Neoadjuvant Interferon alfa-2b Treatment in a Murine Model for Metastatic Ocular Melanoma

A Preliminary Study

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Objectives: To investigate the treatment of metastasis from uveal melanoma and to test the effect of interferon (IFN) alfa-2b in a murine model.

Methods: The B16-LS9 tissue culture melanoma cells were inoculated into the posterior intraocular compartment of 3 groups of C57BL/6 mice. The inoculated eyes were enucleated at 9 days and the mice were euthanized at 26 days after inoculation; the site and number of metastases were determined using standard histologic techniques. Group 1 was the control group; group 2 was given 20000 international units (IU) of IFN alfa-2b intramuscularly 12 hours before enucleation, and group 3 received daily injections of 20000 IU of IFN alfa-2b intramuscularly 12 hours before enucleation.

Results: Pulmonary metastases were detected in 57%, 33%, and 0% of groups 1, 2, and 3, respectively; hepatic micrometastases were detected only in group 1. These results showed a significant decrease in hepatic metastases in mice receiving IFN alfa-2b vs controls ($P = .005$).

Conclusion: Treatment with IFN alfa-2b results in decreased hepatic metastases from intraocular melanoma in a murine model.


Eye-sparing and sight-preserving treatments for primary uveal melanoma have been developed over the past 20 years. One problem is that, when compared with enucleation, these treatments have not affected the rate of metastasis.1,2 Metastases appear in 19% to 35% of patients within 5 years of diagnosis of the primary tumor,3 and the liver is the only site or initial site of metastasis in more than 50% of patients.4 Since many patients fail to exhibit clinically evident metastases until several years after enucleation, it is felt that these patients have “dormant” micrometastases that later become clinically evident. Patients with metastatic uveal melanoma have a poor prognosis; more than half of all patients die within 5 months of diagnosis of the metastasis.5,7 Treatment options for metastatic uveal melanoma include surgery, systemic chemotherapy, chemoembolization, intra-arterial chemotherapy, and chemoimmunotherapy.3,7 Even with therapy, survival is from 1 to 59 months, with a median of 5 to 8 months.3 To investigate 1 potential treatment for hepatic metastasis from uveal melanoma, we examined the effect of inter-}

The results are summarized in the Table and Figure 1. One mouse in group 1 died during enucleation and was excluded. Tumor growth was observed in all eyes. By using the transcorneal inoculation technique, it was possible to achieve relatively large melanomas in the choroid, vitreous, and subretinal space of the murine eye (Figure 2). At the time of enucleation, some tumors had already infiltrated the sclera, subconjunctival space, and orbit. The mean (SD) size of the intraocular melanoma was 1.55 (1.18) × 2.31 (0.43) mm in group 1, 0.96 (0.55) × 2.18 (0.80) mm in group 2, and 1.4 (0.80) × 2.14 (0.55) in group 3. The tumor sizes in the 3 groups were not significantly different ($P = .42$ for tumor height, $P = .95$ for tumor base). There was persistence of melanoma cells in the orbit in 2, 1, and 5 mice in groups 1, 2, and 3, respectively. Occurrences of extraocular melanoma and orbital melanoma at the time of necropsy were not significantly different among the groups. Mice with or-
MATERIALS AND METHODS

MICE
Twelve-week-old female C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, Me). All experiments were conducted according to the Declaration of Helsinki and Guiding Principles in the Care and Use of Animals.9

TUMORS
The B16-LS9 murine melanoma tissue cultures cells were used (Friedrich Miescher Institut, Basel, Switzerland). These cells were derived from hepatic metastases according to the method described by Rusciano and coworkers.9 Frozen cells were thawed and resuspended in 15 mL of minimum essential medium (MEM) supplemented with fetal calf serum, 1-glutamine, and sodium bicarbonate. The cell suspension was centrifuged and the pellet was washed and resuspended in 15 mL of supplemental MEM. The suspension was placed in a 75-cm² tissue culture flask (T-75; Becton Dickinson, Franklin Lakes, NJ) in a carbon-dioxide incubator (Forma Scientific, Marietta, Ohio) at 37°C and grown to confluence in 3 to 5 days. The cells were trypsinized, aliquoted, and washed 3 times in 5 mL of Hanks balanced salt solution. An aliquot of 10 µL of the suspension was placed in a hemocytometer to calculate the concentration of melanoma cells.

INOCULATION OF MELANOMA CELLS INTO THE POSTERIOR COMPARTMENT
Aliquots of 2.5 × 10⁷ cells per 2.5 µL were inoculated into the posterior compartment (PC) of mice, using a transcorneal inoculation technique that allowed us to keep the inoculated cells in the eye.10 Briefly, the mice were anesthetized and a tunnel was prepared from the limbus within the cornea, sclera, and ciliary body to the choroid using a 30-gauge needle under a dissection microscope. The tip of a 10-µL glass syringe with a blunt metal needle (Hamilton Co, Reno, Nev) was introduced into the PC via the needle track and no cells were inoculated until the needle tip was inside the eye. A 2.5-µL suspension of B16-LS9 cells was inoculated. No tumor cell reflux occurred and the subconjunctival space remained free of tumor cells.

