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Inhibition of experimental liver metastasis by combined treatment with tamoxifen and interferon

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The demonstration of estrogen receptors (ERs) in the liver has established it as a hormonally responsive organ. Estrogens have been imputed to have a role in the development of benign and malignant liver tumors. The detection of ERs in samples of normal liver tissue and in hepatocellular carcinomas suggested a treatment strategy with anti-hormonal drugs, i.e. tamoxifen, as used clinically for the treatment of breast cancer. The objective of this study was to test the effect of tamoxifen and tamoxifen in combination with other agents [5-fluorouracil (5-FU) and interferon (IFN)] against experimental liver metastases of human colorectal tumor cells xenografted into nude mice. A human colorectal tumor cell line, LoVo, was injected into the spleens of nude mice. This produces liver metastases in virtually 100% of the mice in 6–8 weeks. One week before tumor cell implantation, all mice were ovariectomized. Treatment was started 3 days after the intrasplenic injections. This consisted of 5 mg tamoxifen pellets (60-day release) implanted s.c., 5-FU given i.p. once a week for 4 weeks on a 46 mg/kg basis and IFN given s.c., daily for 4 weeks, 3×10^5 units/injection. The effect of tamoxifen alone on liver metastases was not significantly different from untreated controls. Tamoxifen in combination with IFN and 5-FU, however, resulted in 50–67% inhibition of liver metastases, as compared with the controls. The effectiveness of the treatment was in the order: tamoxifen + IFN > tamoxifen + 5-FU + IFN > tamoxifen + 5-FU. Thus, IFN may be useful as a potentiating agent in combination with tamoxifen for the treatment of estrogen-dependent tumors.

Key words: Chemotherapy, metastasis, tamoxifen, interferon, LoVo.

Introduction

The liver is generally understood to be a target for estrogens.[1] Numerous reports have suggested an association of estrogens with the development of benign and malignant liver tumors, particularly in women using oral contraceptives.[1–7] The withdrawal of oral contraceptives usually resulted in the regression of benign tumors.[8] In animal studies, the use of estrogens, such as those commonly used by humans as oral contraceptives, promoted the development of experimental hepatocarcinogenesis.[9–10] The estrogen responsive nature of the liver suggested the presence of hepatic estrogen receptors (ER), which have in fact been found in human liver tissue and in human hepatocellular carcinoma.[11–14] This appeared to be analogous to breast cancer where anti-estrogens, i.e. tamoxifen, have been successfully used in therapy. Although reports on the use of tamoxifen in hepatocarcinogenesis are very limited, Farinati *et al.*[15–16] and Martinez-Cerezo *et al.*[17] have been able to prolong survival in patients with unresectable hepatocellular carcinoma by treating with tamoxifen. This stimulated our interest to use tamoxifen for the treatment of experimental liver metastases. An animal model system was used in this study wherein athymic nude mice were inoculated intrasplenically with a cultured human colorectal tumor cell line that metastasizes to the liver.

In addition to exploring the use of tamoxifen in anti-metastatic therapy, another objective was to test the combined effect of tamoxifen and interferon (IFN). IFN has shown anti-proliferative effects both *in vitro* and *in vivo*, and has been used effectively against various cancer cells.[18–20] IFN has also been used in combination with a number of chemotherapeutic agents and has resulted in enhanced antitumor activity over single agents.[21] A number of reports have now described the combined use of tamoxifen and IFN for the treatment of breast cancer.[22–29] The rationale for this combined approach is that IFN induces the expression of ERs and thus enhances the sensitivity of the breast cancer cells toward the action of tamoxifen.[30] Another potential use for tamoxifen is suggested by the observations of Kim *et al.*[31] who used tamoxifen to potentiate the antitumor activity of interleukin-2 (IL-2). Mice bearing pulmonary metastases showed a 95% reduction

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of metastases with combined therapy than with either agent alone.

