Augmentation of Antitumor Activity of 5-Fluorouracil by Interferon α Is Associated with Up-Regulation of p27Kip1 in Human Hepatocellular Carcinoma Cells1

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ABSTRACT

Several clinical trials have demonstrated the effectiveness of combination therapy with 5-fluorouracil (5-FU) and IFN-α in colon cancer, hepatocellular carcinoma (HCC), and other malignancies. In our preliminary clinical studies, we have observed outstanding effects with this combination therapy in patients with advanced HCC. However, the underlying mechanism by which IFN-α modulates the effects of 5-FU is unknown. We, therefore, conducted a mechanistic study using two HCC cell lines, PLC/PRF/5 and HuH7. IFN-α significantly enhanced the growth inhibitory effect of 5-FU in PLC/PRF/5 cells but not in HuH7 cells, and the isobolographic analysis indicated that this effect was synergistic. Flow cytometric analysis showed a delay in the progression of G0-G1 to S phase in PLC/PRF/5, and a sustained gisobolographic analysis indicated that this effect was synergistic. Flow cytometric analysis showed a delay in the progression of G0-G1 to S phase in PLC/PRF/5, and a sustained induction of the cyclin-dependent kinase inhibitor p27Kip1 and down-regulation of cyclin D1 was observed. Moreover, increased expression of p27Kip1 was associated with reduced CDK-2-associated kinase activity. Another difference in the two cell types was that PLC/PRF/5 expressed abundant IFN receptors, but HuH7 did not. Apoptosis assays were not helpful in explaining the mechanism. Our results suggest that the synergistic effects of 5-FU and IFN-α may in part be attributable to alterations in cell cycle progression via up-regulation of p27Kip1.

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INTRODUCTION

Primary HCC3 is one of the most common tumors in Southeast Asia, where the incidence of this disease is ~30 per 100,000 men per year (1). Despite various forms of therapies, including hepatectomy, transarterial embolization, and percutaneous ethanol injection therapy, many patients show disease recurrence and finally progress to the advanced stages with vascular invasion and multiple intrahepatic metastases. The prognosis for HCC is generally poor, and the 5-year survival rate is limited to 25–58% after surgery (2, 3).

Chemotherapy is commonly used for the treatment of human malignancies. Although a single chemotherapeutic agent is usually ineffective in suppressing HCC, previous clinical trials suggested that combination of several chemotherapeutic agents may be effective against advanced HCC. For example, combination therapies such as 5-FU/IFN-α/cisplatin/methotrexate or 5-FU/IFN-α/cisplatin/doxorubicin were useful for patients with advanced, unreatsectable HCC (4, 5). It has also been demonstrated that the regimen of 5-FU and IFN-α induces an enduring partial response in ~30% of patients with HCC who had low levels of serum α-fetoprotein (6).

Combination therapy with 5-FU and IFN-α was initially proposed by Wadler et al. (7) in 1988, using colon cancer cell lines. Subsequently, this combination therapy was applied to various types of human malignancies. In patients with colorectal cancer, esophageal cancer, gastric cancer, or HCC, satisfactory results were obtained (6, 8–12), but contrary data were also reported in the studies for colorectal cancer, gastric cancer, pancreatic cancer, and HCC (13–16). In our department, prospective clinical trials have been in progress using 5-FU and IFN-α in patients with inoperable and extremely advanced HCC. Although these studies should continue for several more years, we have thus far obtained satisfactory results with this protocol. Several in vitro studies have provided some explanations about the cooperative effects obtained with combination of 5-FU and IFN-α. The evidence includes reduced 5-FU clearance and alteration of 5-FU metabolism by IFN-α; increase in the amount of FdUMP that can bind to thymidylate synthetase, resulting in inhibition of conversion from dUMP to dTMP during normal DNA synthesis (17, 18). It appears that IFN-α reduces the uptake of thymidine and the activity of thymidine kinase in conjunction with the action of 5-FU. Moreover, it is suggested that cooperative effects may be exerted through im-

3 The abbreviations used are: HCC, hepatocellular carcinoma; 5-FU, 5-fluorouracil; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Ab, antibody; CDK, cyclin-dependent kinase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling.
munomodulatory actions (19). However, there are only a few studies that have examined the effects of 5-FU and IFN-α on fundamental cell biology, such as the cell cycle and apoptosis (20, 21).

