Interferon Alfa Receptor Expression and Growth Inhibition by Interferon Alfa in Human Liver Cancer Cell Lines

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Type I interferon (IFN-α) receptor consists of two chains (Hu-IFN-αR1 and Hu-IFN-αR2), and Hu-IFN-αR2 takes a soluble (Hu-IFN-αR2a), short (Hu-IFN-αR2b), or long (Hu-IFN-αR2c) form. We examined the expression of type I IFN receptor, the growth-suppression effect of IFN-α, and their relationship in 13 liver cancer cell lines. With reverse-transcription polymerase chain reaction (RT-PCR) analysis, the expressions of Hu-IFN-αR1, Hu-IFN-αR2a, and Hu-IFN-αR2c were confirmed in all cell lines, and that of Hu-IFN-αR2b in 12 cell lines. All cell lines expressed mRNAs of a transcriptional activator, interferon regulatory factor (IRF)-1, and its antagonistic repressor (IRF-2). Flow cytometry revealed weak expression of Hu-IFN-αR2 on the cell surface in 12 cell lines. The soluble-form protein of Hu-IFN-αR2 was detected at varying levels in culture supernatants of all cell lines with enzyme-linked immunosorbent assay (ELISA). Cell proliferation was suppressed in proportion to the dose of human natural IFN-α at 96 hours of culture, but it was not clearly related to the expression of Hu-IFN-αR2 protein on the cell surface. Investigations on the morphology, DNA, and cell cycle presented four growth suppression patterns as a result of IFN-α: 1) induction of apoptosis and blockage of cell cycle at the S phase (9 cell lines); 2) blockage at the G2/M phase (2 cell lines); 3) induction of apoptosis and blockage at the G2/M phase (1 cell line); and 4) blockage at the G1 phase (1 cell line). There was no evidence showing that changes in the expressions of Bcl-2, Bcl-xL, Bak, and Bax lead directly to IFN-α-mediated apoptosis. Our findings demonstrated that IFN-α would express growth-suppression effects at varying degrees by inducing inhibition of cell-cycle progression with or without apoptosis, regardless of the expression level of Hu-IFN-αR2 protein on the cell surface. (HEPATOLOGY 1999;29:1708-1717.)

Interferon alfa (IFN-α) has been shown to possess antiviral activity, antiproliferative activity, and various immunoregulatory activities including: 1) stimulation of cytotoxic activities of lymphocytes and macrophages, and of natural killer cell activity; and 2) induction of class I major histocompatibility complex antigens. The effects of IFN-α are mediated through interaction with the specific cell-surface receptor, type I IFN receptor. This receptor consists of two chains, Hu-IFN-αR1 and Hu-IFN-αR2, which can be present in different forms.² The Hu-IFN-αR1 chain is present as either the full chain (Hu-IFN-αR1) or a splice-variant (Hu-IFN-αR1-s) lacking exons 4 and 5. Hu-IFN-αR2 chain exists in soluble, short, and long forms (Hu-IFN-αR2a, Hu-IFN-αR2b, and Hu-IFN-αR2c, respectively). Most likely, the Hu-IFN-αR1 and Hu-IFN-αR2 chains represent the predominantly active form.² Binding of the receptor and IFN-α induce transcription of IFN-inducible genes through the activation of the Jak/signal transducer and activator of transcription (STAT) signaling pathway. Interferon regulatory factor (IRF)-1, a transcriptional activator, and its antagonistic repressor, IRF-2, have been identified as regulators of type I IFN (mainly IFN-α and IFN-β) and IFN-inducible genes.¹⁰ ¹¹ The IRF-1 gene itself is IFN-inducible and may thus be one of the critical target genes mediating IFN action.¹¹

Antivirus activity of IFN-α has attracted a great deal of attention, and IFN-α has been applied in treatment for hepatitis B virus (HBV)- and hepatitis C virus (HCV)-related chronic hepatitis in several countries (reviewed in Gutterman). In the liver of HCV-infected patients, expressions of Hu-IFN-αR1 and Hu-IFN-αR2 chains were investigated in terms of mRNA level, and the relationship between their expression levels and response to IFN-α therapy was reported. Although IFN-α has been proven to have a curative potential in treatment of HBV- and HCV-associated chronic liver diseases, its effect on hepatocellular carcinoma (HCC), which is a common and often fatal complication of HBV- and HCV-related chronic liver diseases,¹¹ is not well known. Clinical trials of IFN-α in treatment of HCC did not achieve consistent results: one study showed beneficial effects, and the other studies did not show significant antitumor effects. In contrast, IFN-α has been shown to be useful for the treatment of several malignant diseases, including hairy-cell leukemia and chronic myelogenous leukemia (reviewed in Gutterman). Experimental studies showed that IFN-α can inhibit the growth of various normal and malignant cells in vitro by inducing cell-cycle changes (e.g., induction of G0/G1 arrest and prolongation of the S phase) and/or apoptosis.
is. To date, antiproliferative effects of IFN-α against a few HCC cell lines have been reported in vitro; however, the mechanism of growth inhibition by IFN-α has not been studied in HCC cells in detail. Furthermore, no studies have been conducted to clarify whether cancer cells express type I receptors, or whether IFN-α suppresses the proliferation of cancer cells in proportion to the expression of the receptors on the cell surface. It is important to deepen the understanding of the action of IFN-α on HCC cells, because some patients with HBV- and HCV-related liver diseases may already have a small, clinically undetectable HCC during IFN-α therapy. In the present study, we examined: 1) mRNA expressions of type I IFN receptor and IRFs; 2) protein expression of Hu-IFN-αR2; 3) in vitro growth-inhibitory effects and the mechanism of actions of IFN-α in human liver cancer cells, i.e., 11 HCC cell lines and 2 combined hepatocellular and cholangiocarcinoma (CHC) cell lines; and 4) the relationship between (2) and (3).

