Expression of a Transfected $H-2K^b$ Gene in B16 Cells Correlates with Suppression of Liver Metastases in Triple Immunodeficient Mice


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ABSTRACT

In vivo experiments performed with NIH (nu/nu, bg/bg, xid/xid) triple immunodeficient (TD) mice revealed the striking ability of i.v. injected B16-F1 and B16-F10 murine melanoma cells to colonize not only the lungs but also the liver of TD mice. Subsequently, B16 melanoma cell cultures, which express very low levels of H-2K$^b$ antigen, were cotransfected with plasmids pRSVneo, containing the neomycin resistance gene, and 6-2B1pMT, expressing the H-2K$^b$ complementary DNA under the control of the metallothionein enhancer-promoter. Several neomycin-resistant clones were analyzed for H-2D$^b$ and H-2D$^d$ expression by RNase protection and flow cytometry assays. All parental lines and transfected clones expressed normal levels of H-2D$^b$ mRNA, while only some of the transfected clones expressed easily detectable levels of H-2K$^b$ mRNA. Moreover, in these clones H-2K$^b$ expression could be enhanced in the presence of Zn$^{2+}$, indicating that the metallothionein enhancer was functioning properly. Parental cells and transfected clones were injected i.v. in TD mice to assess the possible involvement of H-2K$^b$ antigen in regulating the metastatic potential of B16 melanoma cells. We observed a remarkable correlation between expression of H-2K$^b$ antigen and suppression of liver-specific metastases in TD mice. Identical results were obtained when we gave TD mice injections of mixed populations of transfectants expressing H-2K$^b$ antigen, obtained by fluorescence-activated cell sorting. These experiments allowed us to rule out the possibility that the observed changes in metastatic potential were due to clonal variability among individual transfected clones. Taken together, the results of our in vivo studies with immunodeficient mice support the notion that specific major histocompatibility complex Class I molecules modulate the metastatic potential of malignant cells also by mechanisms which are independent of their well-established role in antigen presentation.

INTRODUCTION

Metastasis formation is the end result of a very complex multistep process during which malignant cells progressively acquire new phenotypic properties which are associated with invasion of surrounding tissues, extravasation, migration to distant sites via the bloodstream, and colonization of distant organs. A better understanding of the metastatic process is a crucial necessity, since metastatic disease is the major cause of death for cancer patients. In the last few years considerable progress has been made in elucidating some of the biochemical changes underlying the metastatic process. More recently, the use of molecular techniques has allowed the characterization of genetic changes which are associated with the metastatic phenotype (for review, see Ref. 1). Transfection experiments have been particularly valuable in demonstrating the ability of several oncogenes in augmenting metastatic potential as well as in identifying at least four different metastasis suppressor genes (for review, see Ref. 2).

Some of these transfection studies originated from the long-standing observation that many experimental as well as spontaneously occurring malignant tumors exhibit much reduced expression of MHC Class I antigens (for review, see Ref. 3). Since MHC Class I antigens mediate and restrict lysis of target tumor cells by CTL (4), the observed loss in MHC Class I expression by malignant cells is assumed to allow tumor cells in general, and metastatic cells in particular, to evade the host immune response (5). Indeed, the metastatic competence of Lewis lung carcinoma cells was correlated with the level of suppression of H-2K$^b$ gene expression (6) and restoration, by DNA-mediated gene transfer, of H-2K$^b$ antigen expression suppressed the metastatic phenotype (7). These in vivo studies, performed with immunocompetent syngeneic mice, established a causal relationship between diminished MHC Class I expression and metastatic phenotype, and demonstrated the importance of restored immunogenic properties in allowing effective targeting and destruction of malignant cells by CTL.

More recently, we have reported that expression of specific MHC Class I genes in human and rodent tumor cell lines correlated with a significant alteration in tumor growth and formation of spontaneous metastases in immunodeficient mice (8–11). Thus, our experimental observations indicated that, in addition to their well-established function in antigen presentation and immune recognition, MHC Class I antigens play a nonimmune role in the modulation of cell proliferation and metastatic growth. In order to obtain specific information on the molecular mechanisms by which restored expression of specific MHC Class I antigens can modulate metastatic potential within a nonimmune context, we have developed an animal model based on NIH (nu/nu, bg/bg, xid/xid) TD mice, injected i.v. with various derivatives of B16 murine melanoma cells.

