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Inhibition of liver metastases in nude mice by the combined action of 5-fluorouracil and interferon

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Liver metastasis in nude mice was studied using LoVo, a cell line developed from a patient with adenocarcinoma of the colon. LoVo cells injected into the spleen of 4-week-old nude mice showed 100% tumorigenicity in the spleen and metastases to the liver. This system was used to study ways in which to improve the efficacy of 5-FU, which was used in combination with alpha-2a interferon. Our results show that interferon can potentiate the effect of 5-FU in inhibiting liver metastases. Either drug given alone did not show comparable inhibition.

Key words: Chemotherapy, 5-FU, interferon, LoVo, metastases, nude mice.

Introduction

Ever since the introduction of 5-fluorouracil (FU) by Heidelberger in 1957, there has been no other major chemotherapeutic advance in the treatment of metastatic colon cancer. Statistics reveal that FU has produced objective responses in only 15–20% of patients with advanced colorectal cancer and no evidence of overall improved patient survival.[1] Efforts are being directed to increase the chemotherapeutic effectiveness of FU by combining its usage with other agents which may potentiate or enhance the activity of FU. One agent which has been singled out in a number of recent studies is interferon (IFN) which has attracted interest because of its antiproliferative effects on cells in culture.[2] IFN has been used against a variety of different tumors and, indeed, some reports indicate promising antitumor effects.[3–5] Its mechanism of action, however, is unknown. Another problem with using IFN is that there are many different types and subtypes of human IFNs, depending on its source and owing to the technology of genetic engineering. At least 10 different subtypes of alpha (leucocyte) IFN, four of beta (fibroblast) IFN, and an unknown number of gamma (antigen- or mitogen-stimulated lymphocytes) IFNs exist.[6] Partly alleviating the dilemma, however, is the species specificity of human IFNs, i.e., human IFNs are not effective on mouse cells and vice versa. This allows one to dissociate the direct effects of human IFN on human tumor cells which have been xenografted in nude mice from indirect effects on the murine host.

Morikawa *et al.*[7] reported that FU used with mouse gamma IFN produced therapeutic effects against the growth of human tumor cells in the spleen and liver of nude mice. The antitumor effects were ascribed to the direct antitumor effects of FU and the modulation of host immune mechanisms by the mouse gamma IFN. Using the same animal model system to study metastases, we have injected a human colorectal tumor cell line into the spleens of nude mice. Liver metastases result in 6 weeks in the untreated mice. We have studied the antimetastatic potential of the combined use of FU and human alpha IFN. A treatment schedule is described which is characterized by continuous injections of IFN over a period of 4–5 weeks, together with once a week doses of FU: marked inhibition of liver metastases resulted. These findings may be useful in the treatment of disseminated colorectal cancer.

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Materials and methods

Recombinant alpha-2a interferon (Roferon A) was a gift from Roche Laboratories, Hoffmann-La

Roche, 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.

Athymic nude mice were purchased from Harlan Sprague Dawley, Indianapolis, IN. All mice in this study were males and were used at 4 weeks of age. The mice were housed in laminar flow air filtered isolator units under specific pathogen-free conditions. The Public Health Service Policy on humane care and use of laboratory animals was followed, administered by the Institution's Animal Care and Use Committee. All mice were injected intrasplenically with tumor cells by the technique reported by Kozlowski *et al.*[8] and which we have also used and reported earlier.[9]

Tumor cell lines used in this study were Colo 205 and LoVo. These were both derived from human patients with carcinomas of the colon. Colo 205 was isolated from the ascitic fluid and LoVo from a metastatic tumor nodule in a lymph node. The cell lines were obtained from the American Type Culture Collection (ATCC), and maintained in the laboratory using media recommended by the ATCC. Tumor cells were injected in a volume of 0.050 ml/injection into the spleen. The concentration of cells injected was 1.5×10^6.

FU was given on the basis of 80 mg/kg. The average mass of the mice was 20–22 g. Each mouse received 1.76 mg/injection, i.p. In general, experiments were begun on a Friday, and drug injections started on the third day thereafter. IFN injections were given for five successive days from Mondays through Fridays, the mice were rested for 2 days, and then injections were resumed again. FU injections were usually given once per week, on Mondays. IFN doses were $(2–3) \times 10^5$ units/injection, s.c. Specific doses and schedules are indicated in the text.