Materials and methods

LoVo, the human tumor cell line used in this study, was originally derived from a metastatic tumor nodule in a lymph node from a patient with adenocarcinoma of the colon. 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. Athymic female nude mice were purchased from the NCI-Frederick Cancer Research and Development Center. All mice were housed and maintained under specific pathogen-free conditions in the Nude Mouse Facility of the Mercy Cancer Center. Mice (6–8 weeks old) were injected intrasplenically with LoVo cells in a volume of 0.050 ml/injection containing 1.0×10^6 cells 7 days after ovariectomy.

Tamoxifen was administered to the mice in (5 mg) pellet form, implanted s.c. with a trochar 3 days after injection of LoVo cells. These pellets, purchased from Innovative Research of America (Toledo, OH), have been designed for use in nude mice and result in blood levels of 3–4 ng/ml over a 60-day release time. Recombinant IFN-α2a was obtained as a gift from Roche Laboratories (Hoffmann-La Roche, Nutley, NJ). IFN was given s.c. at a dose of 3×10^5 units/injection in 0.05 ml. 5-Fluorouracil (5-FU) was a generic form obtained from the hospital pharmacy and was injected i.p. based on 46 mg/kg.

Experiments were started on Fridays with the intrasplenic injections of LoVo cells into mice that had been ovariectomized 7 days earlier. Three days later, tamoxifen pellets were implanted and injections of IFN started. IFN was given daily, five times/week, for 4 weeks. When 5-FU was used in an experiment, it was administered on the same day that the tamoxifen pellet was implanted. Thereafter, 5-FU was given once weekly for 4 weeks. Experiments were terminated at 8 weeks. Mice were sacrificed by anesthetic overdose and autopsies conducted. Gross observations of abdominal metastases, particularly for the liver, were recorded. Lungs were excised and processed for histological analysis in order to score for the presence of metastatic nodules.

Results

All female mice used in the experiments reported here were ovariectomized 7 days before being inoculated intrasplenically with tumor cells. The tumor cells used in this study, LoVo, have been grown in cell culture in our laboratory for the past 5 years. In our previous experience with the model system used here with athymic nude mice, intrasplenic injections of LoVo cells resulted in metastases to the liver in 100% of the mice.[32] The incidence of lung metastases is usually around 80–90%. Figure 1 shows a typical example of an untreated control mouse at autopsy with liver metastases.

The incidence of liver and lung metastases is shown in Table 1. This records the effect of treatment with tamoxifen alone and in combination with IFN and 5-FU. The percent inhibition of liver and lung metastases and the statistical comparison with the control mice (ovariectomized only) are shown in Table 2. Tamoxifen, when combined with IFN, was most effective in inhibiting liver metastases. IFN also appeared to potentiate the effect of tamoxifen + 5-FU from 50 to 57% inhibition when IFN was added to the treatment regimen. However, it is clear that the most effective treatment group for inhibiting liver metastasis was tamoxifen + IFN. For lung metastasis, the tamoxifen + IFN + 5-FU group showed a slightly higher percent inhibition than the tamoxifen + IFN group. This difference,however, was not statistically significant. Both groups were, nonetheless, significantly different when compared to the controls. The tamoxifen + 5-FU group was

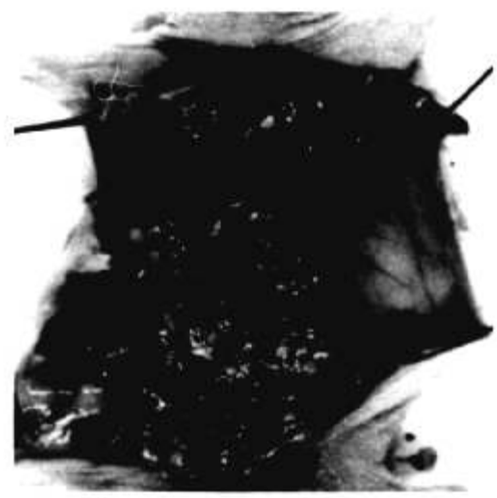

Figure 1. Extensive metastatic nodules in untreated control mouse at autopsy.

