Interaction of Fluorouracil and Interferon in Human Colon Cancer Cell Lines:
Cytotoxic and Cytokinetic Effects

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ABSTRACT

Fluorouracil (FUra) is the most active agent in advanced colorectal carcinoma, and this activity can be enhanced by various modulating agents both in vitro and in vivo. To determine whether interferon (IFN) is capable of augmenting the cytotoxic and cytokinetic effects of FUra, combinations of FUra and IFNα, -β, and -γ were tested against 2 human colon cancer cell lines in vitro. In a clonogenic assay, IFNα and -β, at concentrations that produced less than 1 log cell kill, significantly increased the cytotoxic effects of FUra in both cell lines. IFNγ also enhanced the cytotoxic effects of FUra, but unlike IFNα and -β, only at the highest concentrations tested. Median effects analysis demonstrated that all 3 IFNs exhibited synergy with FUra. Combinations of IFNs were no more effective at modulating FUra activity than single agent IFN. Flow cytometric studies indicated that these effects did not correlate with cytokinetic alterations. Only the combination of FUra and IFNγ produced cytokinetic effects different from those of FUra alone. Incubation with IFNα or IFNγ for 24 h resulted in only modest cytokinetic alterations, and they did not modify the effects of FUra. These results indicate that IFN is capable of increasing the cytotoxic actions of FUra and that this is separable from any cytokinetic effects produced by the interferons.

INTRODUCTION

The fluorinated pyrimidine, FUra, is the most active single agent in the treatment of metastatic colorectal carcinoma; nevertheless, only 7–20% of patients will respond to therapy with few complete responders and no improvement in overall survival (1). Standard strategies for improving the therapeutic results obtained with this agent, such as administering FUra in combination with other cytotoxic drugs or intensifying the dose, have been limited by unacceptable toxicities and no improvement in efficacy (1). Thus, other approaches appear to be warranted.

The cellular metabolism of FUra and the interaction of its active products with intracellular targets have been extensively investigated (2–4). Furthermore, multiple agents have been identified that are capable of modulating the biochemistry of FUra and its metabolites and enhancing their antitumor effects (5–7). Clinical trials have recently demonstrated that this strategy is capable of improving both response to therapy and survival (8, 9). Nevertheless, the benefits have been limited, and other modulating agents may improve these early results.

One family of compounds that is logical to investigate in this regard is the IFNs. These multifunctional cytokines are classified by their cellular origin, stability in acid, receptor, and more recently by their molecular structure (10). Their activity is mediated by trans-acting factors that interact with interferon-responsive enhancers on interferon-activatable genes (11, 12). End points of IFN activation on cell structure and function are nearly global, and include effects on nucleotide pools, enzyme activities, protein expression, and gene transcription (13), as well as the ability to modify the cytotoxic or antiproliferative effects of various cancer chemotherapeutic drugs (14).

To determine whether IFNs are capable of modulating the activity of FUra against human solid tumors, the effects of the combination on cell survival and cell cycle traverse were studied using the human colon cancer cell lines HT-29 and SW480. Synergy was assessed by median effects analysis. Because synergy has been reported between different classes of IFNs, combinations were also tested to determine whether the modulatory effects may also be affected by combining cytokines.

MATERIALS AND METHODS

Cells. The human colon cancer cell lines, HT-29 and SW480, initially isolated by Fogh and Trempe (15), were the generous gift of Dr. Leonard Augenlicht. Cells were cultured at 37°C in 5% CO2 in RPMI 1640 with 10% fetal calf serum (Grand Island Biological Co., Grand Island, NY).

Materials. Recombinant α2a-IFN (Roferon) was supplied by Hoffman-LaRoche Inc., Nutley, NJ. Recombinant β-interferon (Betaseron) was supplied by Triolon Biosciences, Alameda, CA. Recombinant γ-interferon was purchased from Boehringer-Mannheim (Indianapolis, IN). FUra (Adrucil; Adria Laboratories, Columbus, OH) was obtained commercially.

Clonogenic Assay. HT-29 or SW480 cells in midexponential growth were counted and plated in RPMI 1640 with 10% fetal calf serum in 100-mm plastic Petri dishes and allowed to attach overnight. Cells were washed 3 times with phosphate-buffered saline, and fresh media were added. The cells were incubated for 14 days, then fixed and stained with Coomassie blue in a one-step process. Colonies, defined as aggregates of at least 33 cells, were counted under low-power magnification. Differences between treatments were evaluated by analysis of variance. All results represent the mean of at least 3 experiments.

