Relationship Between Natural Killer Cell Susceptibility and Metastasis of Human Uveal Melanoma Cells in a Murine Model

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**Purpose.** The purpose of this study was to determine the susceptibility of human uveal melanoma cells to in vitro and in vivo natural killer (NK) cell-mediated cytolysis and to determine if NK cells influence metastasis from the eye.

**Methods.** Four human uveal melanoma cell lines and one melanoma cell line derived from a metastatic lesion from a patient with uveal melanoma were tested for in vitro and in vivo NK cell-mediated lysis in a mouse model. Major histocompatibility complex (MHC) class I antigen expression was evaluated by flow cytometry. The role of NK cells in controlling the metastasis of uveal melanoma cells from the eye to the liver was examined in nude mice.

**Results.** Sensitivity to in vitro and in vivo lysis by human and murine NK cells was correlated with reduced expression of MHC class I antigens. Uveal melanoma lines expressing normal MHC class I antigen expression were insensitive to NK cell-mediated lysis, both in vitro and in vivo. Metastasis of uveal melanoma cells was inhibited by NK cell activity because disruption of in vivo NK function produced a sharp increase in the spontaneous metastasis of intraocular melanomas in nude mice.

**Conclusions.** There is considerable variation in the susceptibility of human uveal melanomas to NK cell-mediated cytolysis. Susceptibility is closely correlated with reduced expression of MHC class I antigen expression. Disruption of NK cell function significantly increases the development of hepatic metastases from human uveal melanoma cells. Invest Ophthalmol Vis Sci. 1995;36:435-441.

Uveal melanoma is the most common primary intraocular tumor in adults. Although primary uveal melanoma can be successfully treated by enucleation or radiotherapy, approximately 50% of patients with large uveal tumor will die of metastatic disease within 5 years. The liver is the most frequently affected organ in patients with metastatic melanoma from the uveal tract, and hepatic metastasis remains the leading cause of death in patients with uveal melanoma. Thus, preventing and treating hepatic metastases could have an important impact in the treatment of uveal melanoma.

In recent years, there has been a rekindled interest in the application of immunotherapy for a variety of tumors, especially cutaneous melanoma. Clinical trials involving the use of interleukin-2 (IL-2) and the adoptive transfer of tumor-infiltrating lymphocytes (TIL) have provided evidence attesting to the significant, albeit limited, efficacy of immunotherapy for metastases arising from cutaneous melanomas. Although cutaneous melanoma has received considerable attention, few studies have focused on the potential of immunotherapy of uveal melanoma. Ksander and coworkers have shown that antigen-specific cytolytic T lymphocyte (CTL) activity was demonstrable in TIL isolated from human choroidal melanoma. Kan-Mitchell and coworkers were able to isolate and demonstrate melanoma-specific CTL from the peripheral blood of patients with choroidal melanoma. Although cells with natural killer (NK) activity are...
present in TIL populations isolated from human choroidal melanomas, no studies to date have examined the relative susceptibility of human uveal melanomas to NK cell-mediated cytolysis or the capacity of NK cells to prevent the metastatic spread of uveal melanoma cells. The present study was designed to address both of these crucial questions.

MATERIALS AND METHODS

Mice

Athymic nude BALB/c (H-2d) mice were purchased from the Jackson Laboratories (Bar Harbor, ME). The use of animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Tumor Cell Lines

Four human uveal melanoma cell lines, designated OCM1, OCM3, OCM8, and EOM3, were used. OCM1, OCM3, and OCM8 were generously provided by Dr. June Kan-Mitchell (University of Southern California School of Medicine, Los Angeles, CA). OCM1 is a predominantly spindle morphology, whereas OCM3 and OCM8 are predominantly epithelioid tumors. OMM1 was isolated from a subcutaneous metastasis in a patient with uveal melanoma and displays a mixed cell morphology.12 The EOM3 cell line displays an epithelioid morphology in vitro but was derived from a posterior choroidal melanoma of mixed cell morphology. OCM1, OCM3, and OCM8 cells were cultured in Ham’s F-12 medium containing 10% heat-inactivated fetal calf serum, 1% L-glutamine, 1% sodium pyruvate, 1% nonessential amino acids, 1% HEPE’s buffer, and 1% antibiotic-antimycotic solution. OMM1 and EOM3 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal calf serum, 1% L-glutamine, 1% sodium pyruvate, 1% vitamin solution, and 1% antibiotic-antimycotic solution.

