Positive Interactions between Interferon and Chemotherapy Due to Direct Tumor Action Rather Than Effects on Host Drug-metabolizing Enzymes


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ABSTRACT

The mechanism of increased antitumor activity when human lymphoblastoid interferon [HuIFN-α(Ly)] and the drugs cyclophosphamide and Adriamycin are used in combination on a human tumor xenograft in nude mice has been investigated. HuIFN-α(Ly) did not affect hepatic levels of the drug-metabolizing enzymes cytochrome P-450 or the glutathione S-transferases. In contrast, mouse interferon caused significant and differential changes in the isozymic forms of these enzymes. However, addition of mouse interferon to the HuIFN-α(Ly)/cyclophosphamide or Adriamycin combinations had no effect on the final result, and did not increase the toxicity of the combination therapy. These data provide evidence that the increased activity of the combination therapy is due to effects on the tumor rather than on the host.

Further studies showed significant perturbations in the tumor cell cycle after in vivo combination therapy. Cyclophosphamide caused an accumulation in G2 and the addition of HuIFN-α(Ly), which alone caused little change in cycle distribution, delayed this G2 block and strongly increased the number of cells in S phase. A similar, although less pronounced, effect was seen with HuIFN-α(Ly)/Adriamycin therapy. The increase in S phase seen in combined therapy may account for the synergy seen.

INTRODUCTION

It is now well established that IFNs can have antitumor activity in animals and humans (1, 37). Although they do not appear to be effective in most tumors as single agents when the tumor load is high, low-dose therapy with low tumor load can be of therapeutic benefit in, for instance, renal cell carcinoma, non-Hodgkin's disease lymphoma, chronic myelogenous leukemia, and some forms of myeloma (26, 31, 36, 37). Although the mechanism of this antitumor effect is not fully understood, it is possible that direct cytostatic action on tumor cells, alterations of tumor cell surface antigen expression, and indirect stimulation of host defenses are all involved (1, 3, 7, 39), thus making IFN action very different from cancer chemotherapeutic agents, and the combination of the two an interesting possibility. We have reported recently that a combination of HuIFN with CY or ADR had synergistic inhibitory activity on a human breast cancer xenograft (2), and similar positive interactions have been seen between MulFN and CY or 1,3-bis-2-chloroethyl-nitrosourea in experimental murine cancer (12, 15). However, some experimental studies in animals have shown antagonism between IFN and CY or 5-fluorouridine (22, 23), which has been attributed to effects of IFN on drug metabolism by the tumor-bearing host, or to effects of IFNs on the cell cycle of the tumor cells which may prevent cycle- or phase-specific drugs working effectively. The nude mouse/human tumor xenograft model allows us to dissociate between effects of IFN on the organism and on the tumor using the appropriate species-specific HuIFNs and MulIFNs (3).

Therefore, in this paper, we have studied the effects of HuIFN and MulIFN on hepatic drug-metabolizing enzyme systems to determine whether the therapeutic effects seen during drug/IFN administration could be attributed to IFN-induced alterations in drug metabolism, and investigated the IFN/drug synergy further in the nude mouse model by studying the effects of combination therapy on cell cycle progression of tumor cells.

MATERIALS AND METHODS

Mice. Female nu/nu (nude) mice of mixed genetic background were bred by A. Sebesteny and J. Menzies (ICRF Laboratories, Mill Hill, United Kingdom) and maintained as described previously (3) with the exception that 6- to 8-week-old specific-pathogen-free mice were used.

Tumor. The tumor used in all these experiments was derived from a mucoid carcinoma of the breast in an untreated postmenopausal woman. Details of its morphology and growth characteristics are given elsewhere (3). The tumor was used between passages 18 and 23 in the nude mouse.

Experimental Procedure. Tumors were excised, chopped with scissors until a fine suspension was obtained, and then implanted into 6- to 8-week-old experimental mice as described previously (3). After 2 to 3 weeks, mice bearing tumors of near-identical size were divided into groups of 4, and therapy commenced. Mice were given IFN s.c. at a site distant from the tumor daily, and ADR or CY i.p., once a week. Tumor growth was assessed weekly by measuring the 2 largest diameters at right angles to each other, and the tumor size index as shown in the Figs. was a multiplication of these 2 measurements. Each point on the graph represents a mean of 4 values from different animals.

