Enucleation in Consort with Immunologic Impairment Promotes Metastasis of Intraocular Melanomas in Mice

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A series of investigations was employed to determine if metastases of intraocular melanomas could be induced by experimental manipulations. Syngeneic B16F10 melanoma cells transplanted intracameraly into C57BL/6 mice produced progressively growing intraocular tumors, yet formed only occasional pulmonary metastases. Neither enucleation nor mechanical manipulation of the melanoma-containing eye promoted a significant increase in the incidence of metastases. Likewise, immunologic impairment in the form of natural killer cell deficiency, T-lymphocyte deficiency, or γ-irradiation-induced lymphopenia failed to produce spontaneous metastases in intraocular melanoma-bearing mice. However, enucleation in consort with immune impairment (T-cell deficiency) produced a sharp increase in the incidence and number of pulmonary metastases in intraocular melanoma-bearing mice. Further studies showed that external pressure to the tumor-containing globe (without enucleation) produced extensive metastases in athymic, nude mice. By contrast, atraumatic enucleation of rapidly frozen eyes prevented metastasis of intraocular melanomas in similar hosts. Collectively, the results indicate that induction of distant metastases in hosts harboring intraocular melanomas requires two simultaneous processes: (1) mechanical manipulation of the melanoma-containing eye, and (2) concomitant impairment of T-cell-dependent immune processes. The data strongly suggest that mechanical manipulation of melanoma-containing eyes produces intravascular showers of melanoma cells that are rejected by T-cell-dependent immune processes in the immunocompetent host. In the absence of these normal T-cell-dependent immune mechanisms, enucleation-induced showers of blood-borne melanoma cells gain a foothold in the lung and form progressive metastases.

In recent years, attention has been devoted to development of new therapeutic approaches to intraocular melanomas. Enucleation, which served as the time-honored method for treating intraocular neoplasms, came under criticism recently.1,2 Zimmerman et al1 have argued that enucleation increases greatly the risk of metastatic spread of intraocular tumors by promoting intravascular showering of melanoma cells. Others have raised compelling rebuttals to this hypothesis and continue to favor early removal of a tumor-containing eye.3-5 Controversy continues to surround this issue in part because experimental evidence in support of either position is slim.

In an attempt to address the role of enucleation in metastatic spread of primary intraocular tumors, investigators have turned to animal models. Using Green hamster melanoma, Fraunfelder et al6 found that hamsters subjected to atraumatic enucleation had longer survival times than similar animals subjected to traumatic enucleation. The authors implied that traumatic enucleation led to increased metastases, however, no data were given regarding number, size, or organ distribution of distant metastases. Moreover, it is unclear as to whether the Green hamster melanoma cells were syngeneic with the host, since there was no reference to the specific hamster strain or the major histocompatibility complex haplotype of the host. We have reported recently that enucleation of virally induced uveal melanomas in cats resulted in a sharp increase in the frequency and size of secondary tumors at distant body sites and a concomitant reduction in serum antibody titers directed against tumor-specific transplantation antigens expressed on the tumor cell membranes.7 However, the feline uveal melanoma model has serious disadvantages, including the observation that the secondary tumors in this model are fibrosarcomas and not melanomas and, therefore, do not represent valid metastases. Thus, there is an obvious need for an appropriate animal model for studying spontaneous metastasis of intraocular melanomas.
The B16 melanoma cell line has been used extensively for investigating the metastatic process and the immunologic parameters that influence metastatic tumor development in mice (see reference 8). In the present report, the B16 melanoma cell line was used to explore the roles of enucleation and immunologic impairment on the development of spontaneous metastases of murine intraocular melanoma.

Materials and Methods

Experimental Animals

Adult female C57BL/6 mice and the beige mutant (bg/bg) of C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). Female athymic, nude (nu/nu) BALB/c mice were purchased from Life Sciences, Inc. (St. Petersburg, FL). All mice were used as experimental subjects between 3 and 5 months of age. The present investigations conform to the ARVO Resolution of 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. All surgical procedures were performed using ketamine hydrochloride anesthesia.

Tumor Cells

B16F10 melanoma cells (C57BL/6 origin; H-2b) were grown in monolayer cultures in Falcon 75-cm² tissue culture flasks (Falcon Plastics; Oxnard, CA) using Dulbecco’s modified Eagle’s minimal essential medium (MEM; GIBCO Laboratories; Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS; GIBCO), gentamicin (0.05 mg/ml; Schering Corp., Kenilworth, N.J.), and vitamin solution (GIBCO). The origin and properties of the B16F10 subline of B16 melanoma have been described in detail previously.9,10 Melanoma cells were harvested from tissue culture flasks by gentle trypsinization, washed in MEM containing 10% FCS, and resuspended in MEM without FCS for intracameral inoculations.