Table 1. Incidence of metastases[a]

Treatment	Liver	Lung
Control	13/13	11/13
Tamoxifen	9/12	8/12
Tamoxifen + IFN	4/12	5/12
Tamoxifen + 5-FU	6/12	7/12
Tamoxifen + IFN + 5-FU	6/14	5/14

[a]No. of mice with metastases/no. of mice injected.

Table 2. Percent inhibition of liver and lung metastases

Treatment	Liver (%)	*p*-value[a]	Lung (%)	*p*-value
Control	0	–	0	–
Tamoxifen	25	0.09	33	0.28
Tamoxifen + IFN	67	0.00045	58	0.03
Tamoxifen + 5-FU	50	0.0052	42	0.15
Tamoxifen +IFN + 5-FU	57	0.0014	64	0.013

[a]Fisher's exact test.

clearly weaker in effect. In Figure 2, livers excised from mice with different treatments are shown. They are, from (a) to (e), respectively: (a) ovariectomy control; (b) tamoxifen; (c) tamoxifen + 5-FU; (d) tamoxifen + IFN; (e) tamoxifen + 5-FU + IFN.

Discussion

The objectives of this study were (i) to explore the use of antiestrogens, i.e. tamoxifen, for the treatment of colorectal tumor cell metastases to the liver and (ii) to test the effect of IFN as a potential immunomodulatory agent in potentiating the effect of tamoxifen.

Our experiments utilized female nude mice that had undergone ovariectomy 7 days prior to receiving intrasplenic injections of tumor cells. The incidence of liver metastases in the controls was 100%. The effect of tamoxifen alone resulted in 25% inhibition of liver metastases. Tamoxifen used in combination with IFN resulted in 67% inhibition. Tamoxifen + 5-FU resulted in 50% inhibition, while IFN + 5-FU combined with tamoxifen gave 57% inhibition (Table 2). All of the quoted percentages were confirmed by Fisher's exact test analysis to be statistically significant. Though not shown here, certain other pertinent information must be weighed in evaluating the results of this study. When 5-FU and IFN were used as single agents in this system, there were no significant inhibitory effects with respect to