Flow Microfluorometry Studies. The mice were killed by cervical dislocation at set time intervals after initiation of treatment and the tumor immediately chopped finely with scissors; 5 ml of RPMI 1640 (Flow Laboratories, Irvine, Scotland) were added, and the suspension was shaken well and gently pipetted through a piece of 100-sq μm nylon mesh. The resulting cell suspension was spun down at 1000 rpm for 5 min and then prepared as described in Ref 5. Briefly, nuclei were isolated, treated with RNase A, and then stained with propidium iodide. Stained nuclei were analyzed on a Becton Dickinson Modified FACs 1 fluoroscence-activated cell sorter using a 488 nm argon laser beam with 610-nm-long path optical filter. The percentage of cells in each phase of the cell cycle was analyzed from the histogram by a computer program that counted events between preset positions. The channel numbers used to

1 To whom requests for reprints should be addressed.
2 The abbreviations used are: IFN, interferon; ADR, Adriamycin; CY, cyclophosphamide; HuIFN-α(Ly), human lymphoblastoid interferon; MulIFN, mouse Ehrlich ascites cell interferon; SDS, sodium dodecyl sulfate; HuIFN, human interferon.

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NOVEMBER 1984

5249
IFN and Drugs. The HuIFN was prepared from Namalwa cells induced by Sendai virus as described previously (13). The specific activity ranged from $1.17 \times 10^7$ to $2.20 \times 10^7$ units/mg protein. The MuIFN was prepared from Ehrlich ascites cells as described previously (27) and had a specific activity of $5 \times 10^7$ units/mg. Prior to experiment, IFN samples were assayed at least twice in a viral RNA synthesis assay using WISH cells (HuIFN) and L-cells (MuIFN) challenged with Semliki Forest virus. The assays were standardized against the HuIFN standard 69/19 (obtained from the National Institute of Biological Standards and Controls, London, United Kingdom) or the MuIFN international standard (G002-904-511; National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD). After correction for any loss of activity due to one freeze/thaw cycle, the IFN was aliquoted and stored at $-20^\circ$ in phosphate-buffered saline with bovine serum albumin (3 mg/ml; Sigma). CY (Endoxana; W. B. Pharmaceuticals, Ltd., Bracknell, United Kingdom) and ADR, (Farmatilia Carlo Erba Ltd., Barnet, United Kingdom) were freshly diluted to the appropriate dose at each time point.

Liver Enzyme Assays. Mice were killed by cervical dislocation, and the livers were removed and placed immediately in a large volume of ice-cold 1.15% KCl in 10 mM phosphate buffer, pH 7.4 (KCl buffer). After being rinsed, dried, and weighed, the livers were scissors minced, and 3 volumes of KCl buffer were added. After homogenization, this was spun at 11,000 x g for 20 min at 4°, and the pellet was discarded. The supernatant was then spun at 105,000 x g for 1 hr at 4°, and the supernatant was removed and stored at $-70^\circ$ (cytosol). The pellet was resuspended in KCl buffer, spun for 1 hr further at 105,000 x g, then homogenized in 0.25 M sucrose in 10 mM phosphate, pH 7.4; this homogenate (microsome preparation) was then stored at $-70^\circ$. The following drug-metabolizing enzyme levels and activities were then determined. Microsomal cytochrome P-450 concentration was determined spectrally according to the method of Omura and Sato (28), and cytochrome b5 by the method of Omura and Taubes (29). Cytochrome P-450 reductase activity using cytochrome c as substrate was determined by the method of Masters et al. (25). One unit of activity was taken as 1 nmol of cytochrome c reduced per min at 37°. The rate of oxidation of cytochrome P-450 substrates, 7-ethoxycoumarin and 7-ethoxyresorufin, were determined by using published procedures (10, 38). Cytosolic glutathione S-transferase activities with 2,4-dinitrochlorobenzene or 2,4-dichloronitrobenzene as substrates were measured spectrophotometrically by the method of Habig et al. (17).

Peripheral Blood Counts. Approximately 20 μl of blood were collected from the tail vein of each mouse into a heparinized tube. This was diluted 1:10 in 4% acetic acid solution, and the WBC were counted using a phase-contrast microscope.