Intracameral Inoculations

A modified quantitative technique for inoculating a precise number of tumor cells into the anterior chamber of the mouse eye has been described in detail.11 Mice were anesthetized deeply with 0.66 mg of ketamine hydrochloride (Vetalar; Parke, Davis and Co.; Detroit, MI) given intramuscularly. B16F10 melanoma cells, at a concentration of 10⁵ cells/5 μl, were injected intracameraly (IC) into panels of C57BL/6, beige (C57BL/6 mutant), and athymic, nude BALB/c mice. The eyes were examined daily with a dissecting microscope (×8) and tumor growth scored according to the percent of the anterior chamber occupied by tumor.11

Gamma Irradiation of Mice

Mice were γ-irradiated with 500 rad each, at a rate of 109 rad/minute, in a Gamma cell 40 (Atomic Energy of Canada, Ltd.; Ottawa, Canada) containing a ¹³⁷Cs source. This dose has been shown to abolish the capacity of mice to mount a primary antitumor response.12

Cyclophosphamide Treatment

Cyclophosphamide (Cy) was purchased from Mead Johnson Laboratories (Evansville, IN) and was dissolved in sterile Hanks’ balanced salt solution (HBSS) immediately prior to inoculation. Panels of mice received intraperitoneal (IP) injections of Cy (240 mg/Kg bodyweight) before, or after IC tumor inoculations. This dose of Cy is known to impair natural killer (NK) cell function without depleting T-lymphocyte or macrophage populations.13

Enucleation and Ocular Trauma

Tumor-containing eyes were enucleated 10–12 days after IC tumor inoculation. Mice were anesthetized deeply with ketamine hydrochloride (0.66 mg; intramuscularly, see above) and the tumor-containing eyes enucleated with curved scissors. Mild hemorrhage, when present, was arrested by gentle electrocautery—direct pressure was never applied to hemorrhaging blood vessels or to the orbit. In some experiments, the tumor-containing eyes were subjected to mechanical trauma without removing the eye. Mechanical trauma is operationally defined as pressure exerted to the tumor-containing globe administered by blunt forceps. The globes were squeezed 10 times, in rapid succession, without visibly rupturing the external global architecture. All mice were observed three times per week and necropsied when morbidity indicated imminent death (usually day 30 posttumor inoculation).

Quantification of Spontaneous Metastases

Mice were killed when moribund and the number of lung tumor colonies was determined by inspection with a dissecting microscope.14 The lung colony assay is a simple and accurate method for evaluating spontaneous metastases of B16F10 melanomas since these tumor cells localize at the lung surface following intravenous inoculation.15 Moreover, B16F10 melanoma cells metastasize selectively to the lungs and form discrete pulmonary tumors following subcutaneous, intramuscular, intravenous, or intradermal inoculation.16
Spontaneous Metastasis of Intraocular Melanomas

The following experiments were designed to determine if impairment of NK cell activity would lead to spontaneous metastasis of intraocular B16F10 melanoma.

Panels of C57BL/6 mice were rendered NK cell deficient by treatment with cyclophosphamide\(^{13}\) given as a single intraperitoneal injection (240 mg/Kg body weight) on days -1, +9, or +12. The role of NK cells also was evaluated in the NK cell-deficient beige mutant (bg/bg) strain of C57BL/6.\(^{18,19}\) B16F10 melanoma cells were inoculated IC (1 × 10\(^5\)) cells/eye) into panels of beige mice, cyclophosphamide-treated mice, and untreated C57BL/6 mice on day 0 and the tumor-containing eyes enucleated 10 days later. Parallel experiments included similar groups of mice not subjected to enucleation. As in the previous experiment, there were essentially no pulmonary metastases in either beige mice (mean number of metastases was <2.0) or cyclophosphamide-treated mice (mean number metastases was <2.0, Table 1). Moreover, enucleation did not promote spontaneous metastasis in either beige mice or cyclophosphamide-treated mice. Although beige mice (enucleated and nonenucleated groups) had slightly higher mean numbers of metastases than other groups, these differences were not statistically significant \((P > 0.05)\) when compared with control panels (lines 1 and 2; Table 1). Thus, NK cell impairment does not promote spontaneous metastasis of intraocular B16F10 melanomas. Likewise, enucleation, even in the presence of NK cell deficiency, does not lead to an increased incidence of pulmonary metastases.

