transcription of human AFP gene is controlled by three regulatory regions (promoter, enhancer, and silencer) in the 5′-flanking sequence.[24–28] The hepatocyte-specific enhancers exist in a far upstream regulatory region (−3.7 and −3.5 kb) of the AFP gene, and the position-dependent silencers are located between the enhancer regions and the hepatocyte-specific promoter region.[25] Several hormones, growth factors, cytokines, and differentiation inducers are reported to be involved in the regulation of AFP in human HCC cells.[23,24,29–33] Dexamethasone elevates AFP mRNA in human hepatoma cells through specific interaction with the glucocorticoid-responsive element in the human AFP gene promoter.[24] On the other hand, epidermal growth factor (EGF) synergistically interacts with phorbol ester to suppress the AFP enhancer activity, resulting in a marked depression of AFP gene transcription,[33] whereas transforming growth factor-β1 (TGF-β1) hepatocyte growth factor, and sodium butyrate repress AFP gene expression through reduction of its promoter activity.[30–32] In a future study, the AFP enhancer and promoter activities of KIM-1 cells cultured with PEG-IFN-α2b should be investigated to elucidate the AFP mRNA upregulation mechanism.

The ratio of AFP-L3 in cell lysate was lower than the ratio in culture medium. It was also lower in the culture with PEG-IFN-α2b than in the cells without PEG-IFN-α2b, and the level tended to decrease in a dose-dependent and time-dependent manner. The molecular basis of AFP-L3 is the fucosylation of the biantennary sugar chain,[8,34] and AFP-L3 is the product of α1-6 fucosyltransferase (α1-6 FucT) in the presence of GDP-fucose.[35] The higher ratio of AFP-L3 in the culture medium (outside the cells) suggests that fucosylated AFP is secreted more readily than nonfucosylated AFP. In addition, it was suggested that PEG-IFN-α2b could reduce the enzymatic activity of α1-6 FucT that is involved in the fucosylation of AFP. Noda et al.[36] found that acyclic retinoid treatment of HCC cells significantly increased the activity and mRNA levels of α1-6 FucT and the relative percentage of fucosylated AFP (AFP-L3) in culture medium. Whether or not PEG-IFN-α2b affects the enzymatic activity of α1-6 FucT is also a theme for future study.

For HCC-directed gene therapy, most investigators have used human AFP regulatory sequences.[37–40] Ido et al.[41] found that a retroviral vector expressing the herpes simplex virus-thymidine kinase gene under the control of a human AFP gene promoter provided the cytotoxicity to ganciclovir in high AFP-producing human HCC cells but not in low AFP-producing cells. Accordingly, they considered that specific enhancement of AFP promoter activity is likely to be required to induce enough cytotoxicity in low AFP-producing HCC cells.[41] If it induces the enhancement of AFP promoter activity, PEG-IFN-α2b could be a potent modulator of such HCC-directed gene therapy.

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Original Article

Overexpression of the *myc* target gene *Mina53* in advanced renal cell carcinoma

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The *myc* target gene *Mina53* was reported to be overexpressed in esophageal cancer with a poor prognosis. The purpose of the present study was to examined Mina53 expression and its relationship to clinicopathological parameters in human renal cell carcinoma (RCC). Mina53 and Ki-67 expression was examined on immunohistochemistry for 64 surgically resected RCC and non-cancerous tissue. In addition, the relationship between Mina53 expression and clinicopathological prognostic factors of RCC such as age, stage, microvenous invasion (MVI), histological subtype, Ki-67 labeling index (LI), and prognosis, was examined. Mina53 was expressed in the nuclei of tumor cells and tubular nuclei of normal renal tissue. The expression level of Mina53 was significantly higher in patients with poor prognostic factors (stage IV, MVI-positive, and sarcomatoid RCC, and high Ki-67 LI). The prognosis of high Mina53-expressing tumors was significantly poorer than that of non-Mina53-high tumors ($P < 0.0001$). In conclusion, Mina53 is overexpressed in RCC tissue from patients with poor prognostic factors, suggesting that Mina53 overexpression is one of the factors for poor prognosis in RCC.