**MATERIALS AND METHODS**

**Cell Lines and Cell Culture.** This study used 11 human HCC cell lines (KIM-1, 41 KYN-1, 42 KYN-2, 43 KYN-3, 44 HAK-1A, 45 HAK-1B, 46 HAK-2, 47 HAK-3, 48 HAK-4, 49 HAK-5, and HAK-6) and 2 combined hepatocellular and cholangiocarcinoma cell lines (KMCH-1 47 and KMCH-2 48), which were originally established and characterized in our laboratory. Four HCC cell lines (HAK-3, HAK-4, HAK-5, and HAK-6) were established recently. These 13 cell lines were previously confirmed to retain morphological and functional characteristics of the original tumor. KIM-1, KYN-1, KYN-2, and HAK-3 cell lines were established from surgically resected moderately differentiated HCC nodules; KYN-2 and HAK-6, from surgically resected moderately to poorly differentiated HCC nodules; and KYN-3, HAK-4, and HAK-5, from peritoneal effusion of HCC patients with moderately to poorly differentiated, poorly differentiated, and sarcomatous HCCs, respectively. HAK-1A and HAK-1B were established from a single HCC nodule showing a three-layered structure with a different histological grade in each layer. HAK-1A resembles well-differentiated HCC cells in the outer layer of the original tumor, and HAK-1B resembles poorly differentiated cells in the inner layer. KMCH-1 and KMCH-2 were established from CHC nodules consisting of both HCC and cholangiocarcinoma components.

The cells were cultured in a culture medium consisting of Dulbecco's modified Eagle medium (Nissui Seiyaku Co., Tokyo, Japan) supplemented with 2.5% heat-inactivated (56°C, 30 minutes) fetal bovine serum (Bioseira, 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% CO2 at 37°C. In all experiments, the cells were cultured for 24 hours before the experiments to allow the cells to enter their logarithmic growth phase. In colorimetric cell growth assay and cell-cycle analysis experiments, medium with or without IFN-α was renewed 48 hours after the initial treatments.

**Cytokines and Antibodies.** Natural human IFN-α (OIF) and monoclonal antibody raised against the extracellular domain of Hu-IFN-αR2 (IgG, clone ANOC4866) were kindly provided by the Cellular Technology Institute, Otsuka Pharmaceutical Co., Ltd. (Tokushima, Japan). Anti-bromodeoxyuridine (BrdU) antibody and fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin were purchased from Becton Dickinson Immucytometry Systems USA (San Jose, CA); control normal mouse IgG, and monoclonal mouse anti-human Bcl-2 oncprotein antibody was from DAKO (Glostrup, Denmark); rabbit polyclonal antibodies to human Bax, N-terminal, and human Bak, N-terminal were from Upstate Biotechnology (Lake Placid, NY); rabbit polyclonal anti-human Bcl-xL antibody was from Transduction Laboratories (Lexington, KY); monoclonal anti-β-actin antibody was from Sigma Chemical Co. (St. Louis, MO); and peroxidase-conjugated rabbit IgG fraction to rabbit IgG and peroxidase-conjugated rabbit IgG fraction to mouse IgG were from ICN Pharmaceuticals, Inc. (Aurora, OH).