Drug interactions were quantitated by median effects analysis (16, 17). Dose-effect curves were plotted for each agent and for serially diluted combinations using fixed ratios using the median effects equation:

\[
f(a) = \left( \frac{D}{D_m} \right)^m
\]

where \( D \) is dose, \( D_m \) is the dose required for a desired effect on cell survival, \( f(a) \) is the fraction killed, \( f(u) \) is the fraction surviving, and \( m \) is a coefficient of the sigmoidicity of the dose-effect curve. The dose-effect curve is plotted using a logarithmic conversion of this equation, which determines \( m \) and \( D_m \) values. A combination index was determined from the equation:

\[
\text{Combination index} = \frac{(D_1)}{(D_2)} + \frac{(D_2)}{(D_1)} + \frac{\alpha(D_1)(D_2)}{(D_1)(D_2)}
\]

where \( (D_1) \) is the dose of agent 1 required to produce \( x\% \) effect alone, and \( (D_2) \) is the dose of agent 2 required to produce the same \( x\% \) effect in combination with \( (D_2) \). Similarly, \( (D_2) \) is the dose of agent 2
required to produce \( x\% \) effect alone and \((D)2\) is the dose required to produce the same effect in combination with \((D)1\). If the agents are mutually exclusive (i.e., have a similar mode of action), then \( \alpha = 0 \) (i.e., \( CI \) is the sum of 2 terms). If the agents are mutually nonexclusive, then \( \alpha = 1 \) and \( CI \) is the sum of 3 terms. For the present studies, the drug interactions were analyzed by both methods. \( CI \) was solved for 50%, 70%, and 90% effects. \( CI < 1 \) indicates synergy, \( CI > 1 \) indicates antagonism, and \( CI = 1 \) indicates additivity. Computer programs for the IBM-PC based on the median effect plot and \( CI \) equation were employed for data analysis (18).

Cytokinetic Studies. For cell cycle analysis, flow cytometry was performed on propidium iodide-stained cells. Cells in midexponential growth were exposed to drugs for varying time periods, harvested, washed, and treated with sucrose:edematin:dimethyl sulfoxide buffer as described by Vindelov et al. (19). Pellets were incubated in 100 \( \mu l \) of RNase (Worthington Biochemical Corp., Freehold, NJ) 1 mg/ml, for 30 min at room temperature. Following RNA digestion, 2 ml of the propidium iodide solution, 0.05 mg/ml, was added, the nuclei dispersed, and the cells placed on ice for cell cycle analysis.

Cellular fluorescence was measured on an EPICS C flow cytometer (Coulter Electronics, Hialeah, FL) equipped with a UV-enhanced 5-W argon-ion laser (Coherent, Inc., Palo Alto, CA). High-resolution cell cycle analysis and measurement of DNA ploidy was performed on DNA histograms off-line with ROMP and ROC3D programs (Rochester Multiparameter Data Analysis; J. Leary and R. Robinson, University of Rochester) rewritten for a DEC 11/73 computer. Each experiment was performed in duplicate.

RESULTS

Cell Survival. To determine the effects of IFN on cell survival, HT-29 or SW480 cells were incubated with IFN \( \alpha \), \( \beta \), or \( \gamma \), 50–5000 units/ml, for 24 h. Cell survival was determined by a clonogenic assay. There was no reproducible difference in cell survival between control populations and cells treated with IFN, 50 or 500 units/ml. Treatment with either IFN \( \alpha \), IFN \( \beta \), or IFN \( \gamma \), 5000 units/ml, routinely resulted in a 30–90% decrease in cloning efficiency.

Incubation of HT-29 cells with FUra for 24 h resulted in exponential cell kill with IC \(_{50}\), IC \(_{90}\), and IC \(_{99}\) of 75, 215, and >400 \( \mu M \), respectively (Fig. 1A). Comparable values for SW480 cells were 40, 133, and 400 \( \mu M \) (Fig. 1C). Simultaneous incubation of cells with FUra and 500 units/ml of IFN \( \alpha \) or \( \beta \) for 24 h resulted in an enhancement of cell lethality. The potential interaction increased with higher concentrations of FUra such that IFN \( \alpha \) or \( \beta \) decreased the IC \(_{50}\) for FUra by 1.25- to 2-fold, the IC \(_{90}\) by 2- to 3-fold, and the IC \(_{99}\) by 4-fold (Fig. 1; only IC \(_{50}\) values have been graphically represented). The interaction between FUra and either IFN \( \alpha \) or IFN \( \beta \) was significant in HT-29 cells (\( P = 0.025 \), <0.0001, respectively), and between FUra and IFN \( \beta \) in SW480 cells (\( P < 0.0001 \)). To determine whether the combinations were effective at sublethal doses of FUra, cells were incubated with IFN \( \alpha \) or IFN \( \beta \), 500 units/ml, and FUra, 0.1–5.0 \( \mu M \), for 24 h. At these concentrations, the combinations were ineffective against both cell lines, suggesting that modulation of the FUra effect occurs only at concentrations of FUra which result in at least some diminution of cell survival. Unlike IFN \( \alpha \) and \( \beta \), modulation of FUra effects by IFN \( \gamma \) only occurred at the highest level used, 5000 units/ml, for 24 h in both HT-29 and SW480 cells (\( P < 0.0001 \)) (data not shown). As with IFN \( \alpha \) and \( \beta \), no effect was noted at sublethal doses of FUra.