Key words: immunohistochemistry, Ki-67, Mina53, renal cell carcinoma

In Japan, 6358 individuals (4372 men and 1986 women) developed renal cell carcinoma (RCC) in 1997. The crude incidence rates per 100 000 people for men and women were 7.1 and 3.1, respectively, and the age-standardized incidence rates per 100 000 people for men and women were 4.9 and 1.8, respectively.[1]

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Recently, the 10 year survival rate of all RCC patients has exceeded 65% due to improvements in diagnostic imaging and therapy.[2,3] Because RCC is known to be resistant to chemotherapy, radiotherapy and surgery remain the only effective treatments.[4,5] Tumor stage, tumor grade, and Ki-67 status, together with clinical parameters, can best predict prognosis; but even a small or T1 RCC sometimes metastasizes to distant sites and, in rare cases, distant metastatic lesions spontaneously regress or disappear.[6,7] RCC often has an unpredictable outcome. While much is unknown about RCC, various genes reportedly involved in the progression, recurrence, and proliferation of RCC, such as *epidermal growth factor receptor* (*EGFR*), *p53*, and *c-myc*, have been identified.[8–11] Among these, *c-myc* has been shown to be an oncogene that interacts with various cellular factors to stimulate proliferation, apoptosis induction, and lymphangiogenesis.[11,12] The oncogene *myc* with such diverse functions plays an important role, especially in cell proliferation, and is expressed in many cancers. The *myc* product directly binds to the E-box sequence of genomic genes to increase their expression.[13] Previous studies have shown that genes closely involved in cell proliferation are *myc* target genes, that is, genes whose expression is directly increased by *myc*.[14] Tsuneoka *et al.* reported that *Mina53* (myc-induced nuclear antigen with a molecular weight of 53 kDa) is a novel *myc* target gene involved in cell proliferation. The *Mina53* gene is located on chromosome 3q12.1,[14] and encodes a protein with a molecular weight of 53 kDa, which is localized in the nucleus, and is concentrated, in part, in the nucleolus. Recent studies have reported that Mina53 is expressed in all pathological grades of colon cancer, but not or slightly in non-neoplastic colonic cells,[15] and esophageal cancer with a high expression of Mina53 has a very poor prognosis.[16] The purpose of the present study was to examine the expression of myc gene-related molecules, Mina53 in RCC, on

immunohistochemistry, and clinicopathologically evaluate the potential use of Mina53 expression as a new prognostic factor.

MATERIALS AND METHODS

Antibodies

Mouse monoclonal anti-Mina53 antibody (IgG2a, clone M532) was established at Division of Human Genetic Department of Forensic Medicine, Kurume University School of Medicine, as previously described.[15] Mouse anti-Ki-67 antibody (clone MIB-1) and peroxidase-labeled goat antimouse IgG Fab' were purchased from Dako (Glostrup, Denmark) and Nichirei (Tokyo, Japan), respectively.

Immunoblotting

A renal cell carcinoma cell line (ACHN) that was purchased from the American Type Culture Collection (Manassas, VA, USA) was maintained in modified Eagle's medium (Gibco BRL/Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Bioserum, Victoria, Australia), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco BRL/Life Technologies). For western blotting, cells were collected by treatment with trypsin and EDTA in PBS and washed with PBS. Cells were then suspended in 0.125 mol/L Tris-HCl buffer, pH 6.8, containing 3% sodium dodecylsulfate, 50 mmol/L dithiothreitol, and 20% glycerol and boiled for 10 min before separation on a gradient sodium dodecylsulfate–polyacrylamide gel (4–20%). Proteins were transferred to a polyvinylidene difluoride microporous membrane (Millipore, Bedford, MA, USA), and non-specific binding sites were blocked with 1% skim milk in PBS. After treatment with mouse monoclonal anti-Mina53 and HRP-conjugated goat antimouse IgG, signals were detected using an enhanced chemiluminescence western blotting detection reagent system (Amersham Biosciences, Buckinghamshire, UK).

