Combination of 5-FU and IFNα enhances IFN signaling pathway and caspase-8 activity, resulting in marked apoptosis in hepatoma cell lines

KAZUKO KOIKE, AKINOBU TAKAKI, MASASHI TATSUKAWA, MAYUMI SUZUKI, HIDENORI SHIRAHA, YOSHIAKI IWASAKI, KOHSAKU SAKAGUCHI and YASUSHI SHIRATORI

Department of Gastroenterology and Hepatology, Okayama University Graduate School of Medicine and Dentistry, Okayama city, Japan

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Abstract. Interferon (IFN) combined with 5-Fluorouracil (5-FU) treatment has recently been reported to show beneficial effects in patients with advanced hepatocellular carcinoma. IFNα is usually provided for this combination therapy. In this study, we investigated the molecular mechanisms of apoptosis induction in hepatoma cell lines with IFNα and 5-FU combination therapy from the view point of 5-FU's additive effect on interferon-related signaling pathways. Five hepatoma cell lines (Hep3B, Huh7, HLE, PLC/PRF/5, and HepG2) were tested for apoptosis inducibility by IFNα in the absence or presence of 5-FU. Hep3B was the most apoptosis sensitive to IFN plus 5-FU treatment. The JAK/STAT pathway transcriptional factor ISRE was activated more synergistically when 5-FU was added to IFNα treatments. Caspase-3, -9, and especially caspase-8 activity was higher with IFN α plus 5-FU than IFN or 5-FU alone. Inhibition of caspase-8, -9, c-Jun N-terminal kinase (JNK), phosphatidylinositide 3-kinase (PI3K), and p38 mitogen-activated protein kinase (p38 MAPK) revealed that caspase-8 inhibition was the most effective at decreasing the apoptotic effects of IFN and/or 5-FU. In JAK1 and ISGF3γ-silenced Hep3B cells, the apoptosis induction and caspase-8 activation levels by IFN, even in combination with 5-FU, were abrogated. In conclusion, caspase-8 is the most important factor that controls IFN and 5-FU-induced apoptosis in hepatoma cell lines.

Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent and fatal malignancies in the world, especially in Eastern Asia where hepatitis viruses are endemic (1,2). Chronic infection by the hepatitis C and hepatitis B virus is the main cause of chronic hepatitis, liver cirrhosis, and HCC. However, its prognosis is still poor (3,4), and the tumor recurrence rate is high (>50% at 3 years, even after tumor ablation or surgery) (5). Therapeutic strategies for advanced HCC are limited to transcatheter arterial chemoembolization (TACE) and chemotherapy using 5-FU, cisplatin (CDDP), methotrexate (MTX), mitomycin C (MMC), leucovorin (LV), and interferon (IFN) (6-12).

IFNα is a cytokine that exhibits important biological activities *in vitro* and *in vivo*, including immunomodulatory, antiviral, antiproliferative and apoptosis-inducible effects (13,14). This cytokine is used as an anti-cancer drug for renal cell carcinoma, chronic myelogenous leukemia, and malignant melanoma. There are a number of studies that clarify the molecular mechanisms underlying the anti-cancer effect of IFN, such as cell cycle arrest, apoptosis induction, the immune modulatory effect, and anti-angiogenesis.

IFNα exerts its biological actions by binding to high-affinity cell-surface receptors that stimulate phosphorylation of tyrosine residues on type I receptor components and on the receptor-associated tyrosine kinases, Tyk2 and JAK1. These phosphorylated residues serve as recruitment sites for STAT proteins, which bind the activated receptor and are in turn phosphorylated by the JAKs. The phosphorylated STAT proteins then form homodimers or heterodimers with other STAT proteins and translocate to the nucleus, where they bind specific DNA sequences within the promoter regions of IFN-stimulated genes (ISGs). In the case of signaling via IFNα, phosphorylated STAT1 and STAT2 bind to each other as well as a third component, IFN regulatory factor 9 (ISGF3γ/p48), to form the transcription factor ISGF3, which binds a promoter region called IFN-stimulated response element (ISRE).

In addition to the JAK/STAT signaling pathway, the mitogen-activated protein kinase (MAPK) pathway is also involved in IFN-induced biological effects. Cross-talk between the JAK/STAT and MEK/ERK pathway has been well documented (15-19).

Several reports have revealed that IFN is effective for patients with HCC when used as a biochemical modulator of anti-cancer drugs (20,21). A combination therapy of IFNα with 5-FU has been reported to be effective for patients with advanced HCC (22-25). This combination therapy was conducted for advanced gastrointestinal cancers and led to a favorable outcome (26,27). The mechanisms behind the

Correspondence to: Dr Akinobu Takaki, Department of Gastroenterology and Hepatology, Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikata-cho, Okayama 700-8558, Japan
E-mail: akitaka@md.okayama-u.ac.jp

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additive effect of 5-FU and IFN combination therapy, which have been examined using colorectal carcinoma cell lines and renal cell carcinoma cell lines, are considered as follows: a) IFN suppresses the salvage pathway for deoxythymidine monophosphate induction (28), b) IFN induces DNA damage (29), c) IFN increases the level of thymidine phosphorylase that enhances the conversion of 5-FU to its active metabolite 5-fluorodeoxyuridine monophosphate (FdUMP) (30-32), and d) IFN leads to abrogation of a 5-FU-associated increase in the enzyme thymidylate synthase (TS) (33). Using hepatoma cell lines, IFN has been reported to enhance the cyclin dependent kinase inhibitor (CDKI) that modulates the cell cycle (34). Most studies concentrated on IFN's additive effect for 5-FU's effect, but since IFN itself has several anticancer effects, we examined the effect of 5-FU on IFN signaling related pathways.

In this study, we used HCC cell lines to examine the molecular mechanisms for IFNα combined with 5-FU from the standpoint of the intracellular signaling pathway.

