A Treatment For Metastasis of Murine Ocular Melanoma

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In experiments using cultured cells, LS2616 has been shown to decrease growth of primary tumors and pulmonary metastasis of murine melanoma. In the current study, we examine the efficacy of LS2616 for the prophylactic and therapeutic treatment of metastases from ocular and flank inoculations of the highly aggressive in vivo derived B16F10 melanoma in C57BL/6J mice. Experimental animals were treated with 160 mg/kg/day of this drug in drinking water, until they became moribund or died. When mice were pretreated for 7 days and inoculated subcutaneously (sc) or intracamerally (ic) with \(10^5\) in vivo derived B16F10 tumor cells, the mean number of pulmonary metastases was significantly reduced, and the incidence of pulmonary metastases decreased. In ocular experiments, when pretreatment with drug was combined with enucleation at day 7, the mean number of lung nodules was significantly reduced; the incidence of metastasis to the lung and lymph nodes decreased and survival increased. An apparent cure rate of 31% was observed. Treatment beginning on the day of enucleation (day 7) resulted in a reduction of pulmonary metastases, a decrease in metastasis to the lungs and lymph nodes and no change in survival. LS2616 did not alter tumorigenicity of either sc or ic inoculations. In an in vivo neutralization assay, spleen cells of mice treated for 7 days with LS2616 demonstrated an increase in cytostatic or cytotoxic activity when incubated with B16F10 melanoma cells. Thus, the current study demonstrates that LS2616 is very effective in decreasing metastases of both ocular and extraocular tumors of the highly aggressive in vivo derived B16F10 melanoma and appears to possess immunostimulatory properties in this system. The potential usefulness of LS2616 as a therapeutic agent for treating metastases of melanoma is discussed.

In a recent report, we described the ocular metastasis of in vivo derived B16F10 melanoma cells in syngeneic C57BL/6J mice, and found that melanoma cells passaged in culture exhibit a decrease in metastatic potential compared with that of melanoma cells passaged in vivo. In vivo derived B16F10 tumor cells metastasize extensively to the lungs from flank or ocular sites, and animals die of metastatic disease approximately 6 weeks after receiving tumor. In contrast, metastasis from an ocular site is only observed in 33% of mice inoculated intracamerally with cultured cells.

LS2616, a quinoline 3-carboxamide, is a recently discovered immunomodulator with little or no toxic side effects, that is currently being used in clinical trials with patients in Sweden. It has been examined experimentally using cultured B16F10 tumor cells and shown to decrease primary and secondary tumor growth and metastasis. This drug enhances natural killer (NK) cell activity, the delayed type hypersensitivity reaction to bacterial antigens, and proliferative T cell responses, and provides a therapeutic treatment in some murine autoimmune syndromes.

In the current study, we examine the effectiveness of LS2616 in controlling the growth and metastasis of ocular and flank tumors of the highly metastatic in vivo derived B16F10 tumor. In animals treated with LS2616, we report: (1) a significant decrease in the number of pulmonary metastases from ocular and flank tumors; (2) a decrease in metastases, and an increased survival and cure in enucleated animals, (3) increased activity of cytolytic or cytostatic effector cells in the spleen; and (4) little or no effect on the growth of the primary tumor.

Materials and Methods

Experimental Animals

Five- to seven-week-old C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) of both sexes were used as experimental subjects and to maintain the tumor line in vivo. Tumor bearing animals were examined and handled daily. Animals were judged as...
healthy if their physical appearance (with the exception of the presence of a primary tumor) and responses to handling were normal. Animals not judged to be healthy were classified as moribund. The current investigations conformed to the ARVO Resolution on the Use of Animals in Research. Animals were maintained according to the recommendations outlined in the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council.

Drug Treatment

LS2616 was graciously provided by Dr. T. Stalhandske (AB Leo, Helsingborg, Sweden) and was administered to mice in drinking water at a dosage of 160 mg/kg/day. When compared to control mice receiving drinking water only, this concentration does not affect total daily water intake.

Tumor Maintenance and Preparation

The B16F10 melanoma cell line was obtained in 1984 from Dr. Artemio Ovejera (N.I.H., Bethesda, MD). Tumor cells were maintained in vivo by subcutaneous (sc) implantations of 0.5 mm³ fragments of tumor taken from the periphery of flank tumors carried for 3 weeks. Transfers were made by injecting tumor fragments suspended in 0.2 ml of Hank's Balanced Salt Solutions (HBSS, Sigma Chemical Co., St. Louis, MO) into the flank using a 16 gauge needle.