Patients and specimens

Preserved specimens from 64 consecutive RCC patients who had received surgical treatment at Kurume University Hospital between 2000 and 2005 were analyzed and their clinical records were reviewed. All patients underwent radical nephrectomy or partial nephrectomy with para-aortic lymphadenectomy and renal hilar lymphadenectomy. No patients received preoperative treatment. The patients were staged in accordance with the 1997 Union Internationale Contre le Cancer (UICC)/American Joint Committee on Cancer (AJCC) consensus classification.[17] Of the 64 patients, 36, four, 13, and 11 were in stage I, II, III, and VI, respectively (pT1 = 37, pT2 = 4, pT3 = 21, pT4 = 2). Tumors were grouped according to the histological typing of the World Health Organization (WHO). Nuclear grade was assigned according to the Fuhrman nuclear grading system.[18] Microvenous invasion (MVI), stage of cancer, histological subtype, and nuclear grade were determined by two pathologists, H.I. and H.Y. Survival time was defined as the period from the date of surgical resection of the primary tumor to the date of death or last follow up. For disease-specific survival, the survival time of patients who died from causes other than RCC was defined as the period from the date of surgical resection to the date of death. The cause of death was determined by correspondence with the patient's family or local physician, or by reference to the death certificate. During the follow-up period, ranging from 0.9 to 301 weeks (mean, 147 weeks), two patients died soon after surgery of intraoperative or postoperative complications, making the period of follow up extremely short. There were three recurrences and 10 carcinoma-related deaths. The mean age at surgery was 60.4 years (range, 24–86 years). Forty-four patients were men, and 20 were women. Of the 64 patients, 53 had clear cell RCC, five had papillary RCC, five had sarcomatoid RCC, and one had chromophobe RCC (Table 1). Sarcomatoid RCC was first described by Farrow *et al.* as a tumor exhibiting marked cytological atypia and containing enlarged pleomorphic or malignant spindle cells suggestive of sarcoma;[19] but sarcomatoid RCC is currently regarded as a common, dedifferentiated process in the different subtypes of RCC.[20] In the present study we defined sarcomatoid RCC as an RCC with a sarcomatoid component, and classified all tumors with a sarcomatoid component as nuclear grade 4. Of the five sarcomatoid RCC, three and two contained clear cell and papillary cell carcinoma components, respectively. Their sarcomatoid components occupied >60% of tumor tissue. Pure sarcomatoid tumors, whose histological subtypes could not be identified, were not included in the specimens examined.

Immunostaining

Routinely processed, formalin-fixed, paraffin-embedded serial sections (4 μm) containing cancerous and non-cancerous areas were mounted on 3-aminopropyltriethoxysilane-coated slides (Matsunami Glass, Osaka, Japan), and deparaffinized in xylene/alcohol and graded alcohol. The sections were soaked in 10 mmol/L sodium citrate buffer (pH 6.9), and treated in a microwave oven for 50 min for antigen retrieval. Immunostaining for Mina53 and Ki-67 was performed using streptavidin–biotin peroxidase (SAB-PO) kits (Nichirei,

