A Clinically Relevant, Syngeneic Model of Spontaneous, Highly Metastatic B16 Mouse Melanoma

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Abstract. We report a syngeneic model of spontaneous metastatic B16-F10 mouse melanoma in C57/BL6 mice with a very high metastatic frequency that mimics clinical metastatic melanoma. The B16 melanoma cells were injected between the skin and cartilage on the dorsal side of the ear. The model generated lymphatic and visceral metastases in all of the tested animals. In mice with large primary tumors, tumor weight correlated with the tumor growth time and also with the number of metastases in lymph nodes and organs. The dorsal ear space between the skin and cartilage enables both lymphatic and hematogenous metastatic spread. The model should be useful to study the mechanism of melanoma metastasis and to develop therapy for this currently untreatable disease.

Several mouse melanoma models have been developed over the past few decades (1-13) and are used (i) to determine the function of particular proteins in melanoma progression; (ii) to approximate certain biological aspects of human melanomas; and (iii) to critically evaluate novel drugs. However, the main problem with these models has been their clinical relevance with respect to lymph node and visceral metastases (14). A selection of the currently available models includes (i) xenograft models; (ii) syngeneic transplantation models; and (iii) models involving genetically-modified animals. Each model has characteristic advantages which may render it more suitable for answering a respective scientific question. Syngeneic transplantation models were first established. Models such as the Harding-Passey melanoma in BALB/c-DBA/2F1 mice (15), the Cloudman S91 melanoma in DBA/2 mice (16) or the B16 melanoma in C57BL/6 mice (17, 18) have been used for approximately half a century. These model have an intact immune system. The cell lines and sublines used for generation of syngeneic models display a wide degree of heterogeneity with respect to tumor growth rate, tumor take and metastasis formation (18-20).

The syngeneic murine B16 melanoma model usually involves transplantation into the foot pad, intradermally or subcutaneously, in C57/BL6 mice (18, 21) These models usually need resection of the primary tumor, or resection of the foot pad with the primary tumor, in order for formation of distant metastases to occur (22). The second type of syngeneic melanoma model represents experimental metastases occurring following intravascular injection. Intravenous injection into the lateral tail vein is the most commonly used model resulting generally in experimental lung metastases (23). Injection into the peritoneum can generate metastatic lymph nodes (24).

Based on previously reported experiments with the syngeneic Lewis lung carcinoma metastasis model, generated by injection of the cancer cells into the ear area (25), we
report here a syngeneic model of spontaneous highly metastatic B16-F10 mouse melanoma in C57/BL6 mice developed by injection of cancer cells between the skin and cartilage on the dorsal side of the ear. This model faithfully represents metastatic clinical melanoma.

Materials and Methods

Subcutaneous tumor growth. Three female mice (C57/BL6), 6 weeks of age, were injected subcutaneously with $2 \times 10^6$ B16 mouse melanoma cells. All of the animals were maintained in a barrier facility. All animal experiments were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals under assurance of Directive 86/609/EEC on the protection of animals used for scientific purposes in the Czech Republic.

The mouse melanoma cell line B16-F10 was obtained from AntiCancer, Inc. The cells were grown and maintained in RPMI medium, supplemented with with 10% heat-inactivated fetal bovine serum and 1% penicillin and streptomycin. The cells were first harvested from culture by trypsinisation and washed three times with cold serum-free medium before subcutaneous injection.

Ear transplantation of C57/BL6 mice. Tumors derived from s.c.-growing B16-F10 mouse melanoma in female mice were disassociated and a single-cell suspension in PBS was prepared. A total of $5 \times 10^6$/ml cells in 0.1 ml were injected between the skin and cartilage on the dorsal side of the ear. The female mice (C57/BL6, n=20) were anesthetized by ketamine and xylazine during transplantation.

Analysis of metastases. Mice were divided into two groups. Animals from group 1 were sacrificed 4 weeks after B16-F10 mouse melanoma cell injection (group 1: mouse numbers 1-10). The rest of the animals were sacrificed 6 weeks after the tumor cell injection (group 2: mouse numbers 11-20). The size of the
Table I. Location of observed micro- and macro-metastases volume. Micro- and macro-metastases detected in lymph nodes and organs of the mice transplanted with B16 melanoma are described in the table below. They are shown according to the site of metastasis detection (see description of examined locations [no. 1-25] in legend). There was no metastasis detected in locations no. 17, 20, 21, 24, which correspond to heart, cerebellum, brain and kidney.