Several clinical trials have demonstrated the considerable effectiveness of 5-FU combined with IFN-α in patients with advanced HCC (4–6, 22), but it has not yet been clarified how IFN-α modulates antitumor activity of 5-FU in HCC cells. In the present study, we examined several aspects of cell biology, including cell proliferation, apoptosis, and changes in the cell cycle, in HCC cell lines PLC/PRF/5 and HuH7, during treatment with 5-FU and IFN-α. In addition, expression of the IFN receptor was examined. Because IFN-α clearly enhanced the growth-inhibitory effect of 5-FU in PLC/PRF/5 cells but not in HuH7 cells, comparison of several aforementioned features may provide evidence associated with the underlying mechanism of this combination therapy.

MATERIALS AND METHODS

Reagents and Cell Lines. Purified human IFN-α was obtained from Otsuka Pharmaceutical Co. (Tokyo, Japan), and 5-FU was obtained from Kyowa Hakko Co. (Tokyo, Japan). The two human HCC cell lines, PLC/PRF/5 and HuH7, were purchased from the Japanese Cancer Research Resources Bank. They were maintained in DMEM supplemented with 10% FBS at 37°C in a humidified incubator with 5% CO₂ in air.

Growth Inhibitory Assays with 5-FU and IFN-α. Cells (3 x 10⁴) per well were added in triplicate to a 96-well microplate, and 24 h later, the medium was replaced by 0.1 ml of fresh medium containing various concentrations of 5-FU and/or IFN-α. Concentrations of 5-FU tested were 0.05, 0.5, and 5 μg/ml, and those of IFN-α were 50 and 500 units/ml. Tumor cells suspended in complete medium were used as a control for cell viability. The medium was changed every 48 h, and 4 days after the addition of 5-FU and/or IFN-α, the numbers of viable cells were assessed by MTT (Sigma Co, St. Louis, MO) assay. Briefly, 10 μl (50 μg) of MTT were added to each well. The plate was incubated for 4 h at 37°C. Unreacted MTT was then removed, leaving the resultant formazan crystals at the bottom of the well. Then, 0.1 ml of 2-propanol was added to each well to dissolve the crystal. The absorbance of the plate was measured in a microplate reader at a wavelength of 570 nm. These assays were repeated, and similar results were obtained. Also in other parts of the present study, experiments were repeated at least twice, and no discrepant results were obtained.

Growth Curves. Cells were uniformly seeded (5 x 10⁴/well for PLC/PRF/5 and 2 x 10⁵/well for HuH7) in triplicates into 24-well dishes. Twenty-four h later (day 0), the culture medium was removed and replaced by 1 ml of fresh medium with or without 0.5 μg/ml 5-FU and 500 units/ml IFN-α. Medium was changed every 48 h, and on days 2, 4, 6, 8, and 10, viable cells were counted using a hemocytometer by trypan blue dye exclusion. The doubling time was calculated using growth curves between days 4 and 8, as described previously (23).

Cell Cycle Analysis. Flow cytometric analysis was performed, as described previously (23). Briefly, cells were washed twice with PBS and then fixed in 70% cold ethanol for 4 h before being washed and resuspended in 1 ml of PBS. Propidium iodide (Sigma; 50 μl of 1 mg/ml solution in PBS) and RNase (Sigma; 1 μl of 10 mg/ml solution) were added for 30 min at 37°C. Samples were filtered through 44 μm nylon mesh, and data were acquired with a FACSort (Becton Dickinson Immunocytometry Systems, San Jose, CA). Analysis of the cell cycle was carried out using ModFit software (Becton Dickinson Immunocytometry Systems).

Western Blot Analysis. Cells were washed twice with ice-cold PBS and collected with a rubber scraper. After centrifugation, the cell pellets were resuspended in lysis buffer [50 mM HEPES, 150 mM NaCl, 2.5 mM EGTA, 1.0 mM EDTA, 1.0 mM DTT, 0.1% Tween 20, 10% glycerol, and protease inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotinin, 1.0 mg phenylmethylsulfonyl fluoride; pH 7.5)]. After sonication, the extracts were clarified at 15,000 × g for 5 min at 4°C, and the supernatant fraction was collected. Western blot analysis was performed, as described previously (23).

