Role of Natural Killer Cells in Intraocular Melanoma Metastasis

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The authors studied the role of natural killer (NK) cells in spontaneous metastasis of murine intraocular melanoma by transplanting murine B16 melanoma cells into the anterior chamber of the eye in syngeneic C57BL/6 mice and determining the number of metastatic lung tumor colonies after 40 days. Depletion of NK activity by anti-asialo GM1 serum dramatically enhanced metastasis and augmentation of NK activity by interferon inhibited it. The strong correlation between host NK activity and intraocular melanoma metastasis indicated that NK cells have an important role in spontaneous metastasis of intraocular melanoma.


Metastasis is one of the most serious problems of intraocular malignant melanoma. The prevention of metastasis is obviously a great factor for improving the prognosis. The immune system is regarded as one of the host defense mechanisms against tumor growth and spread. Recently, natural killer (NK) cells have been shown to be important in preventing the intravascular dissemination of malignant tumor cells that were experimentally transplanted subcutaneously or in foot-pad. However, Niederkorn reported that impaired NK cell function did not promote spontaneous metastasis in mice with intraocular melanoma.

NK cells are morphologically identified as large granular lymphocytes that have asialo GM1 as a specific surface marker. Reports show that the activity of murine NK cells is augmented by interferons (IFNs) and IFN inducers and abolished by intravenous injection of anti-asialo GM1 serum. Human recombinant IFNaA/D, a product of the newly developed gene technology, has been reported to be as effective on murine cells as on human cells. In the present study, we employed anti-asialo GM1 serum and IFNaA/D to determine the role of NK cells on spontaneous metastasis in mice with intraocular melanoma.

Materials and Methods

Mice

Specific pathogen free, 6-wk-old male C57BL/6 mice were purchased from Shizuoka Cooperative for Experimental Animals, Hamamatsu, Japan. Studies using these animals were performed in conformance with the ARVO Resolution on the Use of Animals in Research.

Tumors

B16 murine melanoma, syngeneic to the C57BL/6 mouse, were maintained in vitro in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, streptomycin, and penicillin. Tumor cells within four to seven passages after recovery from frozen stocks were used for metastatic assay. The cells were removed from plastic surfaces by 0.2% trypsin-0.02% EDTA treatment and extensively washed before intracameral inoculation. YAC-1 lymphoma cells were also maintained in vitro.

Intracameral Inoculation of Melanoma

B16 melanoma cells were inoculated into the anterior chamber of the mouse eye by a modified technique of Boone and DuPree. Briefly, mice were anesthetized by ether inhalation. The cornea was punctured with a 30-gauge needle under the low power magnification of a dissecting microscope. The aqueous fluid was absorbed with a filter paper. Then 2 μl of tumor cell suspension in PBS containing 5 × 10⁶ tumor cells was injected into the anterior chamber with a 10-μl Hamilton microsyringe.

Interferon and Anti-Asialo GM1

Pure recombinant human interferonαA/D (IFNaA/D; specific activity; 1.5 × 10⁸ units/mg) was kindly provided by Japan Roche Institute, Kanagawa, Japan. Anti-asialo GM1 serum was purchased from Wako...
Pure Chem, Tokyo, Japan. Anti-asialo GM1 was diluted 1:10 with sterile saline before intravenous injection.

**Assay for NK Activity**

Murine splenic NK activity was determined by a standard $^{51}$Cr release assay. Non-adherent spleen cells were cocultured with $^{51}$Cr-labeled YAC-1 lymphoma cells at various E/T ratios in a total volume of 200 µl in the wells of round bottom, 96 multiple-well plates. The plates were incubated at 37°C for 4 hr in humidified atmosphere of 5%CO$_2$ and 95% air. The plates were centrifuged at 200 × g for 5 min, 100 µl of supernatant was removed, and radioactivity was determined by a gamma counter. All assays were carried out in triplicate. Percent cytotoxicity was calculated as follows:

\[
\text{% Cytotoxicity} = \left( \frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Maximum release} - \text{Spontaneous release}} \right) \times 100
\]

Maximum release or spontaneous release was determined as the radioactivity from target cells incubated with 0.1 N HCl or the culture medium, respectively.

**Assay for Metastasis**

Development of spontaneous metastasis from intraocular melanoma was determined by counting the number of lung tumor colonies. Mice were killed 40 days after intracameral melanoma inoculation. Lungs were removed and fixed in 10% formaldehyde solution. Tumor nodules on the surface of the lung were counted under a dissecting microscope.

**Statistical Analysis**

The Student t-test was used.

**Results**

**Effects of in Vivo Treatment of IFNαA/D and/or Anti-Asialo GM1 Serum on Murine Splenic NK Activity**

As shown in Table 1, natural cytotoxic activity in spleen cells against YAC-1 cells was markedly increased 24 hr after intraperitoneal injection of $2 \times 10^3$ units of recombinant human IFNαA/D. The activity was mediated by NK cells, since it was abolished completely by the intravenous injection of anti-asialo GM1 serum, which is known to deplete NK cell activity selectively without any detectable change in T cell mediated immunity.$^8,^1^0$

**Table 1. Effects of IFNαA/D and/or anti-asialo GM1 on murine splenic NK activity**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% cytotoxicity E/T ratio</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>100:1</td>
</tr>
<tr>
<td>None</td>
<td>4.7 ± 2.3</td>
</tr>
<tr>
<td>IFNαA/D</td>
<td>15.9 ± 2.5</td>
</tr>
<tr>
<td>anti-asialo GM1</td>
<td>-2.4 ± 1.4</td>
</tr>
<tr>
<td>IFNαA/D + anti-asialo GM1</td>
<td>0.3 ± 1.9</td>
</tr>
</tbody>
</table>

* 2 × 10$^3$ units of IFNαA/D was given intraperitoneally and 0.2 ml of anti-asialo GM1 serum (X10 dilution) was given intraperitoneally. Splenic NK activity was determined 24 hr after these treatments by a standard $^{51}$Cr release assay using YAC-1 as target cells.