**Enucleation in Consort with Immune Impairment Promotes Spontaneous Metastases**

The most obvious explanation for the absence of spontaneous pulmonary metastases in intraocular
Table 2. Spontaneous metastasis of intraocular melanomas in immunologically impaired C57BL/6 mice.

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Pulmonary metastases$^{a}$</th>
<th>Range</th>
<th>Median</th>
<th>Mean*</th>
<th>Frequency**</th>
<th>P††</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole-body $\gamma$-irradiation†</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>—</td>
<td>Enucleation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>day +10</td>
<td>0–8</td>
<td>0</td>
<td>1.0 ± 0.79</td>
<td>3/10</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>day +10</td>
<td>0–1</td>
<td>0</td>
<td>0.33 ± 0.24</td>
<td>2/9</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>(trauma without enucleation)$^#$</td>
<td>Day +12</td>
<td>—</td>
<td>0–1</td>
<td>0</td>
<td>0.64 ± 0.39</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Day +12</td>
<td>day +10</td>
<td>0–118</td>
<td>7</td>
<td>35.0 ± 10.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Day +12</td>
<td>day +10</td>
<td>0–1</td>
<td>0</td>
<td>0.25 ± 0.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Day +12</td>
<td>day +10</td>
<td>(normal eye)</td>
<td>0–1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Day +24</td>
<td>(“no touch” enucleation)$^\xi$</td>
<td>0–7</td>
<td>0</td>
<td>0.43 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Day +24</td>
<td>—</td>
<td>0–7</td>
<td>0</td>
<td>0.9 ± 0.71</td>
</tr>
</tbody>
</table>

* $1 \times 10^9$ B16F10 melanoma cells inoculated intracamerally into the left eyes of C57BL/6 mice on day 0.
† Whole body $\gamma$-irradiation = 500 rads.
‡ Tumor-containing eyes were subjected to gentle pressure with blunt forceps without enucleation as described in Materials and Methods.
§ Tumor-containing eyes were subjected to “no touch” enucleation by cryofixation immediately prior to standard enucleation as described in Materials and Methods.

— Metastases (mean number of metastases < 1.0).

$^{a}$ Pulmonary tumor metastases determined by lung colony assay on day 30 posttumor inoculation as described in Materials and Methods.
* Mean ± SEM.
** Frequency = number of mice with one or more metastases per number of mice examined.
†† $P$ value determined using Student’s $t$-test by comparing experimental groups with control group (line 1 of this Table). NS = not significant; $P > 0.05$. 

T-Cell-Dependent Resistance to Enucleation-Induced Metastases

Athymic, nude mice were used to determine if the resistance to enucleation-induced metastases was T-cell dependent. B16F10 cells ($1 \times 10^5$) were inoculated into the left eyes of nude mice on day 0. Ten days later, the tumor-containing eyes were subjected to enucleation or mechanical trauma. Mice were observed three times per week and necropsied when moribund. Examination, at necropsy, revealed that nude mice subjected to enucleation had significantly greater ($P < 0.05$) numbers of pulmonary metastases (mean = 18.8) than the nonenucleation group (mean = 0.83). To rule out the possibility that nonspecific effects of surgery contributed to spontaneous metastases, the contralateral, normal eyes (ie, the right eyes) were enucleated in a panel of nude mice harboring intraocular melanomas in the left eyes. These mice, like the nonenucleated group, developed only occasional metastases (mean = 1.0).

The fact that enucleation induced markedly greater numbers of spontaneous metastases, compared with
Table 3. Spontaneous metastasis of intraocular melanomas in athymic, nude mice

<table>
<thead>
<tr>
<th>Enucleation*</th>
<th>Pulmonary metastases†</th>
<th>N</th>
<th>Mean‡</th>
<th>Frequency§</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td>Range</td>
<td>Median</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OS†</td>
<td>0–5</td>
<td>0</td>
<td>0.83 ± 0.83</td>
<td>1/6</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>OD** (normal eye)</td>
<td>0–54</td>
<td>17</td>
<td>18.8 ± 9.13</td>
<td>4/7</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>OS (trauma without enucleation)</td>
<td>0–100</td>
<td>13</td>
<td>34.8 ± 11.6</td>
<td>10/11</td>
<td>P &lt; 0.025</td>
</tr>
<tr>
<td>OS (“no-touch”)</td>
<td>0–3</td>
<td>0</td>
<td>0.27 ± 0.27</td>
<td>1/11</td>
<td>P &gt; 0.05</td>
</tr>
</tbody>
</table>