Analysis of MHC Class I Expression

The expression of major histocompatibility complex (MHC) class I antigens was assessed by flow cytometry using a fluorescence-activated cell sorter (FACS) and was detected using a mouse monoclonal antibody directed against nonpolymorphic MHC class I determinants (IgG, clone BL-3D3; Accurate Chemical and Scientific, Westbury, NY). Mouse anti-H-2d monoclonal antibody (IgG; clone 11-4.1; PharMingen, San Diego, CA) served as an isotype-matched negative control antibody. Single cell suspensions were prepared and washed in FACS buffer consisting of phosphate-buffered saline (PBS; Ph 7.4) with 1% BSA and 0.02% sodium azide. Cells (10^6) were incubated with primary antibodies for 30 minutes on ice, washed three times, and then incubated with FITC-labeled secondary antibodies for 20 minutes at 0°C and washed an additional three times. Cell suspensions were fixed in 1% paraformaldehyde and assayed for positive staining on Epics Profile Analyzer (Coulter Electronics, Hialeah, FL). Gates were set at 1% of total cells, based on staining by secondary antibodies alone.

In Vitro Natural Killer Cell Assay

Natural killer cell-mediated cytolysis was evaluated by a conventional 4-hour ^51Cr-release assay as described previously.13 Human peripheral blood was used as a source of NK cells. Heparinized blood from healthy donors was layered onto Histopaque-1119 (Sigma Chemical, St. Louis, MO) and centrifuged for 30 minutes at 2,200 rpm at room temperature. The mononuclear cell layer was collected. BALB/c mouse spleens served as a source of murine NK cells. Spleen cells were pressed through sterile stainless steel screens, washed twice in Hanks’ balanced salt solution (HBSS), passed through sterile nylon mesh (Tech, Elmsford, NY), and washed in HBSS an additional time. Total release counts per minute were determined by treatment of target cells with 0.05 ml Hematall LA-Hgb Reagent (Fisher Scientific, Pittsburgh, PA) and spontaneous release by incubating target cells with complete medium in the absence of effector cells.

Liver Localization Assay

Assessment of hepatic NK cell-mediated immunity was performed by an in vivo clearance assay as described previously.14 Briefly, ocular tumor cells were labeled in vitro with 100 μCi of Na_2^51CrO_4 for 1 hour (37°C), washed three times with HBSS, and resuspended in HBSS at a concentration of 1 x 10^6 cells/ml. ^51Cr-labeled melanoma cells (1 x 10^7 in 0.1 ml) were injected into the lateral tail vein. Clearance of ^51Cr-labeled tumor cells was determined by counting the radioactivity (gamma emission) of livers, lungs, spleens, kidneys, and 0.5 ml of blood removed from each animal 24 hours after intravenous tumor injection. Percent of radiolabel recovered was determined by dividing the counts per minute (cpm) for each liver divided by the total cpm recovered for the liver, lungs, kidneys, spleen, and 0.5 ml of blood for each animal. The total cpm for each animal ranged from 35,000 to 45,000 cpm. In vivo NK cell-mediated elimination of radiolabeled tumor cells is reflected by a reduction in the radioactivity of the liver.14

Intracameral Transplantation and Hepatic Metastasis Determination

A modified quantitative technique for the orthotopic intracameral (IC) transplantation of precise numbers of tumor cells into the mouse eye has been described
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FIGURE 1. Susceptibility of five human uveal melanoma cell lines to cytolysis by murine NK cells. Cytolysis was measured in a conventional 4-hour 51 Cr-release assay using murine spleen cells at an effector-to-target ratio of 50:1. Results are expressed as mean ± standard deviation. NK = natural killer.

previously. Mice were deeply anesthetized with 0.66 mg of ketamine hydrochloride (Vetalar; Parke-Davis, Detroit, MI) given intramuscularly. Tumor cells (10^5/0.5 µl) were inoculated IC using a 1.0-ml Hamilton syringe fitted with a 35-gauge glass needle. Metastatic hepatic tumor foci were readily demonstrable by histopathologic examination of the liver and were scored as previously described. Severity of metastases was scored as: clear (0 = no discernible foci); minimal involvement (1+ = metastatic tumors involved less than 10% of the liver); moderate (2+ = metastatic tumors involved 10% to 25% of the liver); or extensive (3+ = metastatic tumor mass involved ≥25% of the liver).