Table 1 Mina53 expression and clinicopathological factors in RCC

Factors	Mina53 expression		Total
	Negative and low-expression tumors	High-expression tumors	
No. patients (%)	55	9	64
Average age (years)	60.9	57.3	60.4
Gender			
Male	38	6	44
Female	17	3	20
MVI			
Negative	49	3	52
Positive	6	6‡	12
Stage			
Stage I	36	0	36
Stage II	4	0	4
Stage III	10	3	13
Stage IV	5	6§	11
Histological subtype			
Clear cell RCC	49	4	53
Papillary RCC	4	1	5
Chromophobe RCC	1	0	1
Sarcomatoid RCC	1	4*	5
Nuclear grade			
1	9	0	9
2	30	1	31
3	14	3	17
4	2	5**	7
Ki-67 LI			
<10%	50	0	50
≥10%	5	9***	14
Lymph-node metastasis			
Negative	53	5	58
Positive	2	4††	6
Distant metastasis			
Negative	54	4	58
Positive	1	5††	6

*P < 0.001–0.0001, vs sarcomatoid RCC or the other subtypes. **P < 0.01–0.0001, versus nuclear grade 1, or nuclear grade 2, or nuclear grade 3, or nuclear grades 1–3. ***P < 0.0001, vs <10%.

‡P < 0.0001, versus MVI negative. §P < 0.05–0.0001, vs stage I, stage III, or stages I–III. ††Not significant, vs negative.

LI, labeling index; MVI, microvenous invasion; RCC, renal cell carcinoma.

Tokyo, Japan) according to the manufacturer's protocol. After treatment with avidin and rabbit serum, the sections were incubated with primary antibodies at 4°C overnight. The peroxidase reaction was developed by the addition of 3,3-diaminobenzidine-H_2O_2 substrate solution, followed by incubation for 5 min (Mina53) or 2 min (Ki-67). After light counterstaining with hematoxylin, the slides were dehydrated, coverslipped, and observed under a microscope (Olympus BX41, Olympus Optical, Tokyo, Japan). Negative controls were prepared by replacing the primary antibody with normal mouse IgG. The Mina53 expression level in the nuclei of renal tubular epithelium in non-tumorous areas was used as an internal positive control.

Microscopic assessment of Mina53 and Ki-67 expression

Two pathologists, H.I and H.Y., who did not know the clinical status of each patient, independently evaluated and interpreted the results of immunostaining. The expression (staining) levels of Mina53 were classified into three categories. Tumors containing no identifiable Mina53-positive tumor cells were classified as Mina53-negative; tumors that contained focally or diffusely Mina53-positive tumor cells stained as intensely as, or less intensely than, non-cancerous normal renal tubules, were classified as low Mina53-positive; and tumors containing diffusely Mina53-positive tumor cells that were clearly more intensely stained than non-cancerous normal renal tubules were classified as high Mina53-positive. Proliferative activity was assessed by detecting Ki-67 protein. The Ki-67 antigen represents a nuclear cell proliferation-associated protein expressed in the G_1, S, G_2, and M phases of the cell cycle, but not in non-proliferative G_0 cells. The Ki-67 staining results of tumor cells, as expressed by the Ki-67 labeling index (LI), were evaluated as follows. In Mina53-positive tumors, Ki-67 expression was assessed in the area containing Mina53-positive cells and having the highest Ki-67 LI. In Mina53-negative tumors, Ki-67 expres-

sion was assessed in the area having the highest Ki-67 LI in tumor tissue. In each tumor an appropriate area was photographed at a magnification of ×200 with a digital camera, and printed. To estimate the percentage of stained cells, the numbers of positive and negative tumor cells in a field were counted, and the ratio of positive to total cells was expressed as a percentage.

Statistics

The relationships between Mina53 expression levels in negative, low-positive, and high-positive tumors and Ki-67 LI were analyzed on Mann–Whitney *U*-test. In addition, associations between Mina53 expression in RCC and poor prognostic factors such as MVI, stage IV, sarcomatoid RCC, nuclear grade 4, and Ki-67 LI ≥ 10% were examined on χ^2 test. Disease-free survival rates were calculated using the Kaplan–Meier method. *P* for each survival rate as well as prognostic factors (high Mina53 expression, MVI-positive, stage IV, sarcomatoid RCC, nuclear grade 4, and Ki-67 LI ≥ 10%) were analyzed using the log–rank test.[21] Cox's multivariate analysis was used to examine whether Mina53 expression is a prognostic factor independent of other established factors such as cancer stage.[22] All statistical analyses were performed using StatView software (SAS Institute, Cary, NC, USA). $P < 0.05$ were considered significant.

