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Interferon modulation of 5-fluorouracil: use in neoadjuvant therapy inhibits experimental liver metastases in nude mice

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Experimental liver metastasis was studied in 4–5 week old athymic nude mice that were injected intrasplenically with a human colorectal tumor cell line (LoVo). A treatment schedule combining 5-fluorouracil and interferon (IFN) was previously shown to inhibit liver metastases. When this treatment was delayed until after splenectomy at 1, 2 and 3 weeks after tumor cell injections, liver metastases were not inhibited. However, when IFN was given during the interval between tumor cell injections and splenectomy (as neoadjuvant therapy), liver metastases were inhibited in the 2 and 3 week groups, but not in the 1 week group.

Key words: 5-Fluorouracil, interferon, LoVo, metastases, neoadjuvant therapy, nude mice.

Introduction

The use of biological response modifiers, or biomodulators, in combination with different chemotherapeutic agents is currently an active area of research in cancer chemotherapy. The objective of this combination is to enhance the antitumor effect and improve the specificity of the antitumor drug, thereby increasing therapeutic efficacy. One of the more studied biomodulators is interferon (IFN) which, when combined with 5-fluorouracil (5-FU), has shown synergistic or additive effects against different cancer cell lines.[1–6] IFN used as a single agent on patients with advanced colorectal cancer in a number of clinical trials showed no activity.[7] Phase I and II clinical trials were conducted by Wadler *et al.*[8,9] with the combined 5-FU and recombinant IFN-α2a(rIFN-α2a)on patients with advanced colorectal carcinoma. Both of these studies have shown that the addition of IFN to 5-FU enhanced the objective response rates in the patients, with toxicities that were tolerable. Overall, the results are promising and are currently being continued by the Eastern Cooperative Oncology Group to obtain data from multi-institutional sources.

Our interest in the use of combination biotherapy has been in its application to the treatment of colorectal tumor cell metastases to the liver. Morikawa *et al.*[10] had shown that 5-FU and mouse rIFN-γ inhibited the growth of human colon carcinoma cells implanted into the spleens of nude mice. The combined therapy was initiated 3 days after the injection of tumor cells. Because mouse rIFN-γ is not reactive against human tumor cells, the inhibitory effects observed were attributed to the direct antitumor effects of 5-FU coupled with the augmentation of host defense mechanisms by the mouse rIFN-γ. We have also been studying experimental liver metastasis in nude mice using a human colorectal tumor cell line (LoVo). We previously reported[11] that an intensive regimen of 5-FU (given once a week) and human rIFN-α (given 5 times per week), begun 3 days after tumor cell inoculation and maintained for up to 4–5 weeks, successfully inhibited liver metastases. We have now applied this combined therapy to mice beginning at different times after tumor cells were injected into the spleen and containing tumors. The aim of these studies was to initiate adjuvant therapy following splenectomies at 1, 2 and 3 weeks after tumor cells were injected.

Materials and methods

Athymic nude mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). All mice used

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in this study were males at 4–5 weeks of age. The mice were housed in laminar flow air filtered isolator units in a room maintained at 80°F and 55% relative humidity. Lighting was automatically controlled, alternating 12 h of light and dark cycles. Feed, water and bedding materials were autoclaved before use. The US Public Health Service Policy on humane care and use of laboratory animals was followed, administered by the Institutional Animal Care and Use Committee. All mice were injected intrasplenically with tumor cells by the technique reported by Kozlowski *et al.*,[12] which we have also used and reported earlier.[13,11]

All animals were sacrificed by cervical dislocation, autopsied and grossly visible tumors recorded. Any suspicious or doubtful liver lesions were taken and fixed in 10% phosphate buffered formalin. All lungs were taken and also fixed in formalin. All tissues fixed were processed for histological examination. Sections were cut at 5 μm from three randomly selected blocks of each tissue, stained with hematoxylin and eosin, and studied for the presence of tumor cells.