**Analysis of Hu-IFN-αR1, Hu-IFN-αR2, IRF-1, and IRF-2 mRNA Expressions by Reverse-Transcription Polymerase Chain Reaction.** Total RNA was isolated from the cell lines by using RNAzol B (Biotechnex Laboratories, Inc., Houston, TX). For first-strand cDNA synthesis, 5 µg of total RNA was incubated at 37°C for 45 minutes in 20 µL reverse-transcriptase buffer (50 mmol/L Tris-HCl [pH 8.3]/75 mmol/L KCl/3 mmol/L MgCl2/0.5 mmol/L deoxyribonucleoside triphosphates) containing 40 U of RNAsin (Promega Corp., Madison, WI), 90 pmol of Random Primer (Takara Shuzo Co., Ltd., Kyoto, Japan), and 200 U of RNase H (reverse transcriptase) (Superscript II, Gibco BRL/Life Technologies Inc.) Reverse-transcriptase (RT) reaction was stopped by heating the RNA mixture at 95°C for 5 minutes. Then, a one-tenth volume of this cDNA solution was added to the polymerase chain reaction (PCR) solution as the template of DNA synthesis. PCR reaction of Hu-IFN-αR1 was made with the following primers: a sense primer, 5'-AGTGTATGTGGGCTTTGAGTTAAGG (primer 1); and an antisense primer, 5'-CTGGTCTTACACAAATATACGATTG (primer 2). Hu-IFN-αR2 gene is reported to produce four different transcripts encoding three different forms of Hu-IFN-αR2. The specific primers (primer pair 3 and 4), which were reported by Lutfalla et al., were used to amplify Hu-IFN-αR2a and Hu-IFN-αR2c cDNAs, and one of the two Hu-IFN-αR2b cDNAs; and the one cDNA that was not enhanced was the Hu-IFN-αR2b cDNA containing a short 3' untranslated region. In addition, another pair of the primers was designed and used to amplify the extracellular domain of Hu-IFN-αR2b and Hu-IFN-αR2c cDNAs, i.e., a sense primer, 5'-GATTGCCGTGATTA- CATCGAGTAA (primer 5); and an antisense primer, 5'-TTTGGCA- GATTCTTCGCTGATTC (primer 6). PCR reaction of human IFN-1R and IFN-2R was made with the specific primers (primer pair 7 and 8 and primer pair 9 and 10, respectively), which were reported by Harada et al. The PCR reaction solution consisted of 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/mL KCl, 1.5 to 2.5 mmol/L MgCl2 (1.5 mmol/L for primer pair 7 and 8; 2.0 mmol/L for primer pair 3 and 4; and 2.5 mmol/L for the others), 0.01% gelatin, 0.2 mmol/L deoxyribonucleoside triphosphate, 0.1% primer (primer pair 5 and 6) or 2 µmol/L appropriate 5' and 3' primers, and 25 U/mL AmpliTaq DNA Polymerase (Perkin Elmer, Branchburg, NJ). PCR reaction was repeated for 33 cycles by using a Thermocycler (Perkin-Elmer Cetus Corp., Norwalk, CT), and each cycle included 94°C (30 seconds), 60°C (2 minutes), and 72°C (3 minutes) for primer pair 1 and 2 and primer pair 5 and 6; 94°C (1 minute), 57°C (1 minute), and 72°C (2 minutes) for primer pair 3 and 4; and 95°C (30 seconds), 57°C (30 seconds), and 72°C (1 minute) for primer pair 7 and 8 and primer pair 9 and 10. PCR product (5 µL) was electrophoresed with a 2% or 4% NuSieve agarose gel (FMC Bioproducts, Rockland, ME). The gel was stained with 0.1% ethidium bromide for 20 minutes, followed by two 15-minute washes with ddH2O, and then specific DNA bands were examined under an ultraviolet transilluminator.

**Flow Cytometry.** For cell-surface staining of Hu-IFN-αR2, cell suspensions (4 × 106 cells per tube) were washed once with a washing-buffer (10 mmol/L phosphate-buffered saline [PBS] [pH 7.4]; 0.2% bovine serum albumin; 0.1% NaN3) and reacted with 10 µL of anti-Hu-IFN-αR2 antibody (final concentration, 10 µg/mL) on ice for 1 hour. Cells were then washed twice with the washing buffer, incubated with 4 µL of fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin on ice for 30 minutes in a dark place, washed twice with the washing buffer, fixed in 4% paraformaldehyde on ice for 10 minutes, washed once with the washing buffer, and analyzed by using a FACS Scan (Becton Dickinson Immucytometry Systems USA).

**Enzyme-Linked Immunosorbent Assay.** Cultured cells (4 × 105 cells per well) were seeded on 100-mm dishes (Falcon 3003), cultured for 24 hours, and then the medium was renewed. After 72 hours, the amount of the soluble form of Hu-IFN-αR2 in the supernatant was measured by using enzyme-linked immunosorbent assay kits manufactured by Cellular Technology Institute, Otsuka
Pharmaceutical Co., Ltd. Cultured cells were washed with PBS twice and collected by a scraper, and the amount of the cellular proteins was determined by using the BCA protein assay reagent (Pierce, Rockford, IL). The amount of the soluble form of the Hu-IFN-αR2 was assessed after making the correction for the amount of the cellular protein. Each experiment was repeated at least three times, and each experiment was duplicated.