Here we report on the striking ability of i.v. injected B16-F1 (12) and B16-F10 (13) murine melanoma cells to colonize not only the lungs, but also the liver of TD mice. We also report experimental evidence showing that expression of a transfected H-2K$^b$ cDNA in B16-F1 and B16-F10 cells, which express low to undetectable levels of the endogenous H-2K$^b$ antigen, correlates with partial to total suppression of liver-specific metastases in TD mice. The results of these transfection and in vivo studies are consistent with the notion that H-2K$^b$ molecules, in addition to their well-established antigen-presenting function, modulate specific cell surface structures (e.g., growth factor...
receptors, adhesion molecules, etc.) responsible for the exquisite liver specificity of B16-derived experimental metastases in TD mice.

MATERIALS AND METHODS

Cell Lines and Transfection Procedure. B16-F1 and B16-F10 cells were generously provided by Dr. I. J. Fidler (M. D. Anderson Cancer Center, Houston, TX) and were propagated in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum.

Exponentially growing cell cultures were cotransfected, using the calcium phosphate precipitation technique (14), with plasmids pRSVneo and 6-2BlpMT (Fig. 2) at a 1:5 molar ratio. pRSVneo confers neomycin resistance, while 6-2BlpMT allows constitutive expression of H-2Kb heavy chain. 6-2BlpMT was constructed by replacing the hGH gene of plasmid MThGH (16) with a Bam HI fragment containing the H-2Kb cDNA clone 6-2B1 and the SV40 3' splice and polyadenylation signals from vector pBG312 (17). Neomycin-resistant clones were selected in culture medium containing G418 (GIBCO) at 1 mg/ml, picked up at about 3 wk after transfection, and expanded for further studies.

Transcription Analysis. Total RNA was extracted as described (18). For RNase protection assays (19), 10 µg/sample of total RNA were hybridized to H-2Kb- and H-2Db-specific riboprobes (20), synthesized from α1-specific DNA fragments (395 and 520 base pairs long, respectively), subcloned into the riboprobe vector pGEM (Stratagene).

Immunofluorescent Staining. Hybridomas AF6-88.5.3 and 28-14-8S were obtained from the American Type Culture Collection. Their corresponding MAb are specific for H-2Kb and H-2Db antigens, respectively.

Flow cytometry of immunofluorescence-stained cells was performed following incubation of about 10^6 cells with appropriate dilutions of MAb AF6-88.5.3 and 28-14-8S (specific for K^b and D^b determinants, respectively), followed by incubation with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Tago, Inc.). Samples were then washed, suspended in 1% paraformaldehyde, and analyzed using a Becton Dickinson FACScan II.

Fluorescence-activated cell sorting (Coulter 541) was performed using H-2Kb-specific MAb AF6-88.5.3, in order to isolate high expressors (top 10%) and low expressors (bottom 10%) of H-2Kb antigen from the pool of B16-F10 clones transfected with plasmids pRSVneo and 6-2BlpMT.

In Vivo Studies. NIH (nu/nu, bg/bg, xid/xid) triple immunodeficient mice were bred and hosted in the pathogen-free, virus-free facility of the Department of Radiation Medicine at Massachusetts General Hospital. Single cell suspensions in PBS without Ca^2+ and Mg^2+ were injected into the lateral tail vein of 8-wk-old mice at a concentration of 5 x 10^4 or 10^5 cells in 0.3 ml of inoculum. At about 3 wk postinjection each mouse was sacrificed and subjected to autopsy. Lungs were removed and fixed in Bouin's solution before counting metastatic nodules under a dissecting microscope. In addition, selected specimens (mostly from lungs and liver) were fixed in 10% formalin in PBS and processed for histopathological examination to confirm the malignant nature of the observed lesions.

RESULTS

Organ-specific (Liver) Metastases in TD Mice Given Injections i.v. of B16-F1 and B16-F10 Cells. The study of experimental metastases allows a quantitative assessment of certain parameters of metastatic potential, as metastatic nodules in specific organs can be counted and analyzed in many ways. Experimental metastases also provide information on the nonrandom distribution of some metastatic lesions, since local organ factors may favor the arrest and proliferation of malignant cells disseminated into the bloodstream.