LoVo, the human tumor cell line used in this study, was originally derived from a metastatic tumor nodule in a lymph node.[14] The cell line was obtained from the American Type Culture Collection (Rockville, MD), and maintained in the laboratory using Ham's F-12 medium supplemented with 10% fetal bovine serum and antibiotics. Cultures were maintained at 37°C in a humidified atmosphere with 6% CO_2. Intrasplenic injections were in a volume of 0.050 ml/injection containing 1.5×10^6 cells.

rIFN-α2a (Roferon A) was a gift from Roche Laboratories, Hoffmann-LaRoche (Nutley, NJ). Each vial contained 18×10^6 IU, with a specific activity of 2×10^8 IU/mg protein. Sterile water was used to reconstitute the contents of each IFN vial. 5-FU was a generic form obtained from the hospital pharmacy, packaged at 50 mg/ml in a 10 ml vial. 5-FU was injected i.p. on the basis of 80 mg/kg. The average weight of the mice was 20–22 g. Each mouse received 1.76 mg/injection. IFN was given s.c. at 3×10^5 units/injection. Intrasplenic injections and splenectomies were performed on mice while under methoxyflurane anesthesia.

Results

Our previous study[11] had shown that the combined treatment of 5-FU (started 3 days after tumor cell injections and given 3 more times at 1 week apart) and IFN (also started 3 days after tumor cell injections but given daily thereafter for 4–5 weeks) inhibited the formation of liver metastases. The same treatment schedule was used in this study but with two important differences. (i) The treatment was delayed until after splenectomy, which was performed 1, 2 and 3 weeks after tumor cell injections. (ii) Another series of animals was treated with additional IFN which was administered daily during the interval from injection of tumor cells to splenectomy. Following splenectomy, the same combined 5-FU and IFN injections were given as in group (i).

Table 1 presents the data of the combined 5-FU and IFN treatments following splenectomies at different times after tumor cells were injected. The data were analyzed using Fisher's exact test, and p values for the comparisons of controls and 'post' treatments are included. The combined treatment following splenectomy ('post' series) at 1, 2 and 3 weeks after tumor cell injections did not show inhibition of liver (and lung) metastases. When IFN was administered during the period between tumor cell injection and splenectomy ('pre' series), however, inhibition of liver metastases was demonstrated in the 2 and 3 week groups. The 1 week group failed to show comparable inhibitory activity in liver metastases. It is clear (comparing the controls vs. 'pre' groups) that IFN treatment given during the period leading up to splenectomy exerts

Table 1. Effect of combined 5-FU and IFN on metastases following splenectomy

	No. positive/No. injected		
	spleen	liver	lung
A: 1 week after tumor cell injections			
controls	12/12	11/12	9/12
splenectomy	8/8	7/8	7/8
pre (A)	12/12	10/11	9/11
post (B)	13/13	8/12	5/12
B: 2 weeks after tumor cell injections			
controls	12/13	9/11	7/11
splenectomy	13/13	8/12	11/12
pre (A)	11/12	3/11[a]	4/11
post (B)	13/13	8/12	6/12
C: 3 weeks after tumor cell injections			
controls	12/12	9/12	6/12
splenectomy	13/13	6/10	6/10
pre (A)	14/14	3/13[a]	2/13
post (B)	13/13	9/11	9/11

[a] Controls vs. pre (A): $p = 0.01$.

Figure 1. Spleens removed at 3 weeks post tumor cell injection. Tumors have developed in all of the spleens.

Figure 2. Liver metastases. Mice sacrificed at 7 weeks post tumor cell injections. Metastases present in five of six livers shown.

a difference with respect to improving the therapeutic efficacy of 5-FU. In our previous study[11] we had learned that IFN given as a single agent (regardless of the number of times given) was completely ineffective in inhibiting liver metastases. Also, 5-FU alone was either ineffective or too toxic, depending on the number of doses given.