Antibodies. The following rabbit polyclonal Abs were used at appropriate concentrations as recommended by the manufacturer: anti-p27Kip1 Ab (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-p21waf1/cip1 Ab (Santa Cruz); anti-human IFN receptor α/β Ab (Santa Cruz Biotechnology; Refs. 24 and 25); anti-cyclin D1 Ab (Upstate Biotechnology Inc., Lake Placid, NY); anti-CDK2 Ab (UBI); and anti-human cyclin E Ab (Upstate Biotechnology Inc.).

In Vitro Assay for CDK2-associated Activity. The in vitro CDK2-associated kinase assay was performed as described previously (26). Cells were collected and sonicated in kinase buffer (lysine buffer plus 10 mM β-glycerophosphate, 1 mM NaF, and 0.1 mM sodium orthovanadate). After centrifugation, the supernatant fraction was collected. Immunoprecipitation with 1 μg of CDK2 polyclonal Ab (Upstate Biotechnology Inc.) was performed using protein A-Sepharose beads (Sigma), followed by washing of the beads four times with kinase buffer and twice with reaction buffer [50 mM HEPES (pH 7.5), 10 mM MgCl₂, 1 mM DTT, 2.5 mM EGTA, 10 mM β-glycerophosphate, 1 mM NaF, and 0.1 mM sodium orthovanadate]. The final pellets were resuspended in 45 μl of reaction buffer containing 2 μg of histone H1 (Sigma) and 5 μCi of [γ-^3²P]ATP and incubated for 30 min at 30°C. The reaction mixture was then subjected to SDS-PAGE, and the intensity of phosphorylation of the histone H1 substrate was determined by autoradiography.

Detection of Apoptosis. To detect in situ apoptosis, we used the TUNEL (3) method with the Apop Tag kit (Oncor S7100, Gaithersburg, MD) as described previously (26). This method can detect fragmented DNA ends of apoptotic cells. Cells (5 x 10⁶/well) were uniformly seeded into 10-cm diameter dishes and cultured for 24 h. The medium was removed and replaced by 10 ml of fresh medium with or without 0.5 μg/ml 5-FU and 500 units/ml IFN-α. Cells free in the medium and attached to the dish were harvested 72 h after the addition of 5-FU or IFN-α and then fixed with 10% buffered formalin for detection of apoptotic cells. Terminal deoxynucleotidyl transferase was omitted from the nucleotide mixture for the negative control. As a positive control, we used paraffin-embedded sections of 10% buffered, formalin-fixed rodent mammary glands. For quantification of apoptotic cells, 10 microscopic fields were randomly selected at ××25, >700 total cells were counted in...
ing of the gel also confirmed equal loading of the total protein and served as loading controls. Coomassie blue staining of the gel confirmed equal loading of the total protein (data not shown).

Expression of IFN receptors in the two cell lines. PLC/PRF/5 displayed prominent bands of IFN receptors at M, 40,000 (soluble form), M, 51,000 (short form), and M, 102,000 (long form). Coomassie blue staining of the gel confirmed equal loading of the total protein (data not shown).

**RESULTS**

**Detection of IFN Receptors.** With antihuman IFN α/β receptor Ab, PLC/PRF/5 displayed prominent bands at M, 40,000 (soluble form), M, 51,000 (short form), and M, 102,000 (long form), which were about 7.7-, 2.8-, and 2.4-fold more intense, respectively, than the corresponding bands expressed in HuH7 (Fig. 1). Nonspecific, cross-reactive bands (M, 140,000 and M, 80,000) appeared at similar densities between the two cell lines and served as loading controls. Coomassie blue staining of the gel also confirmed equal loading of the total protein (data not shown).