* 1 X 10^5 B16F10 cells inoculated into the left eyes on day 0. Tumor-containing eye (OS) or normal eye (OD) were enucleated or traumatized 10 days later. Standard enucleation, trauma, and “no-touch” enucleation procedures are described in the Materials and Methods.
† Pulmonary tumor metastases determined by lung colony assay on day 30 posttumor inoculation as described in Materials and Methods.
‡ Mean ± SEM.
§ Frequency = number of mice with one or more metastases per number of mice examined.
P = Probability (P) values were determined by Student’s t-test. Mean and SEM for each experimental group were compared with control group (line 1 of this table) and the probability value determined.
|| OS = left eye.
** OD = right eye.

the nonenucleation group, lends credence to Zimmerman and McLean’s hypothesis that mechanical manipulation of tumor-containing eyes causes increased intravascular showering of the intraocular tumor cells. If this were true, one would predict that mere pressure to the tumor-containing globe, without surgically removing the eye, would result in an increased intravascular showering of the tumor emboli and, thus, produce extensive pulmonary metastases. This hypothesis was tested in nude mice. B16F10 melanoma cells were inoculated into the left eyes of nude mice on day 0. Instead of enucleating the tumor-containing eyes on day 10, the globes were traumatized with blunt forceps, and the eyes left in place. Each tumor-containing eye was squeezed 10 times in rapid succession. Care was taken not to rupture the globe or to induce hemorrhage—none of these globes were perforated. The results of these experiments show clearly that external trauma (ie, pressure) to the tumor-containing eye induces a dramatic increase in the number of spontaneous metastases originating from the intraocular tumor nidus. As seen in Table 3, mice subjected only to mechanical ocular trauma developed extensive pulmonary metastases (mean = 34.8). This is a sharp contrast to the results found in mice not subjected to enucleation (mean = 0.83) or mice whose contralateral normal eye was enucleated (mean = 1.0).

A prediction of the Zimmerman-McLean hypothesis is that minimizing pressure and physical manipulation of the tumor-containing eye during enucleation should reduce greatly the risk of showering and subsequent metastatic disease. A “no-touch” enucleation procedure in which tumor-containing eyes were frozen rapidly, immediately prior to enucleation was tested next. B16F10 melanoma cells (1 X 10^5) were inoculated into the left eyes of nude mice. Ten days later, “no-touch” enucleations were performed by gently touching the tumor-containing globes with cotton-tip applicator sticks saturated with liquid nitrogen and immediately enucleating the frozen globes. Subsequent observation and necropsies showed that only one mouse subjected to “no-touch” enucleation developed metastases (Table 3). Thus, the development of spontaneous metastases from intraocular melanomas virtually can be eliminated by minimizing the amount of physical manipulation of the tumor-containing eye during enucleation and suggests that intravascular showering of tumor emboli is the basis for enucleation-induced metastases.

Discussion

The dilemma over whether or not enucleation contributes to the metastatic spread of intraocular melanomas is based on interpretations of retrospective studies on human patients. Such investigators are subject to ambiguous interpretation since many crucial parameters cannot be controlled. Particularly important in this regard are: (1) the immunologic status of the patients before and after surgery; (2) the nonspecific immunosuppressive effects of surgery and anesthesia; (3) the role of the patient’s genetic constitution; (4) the immunogenicity of intraocular melanoma cells for each patient; and (5) the time course of extraocular metastasis development—that is, are subsequent metastases the result of preexisting nests of extraocular tumor foci or the result of recently disseminated melanoma cells released at the time of surgery?

By employing a murine intraocular melanoma model, it was possible to explore this problem in a prospective setting in which these parameters could be controlled and analyzed independently. However, it should be emphasized that the present findings are from an animal model in which syngeneic melanoma cells were transplanted directly into the anterior segment of the mouse eye. Even though these melanomas invaded the ciliary body and choroid (unpublished observations), caution should be exercised in interpreting these results in the context of human uveal
melanoma. It is possible that the behavior of transplanted murine intraocular melanomas does not parallel the behavior of spontaneous, uveal melanoma in humans. Nonetheless, the present studies have produced a number of important fundamental concepts regarding the biology and immunology of intraocular neoplasms.