RESULTS

Effect of Combined IFN/Drug Therapy. Chart 1 shows a typical experiment using combinations of $2 \times 10^6$ units of HuIFN-α(Ly) given daily and 0.25 mg of CY or 0.04 mg of ADR given once weekly. As we described in a previous paper (2), the interaction between IFN and chemotherapy was defined as positive or synergistic using the calculations of Berenbaum (9). The results obtained with IFN and CY were statistically better than those obtained with either IFN or CY alone ($p < 0.01$), and those obtained with IFN and ADR were better than either alone ($p < 0.01$ or $p < 0.05$). Similar positive interactions between HuIFN-α(Ly) and CY have recently been seen with a human bowel carcinoma xenograft. 3

Effect of Homologous IFN on Drug-metabolizing Enzymes of the Liver. Because drug-metabolizing enzyme systems are intimately involved in the mode of action of drugs such as ADR and CY, experiments were carried out to determine whether HuIFN-α(Ly) induced changes in these enzyme activities in the mice. Enzyme levels and activities of the hepatic microsomal...
Mechanism of Synergy between IFN and Chemotherapy

Table 1

<table>
<thead>
<tr>
<th>Yield of microsomal protein (mg/g tissue)</th>
<th>Enzyme concentration</th>
<th>Monooxygenase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 11.7 ± 2.5等</td>
<td>P-450 (nmol/mg protein)</td>
<td>Cytochrome b5 (nmol/mg protein)</td>
</tr>
<tr>
<td>HulFN 12.0 ± 4.5</td>
<td>3.7 ± 0.19</td>
<td>0.59 ± 0.04 (96)</td>
</tr>
<tr>
<td>MulFN 10.9 ± 3.3</td>
<td>2.3 ± 0.30 (109)</td>
<td>0.12 ± 0.30 (75)</td>
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* Mean ± S.D. of values obtained from 3 separate livers.
* Numbers in parentheses, percentage of control values.
* Statistically significant from control (p < 0.01).

fractions derived from nude mice treated daily for 5 weeks with 1 x 10^8 units of HulFN or HulFN are shown in Table 1. HulFN-α(Ly) had no effect on any of the parameters measured, but HulFN therapy caused marked changes. Table 1 shows that cytochrome P-450, which is responsible for the oxidation of a vast number of drug substrates (40), including CY, was reduced 18% following treatment with HulFN. This was statistically significantly lower than control values (p < 0.01). It was of interest that the cytochrome P-450-mediated deethylation of 7-ethoxycoumarin was reduced to a much larger extent (49%) (p < 0.01). 7-Ethoxyresorufin activity was reduced by 26%, but this value was not significantly different from that of the controls. Cytochrome P-450 reductase, which is an enzyme active in the one electron reduction of compounds such as ADR (24) as well as cytochrome b5, was also reduced in parallel with the cytochrome P-450. These reduced enzyme levels could not be accounted for by a change in the yield of microsomal protein, which in the HulFN-treated groups of animals were not significantly different from controls. Table 1 shows a typical experiment where values are the mean of 3 separate mouse liver preparations. Similar results were found in 2 further experiments with 3 mice in each treatment group. SDS-polyacrylamide slab gel electrophoresis of microsomal samples from the various treatment groups is shown in Fig. 1a and b. No differences in the composition of microsomal proteins between control mice and those treated with HulFN-α(Ly) were observed, however in animals treated with MulFN, significant changes were seen. IFN induced the synthesis of a protein with a mobility in the region associated with cytochrome b5, was also reduced in parallel with the cytochrome P-450. These reduced enzyme levels could not be accounted for by a change in the yield of microsomal protein, which in the MulFN-treated groups of animals were not significantly different from controls. Table 1 shows a typical experiment where values are the mean of 3 separate mouse liver preparations. Similar results were found in 2 further experiments with 3 mice in each treatment group. SDS-polyacrylamide slab gel electrophoresis of microsomal samples from the various treatment groups is shown in Fig. 1a and b. No differences in the composition of microsomal proteins between control mice and those treated with HulFN-α(Ly) were observed, however in animals treated with MulFN, significant changes were seen. IFN induced the synthesis of a protein with a mobility in the region associated with cytochrome P-450 isozymes at molecular weights of 50,000 was also increased. Proteins with mobilities at M, 46,800; 51,000; and 54,000 appear to be present at reduced concentrations. Cyto¬