Materials and methods

Cell lines and reagents. The hepatoma cell lines Hep3B, PLC/PRF/5, Huh7, HLE, and HepG2 were maintained in Dulbecco's modified Eagle's medium (D-MEM) (Invitrogen Co., Carlsbad, CA). All media were supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Vitromex, Vilshofen, Germany), 1% non-essential amino acid (Sigma chemical, MO), 1% sodium pyruvate (Sigma-Aldrich Co., St. Louis, MO), and 1% penicillin/streptomycin solution (Sigma-Aldrich Co.). The cells were cultured at 37˚C in a humidified atmosphere of 5% CO_2.

Human IFNα2 was kindly provided by Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan), and 5-FU was provided by Kyowa Hakko Kogyo Co. Ltd. (Tokyo, Japan). Z-LEHD-FMK, a caspase-9 inhibitor, and Z-IETD-FMK, a caspase-8 inhibitor, were purchased from BD Biosciences (San Diego, CA). The p38 MAPK inhibitor (SB202190), p38 MAPK inhibitor negative control (SB202474), selective PI3K inhibitor (wortmannin), PI3K inhibitor negative control (LY303611), JNK inhibitor, and JNK inhibitor negative control were obtained from Calbiochem (La Jolla, CA).

Assessment of apoptosis by propidium iodide (PI) staining. Cells were loaded into 6-well plates ($2x10^5$ per well) containing D-MEM with 10% FCS and treated with IFNα (1000 IU/ml) and/or 5-FU (10 μg/ml). After 72 h, the cultured cells were collected and washed with phosphate-buffered saline (PBS). RNaseA (1 mg/ml) (Sigma-Aldrich Co.) was added for 30 min at 37˚C following propidium iodide (PI) (1 mg/ml) (Sigma-Aldrich Co.) staining. Samples were acquired with FACScan (Becton Dickinson Immunocytometry Systems, CA) and were analyzed using Cell Quest software (Becton Dickinson Immunocytometry Systems).

Western blot analysis of JAK/STAT and p38 MAPK pathways. The IFN-induced apoptosis-sensitive Hep3B cells were cultured in 6-well plates ($3x10^5$ per well) for 48 h and then treated with IFNα (1000 IU/ml) and/or 5-FU (10 μg/ml). Cultured cells were washed twice with ice-cold PBS and lysed with lysis buffer (0.1 M Tris-HCl, 4% SDS, 10% Glycerol, 0.004% bromophenol blue, 10% 2-mercaptoethanol). The lysates were collected and boiled for 5 min. Samples were electrophoresed in a 7.5% or 10% sodium dodecyl sulfate-polyacrylamide gel, and transferred to a transfer membrane (Millipore Co. Bedford, MA). Membranes were blocked in 5% BSA in 20 mmol/l Tris-HCl (pH 7.6), 137 mmol/l NaCl, and 0.1% Tween-20 (TBS-T) for 1 h at 37˚C, and then probed at 4˚C overnight with antibodies in TBS-T containing 1% BSA. After washing, the membranes were incubated with a peroxidase-conjugated secondary antibody (Amersham Biosciences Co., Piscataway, NJ) at room temperature for 1 h, and visualized with an enhanced chemiluminescence detection system (Amersham Biosciences Co.).

The antibodies used for Western blotting were rabbit anti-phospho-STAT1 antibody (Ab), anti-phospho-JAK1 Ab, rabbit anti-phospho-Tyk2 Ab, rabbit anti-p38 MAPK Ab, rabbit anti-p42/44 MAPK Ab, mouse anti-phospho-p38 MAPK Ab, mouse anti-phospho-p42/44 MAPK Ab (Cell Signaling Technology, Beverly, MA); and mouse anti-STAT1 Ab, mouse anti-JAK1 Ab, mouse anti-Tyk2 Ab, and mouse anti-ISGF3γ Ab (Becton Dickinson).

Luciferase reporter assays of Elk-1 and Interferon-stimulated response element (ISRE). To measure Elk-1 activity levels, we used a reporter system (Pathdetect, Stratagene, La Jolla, CA) with fusion proteins comprised of a GAL4 DNA binding domain fused to the activation domain of Elk-1. Hep3B was cultured in 10-cm dishes, harvested, and resuspended in 1 ml of cold PBS. Thirty μg of fusion trans-activator plasmid (pFA2-Elk1), 30 μg of reporter plasmid (pFR-Luc), and 10 μg of control plasmid-encoding renilla luciferase (pRL-TK) were added to the cells. The mixture was transferred into a 4 mm electroporation cuvette (Bio-Rad, Hercules, CA). Electroporation was carried out with an Electroporator (Gene pulser, Bio-Rad) using a single electric pulse (voltage, 300 V; capacitance, 950 microfarad; cuvette gap, 4 mm). Immediately after transfection, the cells were transferred to a 6-well plate. After 36 h, the cells were cultured with IFNα (1000 IU/ml) in the presence or absence of 5-FU (10 μg/ml) for 12 h. The cells were lysed using the passive lysis buffer (Promega, Madison, WI), and the activity of firefly and Renilla luciferases was measured with a luminometer (Berthold Technologies, Bad Wildbad, Germany).

For the ISRE activity reporter assays, we used a luciferase reporter plasmid vector, 30 μg of ISRE-luc (CLONTECH Laboratories, Palo Alto, CA), and 10 μg of control plasmid-encoding renilla luciferase (pRL-TK). After 12 h, the cells were cultured with IFNα (1000 IU/ml) and/or 5-FU (10 μg/ml) for 24 h.