Experiments using mice with selective immune deficiencies made it possible to examine the role of specific, immune components in preventing spontaneous metastasis of intraocular melanomas. Surprisingly, immune depression in the form of NK-cell deficiency, T-cell deficiency, or gamma-irradiation-induced lymphopenia was insufficient in its own to promote spontaneous metastases. Likewise, neither enucleation nor mechanical manipulation of the tumor-containing globe alone promoted progressive metastatic tumor development. However, enucleation in consort with either congenital T-cell deficiency (ie, nude mice) or gamma-irradiation-induced lymphopenia produced a sharp increase in the frequency and number of pulmonary metastases. By contrast, enucleation did not promote metastasis of intraocular melanomas in NK-deficient mice even though this tumor line is NK-sensitive. Collectively, these results indicate that induction of distant metastases in hosts bearing intraocular melanomas depends upon two conditions prevailing: (1) a surgery-dependent phase and (2) impairment of T-cell-dependent immune processes. The surgery-dependent component of metastasis induction is presumably due to intravascular showering of tumor emboli. This conclusion is based on the observation that mere mechanical pressure to the tumor-containing eye, without surgically removing the globe, leads to extensive pulmonary metastases in athymic, nude mice. The fact that neither enucleation nor external ocular trauma leads to pulmonary metastases in immunologically intact, normal mice supports the conclusion that metastasis induction is a two-step process and suggests further that intravascular showers of melanoma cells (induced by either enucleation or mechanical trauma) are rejected by T-cell-dependent, radiosensitive immune mechanisms in the immunocompetent host. Studies with beige mice and cyclophosphamide-treated euthymic C57BL/6 mice demonstrated that this resistance to enucleation-induced metastases was not NK cell-dependent.

The biology of B16F10 melanoma provides insights into the nature of this immune rejection. For example, B16F10 melanoma cells invade the pulmonary blood vessel walls and extravasate within 2–4 hr of intravenous injection. Over 99% of the B16F10 melanoma cells that do not extravasate within this brief time period perish rapidly due to nonimmunological causes. Results from the present time-course studies with whole-body gamma-irradiation show that hosts subjected to irradiation 2 days after enucleation (ie, day +12 posttumor inoculation) developed extensive pulmonary tumors, whereas mice irradiated 14 days after enucleation (ie, day +24 posttumor inoculation) did not form progressive metastases. If immune rejection were to occur intravascularly, then mice subjected to gamma-irradiation 2 days after enucleation already would have eliminated the blood-borne melanoma cells in the 48 hr prior to gamma-irradiation. Even though these mice were immunocompetent during this interval, they did not reject the disseminated tumor cells. Since these hosts developed fulminant metastases, one must conclude that a significant number of blood-borne melanoma cells escaped immune rejection within the vascular compartment. By contrast, immunocompetent, intraocular melanoma-bearing hosts (subjected to enucleation) reject disseminated melanoma cells and do not develop progressive metastases. Based on the data discussed above, we would assume that immune rejection occurs at extravascular pulmonary sites in the immunocompetent host.

It is reasonable to conclude that in the immunocompetent host, these extravascular tumor cells are rejected within 2 weeks of enucleation since no pulmonary tumors developed in hosts subjected to immune impairment (ie, gamma-irradiation) 2 weeks after enucleation. If immune rejection did not occur within this 2-week period, these mice would have developed significant numbers of pulmonary tumors. However, metastases were found in only two of these mice with mean of <1.0 metastasis per mouse in this experimental group.

Collectively, these data demonstrate that the immune rejection of disseminated melanoma cells in the intraocular melanoma-bearing mouse is swift, compartmentalized, and occurs within a predictable window of time.

Perhaps the most impressive finding is that external pressure to the globe induces extensive metastatic spread of intraocular melanoma in immunologically impaired hosts. Although this finding may come as no surprise, it is still noteworthy since it documents a widely held hypothesis that, until now, has not been proven unequivocally. Others have shown that increased numbers of tumor cells enter the peripheral circulation during and after surgery. However, it is unclear whether the number, viability, and tumorigenicity of such tumor cells are sufficient to develop into progressive metastases in human patients subjected to surgery. The mere presence of blood-borne tumor cells is not tantamount to progressive metastatic disease. For example, Fidler has shown that 99.9% of blood-borne B16 melanoma cells perish without forming progressive metastases. The present data clearly
show that intravasated tumor emboli, released during surgery, are tumorigenic in the immunosuppressed host and, thus, are an important consideration when performing therapeutic enucleation. However, caution must be exercised before applying these findings directly to human uveal melanomas. The present tumor model differs significantly from human intraocular melanoma in that the murine melanomas were transplanted into the anterior segment of the mouse eye and, therefore, did not originate by in situ transformation within the uveal tract as occurs in the human counterpart. It is entirely possible that melanomas and, therefore, did not originate by in situ transformation with rational surgery, the rate of metastasis of metastatic disease. By combining selective immunopotentiation with rational surgery, the rate of metastasis of intraocular melanomas in human patients may decline even further in the future.

Key words: intraocular melanoma, enucleation, metastasis, immunologic impairment

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References