RESULTS

Expression of Mina53 in renal cell carcinoma cells on western blot

Anti-Mina53 mouse monoclonal antibody (M532) recognized a single band with a molecular weight of 53 kDa on western blot (Fig. 1). The result indicates that ACHN cells express Mina53 and that anti-Mina53 antibody specifically recognizes Mina53 protein in RCC cells with no cross-reactivity to other proteins.

Immunohistochemical expression of Mina53 and Ki-67 in RCC and non-cancerous tissues

Low level of Mina53 expression was observed in the nuclei of normal tubules in all non-cancerous renal tissues (Fig. 2a,b). Mina53 expression in the nuclei of RCC cells varied from negative to high, and Mina53-negative (Fig. 2d,e), low Mina53-expressing (Fig. 2g,h), and high Mina53-expressing tumors (Fig. 2j,k) accounted for 32.8% (21 tumors), 53.1% (34 tumors), and 14.1% (nine tumors) of all cancers. The distribution of Mina53-positive RCC cells was uniform in the tumor nodule but in some cases of low-Mina53 expression, Mina53 expression was focally or predominantly observed in the periphery of the tumor. In all cases the expression level of Mina53 was uniform in the tumor nodule. In three high-Mina53 expressing tumors (two sarcomatoid RCC and one papillary RCC cases), Mina53 expression was observed in the nucleus with concentrated amounts in the nucleolus. On histology Mina53 was expressed in 33 clear cell RCC (62.3%), four papillary RCC (80%), five sarcomatoid RCC (100%), and one chromophobe RCC (100%; Table 1). Nuclear Ki-67 expression was observed in all 64 RCC at various levels (Fig. 2f,i,l), but few or no renal tubules were positive for Ki-67 (Fig. 2c).

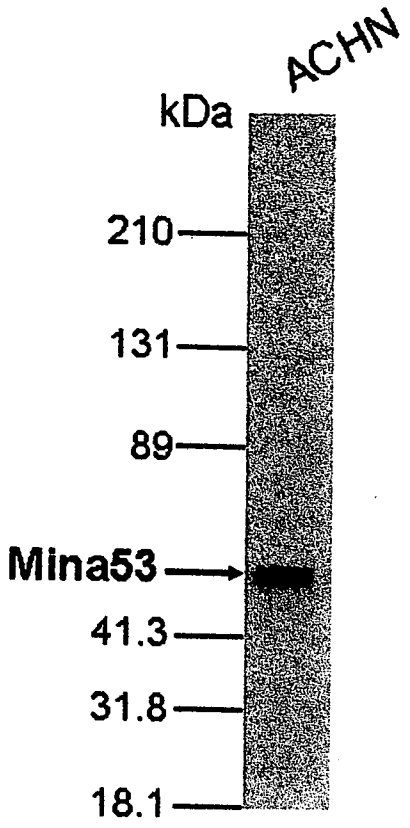

Figure 1 Western blot analysis of Mina53 protein in cultured renal cell carcinoma (RCC) cells. Proteins were subjected to electrophoresis in the 4–20% gradient sodium dodecylsulfate–polyacrylamide gel, transferred to polyvinylidene difluoride transfer membrane, and probed with anti-Mina53 (clone M532) monoclonal antibody. Anti-Mina53 mouse monoclonal antibody (M532) recognized a single band with a molecular weight of 53 kDa on western blot. The result indicates that ACHN cells express Mina53 and that anti-Mina53 antibody specifically recognizes Mina53 protein in RCC cells with no cross-reactivity to other proteins.

Association between Mina53 expression and clinicopathological parameters in RCC

The patients were 44 men and 20 women. There was no association between Mina53 expression and gender or age.

