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

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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. IFNs 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 and 5-FU when 5-FU was added to IFNα treatments. JAK/STAT pathway transcriptional factor ISRE was activated more synergistically than IFNα plus 5-FU treatment. The JAK/STAT pathway and caspase-8 activity, resulting in marked 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), and 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 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

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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 deoxycytidine 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% CO2.

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 (LY333611), 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). RNase A (1 mg/ml) (Sigma-Aldrich Co.) was added for 30 min at 37˚C following propidium iodide (PI) (1 μg/ml) (Sigma-Aldrich Co.) staining. Samples were acquired with FACSscan (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 IFNα-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
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 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 (1.0x10⁵ per well). After overnight incubation, 4 μl of Lipofectamine™

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**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.
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 pathway proteins, i.e., JAK1, Tyk2, and STAT1, by Western blotting (Fig. 2). Tyk2 and STAT1 proteins were phosphorylated by IFN. 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.
The 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 IFNαonly 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 IFNα.

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
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 (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 IFNα 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...
were phosphorylated by IFN-γ, the main pathway for IFN signaling. Tyk2 and STAT1 proteins are phosphorylated by Western blotting, as the JAK/STAT pathway is involved in the effects on both signaling pathways.

The combination treatment of 5-FU and IFN-γ suggests that 5-FU also enhances the IFN signaling pathway. The induction of ISRE was enhanced by the addition of 5-FU. This data indicates that the combination of 5-FU and IFN treatment was more strongly induced by IFNγ plus 5-FU than IFNγ 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-independent 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 p44/42 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 apoptosis.
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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