**Growth-Inhibitory Assays.** To investigate whether 5-FU and IFN-α have cooperative effects on inhibition growth of HCC cells, growth-inhibitory assays were performed. Cells were exposed to 5-FU and/or IFN-α for 4 days at various concentrations. 5-FU alone (Fig. 2A), 0.5 μg/ml 5-FU inhibited cell growth by 23.2 ± 0.06% in PLC/PRF/5 (left panel) and 24.0 ± 0.05% in HuH7 (right panel). At 5 μg/ml, 5-FU reduced cell growth to 49.2 ± 0.03% and 49.2 ± 0.08% of control cells, in PLC/PRF/5 and HuH7, respectively. No growth-inhibitory effect was observed in cells with IFN-α alone at 50 or 500 units/ml. When 5-FU and IFN-α were administered simultaneously at various concentrations, significant cooperative effects were observed at 0.5 μg/ml 5-FU plus 50 or 500 units/ml IFN-α in the PLC/PRF/5 cell line (P = 0.010 and 0.046, respectively), and the isobolographic analysis indicated that these cooperative effects were synergistic (Fig. 2B). However, these effects were not observed with 5 μg/ml 5-FU plus IFN-α. In HuH7 cells, the growth-inhibitory effects induced by 0.5 or 5 μg/ml 5-FU were not affected by the addition of IFN-α.

**Growth Curves.** Growth curves were drawn up to 10 days (Fig. 3). 5-FU (0.5 μg/ml) and IFN-α (500 units/ml) were selected for treatment, because under these conditions IFN-α enhanced the growth-inhibitory effects of 5-FU in the PLC/PRF/5 cell line. In PLC/PRF/5 cells, the doubling time was 41.8 ± 2.5, 54.3 ± 5.9, 72.2 ± 14.2, and 144.8 ± 34.6 h for cultures of control, IFN-α, 5-FU, and 5-FU plus IFN-α, respectively. The difference between the latter two groups was significant (P = 0.021). In HuH7 cells, the doubling time was 29.4 ± 4.0, 35.6 ± 3.7, 107.2 ± 6.7, and 145.5 ± 8.9 h for each treatment, and no significant difference was found between 5-FU alone and 5-FU plus IFN-α. A significant difference was also observed in cell numbers on day 10 between the 5-FU group and combination group in PLC/PRF/5 cells (P = 0.020) but not in HuH7 cells.

**Cell Cycle Analysis.** We then performed flow cytometric analyses to examine changes in cell cycle progression when cells were treated with or without 0.5 μg/ml 5-FU and/or 500 units/ml IFN-α. These studies were performed in cultures that were grown previously in serum-free medium for 72 h to arrest the cells in G0-G1, because after re-addition of 10% FBS, the cells would synchronously progress through the cell cycle. Cells were then collected 6, 12, 24, 48, 72, and 96 h later. Flow cytometric data confirmed that after serum starvation, the majority of cells (PLC/PRF/5, 69.0%; HuH7, 74.0%) were in G0-G1. After refeeding the serum-starved cultures of PLC/PRF/5 with 10% FBS minus drugs (control cultures), cells remained in G0-G1 until 12 h and then progressed into the S (34%) and G2-M (34%) phases within 24 h (Fig. 4B), and one full cell cycle was completed at 48 h (Fig. 4A). In cultures refed with serum plus 5-FU, the distribution of the cell cycle was similar until 12 h, compared with control cultures. At 24 h, the cells progressed into S phase, but there was an appreciable number of cells with S-phase DNA content (48%; Fig. 4B) with a right-sided downward slope (Fig. 4A), perhaps because of interruption of the normal process of DNA synthesis. In turn, this caused a delay in progression to G2-M phase (15%). The combination of 5-FU and IFN-α apparently delayed the cell cycle. At 24 h, there was still a marked accumulation of cells in G0-G1 phase (63%), although at the same time point the per-
percentage in G0-G1 phase decreased to approximately one-half in the other groups (Fig. 4B). There was a gradual increase in the S-phase fraction from 48 to 72 h (Fig. 4B), but even by 72 h the cell cycle had not returned to the initial distribution pattern and remained significantly different from control or 5-FU-treated cultures (Fig. 4A). In cultures refed with serum plus IFN-α, the percentages of cells in each phase were not largely different from those of the control cultures (Fig. 4B).

In experiments conducted in HuH7 cells, the effect of 5-FU or IFN-α on cell cycle progression was similar to that observed in PLC/PRF/5 (Fig. 4C). In cultures subjected to combination therapy, when compared with cultures of 5-FU alone, there was no difference in cell cycle distribution at 24 h, although there was a slight increase in S-phase fraction (42%) at 48 h. At 72 h, unlike PLC/PRF/5, the distribution of the cell cycle was not different from that of other groups.