Cyclophosphamide Treatment

Cyclophosphamide was purchased from Mead Johnson Laboratories (Princeton, NJ) and dissolved in sterile HBSS. Panels of mice received intraperitoneal injections of (240 mg/kg) 24 hours before their sacrifice and use in the in vitro NK assays. For in vivo experiments, NK activity was inhibited by intraperitoneal injection of cyclophosphamide (240 mg/kg) on day 30 after tumor transplantation.

Poly I:C Treatment

Polyinosinic acid (poly I:C) was purchased from Sigma and dissolved in sterile HBSS before treatment. Natural killer cell activity was stimulated in experimental animals by injecting poly I:C (100 µg/mouse) intraperitoneally at weekly intervals after IC tumor transplantation and continuing until the time of necropsy.

Anti-asialo GM1 Treatment

Anti-asialo GM1 antibody was purchased from WAKO Chemicals (Dallas, TX) and used for in vivo depletion of NK cells. BALB/c nude mice were treated intravenously with anti-asialo GM1 (0.2 ml of a 1:10 dilution/mouse/injection) on days -3 and day -1 before the in vitro and in vivo NK assays. For metastases experiments, anti-asialo GM1 antibody was injected once per week until the time of necropsy.

Statistics

Student’s t-test was used to test the statistical significance of the data. Differences were considered significant when P < 0.05.

RESULTS

Human Uveal Melanoma Cells Differ in Their Susceptibility to NK Cell-Mediated Cytolysis

Although lymphoid cells bearing NK-associated cell membrane markers have been identified in uveal melanomas, no studies to date have specifically examined the susceptibility of uveal melanoma cells to NK cell-mediated cytolysis. Therefore, five human uveal melanoma cell lines were examined for their susceptibility to in vitro cytolysis by murine and human NK cells. Two human uveal melanoma cells lines, OCM3 and EOM3, were susceptible to cytolysis by both murine (Fig. 1) and human NK cells (data not shown).

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Control</th>
<th>Poly I:C</th>
<th>Cyclophosphamide</th>
<th>Anti-asialo GM1</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCM1</td>
<td>70.5</td>
<td>57.7</td>
<td>80.3 (P = 0.057)</td>
<td>81.8 (P = 0.14)</td>
</tr>
<tr>
<td>OCM3</td>
<td>64.2</td>
<td>52.3</td>
<td>81.2 (P = 0.034)</td>
<td>83.7 (P = 0.02)</td>
</tr>
<tr>
<td>OCM8</td>
<td>72.1</td>
<td>68.4</td>
<td>76.2 (P = 0.54)</td>
<td>75.4 (P = 0.63)</td>
</tr>
<tr>
<td>OMM1</td>
<td>67.5</td>
<td>64.3</td>
<td>74.5 (P = 0.29)</td>
<td>73.6 (P = 0.37)</td>
</tr>
<tr>
<td>EOM3</td>
<td>63.3</td>
<td>59.6</td>
<td>79.4 (P = 0.035)</td>
<td>78.8 (P = 0.046)</td>
</tr>
</tbody>
</table>

* Percent radiolabel recovered in the liver based on total counts recovered from lungs, liver, spleen, kidneys, and 0.5 ml of peripheral blood. Probability (P) values for each group compared to untreated controls are shown in parentheses.
FIGURE 2. Inverse relationship between expression of MHC class I antigen expression and susceptibility of human uveal melanoma cell lines to NK cell-mediated cytolysis. Human peripheral blood served as a source of NK cells. For FACS profiles, the abscissa is a logarithmic scale that represents fluorescence intensity with specific antibody to MHC class I antigen. The ordinate is a linear scale representing the relative number of cells staining positively with the same antibody. The bar graphs represent the mean NK-mediated cytolysis (± standard deviation) of the respective target cell in a conventional 4-hour 51Cr-release assay using human peripheral blood lymphocytes as effector cells. Effector-to-target ratio was 50:1. MHC = major histocompatibility complex; NK = natural killer; FACS = fluorescence-activated cell sorter.