Measurement of caspase activity. To measure caspase-3, -8, and -9 activity, a caspase colorimetric protease assay kit (Biovision, Palo Alto, CA) was used according to the manufacturer's protocol. In brief, the cells ($3x10^5$ per well) were seeded on 6-well dishes, and treated with IFNα (1000 IU/ml) and/or 5-FU (10 μg/ml) for 48 h. After washing twice with ice-cold PBS, the cells were lysed with cell lysis buffer and incubated on ice for 10 min. Cells were harvested by scraping and then

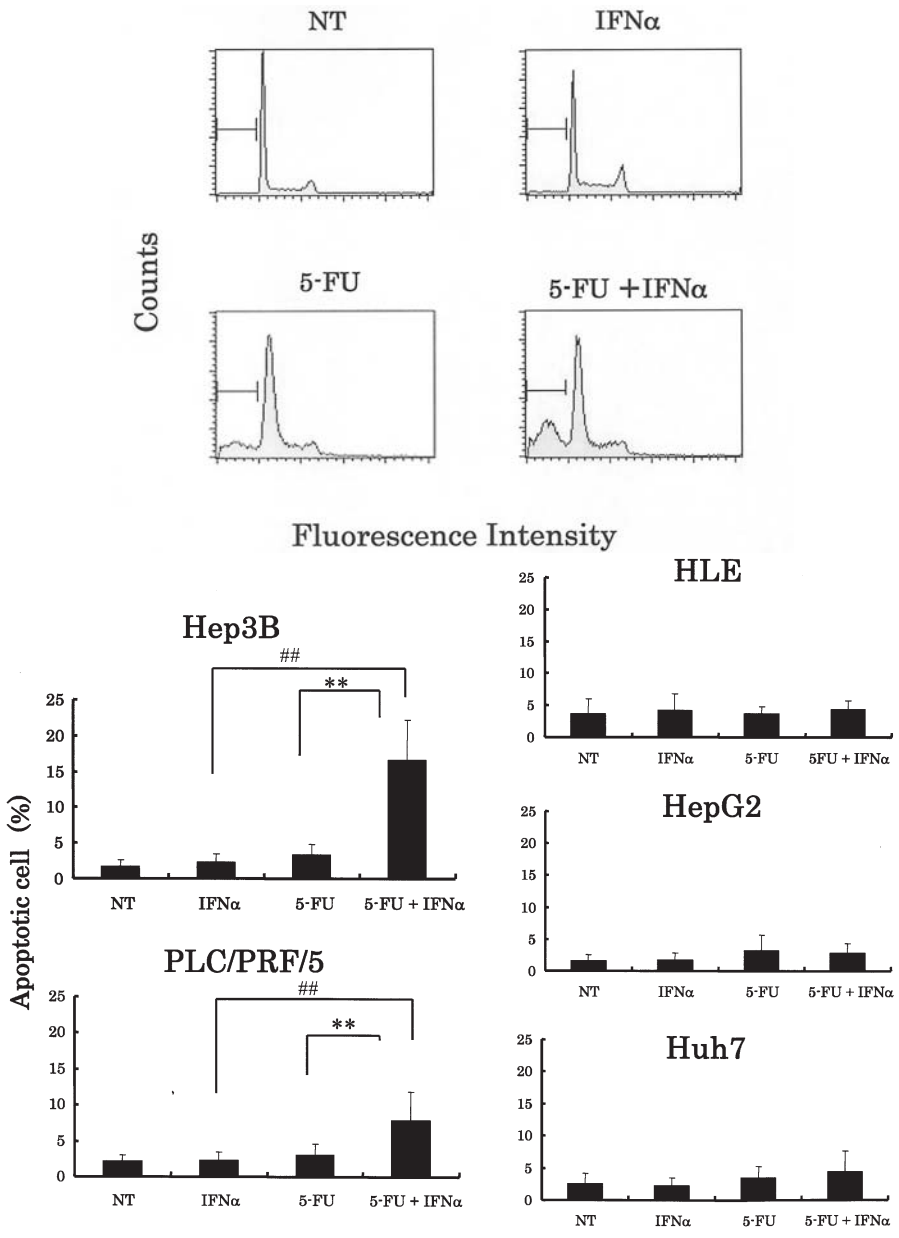

Figure 1. Apoptosis of human hepatoma cells by IFNα and/or 5-FU treatment. (A) Flow cytometric analysis of Hep3B cells. Hep3B was treated with 1000 IU/ml of IFNα and/or 10 μg/ml of 5-FU for 72 h. Cells were then stained with propidium iodide and subjected to DNA content analysis by flow cytometry. NT, no treatment. (B) Summary of IFNα- and/or 5-FU-induced apoptosis of human hepatoma cells. Hep3B, PLC/PRF/5, HLE, HepG2, Huh7 cells were treated with 1000 IU/ml of IFNα and/or 10 μg/ml of 5-FU for 72 h. The percentage of G0-G1 phase cells was estimated by point analysis. Bars represent means ± SD; $^{**}p<0.01$ vs. cells treated with 5-FU only; $^{\#\#}p<0.01$ vs. cells treated with IFNα only.

centrifuged at 13200 rpm for 1 min. The supernatants were transferred to fresh tubes. After adding reaction buffer (containing DTT) and DEVD-pNA substrate to a 96 U assay plate, samples were incubated at 37˚C for 3 h, and read at 405 nm in a microplate reader (Bio-Rad).

Effect of caspase inhibitor on IFN-related apoptosis. To inhibit caspase activity, a specific caspase-9 inhibitor, Z-LEHD-FMK (BD Biosciences), or a specific caspase-8 inhibitor, Z-IETD-FMK (BD Biosciences), was added to cell cultures at a final concentration of 10 μM 1 h prior to the addition of 5-FU and/or IFNα.

Effect of MAPK, JNK, and PI3K inhibitors. Hep3B cells were pretreated with 10 μM of p38 MAPK inhibitor, 10 μM of a MAPK inhibitor negative control, and 10 μM of JNK inhibitor, and 10 μM of a JNK inhibitor negative control for 1 h, and then cultured with IFN and/or 5-FU for 72 h. A PI3K inhibitor (wortmannin) was added to cell cultures at a final concentration of 1 μM 1 h prior to the addition of IFNα and/or 5-FU. After 72 h of culture, the cells were collected and stained with propidium iodide (PI) (1 mg/ml). Samples were analyzed by flow cytometry.

Effect of JAK1 and ISGF3γ gene silencing with small-interfering RNA (siRNA). siRNA duplexes targeting JAK1 and ISGF3γ sequences and stealth negative control were obtained from Invitrogen Co. (Carlsbad, CA).