Potentiation of cytotoxicity by combining IFN and FUra was greater at higher concentrations of IFN (Fig. 1, B and D). Three log cell kill was observed in both cell lines at the highest concentration of IFN tested in combination with FUra.

The effect of shorter exposures to combined IFN and FUra was examined in HT-29 or SW480 cells. There was no enhancement of the cytotoxicity of FUra effects for either cell line at IFN \( \alpha \) concentrations of 50-, 500-, or 5000 units/ml with a 2-h treatment.

Synergistic growth inhibition has been reported between type I and type II IFNs (20–24). Cells were incubated with combinations of IFN \( \alpha \), IFN \( \beta \), and IFN \( \gamma \) at concentrations of 50, 500,
FUra AND IFN IN HUMAN COLON CANCER CELL LINES

Fig. 2. Median effects plot of interaction of FUra and IFNα (A), IFNβ (B), or IFNγ (C) in HT-29 (A and C) or SW480 (B) cell cultures. No differences were noted whether assumptions of mutual exclusivity or non-exclusivity of action were made. Interactions of FUra and IFNα and -β are strongly synergistic (plot below the horizontal dotted line) over nearly the entire range of concentrations tested, while the interaction between FUra and IFNγ is antagonistic (plot above horizontal dotted line) over the lower concentrations of drugs tested and synergistic at higher concentrations.

or 5000 units/ml divided equally on a unit-for-unit basis between the IFNs. Incubation of either HT-29 or SW480 cells with the 2 lowest dose levels of IFN combinations resulted in no cytotoxic effect. Incubation with IFN combinations at 5000 units/ml resulted in a consistent 40–60% cell kill, comparable with that noted with IFNα or -β used individually. All combinations of IFNs significantly augmented the cytotoxic effects of FUra ($P < 0.0001$ for all combinations of IFNs); however, no combination, including the combination of all 3 IFNs with FUra, was more effective at enhancing the cytotoxic effects of FUra than IFNα or IFNβ used individually.

To determine whether the interaction between FUra and IFN was additive or synergistic, median effects analysis was employed. All 3 IFNs exhibited synergy when combined with FUra against either HT-29 or SW480 cells (Fig. 2). For IFNα, the effects were highly synergistic over nearly the entire range of concentrations tested in HT-29 cells, but were only synergistic in SW480 cells for those concentrations for which the $f(a)$ exceeded 0.7. For IFNβ, the interaction with FUra was highly synergistic over nearly the entire range of concentrations tested against SW480 cells, but only synergistic for higher concentrations [$f(a) > 0.6$] tested against HT-29 cells. For IFNγ, synergy was only noted at the higher concentrations of drugs tested [$f(a) > 0.8$] for both cell lines. These conclusions were unchanged whether the assumption was made of mutual exclusivity or non-exclusivity of action.

Because synergy has been observed in other cell lines with combinations of IFN and IL-2 (25–28), HT-29 or SW480 cells were incubated with the combination of FUra and either IL-2 alone (5–50 ng/ml) or IL-2 in combination with IFN. Neither incubation with IL-2 alone nor IL-2 in combination with any IFN for 24 h was cytotoxic for HT-29 or SW480 cells. There was no enhancement of FUra cytotoxicity when FUra was combined with IL-2 alone for 24 h. When cells were incubated with FUra plus the combination of IL-2 and either IFNα, β, or γ for 24 h, there was no enhancement of FUra lethality above that of combined FUra and IFN.

Cytokinetic Effects. To ascertain the effects of IFN, FUra, and the combination on cell cycle traverse of HT-29 and SW480 cells, cells were incubated with drug for various time periods, stained with propidium iodide, then analyzed by flow cytometry. As shown in Fig. 3, incubation with 50 μM FUra for 2–24 h resulted in only modest cytokinetic changes with the most prominent being partial depletion of G2 + M in both cell lines and approximately doubling of the percentage of cells in S phase at 9 h (HT-29, Fig. 3A) or 24 h (SW480, Fig. 3B).

In contrast, incubation of cells with IFNβ, 500 units/ml, or the combination of FUra and IFNβ for 2–24 h resulted in greater cytokinetic effects than with FUra alone. As shown in Fig. 3A and 3B, SW480 cells treated with the combination had a 3-fold increase of cells in S phase at 24 h with partial depletion of G1 and G2 + M at 18–24 h. The predominant effect of IFNβ alone in this cell line was also partial depletion of G2 + M at 18–24 h. For HT-29 cells, incubation with IFNβ resulted in a rapid (2–4 h) accumulation of cells in G2 + M, followed by a nearly 4-fold increase of cells in S phase at 9–24 h, and steady depletion of cells in G1 (Fig. 3B). The predominant effect of incubation of HT-29 cells with the combination of FUra and IFNβ was a 2-fold increase of cells in S phase at 2 and 9 h.