Tumor cell suspensions were prepared by forcing 1.0 cm³ pieces of 3-week-old flank primary tumor through a 60 mesh stainless steel tissue grid. Cell suspensions were washed twice in HBSS.

Establishment of Primary Tumors

Intracameral (ic) and subcutaneous inoculations: Tumor inoculations were carried out as described previously. Briefly, mice were anesthetized by intraperitoneal (ip) injection with 0.06 mg/g of nembutal (Abbott Laboratories, North Chicago, IL) and the pupil dilated with one drop of 1.0% mydriacil (Alcon Laboratories, Fort Worth, TX). Using the Zeiss OPMI-1 dissecting microscope (×25), 5 μl of a cell suspension containing 10⁵ B16F10 cells in HBSS was inoculated into the anterior chamber.

Flank tumor growth was measured weekly using a vernier caliper.

Enucleation

Anesthetized mice were enucleated 7 days after ic inoculation of a tumor cell suspension and a suture used to close the eyelids. Enucleated eyes were dissected and examined as described previously. When using this protocol, enucleated eyes were always filled with tumor and recurrent growth of tumor in the orbit did not occur.

Assay For Metastasis

In most experiments, mice either died or became moribund and were necropsied. In one protocol noted below, healthy animals were sacrificed at 10 weeks and necropsied. In all experiments, the lungs, spleen, liver, intestine, lymph nodes, brain, heart and kidneys were removed from the animal and examined at ×25 for evidence of metastasis. The number of lung metastases was determined as described previously. Nodule size was quantified and the diameter of nodules 0.1–0.5 mm, 0.6–1.0 mm, and greater than 1.0 mm recorded.

In Vivo Neutralization Assay

A Winn-type neutralization assay modified by Merluzzi et al was used to assess the cytostatic or cytolytic activity of LS2616 activated splenocytes. Suspensions of 10⁵ B16F10 melanoma cells were mixed with spleen cells from animals treated with LS2616 or with splenocytes from untreated animals at an effector/target ratio of 100:1. The suspension containing tumor cells and spleen cells was centrifuged at 30 g for 5 min, resuspended in DMEM with 10% fetal bovine serum (Gibco Laboratories, Grand Island, NY) and incubated at 37°C and 5.0% CO₂ for 5 hr. The cell mixtures were washed three times in HBSS, resuspended to contain 10⁵ tumor cells and 10⁷ effector cells and injected subcutaneously into the flanks of mice.

Statistical Analysis

Differences in the mean number of nodules were analyzed using the student t-test.

Results

The Effect of Pretreatment With LS2616 on Spontaneous Metastasis From an Ocular Site in Unenucleated Mice

In order to examine the effect of LS2616 on metastasis of ocular tumor, 11 animals pretreated with 160 mg/kg/day of LS2616 for 7 days were then inoculated with 10⁵ in vivo derived B16F10 melanoma cells, and given daily treatment with LS2616 for the duration of the experiment. Twelve animals were inoculated with tumor and received no drug treatment. All animals died or became moribund during the sixth week of the experiment. Thus, survival of animals treated with LS2616 (0%, 0/11) was similar to that of the controls (0%, 0/12, Table 1). The effect of drug treatment on the frequency and extent of metastasis was also examined. All animals in the untreated group had extensive pulmonary metastasis and tumor in the
Table 1. The effect of LS2616 treatment on tumor growth and metastasis of B16F10 melanoma inoculated subcutaneously and intraocularly

<table>
<thead>
<tr>
<th>Experimental protocol</th>
<th>Tumorigenicity (%)</th>
<th>Survival (%)</th>
<th>Mean ± SEM</th>
<th>Incidence (%)</th>
<th>SMLN Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1-0.5 mm</td>
<td>0.6-1.0 mm</td>
<td>&gt;1.0 mm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SC Inoculations</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No LS2616</td>
<td>100 (7/7)</td>
<td>0 (0/7)</td>
<td>195 ± 19.1 (99-316)</td>
<td>140</td>
<td>40</td>
</tr>
<tr>
<td>LS2616</td>
<td>100 (10/10)</td>
<td>0 (0/10)</td>
<td>22 ± 12.1 (0-102)**</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td>IC Inoculations</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No LS2616, no enuc.</td>
<td>100 (12/12)</td>
<td>0 (0/12)</td>
<td>172 ± 71.2 (21-289)</td>
<td>102</td>
<td>59</td>
</tr>
<tr>
<td>LS2616, no enuc.</td>
<td>100 (11/11)</td>
<td>0 (0/11)</td>
<td>21 ± 15.8 (0-182)**</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>No LS2616, enuc.</td>
<td>100 (7/7)</td>
<td>0 (0/7)</td>
<td>191 ± 15.4 (142-256)</td>
<td>132</td>
<td>34</td>
</tr>
<tr>
<td>LS2616, enuc.</td>
<td>100 (13/13)</td>
<td>31 (4/13)</td>
<td>8 ± 3.9 (0-46)**</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Enuc., LS2616</td>
<td>100 (13/13)</td>
<td>0 (0/13)</td>
<td>58 ± 15.2 (0-178)</td>
<td>39</td>
<td>11</td>
</tr>
</tbody>
</table>