By contrast, the other three cell lines displayed insignificant susceptibility to NK cell-mediated lysis. Specific lysis by human NK cells was less than 10% for OCM1, OCM8, and OMM1 (data not shown).

The susceptibility of human uveal melanoma cells to NK cell-mediated lysis in vivo was tested using a previously described clearance assay. The elimination of intravenously injected radiolabeled tumor cells is a well-established method for evaluating NK cell-mediated lysis in vivo. We have previously shown that human uveal melanoma cells preferentially localize in the liver after intravenous injection in nude mice. Therefore, the in vivo elimination of human uveal melanoma cells was evaluated by determining the residual radioactivity of livers removed from nude mice 24 hours after intravenous injection of radiolabeled tumor cells. In some mice, NK function was stimulated by i.p. injection of 100 µg of poly I:C 24 hour prior to the assay. In other groups of mice, NK cell activity was impaired by i.p. injection of either cyclophosphamide (240 mg/kg) or anti-asialo GM1 antibody.

The in vivo clearance of intravenous injected uveal melanoma cells paralleled the in vitro results. That is, NK-sensitive OCM3 and EOM3 melanoma cells were eliminated more efficiently from the liver than were NK-insensitive OCM1, OCM8, and OMM1.
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TABLE 2. Correlation Between MHC Class I Antigen Expression and NK Cell Sensitivity

<table>
<thead>
<tr>
<th>Melanoma Cell Line</th>
<th>MHC Class I Expression (% Positive Cells)</th>
<th>In Vitro NK Cell Activity (% Specific Cytolysis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCM1</td>
<td>90.12</td>
<td>3.5 ± 3.7</td>
</tr>
<tr>
<td>OCM8</td>
<td>84.10</td>
<td>3.6 ± 4.6</td>
</tr>
<tr>
<td>OMM1</td>
<td>76.85</td>
<td>5.9 ± 5.3</td>
</tr>
<tr>
<td>EOM3</td>
<td>8.55</td>
<td>31.8 ± 9.8</td>
</tr>
<tr>
<td>OCM3</td>
<td>4.71</td>
<td>35.2 ± 11.9</td>
</tr>
</tbody>
</table>

MHC = major histocompatibility complex; NK = natural killer.

NK Sensitivity of Human Uveal Melanomas Is Inversely Correlated with MHC Class I Antigen Expression

The NK sensitivity of murine fibrosarcomas and murine lymphomas has been shown to be inversely correlated with MHC class I antigen expression. Accordingly, the expression of MHC class I antigens by human uveal melanoma cells was evaluated by flow cytometry. The results indicated that NK cell-mediated cytolysis was significantly reduced in human uveal melanoma cell lines in which more than 75% of the cells expressed class I MHC antigens (Fig. 2). By contrast, uveal melanoma cell lines less than 10% positive for MHC class I expression were vulnerable to NK cell-mediated cytolysis (Fig. 2). Thus, the NK sensitivity of human uveal melanoma is inversely correlated with expression of MHC class I antigens (Table 2).

NK Cells Prevent the Metastasis of Uveal Melanoma Cells

The possibility that NK cells can limit or even prevent the metastasis of uveal melanoma cells was examined in mice. OCM3 human uveal melanoma was selected for further study based on its in vitro and in vivo susceptibility to NK cell-mediated lysis. OCM3 cells were transplanted into the anterior segments of BALB/c nude mice on day 0. In some mice, NK function was disrupted by either injecting cyclophosphamide intraperitoneally on day 30 or through weekly injections of anti-asialo GM1. Other groups of mice had their NK activity stimulated by weekly injections of poly I:C. All mice were necropsied on day 50. Hepatic metastases were detected in only one of the five untreated nude mice and none of the poly I:C mice (Table 3). However, 8 of the 10 nude mice with impaired NK cell activity harbored hepatic metastases of OCM3 human uveal melanoma.