Results

This study started with a comparison of the metastatic potential of the two cell lines, Colo 205 and LoVo. Colo 205 had been used in an earlier study,[9] in which we had noted that there was no evidence of liver metastases after intrasplenic injections. It was not clear at that time whether this absence of liver metastases was the effect of the drug combination used or an intrinsic property of the cells. Table 1 compares tumor formation in the spleen, liver and lung by Colo 205 and LoVo cells. While both cell lines were tumorigenic in the spleen, there were no metastases in the liver and lung by Colo 205, whereas LoVo displayed strong metastatic ability to the liver and, to a lesser degree, in the lung.

Table 1. Tumors produced by Colo 205 and LoVo cells

	Positive/injected	
	Colo 205	LoVo
Spleen	7/11	10/12
Liver	0/11	11/12
Lung	0/11	3/12

Other tests for tumorigenicity were applied to both cell lines. The ability to grow in clonogenic soft (0.3%) agar culture has been correlated with tumorigenicity of transformed cells in nude mice.[10] The invasive behavior of malignant cells applied to chick embryo skin has been suggested as a suitable test for tumorigenicity.[11,12] Both Colo 205 and LoVo cells displayed growth in these two test systems, as indicated in Table 2. Li *et al.*[13] recently found that growth of tumor cells in dense agarose, e.g., >0.6%, could be used as a predictor of the metastatic potential. We tested this by culturing both cell lines in 1.2% agar, and found that Colo 205 showed no growth, whereas LoVo cells displayed small colonies. These data are summarized in Table 2.

Thus it was clear from these preliminary tests that LoVo cells would be suitable for studies of metastasis from the spleen to the liver. For the work shown in Table 1, 1×10^6 cells were injected intrasplenically. A test of different concentrations of

Table 2. Comparative features of Colo 205 and LoVo cells

	Colo 205	LoVo
Clonogenicity in soft agar (0.3%)	+	+
Invasion in chick embryo skin	+	+
Tumorigenicity in nude mice (s.c., i.s.)	+	+
Metastasis to liver in nude mice after i.s.	–	+
Growth in 'hard' agar (1.2%)	–	+

Table 3. Tumor formation with different doses of LoVo cells

Dose	Positive/injected		
	Spleen	Liver	Lung
250 000 cells	4/5	1/5	0/5
500 000 cells	6/6	2/6	2/6
1 000 000 cells	5/5	3/5	3/5
1 500 000 cells	4/4	4/4	4/4

Table 5. Effect of combined FU and IFN on liver metastases (schedule 2)

	Positive/injected		
	Spleen	Liver	Lung
Controls	7/7	7/7	2/7
FU	2/5	3/5	0/5
IFN	6/6	6/6	0/6
FU + IFN	3/5	5/5	0/5

LoVo cells was made to determine the optimum number of cells to inject into the spleens of nude mice to produce liver metastases. The data are shown in Table 3.

While 1×10^6 cells appeared sufficient to induce tumors in the spleen and subsequent liver metastases, we routinely used 1.5×10^6 LoVo cells in 0.050 ml for all subsequent experiments. 6 weeks after intrasplenic injection, all untreated mice showed spleen and liver tumors.

In the first experiment using the combined FU and IFN treatment, IFN was started 2 weeks after tumor cells were injected. From that point, IFN was given every other day (except weekends) for a total of 11 injections. FU was started 3 weeks after the tumor cells were injected, and given 1 week apart, for a total of three doses. The results from this schedule are shown in Table 4 (schedule 1).

This schedule was not effective in inhibiting tumor formation. FU or IFN used singly had no appreciable effect, and this did not change when they were used in combination.

In the next schedule both drugs were started together 1 week after the tumor cells were injected. IFN was given every other day thereafter, for a total of 11 injections. FU was given once a week for 4 weeks. The results from this schedule are shown in Table 5 (schedule 2).

This schedule was also not remarkable in inhibiting tumor formation. IFN used alone, in particular, was completely ineffective.