Detection of Cell Cycle Regulatory Proteins. The above flow cytometric analyses indicated that PLC/PRF/5 cells displayed an apparent delay in G1-S transition between 5-FU and combination treatments. Therefore, we examined the expression of several cell cycle regulatory proteins involved in G1-S transition, including G1 cyclins (D and E type) and the CDK inhibitors p21\textsuperscript{waf1/cip1} and p27\textsuperscript{kip1}. Among these molecules examined, the levels of p27\textsuperscript{kip1} and cyclin D1 changed as the cell cycle progressed. In control cultures of PLC/PRF/5, expression of p27\textsuperscript{kip1} diminished with time and was lowest at 24 h (Fig. 5A, left panel). In the 5-FU or IFN-α group, p27\textsuperscript{kip1} decreased at 24 h and remained relatively low at 48 h. By contrast, p27\textsuperscript{kip1} expression in the combination therapy group remained high until 24 h, and an appreciable amount of p27\textsuperscript{kip1} was still detectable at 48 h. Densitometric analysis indicated that the densities of the band of combination group was almost 3.6-fold higher than that of the 5-FU group. In HuH7 cells, p27\textsuperscript{kip1} expression was high at 6 h, and the level did not vary much with time in the control cultures, nor was any difference induced by the drugs (Fig. 5A, right panel).

The addition of a single drug did not affect cyclin D1 expression until 24 h among the four groups in PLC/PRF/5 (Fig. 2A, growth-inhibitory assays were performed in the two HCC cell lines, PLC/PRF/5 and Hu7, using the MTT method. The proportion of cells incubated without drugs was defined as 100% viability. *p<0.05, statistically significant. Bars, SE. B, the isobolographic analysis indicated that these cooperative effects in PLC/PRF/5 were synergistic.
DISCUSSION

The present in vitro study was performed in an effort to explore the underlying mechanisms of how IFN-α may enhance the tumor-inhibitory effect of 5-FU. In this study, we used two hepatoma cell lines that were both sensitive to 5-FU but insensitive to IFN-α alone. Differences between the cell lines emerged in their responses to the 5-FU and IFN-α combination. When combined with 0.5 μg/ml 5-FU, IFN-α induced growth-inhibitory effects in a dose-dependent manner in PLC/PRF/5. Thus, within the range, 5-FU and IFN-α had synergistic effects on cell growth, as confirmed with the isobolographic analysis. Importantly, the concentration of 5-FU used in our study (0.5 μg/ml) was almost the same level as that in plasma of patients when the drug is clinically administered by continuous infusion (29). Conversely, no cooperative effects were seen in HuH7 cells. The synergistic effects in PLC/PRF/5 were apparent, although the difference was not so dramatic (Fig. 2A). This may be because 4 days of culture was too short a time period. Indeed, the difference in proliferation between the two cell types was more obvious when the cell culture was extended up to 10 days (Fig. 3). It is also possible that the difference in sensitivity to 5-FU might play some role in the cooperative effects; the doubling times in the 5-FU group were 1.7-fold and 3.7-fold longer than the control group in PLC/PRF/5 and in HuH7 cells.

5B, left panel). When drugs were combined, cyclin D1 expression markedly decreased at 48 h. In HuH7 cells, cyclin D1 level was relatively low at 6 h, and at subsequent time points it was slightly increased in the control and IFN-α groups. By contrast, it decreased at 24 and 48 h in the 5-FU and combination groups (Fig. 5B, right panel). The expression of cyclin E and p21<sup>waf1/cip1</sup> in either cell line was uniform, irrespective of the drug used and time course (data not shown).

**In Vitro Assay for CDK2-associated Activity.** To investigate whether high expression of p21<sup>kip1</sup> seen in PLC/PRF/5 was functional, an in vitro kinase assay was performed, using cell extracts that were harvested 24 h after the addition of the drug. Because the major function of p21<sup>kip1</sup> is to bind the cyclin E/CDK2 complex and inhibit its kinase activity, we assayed the CDK2 immunoprecipitates for in vitro CDK2-associated kinase activity using histone H1 as the substrate. Cell extracts prepared from control, 5-FU-, and IFN-α-treated cultures yielded strong phosphorylation of histone H1 (Fig. 6, Lanes 2–4). By contrast, extracts from cultures treated with 5-FU plus IFN-α displayed an ~3.2-fold decrease in kinase activity when compared with 5-FU alone (Fig. 6, Lane 5). When the extract from the control culture was immunoprecipitated with nonimmune rabbit IgG, no kinase activity was detected (Fig. 6, Lane 1).