Hep3B cells were seeded on a 6-well plate ($1.0x10^5$ per well). After overnight incubation, 4 μl of Lipofectamine™

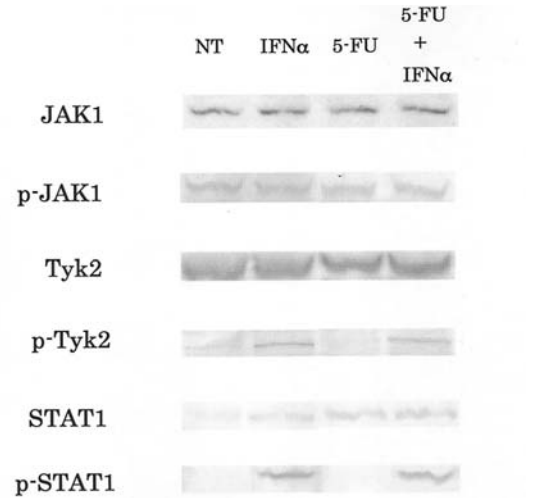

Figure 2. Western blot analysis of JAK/STAT signal transduction pathways in Hep3B cells. Hep3B cells were stimulated with IFNα (1000 IU/ml) and/or 5-FU (10 μg/ml) for 30 min, then harvested for Western blot analysis with the antibodies indicated on the left side of each panel: anti-JAK1, anti-phospho-JAK1, anti-Tyk2, anti-phospho-Tyk2, anti-STAT1, and anti-phospho-STAT1.

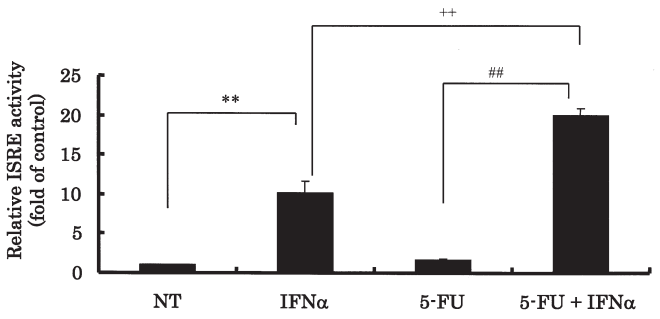

Figure 3. ISRE luciferase assay by IFN and/or 5-FU. Hep3B cells were transfected with the reporter plasmid pISRE-luc, divided into aliquots, and incubated for 24 h. Transfected cells were treated with IFNα (1000 IU/ml) and/or 5-FU (10 μg/ml). Luciferase activity was assayed 24 h after treatment. The data are shown as fold-increase in luciferase activity in response to IFNα and/or 5-FU treatment. The fold-increase in each experiment was calculated by dividing the relative luciferase units in treated samples with the relative luciferase units in untreated samples. The data represent mean ± SD of triplicate measurements. $^{**}p<0.01$ vs. non-treated (NT) cells. $^{\#\#}p<0.01$ vs. cells treated with 5-FU only. $^{++}p<0.01$ vs. cells treated with IFN only.

2000 (Invitrogen) was added to 200 μl of Opti-MEM (Gibco, Invitrogen Corp., Carlsbad, CA) media and incubated at room temperature for 5 min. An aliquot (4 μl) of 20 μM siRNA solution was then added to 200 μl of Opti-MEM, and incubated for an additional 20 min at room temperature, mixing occasionally. Then, an additional solution was added to the cells, and the cells were left for 24 h and then treated with IFNα and/or 5-FU as described previously. Cells were harvested and utilized for the following analysis.

Total RNA was purified from siRNA-transfected cells with Trizol reagent (Invitrogen) according to the manufacturer's protocol. The lamin A/C mRNA expression level was determined by real-time PCR using the QuantiTect gene expression assay system (Qiagen, Valencia, CA) and Light-Cycler (Roche Diagnostics, Basel, Switzerland) in order to verify that non-specific suppression did not occur during siRNA transfection.

The interference of JAK1 protein expression was confirmed by Western blot analysis using an anti-JAK1 antibody and anti-phospho-JAK1 antibody. A luciferase gene reporter assay for ISRE activation was then performed as described above. The interference of ISGF3γ protein expression was confirmed by Western blot analysis using an anti-ISGF3γ antibody.

Statistics. Data were analyzed using the Student's *t*-test. Each set of data represents the mean and SD from at least three independent experiments. A p-value of <0.05 was considered statistically significant.

Results

Apoptosis induction on hepatoma cell lines with IFN and/or 5-FU. In order to investigate the apoptosis sensitivity against IFN alone among the hepatoma cell lines, we tested apoptosis levels of Hep3B, PLC/PRF/5, HLE, HepG2, and Huh7 cells treated with IFNα and/or 5-FU by flow cytometry (Fig. 1). In Hep3B cells, the percentage of sub-G0/G1 apoptotic cells increased with IFNα and/or 5-FU treatment. IFN α or 5-FU alone showed only a slight increase in apoptosis levels compared with no treatment, whereas the combination of IFNα with 5-FU resulted in strong apoptotic effects (Fig. 1A). On the other hand, IFN treatment alone did not induce apoptosis on PLC/PRF/5, HLE, HepG2, and Huh7 cells. Of these 4 cell lines, only PLC/PRF/5 was apoptosis sensitive to IFNα and 5-FU treatment (Fig. 1B).

For the following experiments, we used Hep3B cells, which were the most sensitive to IFNα and 5-FU-induced apoptosis.

Activation of JAK/STAT pathway during IFNα and/or 5-FU treatment in Hep3B. To determine JAK/STAT signaling activation, we assessed the phosphorylation of JAK/STAT signaling pathway proteins, *i.e*. JAK1, Tyk2, and STAT1, by Western blotting (Fig. 2). Tyk2 and STAT1 proteins were phosphorylated by IFNα treatment. The addition of 5-FU had no effect on phosphorylation status.