**Effect of IFN-α on the Proliferation of HCC and CHC Cell Lines.** Effect of natural human IFN-α on cultured cell proliferation was investigated by using colorimetric assays with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide cell growth assay kits (Chemicon, Temecula, CA). Cultured cells (1.5 to 8 × 10^5 cells per well) were seeded on 96-well plates (Falcon, Becton Dickinson Labware, Tokyo, Japan), cultured for 24 hours, and then the medium was replaced by a fresh 100-µL medium alone or medium containing IFN-α (1, 2, 4, 8, 16, 32, 64, 256, 512, or 1,024 U/mL). After 24, 48, 72, or 96 hours, 100 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, 160 µg/mL, was added to each well, cultured for 4 hours, the supernatant was removed, and 100 µL of 40 mmol/L HCl/dimethylsulfoxide was added to each well. Viable cell numbers were estimated by measuring the absorbance with an Easy Reader EAR 400 (SLT Lab Instruments, Salzburg, Austria) by setting the test wavelength at 570 nm and the reference wavelength at 630 nm. Six to eight samples were used in each experiment, and each experiment was repeated at least twice to confirm the reproducibility of the test results.

**Morphological Observation.** In all experiments, cells were observed daily under a phase-contrast microscope (Nikon, Tokyo, Japan). For light-microscopic observation, cultured cells were seeded on Lab-Tek Tissue Culture Chamber Slides (Nunc, Inc., Roskilde, Denmark), cultured with IFN-α (250, 500, or 1,000 U/mL) or without IFN-α for 72 hours, fixed in Carnoy's solution for 10 minutes, and then stained with hematoxylin-eosin.

**Quantitative Analysis of Fragmented DNA Induced by IFN-α.** Cells (1 to 4 × 10^5 cells/dish) were seeded on 60-mm dishes, cultured for 24 hours, and then the medium was replaced by a fresh medium alone or medium containing 1,000 U/mL IFN-α. After 3 days, the amount of fragmented DNA was quantified by using a technique described previously in detail. The percentage of fragmented DNA was calculated as follows: percent of fragmented DNA = supernatant absorbance (supernatant absorbance + pellet absorbance) × 100. The average value was calculated from two independent experiments (n = 7 to 12 in total).

**Expression of Hu-IFN-αR1, Hu-IFN-αR2, IRF-1, and IRF-2 mRNAs in the Cell Lines.** In all 13 cell lines, a 765-bp band that corresponds to the PCR product of Hu-IFN-αR1 was detected (Fig. 1A). In the experiments on Hu-IFN-αR2 expressions using primer pair 3 and 4, a 713-bp band corresponding to the PCR product of Hu-IFN-αR2b was detected in all cell lines except KMCH-2; 481- and 350-bp bands corresponding to the PCR products of Hu-IFN-αR2c and Hu-IFN-αR2a, respectively, were found in all cell lines. The band of Hu-IFN-αR2c showed the strongest intensity (Fig. 1B). In KMCH-2 (a CHC cell line), three extra bands appeared in addition to those of Hu-IFN-αR2c and Hu-IFN-αR2a. In the analysis for the extracellular domain of Hu-IFN-αR2 using primer pair 5 and 6, the expected 642-bp band was detected in all cell lines; however, in the KMCH-2 cell line, a band was detected at approximately 480 bp in addition to the normal (intact) band (Fig. 1C). Direct sequence analysis of this short band revealed that it represented 474-bp PCR products with complete deletion of exon 7 (data not shown), and this result suggested that two of the three extra bands found in the...
In all 13 cell lines, the soluble form of Hu-IFN-αR2 in the spent medium was collected, the mean level was the highest with the amount of cell proteins at the time when the spent medium was at the highest level. If KIM-1 was excluded, there was a relatively good correlation between the expression of Hu-IFN-αR2 on the cell surface and that of soluble Hu-IFN-αR2 in the spent medium.

**Effects of IFN-α on the Proliferation of Liver Cancer Cell Lines.** Cell proliferation was clearly suppressed in a dose-dependent manner after 48 hours of the culture in all cell lines except KYN-2, HAK-2, and HAK-3, in which dose-dependent suppression was observed after 72 hours. When chronological changes in the ratio of viable cell numbers in the cultures with 1,024 U/mL of IFN-α to the number in the cultures with medium only were monitored, the ratio in the 5 cell lines (KIM-1, KYN-2, KYN-3, HAK-4, and HAK-5) decreased until 72 hours of culture, but it was at a plateau or increased at 96 hours. In the other 8 cell lines, cell proliferation was continuously suppressed with time, though sensitivity was different between the cell lines. Proliferation of all cell lines was suppressed in a dose-dependent manner at 96 hours, and the suppression was significant in the range of 1 to 1,024 U/mL of IFN-α in all cells except KYN-1, and in the range of 8 to 1,024 U/mL in HAK-5 (P < .05-.0001). In particular, the ratio in the 5 cell lines (KYN-1, HAK-1A, HAK-1B, HAK-6, and KMCH-1) decreased to 50% or lower when 1,024 U/mL of IFN-α was added to the cultures, and their 50% inhibitory concentration was 291.4 U/mL in KYN-1, 330.3 U/mL in HAK-1A, 216.1 U/mL in HAK-1B, 86.3 U/mL in HAK-6, and 465.5 U/mL in KMCH-1. Sensitivity to the growth-suppression effect of IFN-α was not related to the histological grade of the original tumors of each cell line. Both the expressions of Hu-IFN-αR2 on the cell surface (positive cell rate) and the amount of soluble Hu-IFN-αR2 in the spent medium were not clearly related to the growth-suppression effect of IFN-α.