A large body of evidence has shown that murine melanoma B16-F1 and B16-F10 cells colonize almost exclusively the lungs of i.v. injected syngeneic immunocompetent C57Bl/6 mice. Therefore, we wanted to test the possibility that i.v. injection of B16 cells in immunodeficient mice might have allowed the formation of extrapulmonary metastases in addition to lung metastases. We found that B16-F1 and B16-F10 cells (10^5 cells/animal) injected i.v. in nude mice gave rise only to lung metastases (data not shown); however, in all TD mice given injections of B16-F1 or B16-F10 cells we observed, in addition to lung metastases, a significant number of liver metastases, which were also remarkable for their size (Fig. 1).

Restoration of H-2Kb Expression in B16-F1 Cells. In order to study the effects on the metastatic potential of B16-F1 cells following restoration of H-2Kb expression, we cotransfected B16-F1 cultures with plasmids pRSVneo and 6-2BlpMT.
of TD mice. The result of these in vivo experiments (Table 1) indicated that TD mice given injections of B16-F1 and clone 35 cells exhibited both lung and liver metastases. In contrast, the liver of TD mice given injections of clone G and clone 23 cells was found to be clear of metastases, even after detailed histological examination, suggesting that restored H-2Kb expression in B16-F1 cells may downregulate the ability of these cells to colonize the liver in TD mice by mechanisms which are independent of the immune response.

We also wanted to investigate the possibility that in B16-F10 cells increased expression of the H-2Kb antigen correlated with suppression of metastatic lesions in the liver of TD mice. Therefore, we cotransfected B16-F10 cells with plasmids pRSVneo and 6-2BlpMT. Several neomycin-resistant clones were isolated and analyzed for expression of H-2Kb and H-2Db antigens. Fig. 4 shows the result of flow cytometry experiments performed with MAb AF6-88.5.3 and 28-14-8S (specific for Kb and Db determinants, respectively) on B16-F10 parental cells and three neomycin-resistant clones (Nos. 7, 10, 12). All four cell lines exhibited easily detectable levels of H-2Db antigen; in contrast, B16-F10 and clone 7 expressed low levels of H-2Kb, while clones 10 and 12 expressed higher levels of H-2Kb antigen on the cell surface. In parallel, we have carried out RNase protection assays which have confirmed the results obtained by flow cytometry; in particular, only clones 10 and 12 exhibited constitutive as well as inducible expression of H-2Kb mRNA in the presence of Zn2+ (data not shown).

Metastatic Potential of B16-F10 Transfectants. We next wanted to assess the in vivo metastatic potential of B16-F10 parental cells and transfected clones; therefore, we gave TD mice injections i.v. of each cell line (5 × 10^4 cells/mouse), and 3 wk postinjection we sacrificed the animals to evaluate the number and location of metastatic lesions. Table 2 summarizes the results of these in vivo studies. In TD mice higher numbers of liver metastases were observed with B16-F10 and clone 7 cells, compared with clones 10 and 12 (high Kb expressors). Moreover, while there was no significant difference between B16-F10 and clone 7 cells, in terms of their ability to colonize the liver, the difference with clones 10 and 12 was statistically significant. However, no decrease in lung metastases was observed with clones 10 and 12 compared with the parental cells, suggesting that the reduced numbers of liver metastases observed in TD mice given injections of clone 10 and clone 12 cells were not simply the result of a general decrease in metastatic potential, but rather were selective for liver-specific metastases.

![Fig. 3. RNase protection assay of B16 clones. Total RNA extracted from B16-F1 parental cells and three transfected clones (23, 35, and G) was reacted with #32P-labeled riboprobes, specific for K# and D# mRNAs, respectively. Protection is indicated by a specific shift in the size of the probe. The intensity of the signal is proportional to the amount of specific mRNA in the sample. The unmarked lanes contain the unreacted riboprobe. Note that while all cell lines express comparable amounts of D# mRNA (right), K# transcription is barely detectable in B16-F1 and clone 35 (left).](image_url)

Table 1 Incidence, median, and range of metastases to lungs and liver in triple immunodeficient mice given injections of B16-F1 cells and transfected clones (35, G, 23).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>H-2KbAg</th>
<th>Incidence</th>
<th>Median</th>
<th>Incidence</th>
<th>Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>B16-F1</td>
<td>–</td>
<td>15/15</td>
<td>6 (2–17)</td>
<td>15/15</td>
<td>5 (1–31)</td>
</tr>
<tr>
<td>35</td>
<td>+</td>
<td>12/15</td>
<td>2 (0–9)</td>
<td>13/15</td>
<td>6 (3–26)</td>
</tr>
<tr>
<td>G</td>
<td>+</td>
<td>13/15</td>
<td>2 (0–16)</td>
<td>0/15</td>
<td>0</td>
</tr>
<tr>
<td>23</td>
<td>+</td>
<td>6/11</td>
<td>1 (0–2)</td>
<td>0/11</td>
<td>0</td>
</tr>
</tbody>
</table>

* H-2Kb antigen expression on the cell surface was assayed by flow cytometry using H-2Kb-specific MAb AF6-88.5.3.