Activation of ISRE with IFNα and/or 5-FU. To clarify the functional role of the IFN-inducible genes in IFNα and 5-FU induced apoptosis, we measured ISRE activity using luciferase reporter assays. The cells were treated with IFNα and/or 5-FU for 24 h. IFNα treatment induced high levels of ISRE luciferase activity in Hep3B cells. The combination with 5-FU induced approximately 1.5-fold higher luciferase activity of ISRE than IFNα single treatment (Fig. 3).

Activation of caspase-3, -8, and -9 with IFNα and/or 5-FU. The activation level of caspases in IFNα- and/or 5-FU-treated cells was shown by the fold increase in caspase activity compared to no-treatment cells (Fig. 4). Caspase-8 activity increased by 1.2-fold with IFNα, 1.9-fold with 5-FU, and 3.5-fold with 5-FU plus IFNα versus the no-treatment cells. Similar results were obtained from caspase-9 and -3, *i.e*. IFNα and 5-FU combination resulted in significantly higher activation of caspases, especially caspase-8.

Activation of p38 MAPK and ERK1/2 (p42/44) MAPK with IFNα and/or 5-FU. To determine the activation status of the

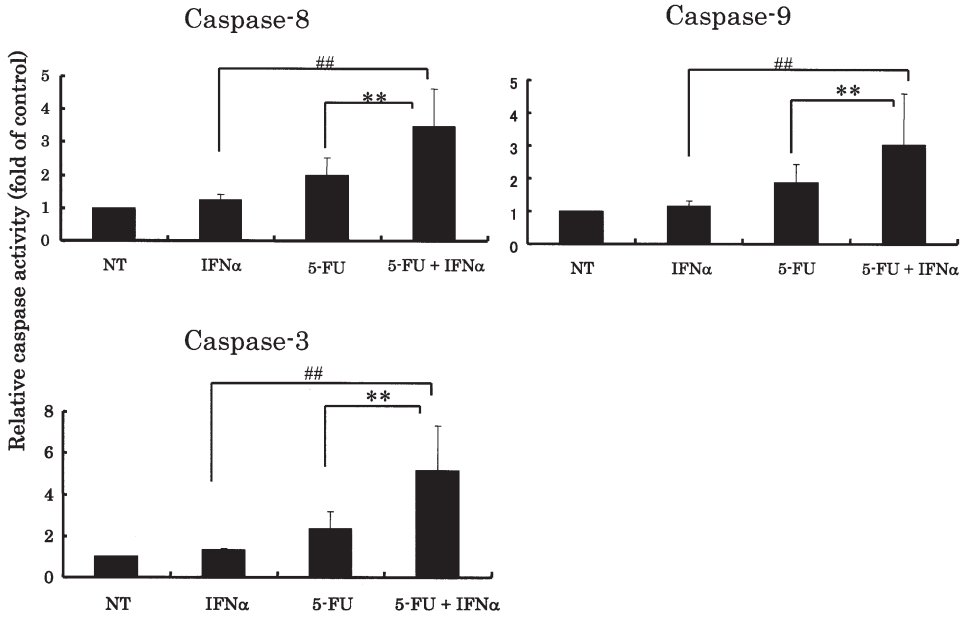

Figure 4. Caspase activity with IFN and/or 5-FU treatment. Hep3B cells were stimulated with IFNα (1000 IU/ml) and/or 5-FU (10 μg/ml) for 48 h. Caspase activity was measured as written in the methods section. The results are expressed in fold-increase activity compared to untreated (NT) cells. The data represent mean ± SD of triplicate measurements. **p<0.01 vs. cells treated with 5-FU only. ##p<0.01 vs. cells treated with IFNα only.

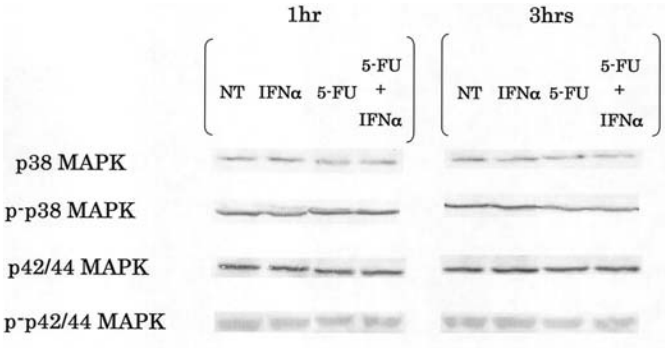

Figure 5. MAPK pathway activation with IFN and/or 5-FU. Western blot analysis of MAPK pathway in Hep3B cells: Hep3B cells were stimulated with IFNα (1000 IU/ml) and/or 5-FU (10 μg/ml) for 1 or 3 h and then harvested for Western blot analysis. The antibodies used are indicated on the left side of each panel: anti-p38 MAPK, anti-phospho-p38 MAPK, anti-MAPK (42/44), and anti-phospho-MAPK (42/44).

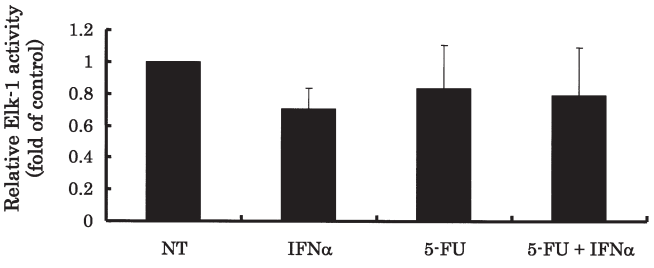

Figure 6. MAPK-related transcriptional factor Elk-1 activity. Hep3B cells were transfected with Elk-1 reporter plasmid and then treated with IFNα (1000 IU/ml) and/or 5-FU (10 μg/ml). Luciferase activity was assayed 24 h after treatment. The data are shown as fold-increase in luciferase activity in response to IFNα and/or 5-FU treatment. The fold-increase in each experiment was calculated by dividing the relative luciferase units in treated samples by the relative luciferase units in untreated samples (NT). The data represent mean ± SD of triplicate measurements.

p38 MAPK pathway, which is known to correlate with the JAK/ STAT signaling pathway, Western blotting was carried out for total and phosphorylated p38. The phosphorylation level of p38 at 1 h and 3 h of IFNα only treatment, 5-FU only treatment, and combination treatment was similar. The same experiment using ERK1/2 (p42/44) antibody was also performed. The phosphorylation level of the ERK1/2 (p42/44) was similar for all treatment groups (Fig. 5).