**Induction of Apoptosis by IFN-α.** With a phase-contrast microscope, some floating dead cells were found at 24 hours after IFN-α addition in KIM-1, KYN-1, HAK-1A, HAK-1B, and KMCH-1. At 48 hours after the addition, the number of dead cell-proliferating units in all cell lines except KYN-2, HAK-2, and HAK-3 decreased at approximately 80% of the number in the control cultures. However, their specificity has not yet been clarified, and this should be examined in future studies.

**Detection of the Soluble Form of Hu-IFN-αR2 in the Culture Supernatants.** In all 13 cell lines, the soluble form of Hu-IFN-αR2 was detected in the spent medium at 72 hours of culture, but the highest amount was approximately 600 pg/mL. When the amount of the soluble form of Hu-IFN-αR2 was corrected with the amount of cell proteins at the time when the spent medium was collected, the mean level was the highest (1,782.6 ± 77.6 pg/mg protein) in KIM-1, and the lowest (59.8 ± 7.0 pg/mg protein) in HAK-4. KIM-1 expressed Hu-IFN-αR2 on the cell surface at an equivalently low level to KYN-1 and HAK-6, but its expression of soluble Hu-IFN-αR2 was at the highest level. If KIM-1 was excluded, there was a

**Expressions of Hu-IFN-αR2 on the Cell Surface in Each Cell Line.** Hu-IFN-αR2 expression on the cell surface was detectable at a low level in all cell lines except HAK-4, whose expression level was equivalent to that in negative controls. The positive cell rate was higher than 10% in 2 cell lines, i.e., 20.8% in HAK-1B and 13.2% in KMCH-1; in the range between 5 and 10% in 3 cell lines; and ≤5% in 8 cell lines.

**Detection of the Soluble Form of Hu-IFN-αR2 in the Culture Supernatants.** In all 13 cell lines, the soluble form of Hu-IFN-αR2 was detected in the spent medium at 72 hours of culture, but the highest amount was approximately 600 pg/mL. When the amount of the soluble form of Hu-IFN-αR2 was corrected with the amount of cell proteins at the time when the spent medium was collected, the mean level was the highest (1,782.6 ± 77.6 pg/mg protein) in KIM-1, and the lowest (59.8 ± 7.0 pg/mg protein) in HAK-4. KIM-1 expressed Hu-IFN-αR2 on the cell surface at an equivalently low level to KYN-1 and HAK-6, but its expression of soluble Hu-IFN-αR2 was at the highest level. If KIM-1 was excluded, there was a

**Fig. 1.** RT-PCR analysis of the expressions of Hu-IFN-αR1 (A), Hu-IFN-αR2 (B and C), IRF-1 (D), and IRF-2 (E) mRNAs in the 13 liver cancer cell lines. PCR products were electrophoresed in a 2% (A and C) or 4% (B, D, and E) NuSieve agarose gel and stained with ethidium bromide. Lane 1: KIM-1. Lane 2: KYN-1. Lane 3: KYN-2. Lane 4: KYN-3. Lane 5: HAK-1A. Lane 6: HAK-1B. Lane 7: HAK-2. Lane 8: HAK-3. Lane 9: HAK-4. Lane 10: HAK-5. Lane 11: HAK-6. Lane 12: KMCH-1. Lane 13: KMCH-2. Lane M4 and lane M9 are øX176/HaeIII digest and øX174/HinfI digest, respectively, and they were used as the DNA molecular-weight markers. Identical volumes of the PCR reaction mixtures without addition of cDNA, which served as negative controls (NC), produced no DNA bands. The positions of the expected size of intact products are indicated.
cells was markedly larger in all cell lines except KYN-3, HAK-4, and KMCH-2 than in the controls, though the number varied among the cell lines. When these cells were cultured for 72 hours with or without IFN-α (250, 500, or 1,000 U/mL), the frequency of the cells showing characteristic features of apoptosis (e.g., cytoplasmic shrinkage, chromatic condensation, and nuclear fragmentation) tended to increase with the increase of IFN-α level (Fig. 2A and 2B). Quantitative analysis of fragmented DNA revealed that the rate of fragmented DNA (appearance of apoptosis) was significantly higher in the cultures with IFN-α than those without IFN-α in all cell lines except KYN-3, HAK-4, and KMCH-2. Agarose gel electrophoresis of DNAs showed the appearance of a clear ladder of fragmented chromosomal DNA that consisted of the multiples of units comprising 180 to 190 bp in the IFN-α-treated KIM-1, KYN-1, KYN-2, HAK-1A, and KMCH-1. In HAK-1B, the ladder was intensified in the IFN-α-treated cells. In HAK-3 and HAK-6, the ladder was present, though the intensity was weak. Two (HAK-2 and HAK-5) of the 10 cell lines that had a significant increase in DNA fragmentation by IFN-α did not show a clear ladder.