Lack of Effect of MulFN on the Synergy between HulFN and CY or ADR. In the experiments shown in Chart 3 and 4, we studied the effect of a daily dose of 1 x 10^8 units of MulFN on the therapeutic effects of 1 x 10^9 units of HulFN-α(Ly) daily plus CY or ADR once weekly. A positive interaction between either IFN and CY (Chart 3) or IFN and ADR (Chart 4) was seen whether or not MulFN was added to this combination. Tumor size indices in all treatment groups were significantly smaller than control sizes (p < 0.05 for single therapy; p < 0.01 for combined therapies). Tumor size indices in all the single therapy groups except ADR were significantly larger than the corresponding combined therapy groups (p < 0.02 or p < 0.05). The addition of MulFN did not result in an increased toxicity as measured by peripheral WBC counts in treated mice. Control mice had a mean WBC count of 9.9 ± 1.7 x 10^a cells/ml (S.D.), and HulFN-α(Ly) plus CY and HulFN-α(Ly) plus ADR were 7.3 ± 3.4 and 8.3 ± 1.8 x 10^a cells/ml, respectively, and the HulFN-α(Ly) plus MulFN plus CY, and HulFN-α(Ly) plus MulFN plus ADR groups gave counts of 6.2 ± 1.3 and 8.0 ± 2.9 x 10^a cells/ml, respectively.

Cell Cycle Distribution of Tumor Cells during Therapy. After the start of therapy in experiments similar to those shown in Chart 1, tumors were removed at daily intervals, and the cell...
cycle distribution was studied using flow microfluorimetry. Throughout the experiment, there was little change in the percentage of cells in each phase of the cell cycle in the control tumors (mean values: G1, 69 ± 4%; S, 12 ± 2%; G2, 19 ± 3%).

Chart 5 shows the tumor cell DNA histogram results for the treated animals expressed by the proportion of cells in each phase of the cell cycle as a percentage of control values for that treatment.
Mechanism of Synergy between IFN and Chemotherapy

phase. No obvious change was seen after one day of therapy in any group. However, at 2 days in the CY-treated group, a pronounced increase in the number of cells in G2 was seen (183% of control values) with a corresponding decrease in G1 (73% of control). At this time, HulFN-α(Ly) therapy alone had little effect on cell cycle distribution in G1, but a small increase in S (133% of control values) was recorded. The G2 accumulation in the combined CY/HulFN-α(Ly) therapy group was not as pronounced (127% of control), but a marked accumulation of cells was seen in S-phase (183% of control). By Day 3, the G2 accumulation was still seen in the CY group (165% of control values) and now became apparent in the CY/HulFN group (182% of control values), but in this combined therapy group there was still a considerable increase in S-phase cells (191% of control values) as compared to the CY alone (91% of control values). In the CY/HulFN-α(Ly) group, the proportion of cells in G1 phase dropped to 48%. Once again, IFN therapy alone showed little effect on the overall distribution of cells in the cycle.

By Day 4, the percentage of cells in S phase was decreased in the CY group (73% of control) but still high (155% of control) in the CY/HulFN-α(Ly) group. Similar results were obtained in 3 separate experiments.

As can be seen in Chart 5, the effects of ADR and ADR/ HulFN-α(Ly) combinations were not as obvious, but a trend similar to that seen with CY was noted. Some G2 accumulation was seen at Days 2 and 3 in the ADR-treated group (133 and 129% of control values, respectively), and this was delayed in the ADR/HulFN-α(Ly) groups where G2 was at control levels at Day 2 and then rose (147% of control values) at Day 3. Also, in the ADR/HulFN-α(Ly) group, an accumulation of cells in S-phase (150 and 155% of control values, respectively) was seen at this time, causing a reduction in the number of cells in G1 phase.

Thus, in summary, combining HulFN-α(Ly) with either of these drugs caused a delay in G2 accumulation and an increase in the number of cells in S phase.