Elk-1 activation status with IFNα and/or 5-FU. Elk-1 is a downstream transcription factor of ERK1/2 (p42/44). We performed luciferase reporter assays for Elk-1 activation. IFNα did not show any effects on Elk-1 activity. Even with the combination of 5-FU, Elk-1 activity did not change (Fig. 6).

Gene silencing using JAK1 and ISGF3γ siRNA. To determine the importance of the JAK/STAT pathway on IFN and 5-FU-induced apoptosis, siRNA of the IFNα receptor binding kinases, JAK1, and a positive regulator of transcription, ISGF3γ, were used. Western blot analysis showed that JAK1 siRNA inhibited JAK1 protein expression by 60% and ISGF3γ siRNA inhibited ISGF3γ protein expression by 57% in Hep3B cells. Lamin A/C mRNA remained constant in Hep3B cells (88±1%, 125±14% as ratio of not siRNA-transfected cells to JAK1 siRNA-transfected cells and ISGF3γ siRNA-transfected cells, respectively).

In the JAK1-silenced Hep3B cells, the JAK1 phosphorylation was not induced by IFN.

JAK1 siRNA inhibited IFN-induced ISRE luciferase activity by 69±10% in Hep3B cells. Similarly, the induction of luciferase activity via the ISRE element was significantly reduced to 69±16% in ISGF3γ silencing compared to negative control cells (Fig. 7A).

In the JAK1-silenced Hep3B cells, the apoptotic effect of IFN in combination with 5-FU was abrogated. In the

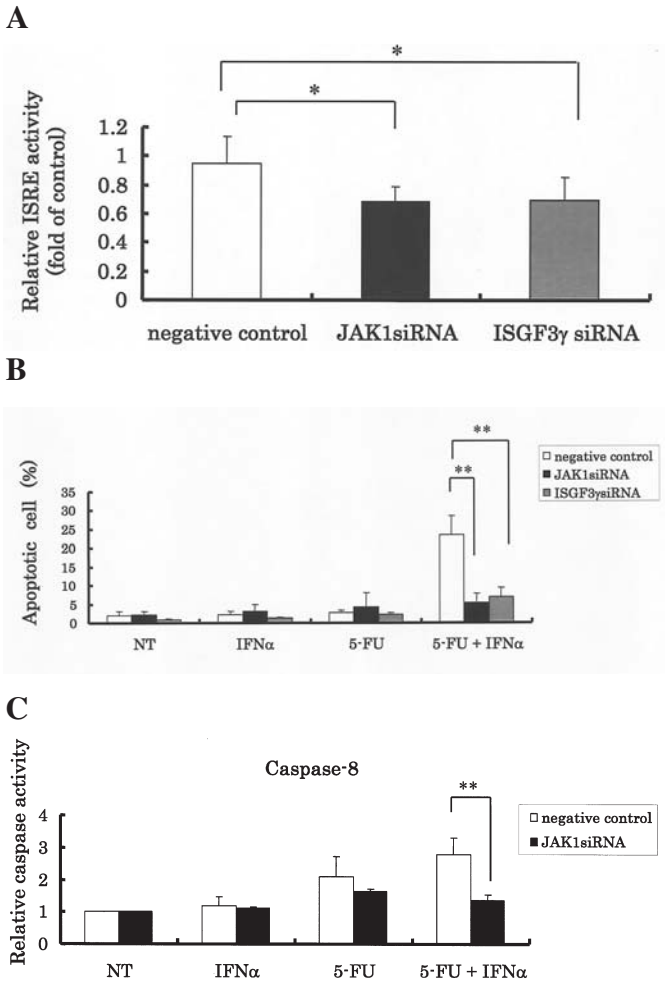

Figure 7. Effects of JAK1 and ISGF3γ silencing on IFN and/or 5-FU treatment. Negative control (nonsilencing) siRNA (40 nM) (white bar), JAK1 siRNA (40 nM) (black bar), and ISGF3γ siRNA (40 nM) (gray bar) were transfected into Hep3B cells and incubated for 24 h. (A) Hep3B cells were transfected with the reporter plasmid pISRE-luc, divided into aliquots, and incubated for 24 h. Then, negative control (nonsilencing) siRNA (40 nM), JAK1 siRNA (40 nM), and ISGF3γ siRNA (40 nM) were transfected, respectively. After 24 h, cells were treated with IFNα (1000 IU/ml). Luciferase activity was assayed 24 h after treatment. The data are shown as fold-increase in luciferase activity in response to IFNα. The fold-increase in each experiment was calculated by dividing the relative luciferase units in siRNA-transfected samples with the relative luciferase units in non-transfected samples. (B) The effect of JAK1 and ISGF3γ gene silencing on IFN- and 5-FU-induced apoptosis: Hep3B cells were treated with IFNα (1000 IU/ml) and/or 5-FU (10 μg/ml) for 72 h. Apoptosis was determined by the flow cytometric analysis of propidium iodide-stained DNA content. (C) Caspase-8 activity in JAK1 gene silencing: Hep3B cells were stimulated with IFNα (1000 IU/ml) and/or 5-FU (10 μg/ml) for 48 h. Caspase-8 activity was measured as written in the methods section. The results are expressed in fold-activity compared with untreated (NT) cells. The data represent mean ± SD of triplicate measurements. *p<0.05 vs. negative control siRNA-transfected cells. **p<0.01 vs. negative control siRNA-transfected cells.

ISGF3γ-silenced Hep3B cells, the apoptotic effect was also abrogated (Fig. 7B).

