Biological Behavior of Malignant Melanoma Cells Correlated to Their Survival in Vivo

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SUMMARY

Successive B16 melanoma tumor lines were selected for their ability to form pulmonary tumor nodules. This was accomplished by injecting tumor cells i.v. into syngeneic C57BL/6 mice and 2 to 3 weeks later collecting the secondary tumor growths and placing them into tissue culture. These tumor cells were then injected i.v. into new syngeneic mice and the process was repeated several times. With each successive tumor line the number of experimental lung tumor nodules was significantly increased. The B16 lines were found to be stable in their metastatic properties even after many subculturings in vitro.

These studies demonstrated that tumor cells that succeed in forming pulmonary tumor colonies also had increased invasive properties into normal tissues when implanted s.c. In addition, the tumor cell lines were prelabeled in vitro with [125I]-5-iodo-2'-deoxyuridine and suspensions of labeled cells were injected i.v. into normal syngeneic hosts. Animals were killed at intervals afterwards, and the lungs and blood were processed and monitored for radioactivity. At any time interval, the lungs of mice treated by injections of cells of high metastatic yield contained more tumor cells. The differences in tumor cell numbers in the lungs were most pronounced immediately following i.v. injection. These results suggest that an increased initial arrest of highly metastatic cells in a capillary bed may be a major factor in their increased survival.

Low numbers of normal lymphocytes or lymphocytes from syngeneic mice immunized to the B16 melanoma, when mixed in vitro with the tumor cells, lead to the formation of multitumor cell-lymphocyte clumps. The degree of clumping was related to both the type of the metastatic tumor line and/or the syngeneic lymphocyte. These results support the hypothesis that the survival of invasive and/or circulating malignant tumor cells is not a random phenomenon; rather it appears that malignant cells possess unique qualities which allow for their survival.

INTRODUCTION

In earlier quantitative studies of the fate of tumor cells, we demonstrated that the mere presence of tumor cells in the blood does not constitute a metastasis since most circulating tumor cells rapidly die with only about 0.1% surviving to form secondary growths (4, 5, 9, 10). Several questions remained unanswered. What affects invasion, embolism, distribution, and survival of tumor cells? Is the metastatic process mainly dependent on host defense or soil factors? Is it also dependent on qualities unique to malignant tumor cells? In other words, do tumor cells invade, circulate, and survive at random to yield metastases, or do the few surviving cells possess properties that are unique to their success?

Evidence supporting the hypothesis that malignant cell properties may determine the outcome of metastasis came from our studies which selected B16 melanoma tumor cell lines for their capacity to successfully form pulmonary tumor colonies in their syngeneic host, the C57BL/6 mouse. All the tumor lines have been maintained in tissue culture and retained their respective low or high metastatic capabilities (6). The development of these B16 melanoma tumor lines is particularly useful for studying cell properties which render one line highly metastatic while the other is not. Moreover, since all the lines are tumorigenic they serve as control for each other, i.e., normal controls are not necessary in comparing tumor cell characteristics for cancer. Recently Boseman et al. (1) reported several major biochemical differences between the low- and high-metastatic B16 tumor lines. The lines were found to differ significantly in their electrophoretic mobility, levels of degradative enzymes, surface glycoprotein glycosyltransferases, and levels of glycosidases and proteases.

This report is concerned with several studies of the biological behavior in vivo and in vitro of the various B16 melanoma lines, demonstrating the importance of tumor cell characteristics to the outcome of experimental metastasis.

MATERIALS AND METHODS

Animals. C57BL/6 mice, 6 to 8 weeks old, purchased from The Jackson Laboratories, Bar Harbor, Maine, were utilized throughout these studies.

Tumor Cell Line Culture. The tumor cell lines have been developed as described in detail previously (6). C57BL/6 mice were given i.v. injections of 50,000 viable B16 melanoma cells and killed 3 weeks later. Their pulmonary melanoma nodules (colonies) were removed aseptically,

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pressed through a stainless steel mesh sieve (E-C Apparatus, St. Petersburg, Fla.), filtered through gauze, and plated in vitro. When the cultures became confluent the cells were harvested and injected into new C57BL/6 mice, and the process was repeated 10 times (11 lines). Previously, these lines have been designated as line 26 and so on. We have now redesignated them to be B16 line F_1, for the 1st passage line and then numbered consecutively, i.e., line F_2, F_3, and so on.