In contrast to IFNβ, the effects observed with single-agent IFNα or γ, or the combinations of these IFNs with FUra were minor. Incubation of either HT-29 or SW480 cells with IFNα, 500 units/ml, resulted in a transient 15% increase of cells in S phase at 2 h, which returned to near baseline levels at 4 h and remained unchanged up to 24 h. There was an accompanying transient partial depletion of G2 + M at 2 h that returned to near baseline levels by 6 h. Incubation of HT-29 or SW480 cells with the combination of FUra and IFNα resulted in essentially the same effects as FUra alone. Incubation of either HT-29 or SW480 cells with IFNγ did not alter cell cycle kinetics, and incubation with the combination of FUra and...
IFNγ resulted in essentially the same cytokinetic changes noted with FUra alone (data not shown).

DISCUSSION

These studies demonstrate that IFNα, β, and γ are capable of modulating the cytotoxic effects of the fluorinated pyrimidine, FUra, against 2 human colon carcinoma cell lines in a dose-dependent fashion. The converse, that FUra is modulating the cytotoxic effects of IFN, is less likely, since these effects were noted at concentrations of IFN that were noncytotoxic. The concentrations of FUra studied were 10- to 40-fold lower than peak concentrations clinically achievable after bolus administration of FUra, 720 mg/m², although the c × t was higher, suggesting that the concentrations used may be clinically relevant (29).

Because distinguishing additive effects from synergy may be difficult if one drug has a shallow dose-response curve (30), formal criteria for drug synergy were used. At the dose-ratio examined, all 3 IFNs exhibited synergy with FUra over at least a portion of the range of concentrations tested. While IFNα and -β interacted synergistically over nearly the entire range of concentrations tested, IFNγ was only synergistic with FUra at the highest concentration tested. Thus, the type I IFNs appear to be more potent modulators of FUra cytotoxicity than type II IFN on a unit-per-unit basis. In vivo, however, combinations of FUra and IFNγ may have more antitumor activity than FUra alone due to mechanisms other than direct enhancement of the FUra cytotoxic effect (31). Other differences between the 3 types of IFN emerge when cytokinetic effects are examined.

Although neither IFNα nor γ had any significant cytokinetic or antiproliferative effects on the 2 cell lines tested, IFNβ markedly increased the proportion of cells in both S and G2 + M either alone (HT-29) or in combination with FUra (SW480), and significantly inhibited cell proliferation in SW480 cell cultures.

Synergy both in vitro and in vivo has previously been reported for combinations of IFNs, and for combinations of IFNs with IL-2 (14, 25–28). Our results did not indicate a synergistic cytotoxic effect for combinations of biological agents against the human colon cancer cells, nor were combinations of biological agents synergistic modulators of the FUra effect. Furthermore, the absence of a modulating role for IL-2 suggests that the effects seen with the IFNs may be specific to that class of agents and not a general property of cytokines.

The mechanism of interaction of FUra and IFN remains unknown. The absence of cytokinetic effects for IFNα or -γ argue against the possibility that these agents are enhancing the effects of FUra cytotoxicity by increasing the proportion of cells in S phase, thus making them more accessible to the cell cycle-specific actions of FUra. Nevertheless, it is possible that late cytokinetic effects, beyond 24 h, may play a role in enhancing FUra cytotoxicity. Recent evidence suggests that IFNα can increase levels of the active FUra metabolite, fluorodeoxyuridine, by 10-fold in HL-60 cells (32). Thus, it is conceivable that IFN can alter the anabolism of FUra, resulting in more thorough inhibition of its target enzyme, thymidylate synthase. This is further supported by the finding that IFN-associated enhancement of FUra cytotoxicity can be reversed with thymidine (33).
IFNs have been demonstrated to enhance the cytotoxic effects of other chemotherapeutic agents, including the anthracyclines, cisplatin, cyclophosphamide, Vinca alkaloids, and nitrosoureas (14). Combinations of IFN with cytotoxic agents have been tested in clinical trials. In general, these combinations have not proven more useful than chemotherapy or IFN used alone (14).

In contrast, the combination of FUra and recombinant IFNα has produced objective responses in 63% of patients with advanced colorectal carcinoma in one clinical phase II trial (34, 35). These clinical results combined with the cell culture studies presented in this report and with early biochemical studies provide a rationale for pursuing further investigations into the mechanism of interaction of IFN and FUra.

REFERENCES

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