INTERFERON
Recombinant human IFN alfa-2b (Intron A; Shering, Kenilworth, NJ) was used. The mice were given 20000 international units (IU) of IFN alfa-2b per intramuscular (IM) injection.

ASSAY FOR METASTASES
Nine days after inoculation, the mice were anesthetized and their right eyes were enucleated under the direction of a dissecting microscope.11 The eyes were processed for light microscopic examination. Serial 5-µm-thick sections of the eyes were stained with hematoxylin-eosin and evaluated for the presence and location of the melanoma. The mice were euthanized 26 days after inoculation and necropsies were performed. Lungs, liver, and cervical lymph-node tissue were grossly examined, submitted in 4% neutral-buffered formaldehyde and processed for light microscopic examination. Three sections through the centers of the organs were microscopically evaluated (Zeiss, Oberkochen, Germany) for the presence or absence of metastases and average number of metastases per section was determined. Orbital tissue was also removed and examined for persistence of melanoma.

TREATED AND CONTROL GROUPS
Three groups of 10 mice each were inoculated with B16-LS9 melanoma cells into the PC. Group 1 was the control group, group 2 was injected with 20000 IU of IFN alfa-2b IM 12 hours before enucleation of the tumor containing eye and group 3 received daily IM injections of 20000 IU of IFN alfa-2b starting 4 days before enucleation. The last injection in this group was given 12 hours before enucleation.

STATISTICAL ANALYSIS
The Kruskal-Wallis test was used to determine if the distribution of number and size of metastatic melanoma differed among combinations of the groups of mice. Both base and height measurements of intraocular melanoma were compared among all 3 groups. Differences in the diameter of cervical lymph-node melanoma in control mice vs those receiving IFN alfa-2b were assessed. The number of hepatic and pulmonary metastases were compared between both control vs IFN alfa-2b and the 2 IFN alfa-2b groups. A value of P<.05 was considered to be significant.

bital melanoma were excluded for determining metastatic rate, since the origin of the metastases could have been the eye or orbit. Pulmonary metastases were detected in 4 (37%) of 7 in group 1, 3 (33%) of 9 in group 2, and 0 (0%) in group 3. Hepatic metastases12,13 were present only in group 1 (4 [57%] of 7). Cervical lymph-node metastases were present in 4 (57%) of 7, 4 (44%) of 9, and 1 (20%) of 5 of groups 1, 2, and 3, respectively. The eyes of all the mice with lymph-node metastases (Figure 3) exhibited melanoma growth into the subconjunctival space, indicating spread via conjunctival lymphatics to the cervical lymph nodes. The diameter of the melanoma in the cervical lymph nodes was on average larger in group 1 (8.5 mm) than in groups 2 and 3 (2.8 mm and 2.0 mm, respectively; P=.01). The number of hepatic metastases (Figure 4) was significantly lower in mice receiving IFN alfa-2b vs controls (P=.005). The number of pulmonary metastases was not significantly different between the IFN alfa-2b–treated mice and the controls (P=.18). The number of hepatic and pulmonary metastases did not change with timing of the IFN alfa-2b injection (12 hours vs 4 days; P>.99 for hepatic, P=.16 for pulmonary).

COMMENT
Between 19% and 35% of patients with uveal melanoma develop metastases within 5 years of diagnosis, mainly to the liver, and virtually all of these patients succumb to the metastatic disease. The median relapse-free inter-
val for patients with uveal melanoma between therapy of the primary tumor and diagnosis of metastasis is 2 to 4 years. These patients include those who have their tumor removed, either by enucleation or resection. Therefore, the metastases are thought to be derived from dormant micrometastases. Treatment of dormant tumor cells may be performed at the time of diagnosis, during the treatment of the primary tumor, or during the relapse-free interval.

Biological response modifiers, such as IFN, have been recognized for their antiviral, immunomodulatory, and antitumor effects. Recombinant human IFN-α has been shown to be as effective in the murine system as murine IFN-α and IFN-β for treating experimental tumors. Of oral, intravascular, and IM injection, IM injection of IFN-α has been shown to give the best results. A study examining tumor uptake of radiolabeled monoclonal antibodies by different doses of IFN has shown maximum tumor uptake in mice receiving 20000 IU of IFN alfa-2b IM; therefore, this dose was chosen for our study. Animals receiving IFN alfa-2b doses greater than 20000 IU failed to demonstrate higher tumor uptake. Several published reports have demonstrated that antibodies to IFN-α can occur in some patients treated with IFN-α. Neutralizing antibodies in immunocompetent mice treated with IFN alfa-2b are first detected after 2 weeks of treatment and are not considered to have had any influence in our study.