The schedule was slightly revised at this point; the drugs were started on the third day after the intrasplenic injections, rather than waiting for a week, as in previous experiments. In this schedule, both FU and IFN were given together, on the third day, and given for five successive days for a total of five combined injections. This schedule proved to be excessive, particularly with respect to the dose of FU. All mice given FU only and those given FU + IFN expired within 10 days. All mice treated with IFN only showed liver metastases, again displaying the ineffectiveness of IFN given alone.

The schedule was modified again; FU was decreased to two injections. In this schedule, we started FU and IFN together on the third day, and IFN was continued for five successive days. This entire sequence was repeated for the second week. The results from this schedule are shown in Table 6 (schedule 3).

This schedule was as ineffective as the previous efforts in attempting to inhibit liver metastases. FU used alone appeared to have an effect on liver metastases, but was not significant.

In our next series, we increased the dose of IFN to 3×10^5 IU/injection, and extended the time of treatment to five injections per week for 3 weeks. FU was given in three different variations, each of

Table 4. Effect of combined FUra and IFN on liver metastases (schedule 1)

	Positive/injected		
	Spleen	Liver	Lung
Controls	3/5	4/5	1/5
FU	4/6	4/6	0/6
IFN	3/5	4/5	0/5
FU + IFN	3/5	4/5	0/5

Table 6. Effect of combined FU and IFN on liver metastases (schedule 3)

	Positive/injected		
	Spleen	Liver	Lung
Controls	6/6	5/6	2/6
FU	5/6	3/6	3/6
IFN	6/6	6/6	1/6
FU + IFN	5/6	6/6	2/6

which started on the third day after tumor cell injections.

(A) FU was given once a week for three successive weeks.
(B) FU was given twice, the first time on the third day, and again 2 weeks later.
(C) FU was given three times, the first on the third day, and twice more after resting 3 days between each injection. The data resulting from this schedule are given in Table 7 (schedule 4).

In this series, schedules A and B appeared to have slight inhibitory effects, but were not significant. We did not include the IFN only series since it was clear from our experience that this was not effective. FU alone was given in one schedule, patterned after the combination schedule of (A).

In the next series, we increased the FU to three and four injections, and the IFN to 4 and 5 weeks. All schedules started on the third day after tumor cells were injected. In schedule A, both FU and IFN were given together on day three. IFN was then continued 5 days per week for 4 weeks. FU was given 1 week apart for a total of four injections. In schedule B, both FU and IFN were started on day three. IFN was then continued daily for 5 days, and continued for 4 weeks. FU was given again 1 week later, then omitted for 1 week, and a final injection given 2 weeks later. In schedule C, both FU and IFN started together on day three. IFN was continued for 5 days, and given for 5 weeks. FU was given two more times, resting a week between, for a total of three injections. The data resulting from these schedules are given in Table 8 (schedule 5).

The data in Table 8 show inhibition of liver metastases with the three schedules used. These are significant as analyzed by Fisher's exact test (compared to the controls; p-values are indicated). Again FU by itself did not produce comparable activity as when combined with IFN. In previous schedules, FU had been given equivalent numbers of times and had not displayed significant inhibitory activity. There was an increase of the IFN dosage in this series, which were given for 4 and 5 full weeks, almost the entire period of the experimental schedule.

Table 7. Effect of combined FU and IFN on liver metastases (schedule 4)

	Positive/injected		
	Spleen	Liver	Lung
Controls	5/5	4/5	3/5
FU (A)	5/6	4/6	3/6
FU + IFN (A)	5/6	3/6	0/6
FU + IFN (B)	6/6	2/6	2/5
FU + IFN (C)	3/3	2/3	1/3

Table 8. Effect of combined FU and IFN on liver metastases (schedule 5)

	Positive/injected		
	Spleen	Liver	Lung
Controls	6/6	6/6	3/6
FU (A)	3/5	3/5	1/5
FU (B)	5/5	5/5	4/5
FU (C)	6/6	5/6	4/6
Schedule A	3/5	2/5 ($p = 0.0606$)	1/5
Schedule B	6/6	1/6 ($p = 0.0076$)	0/6
Schedule C	7/7	2/7 ($p = 0.0163$)	1/7

Discussion

The experiments described here show that experimental liver metastases in nude mice receiving intrasplenic injections of human colon carcinoma cells can be inhibited with the combined regimen of FU and recombinant human alpha IFN. The administration of FU or IFN as single agents was ineffective in inhibiting liver metastases. In order to be effective, IFN had to be given over an extended period, which in our experiments was on a daily basis over a period of 4–5 weeks. Treatment with IFN for shorter periods, e.g., 1–3 weeks, was insufficient.