* Pulmonary metastases determined when moribund or sacrificed at week 10 post inoculation as described in Materials and Methods.
† Nodule size determined at X25; expressed as the mean ± SEM (range) in each of three categories.
‡ Tumorigenicity = percent, and number of mice with palpable tumors at day 21 (sc) or number of mice with tumor in the anterior chamber at day 7 per number of mice examined.
§ Survival = percent, and number of mice alive and healthy at 6 weeks postinoculation per number of mice examined.
§ Survival = percent, and number of mice with metastases to the ipsilateral submandibular lymph nodes per number of mice examined.
* Incidence = percent, and number of mice with one or more nodules per number of mice examined.
** Significantly different (P < 0.01) when compared to controls.

ipsilateral submandibular lymph nodes (SMLN). The frequency of pulmonary metastases decreased from 100% (12/12) in the untreated group to 73% (8/11) in the treated group, whereas the frequency of SMLN metastasis remained unchanged. The mean number of pulmonary metastases in the drug-treated group (21 ± 15.8 nodules/lung) was significantly less (P < 0.01) than that seen in controls (172 ± 71.2 nodules/lung) (Table 1, Fig. 1).

The Effect of Pretreatment With LS2616 on Spontaneous Metastasis in Enucleated Mice

To determine the combined effect of enucleation and drug treatment, 13 animals that began treatment with LS2616 at day −7, were inoculated ic with 10⁵ B16F10 tumor 7 days later and enucleated 7 days postinoculation (day +7). Drug was administered for the duration of the experiment. Control animals received no drug, and enucleation on day 7. Of the 13 drug-treated enucleated mice, nine died 5 weeks postinoculation. The remaining four animals were healthy. These healthy mice were sacrificed 10 weeks postinoculation and showed no evidence of metastasis, and no recurrence of the primary ocular tumor. Thus, survival was increased and 31% of the mice appeared to have been cured. In addition, the incidence of pulmonary metastases was reduced from 100% (7/7) in mice that received enucleation and no...
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WEEKS POST INOCULATION
Fig. 2. Subcutaneous growth of B16F10 primary tumor in control and LS2616-treated mice. Mice received 10^5 in vivo derived B16F10 melanoma cells sc and normal drinking water (—•—) or daily treatment with 160 mg/kg body weight of LS2616 in drinking water (— O —). Growth of the primary tumor was measured weekly. Vertical bars = ±SEM.

treatment to 54% (7/13) in mice that received enucleation and drug treatment. The incidence of metastasis to the ipsilateral SMLN was reduced from 100% (7/7) to 0% (0/13) in drug-treated mice. The mean number of pulmonary metastases in the enucleation and drug-treated group was significantly less (P less than 0.01) than in the group that received enucleation but no drug (8 ± 3.9 nodules/lung compared to 191 ± 15.4 nodules, respectively) (Table 1).

The Effect of Therapeutic Treatment With LS2616 Administered at the Time of Enucleation

To examine the effectiveness of drug treatment in controlling metastasis, when administered after enucleation, 13 animals were inoculated ic with tumor, enucleated on day 7, and given daily drug treatment starting on day 7. Treatment and enucleation resulted in a decrease in pulmonary metastases (58 ± 15.2 nodules/lung) when compared to controls (191 ± 15.4 nodules/lung). However, this decrease was not statistically significant. The incidence of pulmonary metastasis was reduced from 100% (7/7) to 85% (11/13), and metastasis to the ipsilateral SMLN was reduced from 100% (7/7) to 15% (2/13). Two experimental animals died with no evidence of metastasis (Table 1).

The Effect of Pretreatment of LS2616 on Spontaneous Metastasis From a Subcutaneous Site

In order to determine the effectiveness of LS2616 against lymph node and pulmonary metastases, ten mice were pretreated on day -7 with LS2616 (160 mg/kg/day), received sc inoculations of 10^5 B16F10 tumor cells on day 0, and continued to receive drug treatment daily throughout the remainder of the experiment. Eight control mice received sc tumor inoculation but no drug. Treatment of sc inoculated mice resulted in a significant decrease (P less than 0.01) in pulmonary metastases (22 ± 12.1 nodules/lung) when compared to sc inoculated animals that had received no drug treatment (195 ± 19.1 nodules/lung). The incidence of pulmonary metastasis decreased from 100% (8/8) in the untreated group to 50% (5/10) in the sc inoculated and treated mice.