All tumor lines are maintained on plastic in Eagle's minimum essential medium. Supplementation of media and growing conditions have been described previously (6–8).

**Evaluation of Plating Efficiency and Cell Growth Rate in Vitro.** For these in vitro studies 2000 viable (trypan blue-excluding) cells from each of the successive lines were plated into 60-mm Integrid Petri dishes. Twenty-four hr later representative dishes were washed to remove all non-adherent cells, fixed, and stained. Adherent cells were then counted with an inverted microscope. Additional culture dishes (triplicates) were washed, fixed, and stained on each of successive Culture Days 2 to 5. Viable tumor cells were counted in 10 squares (2 x 2 mm) selected at random, and cellular growth per culture dish was thus determined.

**Tumor Cell-lymphocyte Clumping in Vitro.** In previous studies of immune stimulation-inhibition to experimental metastasis we reported that, when tumor cells were mixed in vitro with low doses of sensitized syngeneic lymphocytes, multicell clumps resulted. When these mixtures were injected i.v. into syngeneic mice enhancement of tumor metastasis was observed (9). It therefore became necessary to investigate the relationship of tumor cells and lymphocytes in vitro leading to formation of such multitumor cell-lymphocyte emboli. B16 melanoma cells from line F_1, and/or line F_10 were harvested with a rubber policeman. The cell suspensions were pipetted several times to break up clumps, and the suspension was further filtered through gauze. Non-glass adherent mononuclear lymphocyte suspensions from normal C57BL/6 mice, or those immunized to the B16 melanoma, were prepared as described previously (7). Following all viability tests, tumor cells were mixed with lymphocytes (lymphocytes:tumor cells, 100:1). Control groups of lymphocytes alone or tumor cells alone were included. All mixtures were plated into test tubes and rotated gently for 60 min. Following this rotation, aliquots of the various mixtures were placed on glass slides, fixed, and stained; and the average number of tumor cells per 50 clumps examined at random was determined. The increase in multicell clump size in the test groups was compared to the clump size in tumor cells incubated alone.

**General Procedure for the Study of Experimental Metastasis.** B16 melanoma lines grown in vitro were harvested when cultures were sparse (not confluent). Tumor cell harvesting was routinely carried out by overlaying the cells with a thin layer of 0.25% trypsin:EDTA solution for 1 min. The flask was tapped to facilitate removal of cells from the plastic and media immediately added. The cells were then washed and resuspended in cold 0.9% NaCl solution. The number of viable tumor cells was determined by the trypan blue exclusion test, and the cell suspension was diluted to desired concentration. Inoculum volume in all experiments was 0.2 ml tumor cell suspension per mouse, injected i.v. into the tail vein. Mice were killed 14 days later and the number of their pulmonary metastases was determined with the aid of a dissecting microscope by an independent observer. At the same time, animals were autopsied to determine presence of any extra pulmonary metastases.

**Tumor Cell Invasion and Spontaneous Pulmonary Metastases.** C57BL/6 mice, 6 to 8 weeks old were given s.c. injections of 50,000 viable B16 melanoma from the various lines. Ten days later, when the tumor was growing actively (about 4 to 5 mm in diameter) the tumors were completely excised, and the animals were observed for another 6 to 8 weeks. At this time all mice were killed and presence of any pulmonary or other metastases (resulting from tumor cell invasion prior to excision of the s.c. tumor) was recorded.