Morikawa *et al.*[7] reported previously that the combined usage of FU and mouse recombinant gamma IFN gave the highest inhibitory activity on the growth of human colon cancer cells in the spleen, liver and lung of nude mice. They interpreted the antitumor effects to be the result of direct effects on the tumor cells by FU and indirect effects of the mouse recombinant gamma IFN. The indirect effects were attributed to the activation by mouse recombinant gamma IFN of rodent macrophages to become tumoricidal.

There is an increased interest in exploring novel strategies to improve the efficacy of FU against colorectal cancer. Scheithauer and Temsch[14] have attempted to enhance the cytotoxic activity of FU and leucovorin with the addition of various other

agents, e.g., cisplatin, hyaluronidase or dipyridamole. This resulted in synergistic growth inhibition when tested on several human colon cancer cell lines. Of further interest was the fact that this permitted the use of lower doses of FU, which proved to be more effective. A similar observation was recorded by Miyoshi *et al.*[15] who studied the effects of combined FU and human fibroblast IFN on human neoplastic cell lines. IFN synergistically potentiated the cytotoxic effects of FU and also suggested that each drug reduced the amount of the other needed to treat cancer patients. In another approach, Maehara *et al.*[16] have obtained an augmentation of cytotoxic effects of FU when hyperthermia and dipyridamole were combined *in vitro* on HeLa and B16 melanoma cells. This was interpreted as improving the sensitivity of the tumor cells to FU. Spiegelman *et al.*[17] focused on the idea of metabolic modulation in which FU, an antimetabolite, can be improved as an antitumor agent. FU is known to interact in cellular metabolism via one of two pathways: the conversion to the deoxymonophosphate, which inhibits thymidylate synthetase, leading to the inhibition of DNA synthesis by the depletion of thymidine triphosphate; or, to the incorporation of FU into RNA, which causes problems in various important cellular mechanisms involved in RNA function and processing in the cell. Using two mouse tumors (mammary and colon) they did indeed show antitumor activity when thymidine was added and stimulated the incorporation of FU into RNA.

Several different possibilities can be considered in explaining the mechanism of action of FU effects and that of the combined FU–IFN effects. FU effects encompass activity directly on DNA of the cells and via metabolic modulation, such as described for incorporation into RNA. The action of IFN can be directly on the homologous cells or on the immune system of the host if strict species specificity prevails. In the experiments described here, recombinant human alpha IFN was used and we must assume for the moment that its action was not on the murine host's immune system. It has been pointed out by a number of investigators that repeated and prolonged treatment with IFN is more efficacious than shorter treatments in producing optimal results.[18] Lee *et al.*[19] have shown that the treatment regimen starting on the third day post-tumor inoculation was more effective than treatments starting 1, 5, 7, or 9 days after tumor inoculation. Our results concurred with those observations. We are unable, however, to resolve the critical question of the mechanism of action of IFN, and how its combined usage with FU brought about inhibition of liver metastases. That the use of IFN can improve and increase the lethality of a cytotoxic drug such as FU has attracted clinical interest for use in trials on patients with advanced colorectal carcinoma.[20] Wadler and Schwartz[21] reviewed this area of cancer therapy involving IFN and chemotherapeutic agents and suggested that IFN may have a protective effect on normal host tissues, thus allowing for the delivery of higher doses of cytotoxic agents. Whatever the mechanism, it appears that the approach of seeking methods to improve the efficacy of the available cytotoxic drugs has been fruitful, in that there are now a variety of reports using different systems in which the combined drug approach has been successful in producing antineoplastic agents.[20]

Conclusion

Experimental liver metastasis was studied in an animal model system using 4-week-old nude mice. A cell line (LoVo) established from a metastatic human colon adenocarcinoma, when injected intrasplenically, produced 100% metastases to the liver. When 5-FU or interferon was given as single agents, liver metastases were not inhibited. However, when 5-FU was combined with interferon for an extended period, up to 5 weeks on a daily basis, inhibition of liver metastases could be demonstrated.

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