**Detection of Apoptosis.** To examine the effect of drug combination on the frequency of apoptosis, TUNEL assays were performed 3 days after treatment (Fig. 7). The percentages of apoptosis in PLC/PRF/5 were 1.3 ± 0.1, 4.3 ± 0.3, 6.1 ± 0.5, and 6.6 ± 0.7%, in the control, 5-FU, IFN-α, and drug combination groups, respectively. On the other hand, the frequency of apoptosis was low in HuH7 at 0.6 ± 0.2, 0.7 ± 0.1, 0.9 ± 0.1, and 0.9 ± 0.1%, for each respective group.
respectively. Therefore, 5-FU might be enough to suppress HuH7, which cannot be exceeded by the addition of IFN-α.

Analysis of cell cycle progression provided a clue to the underlying mechanism of the synergistic effects seen in PLC/PRF/5. A marked delay in cell cycle progression was found at 24 h, with G0-G1 accumulation in the combination therapy group in PLC/PRF/5 (Fig. 4B). However, in the HuH7 cell line, the DNA content pattern at the same time point was similar between combination group and 5-FU groups. The difference between the two cell lines could be explained by induction of p27Kip1 (Fig. 5A). PLC/PRF/5 cells exhibited an ~3.5-fold rise in p27Kip1 expression in the presence of both 5-FU and IFN-α at 24 h compared with treatment with 5-FU alone, whereas in HuH7 cells, no significant difference in p27Kip1 expression was observed between the two treatments. Furthermore, a CDK2-associated kinase assay showed considerably lower kinase ac-

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**Fig. 4** A, flow cytometric analyses of PLC/PRF/5 when cells were treated with or without 0.5 μg/ml 5-FU and/or 500 units/ml IFN-α. B, the percentage in G0-G1 (left panel) and S phase (right panel) in PLC/PRF/5 cells at different time points. C, flow cytometric analyses of HuH7 when cells were treated with or without 0.5 μg/ml 5-FU and/or 500 units/ml IFN-α.
tivity in p27 Kip1-overexpressing extracts of PLC/PRF/5. It is possible that the levels of CDK2 or cyclin E and another CDK inhibitor, p21^{waf1/cip1}, may affect the extent of activity of CDK2-associated kinase (23), but the levels of these proteins did not change by drug treatment in either cell line (data not shown). These findings suggest that p27 Kip1 induced by 5-FU and IFN-α in PLC/PRF/5 is functional and may contribute to the delay in progression of the cell cycle from G₁ to S phase.

It is of interest that p27 Kip1 may play a crucial role in chemotherapy against HCC, because recent studies have highlighted the relevance of p27 Kip1 in the progression of various types of human malignancy (30, 31). We showed previously that expression of p27 Kip1 was reduced in subsets of colon cancers (32), and we have found recently that loss of p27 Kip1 is associated with shorter disease-free survival in HCC (33). The present data are consistent with those of the latter report. Thus, it seems that low expression of p27 Kip1 favors sound growth and expansion of HCC cells, and conversely, up-regulated p27 Kip1 may effectively function to suppress HCC.

A linkage between IFN-α and inhibition of the cell cycle has been examined. IFN-α induced G₀-G₁ phase accumulation in NIH-3T3 murine fibroblasts, human Burkitt’s lymphoma Daudi, and the lymphoid cell line U-266 (34, 35). It was also reported that treatment of Daudi cells with IFN-α inhibited the activity of CDK2 but not CDC2 (36), suggesting that a decrease in CDK2 kinase may be responsible for the G₀-G₁ arrest. By contrast, in the present study, we did not observe a significant increase in the G₀-G₁ population nor p27 Kip1 induction with treatment of IFN-α alone. However, p27 Kip1 was strongly induced by simultaneous administration of IFN-α and basal treatment with 5-FU in PLC/PRF/5. Therefore, it is likely that the susceptibility of the cells to IFN-α in inducing p27 Kip1 may differ among cell types, and that 5-FU may aid IFN-α-mediated p27 Kip1 induction, which leads to reduction in CDK2 kinase activity in at least certain HCC cell lines.