DISCUSSION

Numerous studies have demonstrated that NK cells can limit the metastasis of experimental cutaneous B16 melanomas, and more recent studies have suggested that lymphokine-activated killer cells provide protection against metastasis in some patients with cutaneous melanoma. However, to our knowledge, there are no published studies that have examined the susceptibility of human uveal melanoma cells to in vitro and in vivo NK cell-mediated cytolysis. Szalay and coworkers have reported that impairment of NK activity promotes the metastasis of intracamerally transplanted B16 melanoma cells. Using the same murine model, these workers also reported that in vivo stimulation of NK cell activity with the biologic response modifier, LS2616, reduced the metastasis of intraocular B16 melanoma. However, it should be pointed out that B16 melanoma is a murine cutaneous melanoma that may or may not accurately reflect the behavior of human uveal melanoma. As shown here and elsewhere, tumors of the same histologic origin can display vastly different susceptibilities to NK cell-mediated cytolysis, and one must exercise caution when extrapolating results from studies involving heterotopically transplanted tumor cells.

To study the role of NK cells in the prevention of

TABLE 3. Inhibition of Uveal Melanoma Metastasis by Natural Killer Cells

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Severity of Metastatic Foci</th>
<th>Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Poly I/C</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>2*</td>
<td>2*</td>
</tr>
<tr>
<td>Anti-Asialo GM1</td>
<td>2*</td>
<td>3*</td>
</tr>
<tr>
<td></td>
<td>3*</td>
<td>2*</td>
</tr>
</tbody>
</table>

Severity of metastasis based on relative size of metastatic foci detected by microscopic examination of histopathologic liver sections stained with hematoxylin and eosin, as described in Materials and Methods. All mice were necropsied on day 50.
metastases of intraocular melanomas in a prospective manner, a suitable animal model is required. We and others have transplanted B16F10 melanoma into the eyes of syngeneic C57BL/6 mice as a model of intraocular melanoma.20-24 Greene hamster melanoma is perhaps the most widely used animal tumor in ocular melanoma research. However, like B16 melanoma, Greene melanoma arose as a cutaneous melanoma and therefore does not display the same metastatic behavior as uveal melanoma. Human uveal melanomas preferentially metastasizes to the liver,25-29 whereas B16 melanoma characteristically spreads to the lungs.30

It is becoming increasingly clear that tumors transplanted to heteroopic sites often do not display metastatic behavior consistent with the original tumor.20-24 Moreover, many human tumors do not metastasize from heteroopic sites in nude mice but will form metastases after orthotopic transplantation.30-34 The importance of orthotopic transplantation may be due, at least in part, to the influence of local organ-specific factors.35 Thus, the importance of orthotopic, rather than heterotopic, transplantation cannot be overemphasized.

The present model has several attributes that bear noting. Unlike murine B16 melanoma and Greene hamster melanoma, which are both cutaneous melanomas, four of the five tumor lines were derived from patients with uveal melanoma. Thus, the intracamerational transplantations in four of the five tumor cell lines was orthotopic, not heterotopic. Although the in vivo studies involved murine NK cells, it should be emphasized that the in vitro studies demonstrated that murine NK cell-mediated cytolysis paralleled their in vitro counterparts.36 Thus, the hypothesis that NK cells restrict uveal melanoma metastatic spread of uveal melanoma cells.

In summary, the present findings indicate that human uveal melanomas differ in their susceptibility to in vitro and in vivo NK cell-mediated cytolysis. The degree of susceptibility to NK cell-mediated cytolysis is inversely correlated with expression of MHC class I antigen expression. That is, decreased expression of class I antigens correlates with increased NK cell-mediated cytolysis. The results also indicate that disruption of in vivo NK activity can greatly increase the metastatic spread of uveal melanoma cells.

Key Words
uwaeal melanoma, natural killer cells, metastases, liver, major histocompatibility complex

References
Natural Killer Cells and Uveal Melanoma