DISCUSSION

We have found previously that combinations of HulFN-α(Ly) and the drugs CY or ADR are effective therapy against human tumor xenografts in nude mice. There are several papers showing drug IFN synergy in mouse tumor model systems (12, 15), but it is not certain whether IFN-induced alterations in drug metabolism, or direct effects of drug and IFN on tumor cells are responsible for the overall therapy. Because of the species specificity of IFNs, the nude mouse-human tumor xenograft system has allowed us to investigate this question.

The data presented in this manuscript provide strong evidence against the possibility that in drugs, metabolism enzymes explain the increased antitumor effect, since the HulFN- α(Ly) used had no apparent effect on these enzyme systems in the mouse. In agreement with these findings, Singh et al. (35) have made a similar observation following a single dose of a cloned HulFN-α.

There are now several reports in the literature that demonstrate that known IFN inducers, or in one case a pure cloned IFN, suppress cytochrome P-450 monooxygenase activity (18, 32, 33, 35).

The overall activity of cytochrome P-450-dependent monooxygenases measured in microsomal samples is the summation of the individual contribution of many isozyme P-450 forms and is a critical factor in determining overall metabolizing capacity (16, 30, 34). In this study, we provide evidence that homologous IFN has a differential effect on the cytochrome P-450 isozymes. This conclusion is based on 2 observations: (a) the differential effect of MulFN on cytochrome P-450 level and loss of 7-ethoxycoumarin activity; and (b) changes in molecular weight bands between M, 45,000 and M, 60,000. It was interesting to note that several of the protein bands were reduced but one, in fact, increased in intensity. The fact that the protein bands were changed demonstrates that the changes in enzyme activity can be accounted for by changes in protein content rather than changes in the regulation of P-450 heme.

IFN-induced changes in glutathione S-transferase activity have not been reported previously. Similar to the P-450s, this appears due to altered protein synthesis as changes in protein bands on SDS gels occurred in regions where the mouse enzymes are known to migrate (8). The apparent differential regulation of these proteins by IFN, some being induced some being depressed, is intriguing and may be of significance as different glutathione S-transferase isozymes are known to have very different specificities in the deactivation of cytotoxins (14). It is of interest that the protein changes associated with IFN therapy were almost exclusively limited to mobilities in SDS gels associated with drug-metabolizing enzymes. The significance of this is intriguing but unclear. However, it is interesting to speculate that infection may alter susceptibility to cytotoxins or carcinogens.

Thus, continuous therapy with homologous IFN induces changes in drug-metabolizing enzyme activity, and it is possible that the outcome of drug/IFN therapy would also be affected. In the nude mouse-human tumor xenograft model system, we are able to dissociate effects of IFN on drug-metabolizing enzymes in the liver from direct IFN-chemotherapy interactions on the tumor. However, in further studies described here, we have shown that continuous administration of MulFN, at concentrations known to affect the nude mouse liver enzymes, does not alter either the antitumor effect or the host toxicity of the HulFN/ drug combinations.

Therefore, we conclude that the tumor is the most important site for IFN chemotherapy interactions and have provided evidence that the result of such combinations is to increase the proportion of tumor cells in S phase, thus making them maximally susceptible to ADR and CY. The effect of IFNs on the cell cycle appears to be an increase in length of all phases as has been demonstrated previously by in vitro studies with cell lines and strains (4, 5, 20). A variety of cytotoxic drugs cell cycle progression in G2 phase (6, 19).

While IFN-induced slowing of all cell cycle phases could account for the delay in G2 accumulation seen in the combination therapy tumors, it is not clear why an increase in S phase is also seen. However, CY is an alkylating agent, and ADR intercalates with DNA (11); thus, it is reasonable to hypothesize that the S phase accumulation seen in the combination therapy groups is responsible for the enhanced cytotoxicity.

REFERENCES


Fig. 1. SDS-PAGE of hepatic microsomal and cytosolic fractions from nude mice treated with IFN. a and b, hepatic microsomes (30 μg) run on 12.5 and 7.5% polyacrylamide gels, respectively. c, hepatic cytosol (30 μg) run on 12.5% polyacrylamide gels. Arrows, positions where changes in protein bands were observed. C, control animals; Hu, animals treated with human α-IFN; M, animals treated with MuIFN. In a and c, all lanes are samples from individual animals.
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