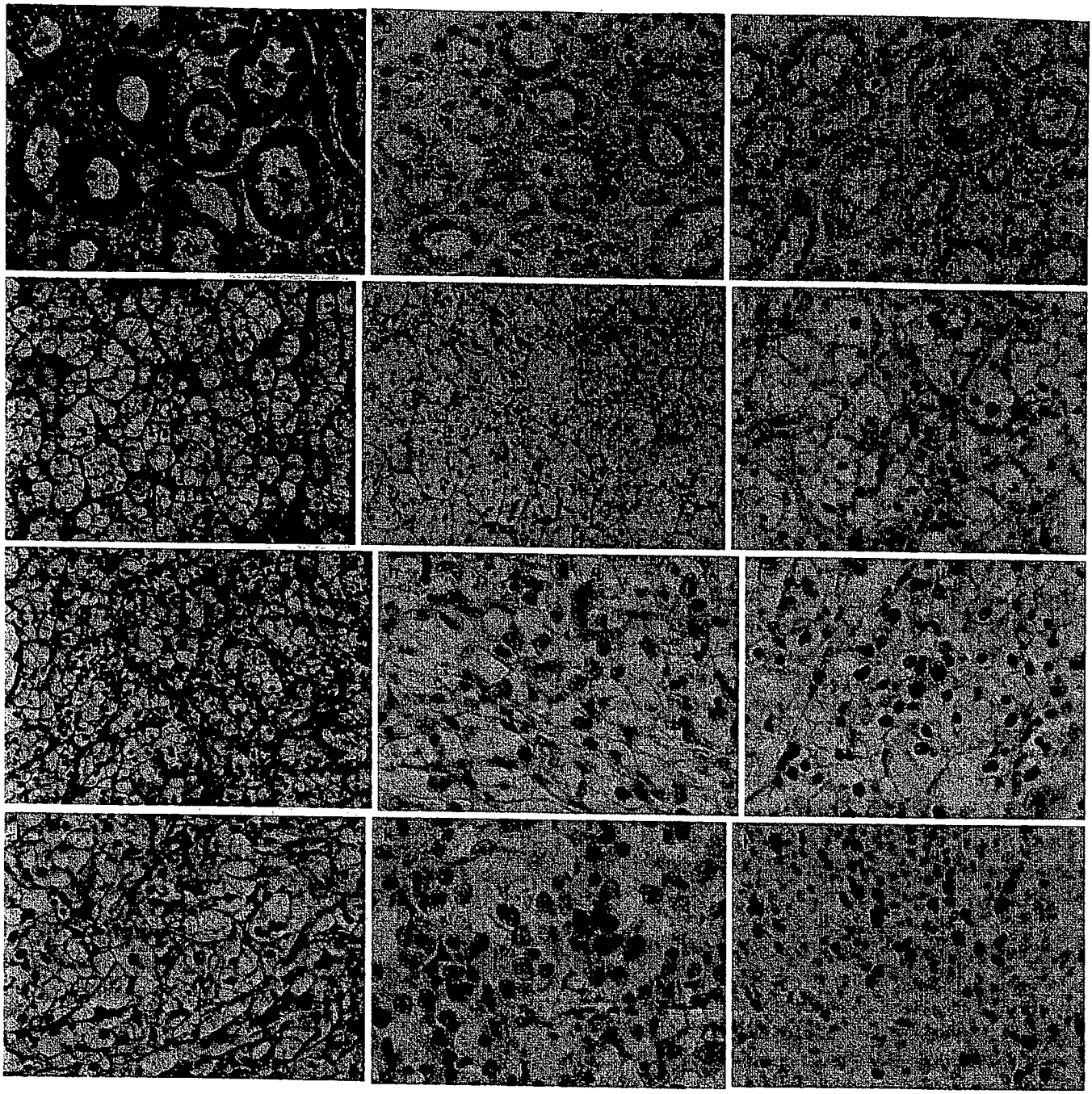

Figure 2 Immunohistochemical staining of Mina53 and Ki-67 in renal cell carcinoma (RCC) and non-cancerous tissues. (**a**) Normal tubules in the non-cancerous area of clear cell RCC (HE). (**b**) Low level of Mina53 expression in the nuclei of tubules in a non-tumorous area (counterstained with hematoxylin). (**c**) Ki-67 expression in the nuclei of tubules in a non-tumorous area. Few or no renal tubules were positive for Ki-67 (counterstained with hematoxylin). (**d**) Mina53-negative clear cell RCC (HE). (**e**) No Mina53 expression in tumor cells (counterstained with hematoxylin). (**f**) Ki-67 expression in the same tumor as in (**d**). Ki-67-positive cells were seen scattered (counterstained with hematoxylin). (**g**) Low Mina53-expressing clear cell RCC (HE). (**h**) Mina53 expression in the same tumor as in (**g**). Tumor cells stained for Mina53 as intensely as renal tubules were noted (counterstained with hematoxylin). (**i**) Ki-67 expression in the same tumor as in (**g**). Ki-67-positive cells were seen scattered (counterstained with hematoxylin). (**j**) High Mina53-expressing sarcomatoid RCC (HE). (**k**) Mina53 expression in the same tumor as in (**j**). Strong Mina53 expression was observed in the nuclei with concentrated amounts in the nucleoli of tumor cells (counterstained with hematoxylin). (**l**) Ki-67 expression in the same tumor as in (**j**). Many Ki-67-positive cells were observed (counterstained with hematoxylin).

Of all 64 tumors, 12 (18.8%) were MVI positive. Of these 12 tumors, six (50%) were high Mina53 positive. The percentage of high Mina53-expressing tumors was significantly higher in MVI-positive than in MVI-negative tumors ($P < 0.0001$, χ^2 test; Table 1).