**Comparative Quantitative Analysis of [%^{125I}]IUDR-labeled Tumor Cells from Various Lines in Lungs and Blood of C57BL/6 Mice.** The procedures and details of the labeling technique have been described previously (4, 9, 10). Actively growing, nonconfluent monolayers of B16 melanoma were labeled with [%^{125I}]IUDR. This is an analog of thymidine that is incorporated into the DNA of proliferating cells and remains there until cell death when it is degraded in the liver. The free radioiodine is excreted in the urine. Reutilization is minimal, and thyroidal accumulation can be prevented by supplementation of the drinking water with 0.1% KI. Tumor cell labeling was accomplished with the addition of [%^{125I}]IUDR, 0.3 μCi/ml media, (specific activity, 200 mCi/mM; New England Nuclear, Boston, Mass.), incubation time of 24 hr. This labeling does not alter the biological behavior of the tumor cells, and practically all cells are labeled as determined by autoradiography (4). Labeled B16 melanoma cells of different lines were harvested and prepared for i.v. inoculation as described above. Several representative inocula doses were placed in test tubes and monitored for radioactivity in a well-type NaI crystal scintillation counter. Each mouse was given an injection i.v. of labeled B16 cells, and 6 mice from each group were sacrificed at intervals ranging from 2 min to 14 days postinjection. Lungs and 0.5 ml of blood were collected from all mice, and the lungs were placed in vials containing 70% ethanol. The ethanol was replaced once a day for 3 days to remove practically all the acid-soluble [%^{125I}]I. The remaining radioactivity is associated with viable tumor DNA (4). The number of viable, originally injected tumor cells was determined from the ratio of cpm to cells in the representative inocula samples. All lung or blood samples were counted 3 times, 5 min each.

**Statistical Analysis.** Statistical analysis was carried out utilizing Student's t-test.

**RESULTS**

**Evaluation of Plating Efficiency and Cell Growth in Vitro.** There were no discernible differences between the plating
efficiencies of the various cell lines (50 to 60% in most experiments). However, with the progressive metastatic lines it appeared that cell doubling time was somewhat decreased. Specifically, line F₁ had a doubling time of 22 hr while line F₁₁ demonstrated a doubling time of 17 hr, agreeing closely with an earlier published report (1).

**Tumor Cell-Lymphocyte Clumping in Vitro.** Several experiments were carried out to determine the factors influencing the phenomenon of tumor cell clumping in vitro when incubated with either normal syngeneic lymphocytes or lymphocytes obtained from syngeneic nude mice. The results of these experiments are shown in Table 2 and demonstrated that tumor clump size was influenced by both the tumor cell line and the lymphocytes in the mixture. When normal lymphocytes were utilized the average number of tumor cells per clump was 5 ± 2 for line F₁ and 13 ± 8 for line F₁₀, and the difference in clump size was significant (p < 0.005). Moreover, lymphocytes obtained from immunized mice were more effective in producing a larger tumor cell-lymphocyte clump. This was demonstrable as line F₁ with immunized lymphocytes yielded an average clump of size of 13 ± 9 tumor cells while in line F₁₀ the average number of tumor cells per clump was 18 ± 10 (p < 0.01).

It would appear that a direct tumor cell-lymphocyte interaction in vitro leads to formation of multicell clumps the sizes of which are probably dependent on cell surface characteristics of both tumor cells and the lymphocytes.

**The Incidence of Pulmonary Tumor Nodules Produced by an i.v. Injection of Tumor Cells from the Successive Lines.** The data demonstrated the following: (a) the incidence of pulmonary tumor colonies significantly increased with successive tumor lines. This finding confirmed our earlier results (6) and those of others (1); (b) the observed increase was not related to any particular tumor cell dose as tumor cells from line F₁₁ yielded more pulmonary tumor nodules than cells from line F₁₀, while tumor cells from F₁ were more metastatic than those from line F₁ (p < 0.001). These results agreed with earlier findings that the number of pulmonary tumor nodules is directly proportional to the number of tumor cells injected i.v. (5, 24, 25).

Lungs from tumor-bearing mice were examined histologically. It appeared that with increasing metastatic capability the pulmonary nodules were larger and consisted of densely growing melanoma cells. On the other hand, nodules produced by tumor cells from line F₁ were smaller and less dense. In addition, mononuclear cell infiltrates were more pronounced around pulmonary nodules produced by cells from line F₁₁ than around nodules produced by cells from line F₁ (Figs. 1 to 4).