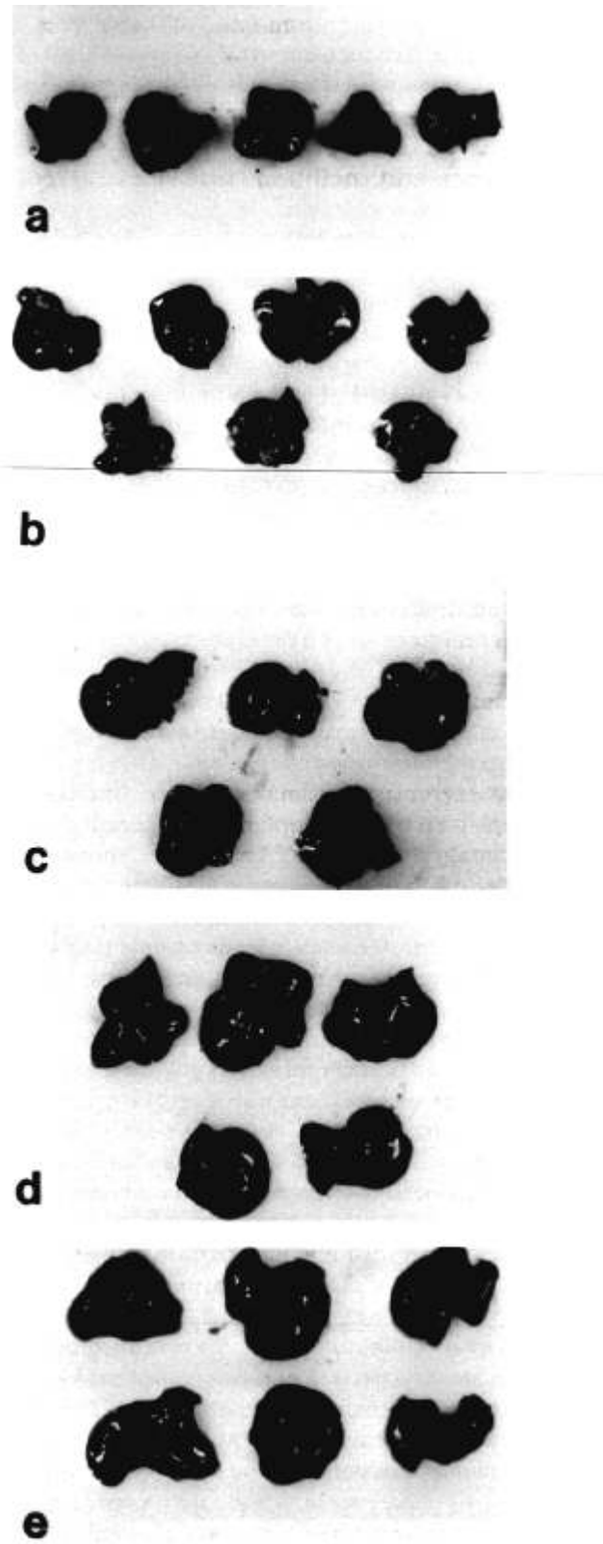

Figure 2. Livers excised from mice treated with different drug combinations. (a) Untreated control; (b) tamoxifen; (c) tamoxifen + 5-FU; (d) tamoxifen + IFN; (e) tamoxifen + 5-FU + IFN.

liver metastases. Others have reported that treatment with IFN increases the expression of ERs in breast cancer cells. Conceivably, this could increase the sensitivity of IFN-treated cells to tamoxifen, followed by inhibition of cellular proliferation. A synergistic effect of IFN and tamoxifen was reported by Bezwoda and Meyer[33] in experiments on MCF-7 cells *in vitro*. When cells were pretreated with IFN-α followed by tamoxifen, there was an enhanced inhibitory effect of tamoxifen on cell proliferation concomitant with a tamoxifen-induced reduction of ER content. With respect to ERs in the liver, Kahn *et al.*[34] demonstrated that the administration of tamoxifen to male rats with regenerating livers following partial hepatectomy resulted in a dose-dependent decrease in hepatic cytosolic ERs. Furthermore, there was no effect of tamoxifen treatment on hepatic regeneration. This is in contrast to another study in male rats by Francavilla *et al.*[35] who showed that the administration of tamoxifen within 6 h of 70% partial hepatectomy resulted in significant inhibition of both DNA synthesis and mitosis. In our study normal mouse liver revealed the presence of ERs by Scatchard plot analysis. Metastatic liver tumors, however, were negative for ERs (data not shown). Recently reported observations of Gagliardi and Collins[36] on the inhibition of angiogenesis by estrogen antagonists such as clomiphene, nafoxidine and tamoxifen may have some relevance here. Antiestrogens such as tamoxifen showed significant inhibition of angiogenesis in experiments on the chick chorioallantoic membrane. Angiogenesis is an important requirement for the metastatic spread of tumor cells,[37] and is rapidly attracting attention as a target for anti-cancer therapy.[38] We have shown that metastases in an estrogen-responsive organ can be inhibited by the combined treatment with an antiestrogenic drug, tamoxifen, and an immunomodulatory agent, IFN. The interactions involved *in vivo* with tamoxifen and IFN that result in the inhibition of metastasis to the liver by colorectal tumor cells appear to be of a complex nature. Further studies are planned to elucidate how tamoxifen and IFN interact to inhibit metastatic growth of tumor cells. Since metastasis is the most feared and dreaded aspect of malignant disease, it is hoped that studies of this nature may contribute to identifying useful drug combinations for chemotherapeutic use.

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