The caspase-8 activity was also abrogated in JAK1 siRNA-transfected cells (Fig. 7C). Similarly, caspase-8 activity was reduced in ISGF3γ silencing compared to negative control cells (data not shown).

Effect of caspase-specific inhibitors. To clarify the importance of caspase-8 and -9, we used inhibitors of caspase-8 and -9

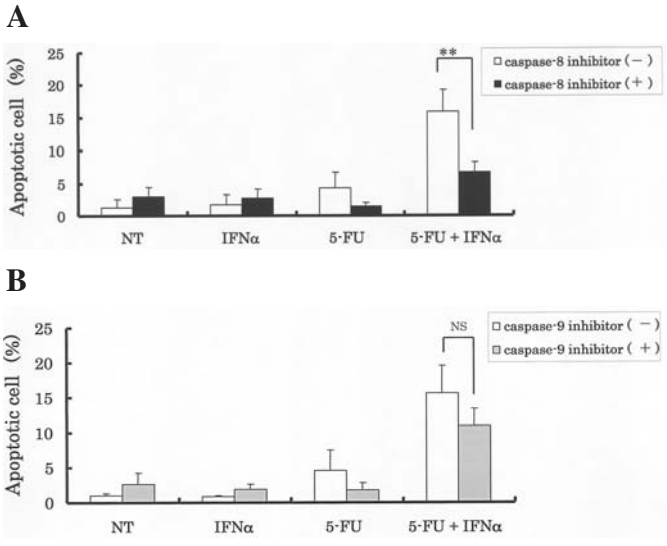

Figure 8. Effect of caspase inhibitor on IFN and/or 5-FU induced apoptosis. (A) Hep3B cells were treated with IFN α (1000 IU/ml) and/or 5-FU (10 μg/ml) for 72 h in the absence (white bars) or presence (black bars) of Z-IETD-FMK caspase-8 inhibitor (20 mM). Apoptosis was determined by the flow cytometric analysis of propidium iodide-stained DNA content. (B) Hep3B cells were treated with IFN α (1000 IU/ml) and/or 5-FU (10 μg/ml) for 72 h in the absence (white bars) or presence (gray bars) of Z-IETD-FMK caspase-9 inhibitor (20 mM). NS, not statistical. **p<0.01 vs. treatment with caspase inhibitor.

(Fig. 8A and B). The caspase-8 inhibitor Z-IETD-FMK and caspase-9 inhibitor Z-LEHD-FMK significantly reduced apoptosis induced by IFNα and 5-FU, while Z-IETD-FMK had more effect on the reduction of apoptosis. These results suggest that caspase-8 plays a more important role on apoptosis induction by 5-FU plus IFNα compared to caspase-9.

Effects of p38 MAPK, JNK, and PI3K inhibitors on IFNα and 5-FU administration. To determine whether the MAPK family was involved in IFN-induced apoptosis, 10 μM of p38 MAPK inhibitor (SB203580), 10 μM of a MAPK inhibitor negative control (SB202474), 10 μM of JNK inhibitor, and 10 μM of a JNK inhibitor negative control were administered. The results showed that the apoptosis-inducible effect of IFNα and/or 5-FU was not affected in the presence of the MAPK inhibitor or JNK inhibitor (Fig. 9A and B). These observations indicate that IFN-stimulated apoptosis did not require p38 MAPK and JNK activation.

PI3K is a direct effector of Ras. The PI3K inhibitor resulted in no effects on apoptosis induced by IFN and/or 5-FU (Fig. 9C).

Discussion

We demonstrated that the Hep3B cell line was the most sensitive to IFN-induced apoptosis out of the five HCC cell lines tested. This effect of IFN on Hep3B apoptosis was enhanced by 5-FU administration, and this additive effect depended strongly on the caspases, especially caspase-8.

Reports show that IFN enhances the effects of 5-FU (28-34). To date, the mechanisms considered to potentially play a role in IFN's additive effect on 5-FU are suppression of the salvage pathway for deoxythymidine monophosphate