Splenectomy alone was not effective in inhibiting liver metastases. Splenectomy in concert with the combined IFN and 5-FU inhibited liver metastases, provided the IFN was given during the period before splenectomy, as in the 2 and 3 week groups. In the 1 week group, it appears that this was not a sufficient pre-treatment period with IFN to modulate the effects of 5-FU in inhibiting liver metastases. Figure 1 shows the spleens removed from mice at 3 weeks after tumor cell injections and demonstrates the extent of tumor formation at the time of removal. Examples of liver metastases are shown in Figure 2.

A survival study was performed with two different schedules in which the question was asked whether or not the lethal effects of 5-FU could be modulated by IFN. In the first schedule, 5-FU was given for 5 successive days. This resulted in all mice dying by day 11. If IFN was given for 5 days during the first week, then followed with 5-FU for 5 days during the second week, survival was extended to 15 days (Figure 3). In another series, 5-FU was given every third day for a total of 5 injections. On the intervening 2 days between 5-FU, IFN was administered. This schedule resulted in all mice dying by 14 days. If we gave IFN for 5 days in the first week before this schedule was started, this extended the survival to 19 days (Figure 4). In both series, when the Kaplan–Meier curves were analyzed by the Mantel–Cox procedure, the administration of IFN *before* the 5-FU treatments resulted in significant differences (p of 0.0001 and 0.0016 in Figures 3 and 4, respectively).

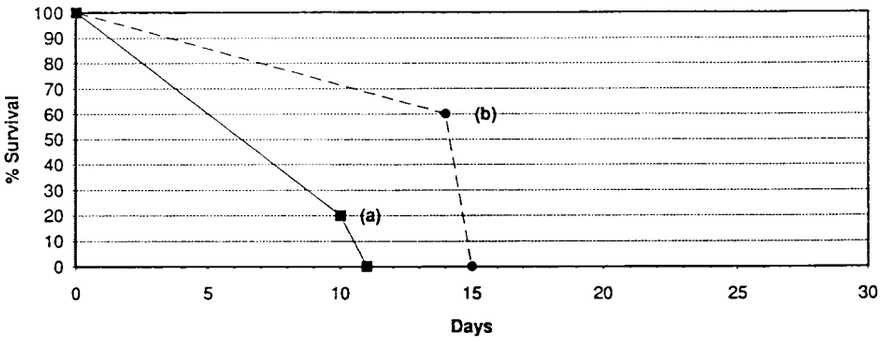

Figure 3. Kaplan–Meier estimates of survival of mice given five successive daily injections of 5-FU (a) and the same schedule *preceded* by five daily injections of IFN (b). $p = 0.0001$.

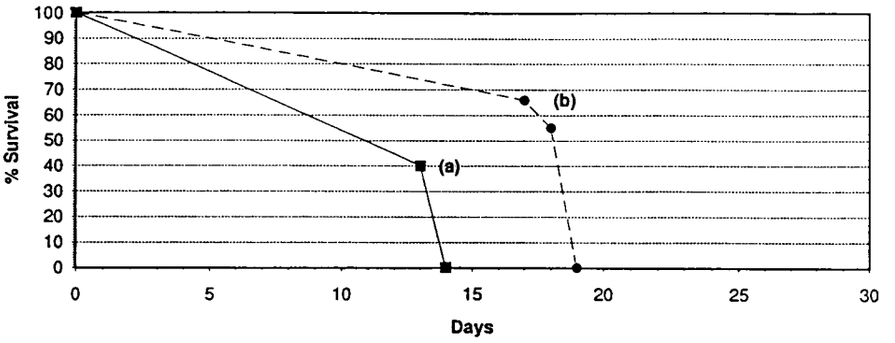

Figure 4. Kaplan–Meier estimates of survival of mice given five injections of 5-FU every third day (a) and the same schedule *preceded* by five daily injections of IFN (b). $p = 0.0016$.