**Effects of IFNαA/D or Anti-Asialo GM1 Serum on Spontaneous Metastasis of Intraocular Melanoma**

The following experiments were designed to determine whether NK cells play any role in the spontaneous metastasis of intraocular melanoma. In preliminary studies, metastatic lung colonies were already established 20 days after melanoma inoculation. We started various treatments from day 7 after melanoma inoculation and determined the number of metastatic lung colonies at 40 days after inoculation. As shown in Table 2, depletion of NK activity by intravenous injection of anti-asialo GM1 serum dramatically increased the number of metastatic lung colonies. On the other hand, augmentation of NK activity by IFNαA/D treatment significantly reduced the number of lung colonies. In contrast with anti-asialo GM1, treatment with silica or carrageenan, which effectively deplete macrophages,$^1^4,^1^5$ had almost no effect on the number of metastatic lung colonies.

**Table 2. Effects of various treatments on spontaneous metastasis in mice with intraocular melanoma**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incidence of metastasis</th>
<th>Number of lung tumor colonies*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± S.D. (range)</td>
<td>Mean + S.D. (range)</td>
</tr>
<tr>
<td>None</td>
<td>6/7</td>
<td>8.1 ± 7.6 (0–21)</td>
</tr>
<tr>
<td>anti-asialo GM1†</td>
<td>5/5</td>
<td>41.4 ± 13.4 (32–65)</td>
</tr>
<tr>
<td>IFNαA/D‡</td>
<td>5/6</td>
<td>1.2 ± 0.8 (0–2)</td>
</tr>
<tr>
<td>Silica§</td>
<td>4/4</td>
<td>6.3 ± 4.6 (3–13)</td>
</tr>
<tr>
<td>Carrageenan¶</td>
<td>5/5</td>
<td>5.4 ± 2.7 (3–10)</td>
</tr>
</tbody>
</table>

* Metastatic lung tumor colonies were counted under a dissecting microscope 40 days after inoculation of $5 \times 10^6$ B16 melanoma cells into the anterior chamber of the eye.

† Once a week 0.2 ml of anti-asialo GM1 serum (X10 dilution) was injected intraperitoneally on day 7 after intracameral melanoma inoculation.

‡ Every 3 days 2 × 10$^6$ units of IFNαA/D was injected intraperitoneally on day 7 after intracameral melanoma inoculation.

§ One milligram of silica was injected intraperitoneally from days 7 to 11 after intracameral melanoma inoculation.

¶ One milligram of carrageenan was injected intraperitoneally on days 7, 9, and 11 after intracameral melanoma inoculation.

| Significantly different from the untreated group (P < 0.01).
lung colonies. These results clearly indicated that NK cells are essential for the prevention of spontaneous metastasis in mice with intraocular melanoma.

Discussion

In the present study, we have shown that spontaneous metastasis of intraocular B16 melanoma was accelerated in NK depleted mice and suppressed in NK activated mice. The correlation between host NK activity and metastatic spread of intraocular melanoma suggests that NK cells play an important role in the prevention of metastasis from B16 melanoma transplanted into the anterior chamber of the eye, similar to that in other body sites. 3 4

Our conclusion differs from the observation of Niederkorn. He showed that the impairment of T cells but not NK cells increased metastasis of experimental intraocular melanoma. The cause of these differences is not entirely clear. We used anti-asialo GM1 to deplete NK cells and confirmed the effect. Niederkorn used cyclophosphamide to deplete NK cells. Compared with our results, the incidence of spontaneous metastasis in his experiments was quite low. The incidence increased only after whole body irradiation to impair T cell function together with mechanical manipulations of the tumor containing eye to facilitate intravenous dissemination of tumor cells. This low metastatic rate may be due to the low rate of tumor cells release from the primary site. Considering these differences, we think that the role of NK cells in metastasis of intraocular melanoma may be different in each tumor system.

Although there are many differences from human choroidal melanoma, our intraocular melanoma model, which showed a relatively high rate of spontaneous metastasis, is obviously valuable to study the therapeutic maneuvers to prevent it. We show that activation of NK cells by IFN treatment significantly reduces the rate of metastasis. IFN is known to activate not only NK cells but also macrophages. 16 Depletion of macrophages, however, did not enhance the rate of spontaneous metastasis in our system. Therefore, the preventative effect of metastasis by IFNaA/D was likely to be mediated mainly by NK cells. It remains to be seen that NK cells have any role in metastatic spread of human choroidal melanoma and activation of NK cells may be beneficial for prevention of metastasis in human patients.

Key words: NK cell, intraocular melanoma, metastasis, interferon anti-asialo GM1

References