Expression of Apoptosis-Related Proteins in IFN-α-Treated and Untreated Cells. Expression of Bcl-2 was confirmed in the 5 cell lines (except KYN-2 and HAK-6) (Fig. 3A), though the level in KYN-1 was low. IFN-α treatment enhanced the expression by 1.6 times in KIM-1 and 2.6 times in HAK-1B, while the enhancement of the expressions in the other 3 cell lines was lower than 1.5 times. Expression of Bcl-xL protein was confirmed in all cell lines, and it was enhanced by 1.5 times or more in 4 cell lines, i.e., 1.9 times in KIM-1, 1.5 times in KYN-1, 2.5 times in HAK-1B, and 1.8 times in KMCH-1, and the expressions in the other cell lines were either mildly enhanced or not changed (Fig. 3A). Expression of Bak protein was enhanced by 1.5 times in HAK-6 and 1.7 times in KMCH-1, but the expression in the other cell lines increased only slightly. Expression of Bax increased, but the enhancement was lower than 1.5 times in all cells (Fig. 3B).

Effects of IFN-α on Cell Cycle. In 11 of the 13 cell lines (excluding HAK-6 and KMCH-2), the ratio of the cells in the S phase increased, and the ratio of the cells in the G2/M phase slightly decreased or did not change, compared with those in the control cells (no treatment) at any time point during 24 to 96 hours after the addition of IFN-α 250 or 1,000 U/mL to the cultures. In 8 of the 11 cell lines (excluding KIM-1, KYN-2, and HAK-2), the ratio of the cells in the S phase tended to increase in a dose-dependent manner (Fig. 4A). In HAK-6, in which the most apparent growth-suppression effect of IFN-α was observed, the cells tended to be in the G2/M phase in a time- and dose-dependent manner (Fig. 4B). In KMCH-2, in which apoptosis was not induced but cell proliferation was suppressed by IFN-α, the cells tended to be in the G1 phase in a time- and dose-dependent manner (Fig. 4C).

Table 1 summarizes expressions of Hu-IFN-αR2 proteins on the cell surface and in the spent medium, and the effects of

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**Fig. 2.** Photomicrographs of KIM-1 cells cultured for 72 hours on a Lab-Tek Chamber Slide with (A) or without (B) 1,000 U/mL of IFN-α in culture medium. IFN-α-induced apoptosis is characterized by cytoplasmic shrinkage, chromatin condensation, and nuclear fragmentation. (Hematoxylin-eosin; original magnification ×100.)
IFN-α on the proliferation, induction of apoptosis, and cell cycle of the 13 liver cancer cell lines.

**DISCUSSION**

The present study investigated the mRNA expression of type I IFN receptor in 13 human liver cancer cell lines, each of which possesses different morphological characteristics. Human type I IFN receptor consists of two chains, i.e., Hu-IFN-αR1 and Hu-IFN-αR2, and a recent study suggests that the Hu-IFN-αR2 is the binding subunit, while the Hu-IFN-αR1 is a necessary unit to form high-affinity receptors. In regard to Hu-IFN-αR2 expression, cloning of the gene for Hu-IFN-αR2 revealed that it produces four different transcripts encoding three different polypeptides that are generated by exon skipping, alternative splicing, and different use of polyadenylation sites. One polypeptide is the soluble form of the receptor (Hu-IFN-αR2a), and the other two are transmembrane proteins with identical extracellular and transmembrane domains but with divergent cytoplasmic tails of 67 (Hu-IFN-αR2b) and 251 amino acids (Hu-IFN-αR2c). Transfection experiments revealed that expression of the Hu-IFN-αR2c, but not Hu-IFN-αR2b, was required for normal interferon binding, activation of the Jak/STAT signal transduction pathway, IFN-inducible gene expression, and antiviral response. Our RT-PCR analysis revealed the expressions of Hu-IFN-αR1, Hu-IFN-αR2c, and Hu-IFN-αR2a in all cell lines, and that of Hu-IFN-αR2b in all cell lines except KMCH-2. These findings demonstrated that liver cancer cells express, at least at mRNA levels, both Hu-IFN-αR1 and Hu-IFN-αR2c, which are necessary for IFN-α to express its effects. In KMCH-2, expressions of variant mRNAs of Hu-IFN-αR2a and Hu-IFN-αR2c, which would be produced by the skipping of exon 7, were found in addition to the expressions of the normal mRNAs. This indicates the presence of a KMCH-2-specific alternative-splicing mechanism; however, the precise mechanism and meaning of these variant expressions should be investigated further.