**The Incidence of Spontaneous Pulmonary Metastases by Tumor Cells from the Successive Tumor Lines.** The process of metastasis involves the invasion of tissues, blood vessels, and/or lymphatics by cells originating from a primary tumor. Tumor cells are then released into the circulation; emboli arrest at distant sites; and tumor cells must invade the parenchyma, establish a microenvironment, and grow into secondary tumors. In the 1st experiment we had tested the ability of the various tumor lines to survive in the circulation, arrest, and develop into secondary growths. It was now necessary to compare their relative survival in the circulation with their initial degree of invasiveness in vivo, a property that is absolutely necessary for the pathogenesis of metastasis. Mice were given s.c. injections of 50,000 viable cells from lines F₁, F₆, and/or F₁₀. Ten days later all tumor nodules were excised and the skin was sutured. The animals were killed 6 to 8 weeks later and examined for metastases. The data demonstrated that the incidence of spontaneous pulmonary metastases markedly differed between the lines. Specifically, only 1 of 11 mice given s.c. injections of line F₁ (low metastases) developed spontaneous pulmonary metastases. In mice given injections of cells from line F₆, 6 of 12 mice developed lung tumors; whereas 9 of 12 mice given s.c. injections with cells from line F₁₁ had pulmonary nodules.

**Quantitative Analysis of Tumor Cells in Lung and Blood.** Several experiments were performed with [³¹P]UdR-labeled B16 melanoma cells from lines F₁, F₆, and F₁₁ injected i.v. into C57BL/6 mice. The data are summarized in Table 3. Most tumor emboli were arrested immediately in the capillary bed of the lung and tumor cell death occurred shortly thereafter. By Day 14 post-i.v. injection about 0.5% of the original injected F₁ line cells survived in the C57BL/6 mice. The survival rate of cells from lines F₆ and F₁₁ was about 10 and 13%, respectively. The survival rate of the line F₁ agreed closely with our previous studies (4, 9, 10). The differences in the number of arrested tumor cells in lungs of the 3 groups were clearly demonstrable by 2 mm postinjection and were statistically significant at any time interval (p < 0.005). At 2 min postinjection 60% of all cells from line F₁ and 100% of all cells from lines F₆ and F₁₁ were arrested in the lung capillary bed. When the blood of the 4 groups was monitored, the difference in tumor emboli arrest was further clarified. Mice given injections of tumor cells from lines F₆ and/or F₁₁ had fewer circulating tumor cells than mice given injections of line F₁, indicating a greater degree of embolic arrest. The immediate decline in lung counts could be due to recirculation of tumor cells, but the decline observed 4 hr following i.v. tumor cell injection was a measure of cell death and not just cell escape (4). The largest difference in lung count was observed by Day 1 post-i.v. injection. The survival rate of cells from line F₁ was about 3%, and lines F₆ and F₁₁ about 50%. By Day 14 after tumor cell injections, gross lung metastases were counted with the

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**Table 1**

<table>
<thead>
<tr>
<th>Source of lymphocytes</th>
<th>B16 line F₁</th>
<th>B16 line F₁₁</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1 ± 1 (1 3)</td>
<td>2 ± 1 (1 4)</td>
</tr>
<tr>
<td>NR C57BL/6</td>
<td>5 ± 2 (3 12)</td>
<td>12 ± 5 (4 18)</td>
</tr>
<tr>
<td>Immune C57BL/6</td>
<td>13 ± 8 (2 30)</td>
<td>18 ± 10 (6 30)</td>
</tr>
</tbody>
</table>

* Glasswool-filtered lymphocytes: lymphocytes: tumor cells, 100:1.
* Fifty clumps counted at random following 90 min interaction of tumor cells and lymphocytes.
* Mean ± S.D.
* Numbers in parentheses, range.
* Mice immunized to the B16 melanoma (see "Materials and Methods").
Table 2
Relationship of the number of viable B16 melanoma cells injected i.v. into C57BL/6 mice to the number of resultant pulmonary metastases