Our results showed a significantly lower incidence of hepatic metastatic disease in the IFN-treated groups in comparison with the control group \((P = .005)\). The frequency of lymphatic spread to regional lymph nodes was also lower in the treated groups, although those lymphatic metastases might have been related to subconjunctival tumor growth and access of tumor cells to subconjunctival lymphatic channels rather than to the IFN treatment. All mice with lymphatic metastases exhibited subconjunctival spread of the melanoma. However, the size of the melanoma in positive cervical lymph nodes in the control group was significantly larger \((P = .01)\) than the tumor in the lymph nodes of the treatment groups, suggesting that IFN might be able to limit lymphatic metastases.

Our study shows that adjuvant recombinant IFN alfa-2b may be effective in a murine ocular melanoma model using B16 melanoma. We used the B16-LS9 melanoma cell line, which has been shown to express low levels of major histocompatibility complex class I antigen and to be susceptible to natural killer (NK) cell-mediated lysis. Several studies have reported that recombinant IFN augments NK cell antitumor activity. The level and time course of augmented splenic
NK activity by human recombinant IFN-α is almost identical to what is reported for a pure preparation of natural murine IFN.²⁴ This indicates that recombinant human IFN-α behaves and functions in mice in a similar way as naturally occurring murine IFN, at least in terms of NK cell activation.²⁵ Natural killer cell depletion with anti-asialo GM1 antibody abrogates the antimetastatic activity of IFN-α.²³ However, 1 clinical trial noted a drop in NK activity when peripheral blood lymphocytes from patients with cancer undergoing treatment with multiple injections of IFN-α were assayed.²⁶ Timing of IFN administration may be a critical factor in effective antitumor therapy. In a study using B16 melanoma injected into the murine footpad, IFN-α/IFN-β administration on days 1 to 5 after tumor excision was not only ineffective, but also decreased mean survival rates compared with untreated controls.²⁷ In another study, maximum increase in NK activity was observed 12 hours after IFN injection and the lowest number of lung metastases was obtained when mice were treated with IFN-α 12 hours before or at the same time as tail vein injection of B16 melanoma cells. No significant effect was observed when the treatment was carried out 24 hours after inoculation.²⁵ These results suggest that B16 melanoma cells are susceptible to NK cells only during a short period after intravascular invasion, possibly before they become attached to capillary endothelial cells and escape from the intravascular space. Treatment of mice with 4 daily IM injections of IFN-α before intravenous injection of B16-F10 melanoma cells resulted in marked inhibition of pulmonary metastases and a rise in NK activity, as measured both in vitro and in vivo.²³ When treatment was extended to 10 daily injections before tumor cell inoculation, the inhibitory effect of IFN on metastases and the augmentation of NK activity were both reduced.²¹ Thus, a maximum treatment effect of IFN alfa-2b may be between 10 days before enucleation and the day of enucleation.

There are differences in mechanisms of metastasis after direct intravascular inoculation of in vitro cultured tumor cells and spontaneous metastases derived from a progressively growing primary tumor.²⁸,²⁹ In this study, we administered IFN alfa-2b 12 hours before enucleation of the eye containing the melanoma. Enucleation may cause a shower of tumor cells into the circulation and the importance of this possibility with regard to the development of metastatic disease is being studied.³⁰,³¹ Prophylactic systemic treatment combined with treatment of the primary tumor is a reasonable management strategy for uveal melanoma. Our study shows that IFN alfa-2b given IM 12 hours before enucleation is an effective prophylactic treatment in an experimental murine intraocular melanoma model. There was no significant difference in the metastatic rate between IFN treatment over 4 days and a single IFN injection before enucleation (P = .18 for any metastases; P > .99 for hepatic metastases; and P = .16 for pulmonary metastases).

Patients with metastatic disease from uveal melanoma have been treated with immunotherapy, including IFN alfa-2b.³²,³³ Combining a 4-drug chemotherapy regimen (dacarbazine, vincristine, bleomycin, and lomustine) with natural leukocyte IFN resulted in a 20% partial response in patients with metastatic uveal melanoma; however, there were no complete responses.³ Systemic IFN is useful in the treatment of metastatic cutaneous melanoma.³⁴ Recombinant IFN alfa-2b has also been used as an adjuvant therapy in patients with cutaneous melanoma without evidence of distant metastatic disease.³⁵ The adjuvant IFN treatment increased the median overall survival rate in the treatment group compared with the untreated control group. Quality-of-life–adjusted survival analysis has shown that, despite the toxicity associated with recombinant IFN alfa-2b therapy, the survival time gained with IFN alfa-2b therapy outweighed the reduced quality of life associated with treatment toxicity and relapse.³⁶ Adjuvant recombinant IFN alfa-2b treatment given before or at the time of enucleation may be a treatment option for patients with uveal melanoma with high-risk factors for developing metastatic disease.

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