Discussion

We had demonstrated earlier[11] that the combined use of 5-FU with IFN was effective in inhibiting liver metastases after intrasplenic injection of a human tumor cell line (LoVo). Starting the treatment 3 days after introducing the tumor cells, 5-FU was given once a week for 4 weeks and IFN was given daily for 4 weeks. This has been a consistently effective regimen to inhibit liver metastasis under the conditions employed. (A number of variations of this schedule were investigated, but none showed equivalent efficacy. For example, 5-FU given in five successive daily injections during the first week resulted in total lethality within 11 days. IFN given without 5-FU resulted in complete absence of inhibitory activity.) We set out to determine whether or not the same treatment regimen would be equally effective in nude mice *after* tumors had developed in the spleen. Accordingly conditions were set up in which LoVo cells were injected into the spleens of nude mice and allowed to develop into tumors for 1, 2 and 3 weeks. At each of these times, splenectomies were performed; 3 days later, the combined 5-FU and IFN treatment that had been effective in our previous study[11] was started. The results of this series showed that liver metastases were not significantly inhibited when compared with the controls. Another series of mice was set up in which *additional* IFN was given during the interval from injection of tumor cells to splenectomy. Thus, in each of the three groups, IFN was given for 1, 2 and 3 weeks prior to splenectomy; then 3 days later, the combined 5-FU and IFN treatment was administered. Under these conditions, liver metastases were inhibited in the 2 and 3 week groups, but not in the 1 week group.

That the combined use of 5-FU with IFN might offer therapeutic potential was suggested by work with cells in culture which showed synergistic or additive effects when the two agents were used together.[1–6] Since then, interest in combining IFN with chemotherapy has increased, as evidenced by the extensive review of 169 papers on this subject covered by Wadler and Schwartz.[15] However, in spite of what appears to be a promising approach for the treatment of cancer, no rational strategy for the combined use of 5-FU and IFN has emerged. As Wadler and Schwartz[15] have pointed out, the interaction between IFN and cytotoxic agents such as 5-FU is complex, and its effectiveness is dependent on concentrations, ratios, duration and sequence of exposure to the two drugs.

We subjected mice to two different schedules of 5-FU that we had confirmed from previous experience would be extremely toxic, resulting in death in about 2 weeks. Injections of 5-FU were started on the third day after tumor cell injections. In the first schedule 5-FU was given daily for a total of five injections (Figure 3a). In the second schedule, 5-FU was given every third day for a total of five injections (Figure 4a). Both of these schedules resulted in complete lethality at 11 and 14 days, respectively. If IFN was administered for a full week (five injections) *before* the two 5-FU schedules, survival was significantly extended ($p = 0.0001$ and 0.0016), as shown in Figures 3(b) and 4(b). In two other schedules, in which 5-FU was given every third day (four injections; Figure 5a) and every other day (three injections; Figure 6a), the administration of IFN *after* 5-FU did not significantly improve the survival of the mice, as shown in Figures 5(b) and 6(b). In the series in which IFN was given *before* 5-FU, it is clear that the IFN did

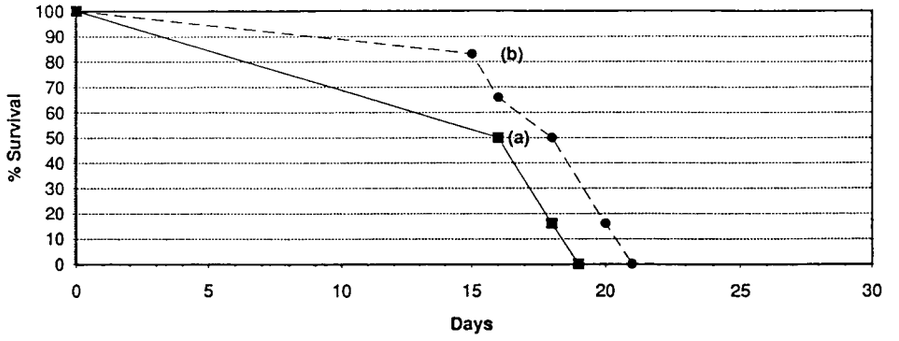

Figure 5. Kaplan–Meier estimates of survival of mice given four injections of 5-FU on every third day (a) and the same schedule *followed* by five daily injections of IFN (b). $p = 0.1616$.