Range.
H-2K<sup>b</sup> ANTIGEN AND MALIGNANT MELANOMA

**Fig. 4.** Flow cytometry of B16-F10 cells and transfected clones 7, 10, and 12. Cell suspensions from individual cell cultures (indicated on top of the figure) were incubated with MAb specific for H-2K<sup>b</sup> (AF6-88.5.3) or H-2D<sup>b</sup> (18-14-8S) determinants. All cell lines expressed the H-2D<sup>b</sup> antigen. In contrast, only clones 10 and 12 expressed easily detectable levels of H-2K<sup>b</sup> antigen.

Metastatic Potential of B16-F10 Cell Populations Transfected with pRSVneo and 6-2BlpMT Plasmids. It could be argued that the decreased ability to generate liver metastases was the consequence of clonal heterogeneity within the B16 cell population, rather than being correlated to restored expression of H-2K<sup>b</sup> antigen. In order to address this possibility we performed a new round of transfections using B16-F10 cells as recipients and pRSVneo and 6-2BlpMT plasmids as donor DNA. This cotransfection gave rise to 35 neo-resistant clones which were pooled and subjected to fluorescence-activated cell sorting using H-2K<sup>b</sup>-specific MAb AF6-88.5.3 (Fig. 5). As a result of this procedure we obtained two subpopulations, F10-K<sup>b</sup>− and F10-K<sup>b</sup>+, based on their ability to express H-2K<sup>b</sup> antigen on the cell surface. As an additional control we transfected B16-F10 cells with pRSVneo alone; this transfection gave rise to 114 clones, which were pooled and processed together. Subsequently we gave TD mice injections of B16-F10 cells and each one of the three subpopulations of F10 transfecants (neo, K<sup>b</sup>−, K<sup>b</sup>+) obtained as described above. The result of these in vivo studies (Table 3) indicates that B16-F10, F10-neo, and F10-K<sup>b</sup>− cells exhibited a comparable ability to metastasize to the liver. In contrast, F10-K<sup>b</sup>+ cells gave rise to liver metastases only in four of ten mice given injections, strongly suggesting that the observed correlation between H-2K<sup>b</sup> expression and suppression of liver metastases could not be the result of clonal variability among B16-F10 transfected cells.

DISCUSSION

Among malignancies, melanomas have a striking tendency toward metastatic spread; moreover, ocular melanoma often exhibits a striking ability to metastasize to the liver (21, 22), that cannot be easily explained on the basis of anatomical or functional considerations.

The finding of liver metastases in TD mice given injections i.v. of B16-F1 and B16-F10 cells is consistent with similar observations in C57Bl/6 (bg/bg) mice given injections i.v. of B16 melanoma cells (23) and supports the notion that NK cells can effectively target and destroy malignant cells in the liver. However, the isolation from B16-F1 cells of a clone (L4) capable of colonizing the liver of immunocompetent C57Bl/6 mice (24) is also consistent with the concept that biological properties intrinsic to the malignant cell are very important in determining organ-specific patterns of metastasis formation. Furthermore, we want to emphasize that the absence of significant NK cell activity in TD mice does not explain the exquisite organ (liver) specificity of the observed lesions nor does it explain their large size.

The results of the in vivo studies performed with B16 cells expressing a transfected H-2K<sup>b</sup> cDNA suggest that restoration of H-2K<sup>b</sup> antigen expression leads to partial or total suppression of liver metastases in TD mice. These experimental data lend support to the notion that restored expression of H-2K<sup>b</sup> is a critical factor in the suppression of liver metastases.