The Effect of Pretreatment of LS2616 on the Growth of Primary Tumors

In order to determine whether treatment of hosts with LS2616 would affect the growth of the primary tumor, animals were pretreated for 7 days with drug, inoculated sc with tumor, and given drug for the duration of the experiment. The primary tumor was measured weekly. Tumor grew in all animals and no differences in the geometric mean tumor diameters were seen between control (N = 8) and experimental (N = 8) animals (Fig. 2). In order to determine if drug treatment could decrease tumor take at a lower tumor burden, six animals were pretreated with drug and inoculated sc with 10^4 tumor cells. Tumorigenicity in the group was again 100%. Furthermore, in all of the ic inoculated drug-treated groups shown in Table 1, tumorigenicity was always 100% (N = 56).

In Vitro Neutralization Assay

In order to determine whether spleen cells from drug-treated animals could effect the tumorigenicity of B16F10 cells, a Winn assay as modified by Merluzzi et al^9 was performed. Animals were treated with drug for 7 days. Control animals received no drug. Splenocytes from either drug treated animals (N = 8) or untreated mice (N = 8) were incubated with tumor cells for 5 hr and injected sc into the flank of recipient mice. In addition, tumor cells in DMEM were also inoculated into a group (N = 8) of recipient mice. None of the animals (0/8) inoculated with tumor cells incubated with splenocytes from drug treated animals had palpable tumor at day 21 postinoculation. In contrast, all recipients inoculated with tumor cells incubated alone in DMEM (8/8), or with splenocytes from untreated mice (8/8), had palpable tumors 21 days after inoculation (Table 2).

Discussion

Our results demonstrate the effectiveness of LS2616 against lymph node and pulmonary metas-
tasis of in vivo derived B16F10 melanoma. When mice pretreated with drug were inoculated with tumor intraocularly or subcutaneously and the primary tumor left intact, there was no effect on the primary tumor. However, a significant decrease in the number of pulmonary metastases was seen. The incidence of metastasis also declined in both groups. Experimental mice, however, showed no increase in survival and eventually died. It is possible that deleterious effects of very large primary tumors contributed to morbidity.

When mice with ocular tumors were enucleated and treatment with LS2616 initiated the day of enucleation, the incidence of pulmonary and lymph node metastasis was reduced. A decrease in the number of pulmonary metastases was seen, but was not statistically significant, and survival was unaffected. In contrast, the combination of pretreatment with LS2616 and enucleation resulted in an apparent cure in 31% of the mice. These mice were healthy and appeared to be completely free of metastatic growth when sacrificed and necropsied 10 weeks postinoculation. In the group as a whole, the extent of pulmonary or SMLN metastasis was markedly decreased in the drug-treated, enucleated mice.

In enucleated mice, pretreatment with LS2616 and enucleation resulted in an apparent cure in 31% of the mice. These mice were healthy and appeared to be completely free of metastatic growth when sacrificed and necropsied 10 weeks postinoculation. In the group as a whole, the extent of pulmonary or SMLN metastasis was markedly decreased in the drug-treated, enucleated mice.

In enucleated mice, pretreatment with LS2616 did not affect the incidence of metastasis to the ipsilateral SMLN. In enucleated mice pretreatment or treatment at the time of enucleation markedly decreased or prevented metastasis to the ipsilateral SMLN and only partially affected the incidence of pulmonary metastasis. This demonstrates varying degrees of susceptibility of different organ metastases to treatment with LS2616 and suggests that the effectiveness of this drug may be dependent upon the tumor burden of the host.

It is interesting to note that in earlier work, the only metastases we observed from primary tumors of B16F10 were in the lungs. In more recent studies, however, we have also been observing metastasis to the ipsilateral submandibular region of both enucleated and enucleated mice. It would appear that there has been an increase in the metastatic capability of this in vivo passaged line.

Previous work by others has demonstrated that LS2616 treatment in C57BL/6 mice results in an increase in NK activity and an immunostimulatory effect on macrophages which indirectly facilitates polyclonal and antigen specific T cell responses. Our modified Winn assay confirms the fact that a drug-stimulated spleen cell population, when incubated with B16F10 melanoma cells at a ratio of 100:1 effector to target cells, is capable of causing a decrease in tumorigenicity.