Hu-IFN-αR2 expression at a protein level was then examined with flow cytometry. As a result, generally weak expression of Hu-IFN-αR2 was detected on the cell surface in all cell lines except HAK-4. IFN-α suppressed proliferation of all cell lines in a dose-dependent manner at 96 hours of culture, but there was no clear relationship between the amount of Hu-IFN-αR2 expression (positive cell rate) on the cell surface and the suppressive effect of IFN-α.

The true reasons for this are unknown at present, but five possible explanations could be suggested. First, the antibody used in the present study is thought to recognize the number of binding sites of Hu-IFN-αR2; however, for the expression of IFN-α effects, not only the number of binding sites, but also the binding affinity of type I IFN receptor to IFN-α is important. Binding affinity could be modulated by the expressions of Hu-IFN-αR1 and additional membrane proteins; therefore, Hu-IFN-αR2 expression would not be related to the growth-suppression effects of IFN-α. Second, soluble Hu-IFN-αR2 could act as an antagonist and neutralize the effects of IFN-α. Soluble Hu-IFN-αR2 was detected at various levels in culture media of all cell lines, and there was a relatively clear relationship between the amount of Hu-IFN-αR2 protein expressed on the cell surface and that of soluble Hu-IFN-αR2 protein, when KIM-1 was excluded from the analysis. This indicated that the majority of the cell lines express Hu-IFN-αR2 on the cell surface and in the soluble form at a certain ratio, but some cell lines overexpress soluble
Hu-IFN-αR2. However, it is questionable whether soluble Hu-IFN-αR2 protein would act as an antagonist because the highest level of the soluble Hu-IFN-αR2 in the present study was approximately 600 pg/mL, which is within the normal range of urinary concentration in humans (0.1 to 1.0 ng/mL) or lower than the blood level in healthy human subjects (1.76 ± 0.74 ng/mL). Third, the antibody used in the present study recognized not only Hu-IFN-αR2c, which mediates the activation of the Jak/STAT pathway, but also Hu-IFN-αR2b, which does not take a direct function in the activation of the Jak/STAT pathway. Fourth, sensitivity to IFN-α could be decreased as a result of the abnormalities of the signaling molecules of the STAT or IRF family of transcription factors. In regard to IRF-1, there have been reports on gene deletion and rearrangement at the DNA level, and the suppression of normal IRF-1 mRNA expression as a result of the increased exon skipping in preleukemic and leukemic cells. RT-PCR analysis revealed that our 13 cell lines express both IRF-1 and IRF-2 mRNAs, and in regard to IRF-1, the expression of intact IRF-1 products was higher than those of the exon-skipped forms of IRF-1. The human IRF-1 gene is mapped on chromosome 5q31.1. In our previous chromosomal analysis for the 13 cell lines, only KYN-2 lacked one chromosome 5, and this was thought to result in the lack of one IRF-1 allele. This could lower the sensitivity of KYN-2 to the growth-suppression effects of IFN-α, because IRF-1 manifests antiproliferative properties and restrains cell growth according to the balance between IRF-1 and its antagonistic repressor, IRF-2. Fifth, the effects of IFN-α could be decreased as a result of the abnormalities of IFN-α-inducible genes that relate to cell growth suppression. In the present study, growth-suppression mechanisms induced by IFN-α varied widely, and even among the cell lines that showed the same pattern, the suppression level was different. Therefore, we hypothesize that each cell line presents different abnormalities or varying degrees of abnormalities in genes that relate to the regulation of apoptosis and cell cycle; these then result in different growth-suppression mechanisms of IFN-α or different sensitivity to IFN-α, and as a result, the growth-suppression effect of IFN-α was not related to Hu-IFN-αR2 expression on the cell surface.

To date, IFN-α has been reported to induce apoptosis in various neoplastic cells other than liver cancer cells and in normal cells, but IFN-α has also been reported to suppress apoptosis in some cells. In the present study, morphological and DNA analyses revealed that IFN-α could induce or enhance apoptosis in 10 of the 13 cell lines (except KYN-3, HAK-4, and KMCH-2). This suggests that IFN-α induces apoptosis in liver cancer cell lines at a relatively high frequency. Interestingly, cell lines with a larger amount of fragmented DNA do not always form a clearer DNA ladder, and this suggested that DNA ladder formation associated with apoptosis is the phenomenon limited to the individual cell line.