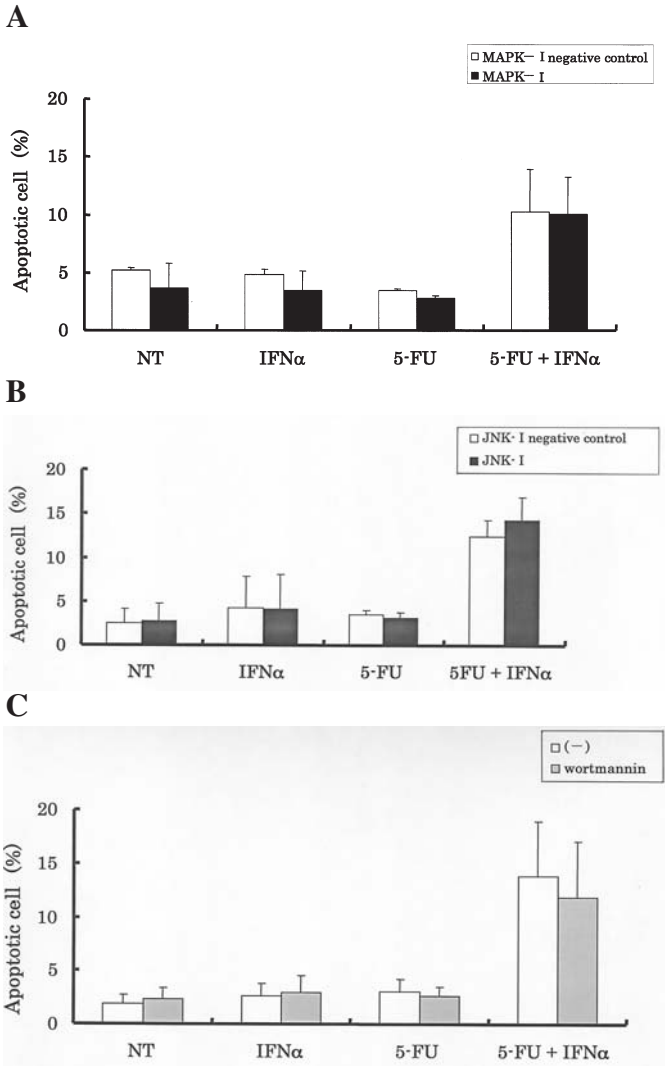

Figure 9. Effect of MAPK inhibitor, JNK inhibitor, and PI3K inhibitor on IFN- and/or 5-FU-induced apoptosis. (A) Hep3B cells were treated with IFNα (1000 IU/ml) and/or 5-FU (10 μg/ml) for 72 h in the presence of a MAPK inhibitor negative control (10 μM) (white bars) or in the presence of a MAPK inhibitor (10 μM) (black bars). (B) Hep3B cells were treated with IFNα (1000 IU/ml) and/or 5-FU (10 μg/ml) for 72 h in the presence of a JNK inhibitor negative control (10 μM) (white bars) or in the presence of a JNK inhibitor (10 μM) (black bars). (C) Hep3B cells were treated with IFNα (1000 IU/ml) and/or 5-FU (10 μg/ml) for 72 h in the absence (white bars) or presence (gray bars) of a PI3K inhibitor, wortmannin (1 μM). Apoptosis was determined by the flow cytometric analysis of propidium iodide-stained DNA content.

induction (28), induction of DNA damage (29), rise in the level of thymidine phosphorylase (30-32), abrogation of the enzyme thymidylate synthase (TS), and enhancement of CDKI (33,34). In this study, the IFN-induced luciferase activation of ISRE was enhanced by the addition of 5-FU. This data suggests that 5-FU also enhances the IFN signaling pathway. The combination treatment of 5-FU and IFNα induced additive effects on both signaling pathways.

We examined the phosphorylation status of the JAK/STAT pathway by Western blotting, as the JAK/STAT pathway is the main pathway for IFN signaling. Tyk2 and STAT1 proteins were phosphorylated by IFNα. Moreover, addition of 5-FU did not change the phosphorylation status of JAK1, Tyk2, and STAT1 as seen by Western blotting. However, the transactivation of ISRE shown by the very sensitive luciferase assay was more strongly induced by IFNα plus 5-FU than IFNα alone. These effects were abrogated by treating the cells with siRNA of JAK1, the upper stream of the JAK/STAT/ISRE signaling pathway, and with siRNA of ISGF3γ the lower stream of the pathway.

Although the JAK/STAT pathway is important to IFN-mediated effects, a number of recent studies suggest that additional signaling pathways are also important for an IFN-dependent biological response. In hematopoietic cells, engagement of the type I IFN receptor leads to activation of JAK kinases, resulting in p38 MAPK activation via the intermediate engagement of MAPKKK and MAPKK (19). The MAPK family comprises ERK, p38, and JNK. In this study, the activation of p38 MAPK and p42/44 MAPK with IFNα treatment, 5-FU treatment, and 5-FU plus IFN treatment was similar for each by Western blotting, and the transactivation of Elk-1 was not induced by any of these treatments. The specific inhibitors of p38 MAPK, JNK, and PI3K showed no effect on IFNα and/or 5-FU-induced apoptosis. Our results showed that the MAPK pathway was not an important pathway in apoptosis induction of the hepatoma cell line by IFNα and 5-FU.

The caspase pathway is involved in IFN-induced apoptosis, and it was reported that the IFN signaling pathway and caspase pathway correlate with each other. Activation of the STAT signaling pathway can cause the activation of caspase and sequential induction of apoptosis (35-37). IFN-mediated apoptosis in liver cancer cells involves the mitochondrial apoptotic pathway and is induced by activating various caspases (38). IFN also upregulates the expression of DR5, the death receptor of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), and downregulates the antiapoptotic molecule survivin (39). Higher expression of DR5 activates caspase-8 and induces apoptosis in the cells. In the colorectal carcinoma cell line and also in the pancreatic cancer cell line, caspase-8 and caspase-3 are reported to play a crucial role in 5-FU and IFN combination-induced apoptosis (40,41). Our results showed that the receptor-type caspase, caspase-8, was more strongly activated by IFNα treatment than the mitochondrial caspase, caspase-9.

Many anti-cancer drugs upregulate the expression of Fas and increase the sensitivity of physiological apoptotic signals (42,43). On the other hand, Hep3B is resistant to Fas-induced apoptosis, but sensitive to IFNγ-induced apoptosis (44). Bid is a pro-apoptotic Bcl-2 family protein that is activated by caspase-8. Hep3B is a 'Bid-abundant cell line' that is more sensitive to 5-FU-induced cytotoxicity than the Bid-insufficient HCC cell line, PLC/PRF/5 (45). These cell line characteristics may explain why Hep3B was the most sensitive cell line to IFNα treatment as well as 5-FU combination treatment in this study.

We found that caspase-8 activity was augmented by the combination of 5-FU and IFNα treatment. Gene silencing of the JAK/STAT pathway revealed that with activation of the receptor binding tyrosine kinases, JAK1 was not enough for IFN's apoptosis induction and caspase-8 activation. Even the silencing of ISGF3γ, the lowest molecule of the JAK/STAT signaling pathway, resulted in almost the same effects as JAK1 silencing. Formation of the transcriptional factor complex STAT1/STAT2/ISGF3γ may be necessary for the induction

of caspase activation and the following induction of apoptosis in the HCC cell line. In recent studies, expression of the caspase-8 gene is shown to be regulated through ISRE by IFNγ (46,47). These data define a new pathway through which IFNs might control sensitivity of the tumor cell to death receptor-mediated apoptosis. Our data showed that ISRE activation was essential for IFN's apoptosis-inducing effect and caspase induction in HCC cell lines. The addition of 5-FU induced much stronger apoptosis than the simple sum of the two drugs, and this synergistic effect also required ISRE activation.

In conclusion, we showed that IFN-induced apoptosis was mainly caspase-8 dependent, and the effect was enhanced by 5-FU. JAK/STAT signaling pathway activation was essential for this apoptosis induction, the effect of which was also enhanced by 5-FU.

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