The importance of NK cell activity in preventing metastasis of murine ocular melanoma remains unclear. Yokoyama et al. reported a significant increase in pulmonary metastases of B16 melanoma when NK cell activity was inhibited with anti-GM1 antibodies and a significant decrease in pulmonary metastases when NK cell activity was stimulated with interferon α. Niederkorn, in earlier reports, suggested NK cell activity did not play a significant role in increasing pulmonary metastases of in vitro cultured B16F10 melanoma. In his system there is evidence that T cell effectors play a critical role in preventing metastasis. Recently, selective T cell depletion experiments continue to support Niederkorn's earlier findings.

In our experiments, pretreatment with LS2616 did not affect tumor take in animals receiving tumor cells sc or intracamerally. Kalland reported a decrease in tumorigenicity in LS2616 pretreated animals. The reason for the discrepancy between our results and that of Kalland's is not known; however, a major difference in our experiments was our use of in vivo derived as opposed to cultured tumor cells. It is possible that the lack of effectiveness of LS2616 in reducing tumor take in our experiments is due to the greater tumorigenicity of in vivo passaged tumor.

Until recently, there have been no chemotherapeutic agents shown to be effective in treating metastases arising from intraocular melanoma. In humans, current chemotherapeutic strategies range in response rates from 17% to 21%; while the response rates of immunotherapeutic strategies vary from 22% to 25%. Niederkorn, using cultured B16F10 tumor cells, has shown that administration of difluoromethylornithine (DFMO) decreases the number of pulmonary metastases in enucleated mice. Little increase in survival was noted in these experiments. It is difficult to compare the usefulness of DFMO with that of LS2616 as a therapy for treating metastases of murine melanoma.

### Table 2. In vivo neutralization of B16F10 inocula by LS2616-activated cytotoxic or cytostatic cells

<table>
<thead>
<tr>
<th>Transferred cells*</th>
<th>10^6 B16F10 melanoma cells†</th>
<th>10^7 Effector cells‡</th>
<th>Tumor incidence§</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>control spleen cells</td>
<td>8/8</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>drug-stim. spleen cells</td>
<td>0/8</td>
</tr>
</tbody>
</table>

* Effector/target cell ratio = 100:1; sc flank injection.
† B16F10 melanoma cell suspension was derived from in vivo passaged tumor in C57BL/6J host.
‡ Effector cells were taken from normal C57BL/6J mice or from mice treated for 7 days with 160 mg/kg of body weight/day of LS2616. Effector and target cells were incubated for 5 h in DMEM before being inoculated at a concentration of 10^5 melanoma/10^7 effector cells into the flank of recipient mice.
§ Tumor incidence = number of mice with palpable tumor at day 21 per number of mice examined.
intraocular melanoma, as there are major differences between the experimental systems used by Niederkorn and coworkers and our laboratory. One major difference between our protocols is the use of cultured versus in vivo derived cells. A second major difference is that in order to obtain baseline or control metastasis of cultured cells, Niederkorn's group renders control mice immunoincompetent by sublethal irradiation and subjects them to traumatic enucleation involving squeezing the globe 10 times before its removal. It is important to note that even these procedures result in fewer pulmonary metastases in control animals: 39 nodules/lung, compared to 191 nodules/lung in our work with immunocompetent mice and in vivo derived B16F10 melanoma. Using in vivo derived tumor obtained from our laboratory, Niederkorn (personal communication) has recently confirmed our earlier observations and found that this line spontaneously metastasizes from the eye of immuno-competent C57BL/6 hosts. In contrast, cultured cells, including those recently obtained from the Frederick Cancer Center, do not metastasize from the eye unless hosts are immunosuppressed or the eyes subjected to trauma.

In summary, LS2616 effectively decreases metastasis from subcutaneous and intraocular primary tumors of in vivo derived B16F10 melanoma. The ability to achieve apparent cures when enucleated mice are pretreated with this drug is promising. It would seem that a combination of LS2616 treatment with other therapeutic strategies may prove to be an effective means of decreasing metastases and increasing survival after enucleation. We are currently examining the effectiveness of LS2616 combined with other immunomodulatory strategies in an attempt to increase survival and effect cures when treatment is begun immediately prior to or at the time of enucleation. Although LS2616 has been shown to have potent immunostimulatory effects, and it is effective against metastases from ocular and flank tumors in mice, its usefulness in humans with uveal melanoma is only speculative at this time.

Key words: ocular melanoma, B16F10, LS2616, therapy

Acknowledgments

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References