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**Table 2** Incidence, median, and range of metastases to lungs and liver in triple immunodeficient mice given injections of B16-F10 cells and transfected clones (7,10,12)

Mice were sacrificed and autopsied 21 days postinjection of 5 x 10<sup>4</sup> cells/animal.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>H-2K&lt;sup&gt;b&lt;/sup&gt;Ag&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Incidence</th>
<th>Median</th>
<th>Incidence</th>
<th>Median</th>
</tr>
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<tbody>
<tr>
<td>B16-F10</td>
<td>−</td>
<td>5/5</td>
<td>25 (17-27)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5/5</td>
<td>37 (25-55)</td>
</tr>
<tr>
<td>7</td>
<td>−</td>
<td>5/5</td>
<td>11 (7-16)</td>
<td>5/5</td>
<td>18 (4-71)</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>5/5</td>
<td>55 (6-102)</td>
<td>5/5</td>
<td>9 (1-12)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>12</td>
<td>+</td>
<td>5/5</td>
<td>42 (18-56)</td>
<td>5/5</td>
<td>1 (1-3)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> H-2K<sup>b</sup> antigen expression was assayed by flow cytometry using H-2K<sup>b</sup>-specific MAb AF6-88.5.3.

<sup>b</sup> Range.

<sup>c</sup> Statistically different from B16-F10 at P < 0.001.
activated cell sorting, two subpopulations expressing low (A*-') or high (A*+') (FIO-Kh). From this second pool of transfectants we isolated, by fluorescence-

efficient mice, consistent with our experimental data (26).

Expression of H-2Kb antigen, measured with MAb AF6-88.5.3, was assayed in B16-F10 cells transfected with pRSVneo alone (FIO-neo) or with pRSVneo and 6-2BlpMT H-2Kb antigen, measured with MAb AF6-88.5.3.

These investigators also observed that H-2Kb+ clones lost their

H-2Kh gene in B16-BL6 cells affects the expression of melanoma antigen in B16 cells may downregulate their liver-specific metastatic potential by mechanisms which are independent of the role of MHC Class I antigens in immune recognition (11, 25). Gorelik et al. (26) have recently reported that transfection of the H-2Kb gene in B16-BL6 cells affects the expression of melanoma-associated antigen and lectin-binding sites. Interestingly, these investigators also observed that H-2Kb+ clones lost their metastatic ability even in immunosuppressed or immunodeficient mice, consistent with our experimental data (26).

Several studies have suggested that MHC Class I antigens may modulate the function of the insulin receptor, as indicated by a structural association between the two molecules (27, 28). Additional studies have shown that a specific peptide derived from the a1 domain of the H-2Dk molecule can regulate the function of the insulin receptor (29), strengthening the argument that an important nonimmune role of MHC Class I heavy chains is to regulate ligand-activated receptors. It is tempting to speculate that B16 cells express a growth factor receptor, whose cognate ligand is present (or synthesized) in the mouse liver. Enhanced expression of H-2Kb antigen in B16 cells may functionally alter this putative growth factor receptor, preventing the formation of metastatic lesions. Alternatively, H-2Kb molecules may be involved in modulating the attachment of i.v. injected B16 cells to liver endothelium. These and other possibilities will be tested experimentally using a variety of biochemical and molecular approaches.

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Table 3 Incidence, median, and range of metastases to lungs and liver in triple immunodeficient mice given injections of B16-F10 and populations of transfected cells (FIO-neo, FIO-Kb-, FIO-Kb+).

<table>
<thead>
<tr>
<th>Cell line/population</th>
<th>H-2KbAg*</th>
<th>Incidence</th>
<th>Median</th>
<th>Incidence</th>
<th>Median</th>
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<tr>
<td>B16-F10</td>
<td>10/10</td>
<td>16(1-27)*</td>
<td>10/10</td>
<td>20(3-61)</td>
<td></td>
</tr>
<tr>
<td>F10-neo</td>
<td>9/10</td>
<td>21(0-77)</td>
<td>9/10</td>
<td>20(0-92)</td>
<td></td>
</tr>
<tr>
<td>F10-Kb-</td>
<td>8/9</td>
<td>16(0-35)</td>
<td>7/9</td>
<td>12(0-41)</td>
<td></td>
</tr>
<tr>
<td>F10-Kb+</td>
<td>+</td>
<td>4/10</td>
<td>0(0-45)</td>
<td>4/10</td>
<td>0(0-18)</td>
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*H-2Kb* antigen expression was assayed by flow cytometry using MAb AF6-88.5.3.

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