The numbers of stage I, II, III, and IV tumors were 36, four, 13, and 11, respectively. Of the 11 stage IV tumors, six (54.5%) were high Mina53 positive. The percentage of high Mina53-expressing tumors was significantly higher in stage IV than in stages I–III tumors ($P < 0.0001$, χ^2 test; Table 1). In addition there were significant differences in the percentage of high Mina53-positive tumors between stage I and IV tumors ($P < 0.0001$, χ^2 test) and between stage III and IV tumors ($P < 0.05$, χ^2 test; Table 1).

Of the five sarcomatoid RCC, four (80%) were high Mina53 positive. The percentage of high Mina53-expressing tumors was significantly higher in sarcomatoid RCC than in other histological subtypes of RCC (clear cell RCC, papillary RCC, and chromophobe RCC; $P < 0.0001$, χ^2 test). In addition, a significant difference was noted in the percentage of high Mina53-expressing tumors between sarcomatoid and clear cell RCC ($P < 0.001$, χ^2 test; Table 1).

The numbers of nuclear grade 1, 2, 3, and 4 tumors were 9, 31, 17, and 7, respectively. Of the seven nuclear grade 4 tumors, five (71.4%) were high Mina53 positive. The percentage of high Mina53-expressing tumors was significantly higher in nuclear grade 4 tumors than in nuclear grades 1–3 tumors ($P < 0.0001$, χ^2 test; Table 1). In addition, there were significant differences in the percentage of high Mina53-expressing tumors between grade 1 and 4 tumors, between grade 2 and 4 tumors, and between grade 3 and 4 tumors ($P < 0.01$, $P < 0.001$, and $P < 0.01$, respectively, χ^2 test; Table 1).