We also demonstrated the possible involvement of cyclin D1 in cell cycle control (37). Decreased cyclin D1 expression was observed in the late cell cycle (at 48 h) in the combination group of PLC/PRF/5 cells but not in the group treated with 5-FU alone (Fig. 5B). This might contribute to the sustained arrest in G₀-G₁ at 48 h (Fig. 4B). On the other hand, although cyclin D1 expression decreased in the combination group in HuH7 cells at 24 and 48 h, similar down-regulation of cyclin D1 was observed also in the group treated with 5-FU alone (Fig. 5B), consistent with a lack of cooperative effect in this cell line.

Recently, it has been reported that expression of the IFN-α receptor varies among hepatoma cell lines and among patients with hepatitis C virus-associated hepatitis (38, 39). HuH7,
which was resistant to combination therapy, exhibited lower IFN receptor expression than PLC/PRF/5. This suggests a possible relationship between resistance to IFN-α and the extent of expression of IFN receptors. Introduction of IFN receptor cDNA into HCC cell lines may clarify the relevance of signal transduction from the receptor to the cytoplasmic and nuclear events involved in cell cycle control. We have performed recently a retrospective study of IFN receptor expression in primary HCC tumors and found that a wide range of expression exists in these tumors (data not shown). A clinical investigation is currently under way in our laboratory in an effort to examine the possible correlation between the extent of expression of the IFN receptor and the effects of combination therapy.

Apoptosis assays did not explain the synergistic effects of 5-FU and IFN-α in PLC/PRF/5 cells. The TUNEL assay indicated that although there was an ∼4–5-fold increase in frequency of apoptosis in the combination group compared with control (6.6 versus 1.3%), IFN-α alone also induced similar levels of apoptosis (6.1%). In HuH7 cells, TUNEL-positive cells were minimal (<1%), irrespective of treatment. Our findings obtained by the TUNEL method matched those measured by flow cytometry in PLC/PRF/5 cells. However, in HuH7, there was a discrepancy between data obtained by the TUNEL method and flow cytometric analysis. It is probable that the increased sub-G1 population seen in HuH7 cells may be attributable to debris of fragmented but not apoptotic cells, because we observed many necrotic cells under the microscope (data not shown).

In addition to the phenomena examined in the present study, several aspects remain to be clarified. As mentioned in

![Fig. 5 Expression of cell cycle regulatory proteins in cells treated with or without 0.5 μg/ml 5-FU and/or 500 units/ml IFN-α by Western blot analysis. A, p27Kip1; B, cyclin D1.](image)

![Fig. 6 In vitro CDK2-associated kinase assay was performed, using cell extracts that were harvested 24 h after drugs were added. N. C., negative control. Nonimmune rabbit IgG was used instead of CDK2 Ab.](image)
the “Introduction,” changes in the 5-FU metabolic pathway could possibly underlie the mechanism of IFN-α synergism. Indeed, we found that the activity of thymidylate synthetase was slightly decreased without an increase in the amount of FdUMP after treatment with 5-FU and IFN-α in PLC/PRF/5 cells (data not shown). It is also possible that the antitumor effect might be exerted via host immunomodification and changes in vascularity in vivo (40–42). These aspects should be addressed in the future. Regardless of the other possibilities, it is apparent from the present study that IFN-α modulated the progression of the cell cycle, possibly via induction of p27^Kip1 in 5-FU-treated PLC/PRF/5 cells. Among the complex and diverse effects of combination therapy (5-FU and IFN-α), our data provide a novel explanation with regard to the intracellular events associated with cell cycle regulation.

REFERENCES


Fig. 7 TUNEL assays were performed 3 days after drugs were added (5-FU, 0.5 μg/ml; IFN-α, 500 units/ml) to detect apoptotic cells. Representative photographs of PLC/PRF/5 cells are shown here, and TUNEL-positive cells are indicated by arrows. ×50.
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