not completely alleviate the severe effect of 5-FU. We point to this, however, as evidence that IFN *can* modulate the toxic effects of 5-FU. That we could demonstrate this with extreme doses of 5-FU raises the possibility that IFN can modulate the toxic effects of 5-FU when appropriately used in combination therapy.

Conceivably, IFN may have a protective effect on the normal host tissues, enabling a tolerance for the known potent cytotoxity of 5-FU. This type of protective effect on the host has been described by Stolfi *et al.*[16] and Stolfi and Martin.[17] These workers found that IFN or IFN-inducers administered to mice following a toxic dose of 5-FU protected the mice from body weight loss, leukopenia and mortality. They hypothesized that the mechanism responsible was the suppression of normal bone marrow cell proliferation by IFN, thus protecting them from the cytotoxic action of 5-FU.

The combined use of 5-FU with IFN and other agents will take on more significance as we develop an understanding of its possibilities. As stated earlier, no rational strategy has yet emerged and equally important questions regarding dosages and times and schedules of administration remain to be elucidated. Much still needs to be learned before we achieve a full understanding of the mechanism of action of the combination and can apply this information for cancer chemotherapy in general.

Conclusion

Experimental liver metastasis was studied in nude mice using a human colorectal tumor cell line (LoVo). LoVo cells injected intrasplenically produce spleen tumors and liver metastases. Our previous study had shown that a combined 5-FU and IFN regimen started 3 days after tumor cell implantation inhibited liver metastasis. This schedule consisted of 5-FU given once a week for 4 weeks and IFN given daily (5 days/week) for 4 weeks. We now report the effects obtained using this same schedule on mice injected intrasplenically with LoVo cells and allowed to develop spleen tumors for 1, 2 and 3 weeks. At each of these times,

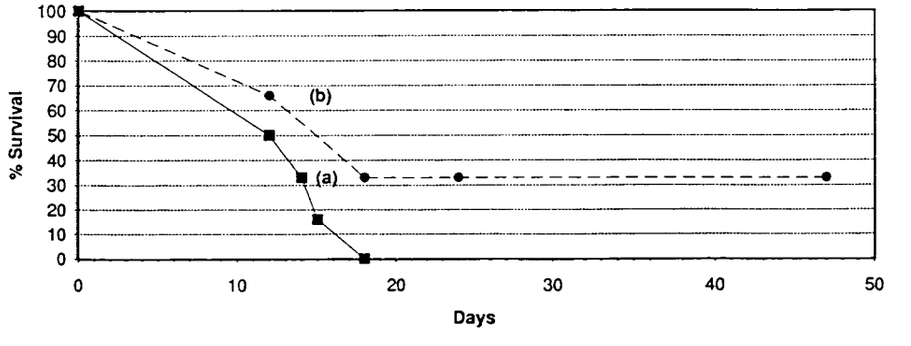

Figure 6. Kaplan–Meier estimates of survival of mice given three injections of 5-FU every other day (a) and the same schedule *followed* by five daily injections of IFN (b). $p = 0.0409$.

splenectomies were performed, and 3 days later, 5-FU and IFN treatment started. When this schedule was used on all three groups, no inhibition of liver metastasis was demonstrated. However, if additional IFN was given during the period from injection of tumor cells to the day of splenectomy, liver metastases were inhibited in the 2 and 3 week groups, but not in the 1 week group. Pre-treatment with IFN (for 2 and 3 weeks) was shown to exert a difference in the inhibitory effect on liver metastasis when used in this situation which was designed to mimic neoadjuvant therapy.

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