<table>
<thead>
<tr>
<th>No.</th>
<th>Location</th>
<th>Location No.</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Supracervical lymph node 1</td>
<td>14</td>
<td>Axial lymph nodes</td>
</tr>
<tr>
<td>2</td>
<td>Supracervical lymph node 2</td>
<td>15</td>
<td>Deep cervical lymph nodes</td>
</tr>
<tr>
<td>3</td>
<td>Contralateral supracervical lymph node</td>
<td>16</td>
<td>Thymus + Mediastinal lymph nodes</td>
</tr>
<tr>
<td>4</td>
<td>Contralateral supracervical lymph node 2</td>
<td>17</td>
<td>Heart</td>
</tr>
<tr>
<td>5</td>
<td>Salivary gland</td>
<td>18</td>
<td>Lung</td>
</tr>
<tr>
<td>6</td>
<td>Contralateral salivary gland</td>
<td>19</td>
<td>Mediastinal nodes</td>
</tr>
<tr>
<td>7</td>
<td>Parathyroid gland</td>
<td>20</td>
<td>Cerebellum</td>
</tr>
<tr>
<td>8</td>
<td>Contralateral parathyroid gland</td>
<td>21</td>
<td>Brain</td>
</tr>
<tr>
<td>9-10</td>
<td>Thyroid gland</td>
<td>22</td>
<td>Liver</td>
</tr>
<tr>
<td>11</td>
<td>Brachial lymph nodes</td>
<td>23</td>
<td>Suprarenal node</td>
</tr>
<tr>
<td>12</td>
<td>Contralateral brachial lymph nodes</td>
<td>24</td>
<td>Kidney</td>
</tr>
<tr>
<td>13</td>
<td>Contralateral axial lymph nodes</td>
<td>25</td>
<td>Spleen</td>
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</tbody>
</table>
primary tumor was measured. The tumor volume was calculated with the formula \( V_T = a(b^2)/2 \), where \( a \) and \( b \) are tumor length and width (mm), respectively (13). The tissue samples from lymph nodes and organs from the neck and chest region were collected and the presence of micro- and macro-metastases was observed. At least two micro-metastatic and one macro-metastatic lesion per organ or lymph node needed to be present for an organ to be considered positive for metastasis. To detect micro-metastases, paraffin-embedded lymphoid tissue sections were stained with hematoxylin–eosin using standard procedures. The total number of metastases was correlated to tumor volume. Correlation analysis of the data was carried out using the Spearman correlation coefficient.

**Results and Discussion**

The number of micro- and macro-metastases observed in each mouse is shown in Table I. The total number of metastases was determined as the sum of observed micro- and macro-metastases. Tumor volume was measured four weeks after s.c. injection in group 1 mice and after six weeks in mice group 2 (see Figure 1 and Table I). Tumor volume correlated significantly with tumor growth time and the number of macro- and micro-metastases in lymph nodes and visceral organs (\( r = 0.65-0.84; \) \( p < 0.001 \)), as well as with the sum of all observed metastases.

Distant micrometastases were observed in a short time (four weeks) in 100% of the transplanted animals after transplantation into the dorsal ear between the skin and cartilage. Twenty-one different metastatic sites, including lymph nodes throughout the body, were identified, similar to the Lewis lung cancer transplanted at this site (25). In the B16F10 melanoma model, cancer cells can be easily visualised due to melanin production.

The biologic mechanism of metastasis has become better understood through the study of the migration and seeding of cancer cells (26-28). The generation of visceral metastases via injection of cancer cells by subcutaneous implantation does not accurately reflect the normal sequence of events in the clinical setting in which lymph node metastasis plays a prominent role. In our study we have observed a high metastatic frequency of lymph node and visceral metastases using the dorsal site of the mouse ear for cancer-cell injection. Tumors growing at this site are drained by the lymph system.

This new metastatic melanoma model provides a valuable tool for studying the mechanisms of metastasis and for developing therapy of lymph and visceral metastases for melanoma.

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**References**