Comparison of Mina53 and Ki-67 expression in RCC tissue

Ki-67 expression was observed in the nucleus of RCC at various levels. The mean Ki-67 LI for all tumors was 9.6%, and the mean LI for Mina53-negative, low Mina53-expressing, and high Mina53-expressing tumors was 5.2%, 5.2%, and 34.7%, respectively. When tumors were divided into high and low Ki-67-expressing groups by the approximate mean Ki-67 LI of 10%, the frequency of high Mina53 expression was significantly higher in the high than in the low Ki-67-expressing group ($P < 0.0001$, χ^2 test; Table 1). Moreover, significant differences in Ki-67 LI were noted between Mina53-negative and high Mina53-expressing tumors ($P < 0.0001$), and between low and high Mina53-expressing tumors ($P < 0.0001$, Mann–Whitney U-test; Fig. 3).

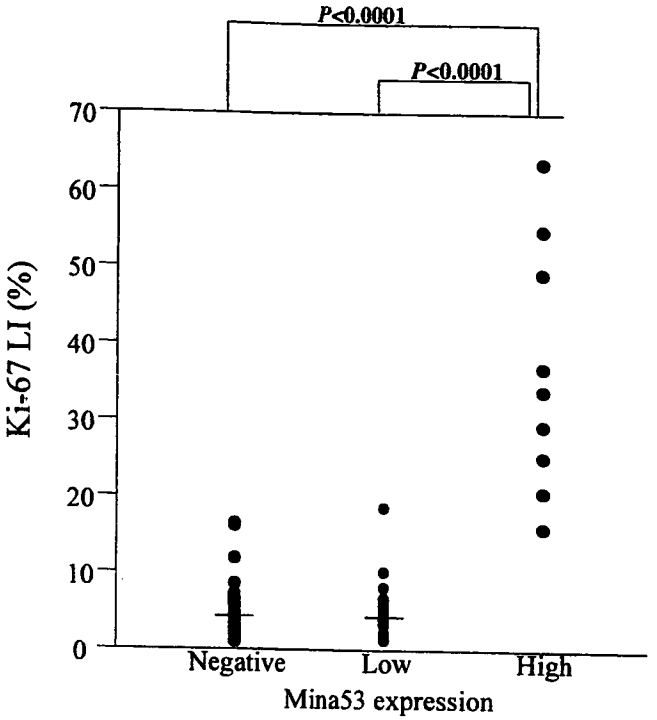

Figure 3 Relationship between Mina53 and Ki-67 expression. Ki-67 labeling indices (LI) were compared in three different groups: Mina53-negative group, Mina53 low-expression group, Mina53 high-expression group. Their respective mean LI were 5.2%, 5.2%, and 34.7%. Their means are indicated by transverse lines. Significant differences in Ki-67 LI were noted between Mina53-negative and Mina53 high-expression groups, and between Mina53 low-expression and Mina53 high-expression groups.

Mina53 and Ki-67 expression in relation to survival time

The survival rates of patients with Mina53-negative, Mina53-low, and Mina53-high tumors were 95.2% (20/21), 91.1% (31/34), and 33.3% (3/9), respectively. The patients were divided into a Mina53-high group and a non-Mina53-high group (consisting of Mina53-negative and Mina53-low groups). Crude survival curves were estimated for each group using the Kaplan–Meier method. The patients with non-Mina53-high tumors had longer survival than those with Mina53-high tumors (Fig. 4). The survival rate was significantly higher in patients with non-Mina53-high tumors than in those with Mina53-high tumors ($P < 0.0001$, log–rank test). The patients with low-Ki-67 LI tumors had longer survival than those with high-Ki-67 LI tumors (Fig. 5). The survival rate was significantly higher in patients with low-Ki-67 LI tumors than those with high-Ki-67 LI tumors ($P < 0.0001$, log–rank test). Possible prognostic factors, including gender, MVI-positive, stage IV, sarcomatoid RCC, and high Mina53 expression, were analyzed using Cox's proportional hazards method. As shown in Table 2, only stage IV and high-Ki-67 LI were found to reflect the survival rate (Table 2).