The mechanism of IFN-α-mediated apoptosis has not yet been clarified. In the 13 cell lines, in which IFN-α induced apoptosis, we examined whether the four members of the Bcl-2 family, which is the important regulator of apoptosis (i.e., Bcl-2, Bcl-xL, Bak, and Bax), were involved in IFN-α-mediated apoptosis. As a result, the expressions of these four proteins were not thought to be the direct inducer of IFN-α-mediated apoptosis. Additionally, normal p53 gene expression is not necessary for apoptosis induced by IFN-α, because mutations at codon 242 in the p53 gene were found in HAK-1A and HAK-1B that showed IFN-α-mediated apoptosis. This point also agreed with the previous findings in the other cell lines. Recent studies reported the involve-
The present study showed that IFN-α directly suppresses the proliferation of liver cancer cells by inducing apoptosis and the inhibition of cell-cycle progression. However, it is still unknown whether IFN-α prevents in vivo oncogenesis by expressing these effects in the very-early-stage, clinically undetectable cancer cells and by suppressing their growth. It is because the peak serum level of IFN-α is only 23 to 53 U/mL with the dose of IFN-α used in the intramuscular injection (500,000 million units) for type C chronic hepatitis, and this level is lower than the 50% inhibitory concentration of HAK-6, i.e., 86.3 U/mL, which showed the accumulation of the cells in the S phase. However, Hobelka et al.,63 and Sangfelt et al.,60 reported the induction of G1 arrest in cells without a normal Rb gene. Therefore, not only a wild-type Rb protein, but also another abnormality in the molecules that regulate cell cycle could be involved.

IFN-α was reported to decrease the frequency of HCC in HCV-related cirrhotic patients,64,65 and among HCV-related chronic hepatitis patients who received IFN-α treatment, frequency of HCC was reported to be lower in responders in whom alanine aminotransferase levels were normalized than in nonresponders.66 It is unknown how HCV contributes to HCC development; however, when hepatocytes continuously receive damage and have replication, the frequency of genetic alteration would increase, and this would lead to the development of HCC.65,66 On the other hand, the mechanisms by which IFN-α suppresses liver cancer development have not been clarified, but possible mechanisms are: 1) the reduced incidence of genetic abnormalities accumulated during cell division, which results from the slowed cell cycle in hepatocytes as a result of the sedation of inflammation; 2) activation of the immune system (e.g., increase of natural killer cell activity); and 3) direct suppressive effects on cancer cells.65

The present study showed that IFN-α on the cell cycle of various normal and tumor cell lines, induction of G1 arrest has been well reported.25,27,34 Additional effects reported are the S phase prolongation or S phase block,27,30 G2/M arrest,31 and others.32 In the present study, blockage of cell cycle at the S phase was found at the highest frequency. The other effects observed were the induction of blockages in the G1 and G2/M phases in 1 cell line each. Known mechanisms in regard to IFN-α-induced G1 arrest are the inhibition of p21/WAF1 and cyclin E- and cyclin D1-dependent kinase 2 activities, suppression of retinoblastoma (Rb) phosphorylation, lowered E2F DNA binding activity, and suppression of c-myc expression.25,57,63 In the liver cancer cells, abnormalities would be present in some of these induction systems. Recently, Qin et al.28 reported proper IFN signaling and loss or inactivation of the normal G1 checkpoint conferred by the Rb protein as a mechanism of IFN-α- and IFN-β-mediated accumulation of the cells in the S phase. However, Hobelka et al.,63 and Sangfelt et al.,60 reported the induction of G1 arrest in cells without a normal Rb gene. Therefore, not only a wild-type Rb protein, but also another abnormality in the molecules that regulate cell cycle could be involved.

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highest sensitivity to IFN-α. In regard to this point, Gutterman\textsuperscript{18} stated that it is highly likely that IFN applied early in the stages of tumor evolution could have a very important clinical effect, whereas its activity in advanced stages in which multiple genetic aberrations are present, would be minimal. Therefore, the sensitivity of early-stage HCC to IFN-α could not be estimated from the sensitivity of the cell lines that possess a larger number of gene abnormalities and higher proliferation capability. Among clinical trials that administered IFN-α to advanced HCC patients, Lai et al.\textsuperscript{22} used a quite higher dose of IFN-α than in the other studies\textsuperscript{23,24} and they obtained higher response rates.\textsuperscript{22} Therefore, such a low dose of intramuscular IFN-α used in treatment for chronic hepatitis could not induce apoptosis or cell-cycle arrest in advanced HCC. If the route of administration is changed and a higher dose of IFN-α is administered to the cancer lesion, e.g., by continuous administration into tumor-feeding arteries, a growth-suppression effect would be expected. In addition, concomitant administration of IFN-α could magnify the antitumor effect of a drug that acts specifically on the cells in the S phase, because IFN-α induces accumulation of S-phase cells at a high frequency.

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