<table>
<thead>
<tr>
<th>No. of viable B16 cells injected i.v.</th>
<th>Av. no. of pulmonary metastases*</th>
<th>B16 line F1</th>
<th>B16 line F6</th>
<th>B16 line F11</th>
</tr>
</thead>
<tbody>
<tr>
<td>6,250</td>
<td>0.25 (0.1)*</td>
<td>10 ± 2 (7.14)</td>
<td>16 ± 3 (12.21)</td>
<td></td>
</tr>
<tr>
<td>12,500</td>
<td>4 ± 1.5 (2.8)</td>
<td>28 ± 6 (18.36)</td>
<td>38 ± 8 (24.49)</td>
<td></td>
</tr>
<tr>
<td>25,000</td>
<td>34 ± 4 (27.38)</td>
<td>150 ± 37 (100.190)</td>
<td>195 ± 23 (160.221)</td>
<td></td>
</tr>
<tr>
<td>50,000</td>
<td>60 ± 6 (52.71)</td>
<td>254 ± 24 (220.290)</td>
<td>370 ± 40 (320.420)</td>
<td></td>
</tr>
</tbody>
</table>

* Six mice/group. Pulmonary metastases were counted on Day 14 post-i.v. injection with a dissecting microscope.
* Number in parentheses, range.
* Mean ± S.D.

DISCUSSION

The most important properties of highly malignant tumor cells is their ability to invade normal tissues and vessels and then survive in the circulation, adhere to a distant vessel...
Figs. 3 and 4. Pulmonary metastases produced by B16 melanoma from Line F1, 14 days following their injection i.v. Note the high density of the melanoma cells within the nodules and mononuclear cell infiltrates around it (Fig. 4). Fig. 3, × 100; Fig. 4, × 450.

wall, penetrate it, and grow into secondary tumor foci (11, 24, 25). We have confirmed results of others (12, 13, 24) that the number of experimental pulmonary metastases is proportional to the number of tumor cells injected i.v. The outcome of metastasis is also influenced by factors such as total number of circulating emboli or their size (5). The selection of our B16 melanoma lines for their metastatic capability tends to suggest that unique tumor cell properties must determine their success in the host. It has been demonstrated that cell suspensions from various types of tumors injected at same sites into rats, consistently established their own pattern of metastasis (20). Some tumor cells could traverse different organs at different rates (11), while cells from different tumors interacted in unique and different patterns with a capillary bed of the same organ (26). The rate by which tumor cells pass through capillaries was not related to their size but was attributed to their plasticity and ability to distort their shape (25).

Malignant tumors are characterized by their ability to invade normal tissues and metastasize. In these experiments we investigated the incidence of spontaneous metastasis by tumor cells from low- and high-metastatic lines. The degree of spontaneous metastases was directly related to the metastatic potentiality of the various tumor lines. This presumably could be taken as a measure of local tumor cell invasion into s.c. tissues. Alternatively, it is conceivable that an equal degree of local invasiveness by tumor cells from all lines occurred, and the incidence of spontaneous pulmonary metastasis reflected only the relative ability of tumor cells to survive in the circulation. It has been suggested that the invasiveness of malignant tumor cells is due to their reduced adhesiveness (2, 15, 24). Tumor cells may be deficient in calcium (3) which could lead to decreased cellular adhesiveness (2, 15). Moreover, malignant transformation could be associated with general enzymatic changes leading toward increased proteolytic and/or fibrinolytic activity in tumor cells as compared to normal cells (16, 17, 21). Invasiveness of tumor cells could be enhanced by such increased levels of degradative enzymes and recently Boseman et al. (1), utilizing our B16 melanoma lines, reported that line F10 had
The arrest of tumor cell emboli in a capillary bed could be determined by a variety of factors, such as tumor cell surface charge (14, 23), levels of tumor cell surface enzymes (1), embolic size and homogeneity (5, 11), plasticity of tumor cells (25), the interaction between tumor cells and host immune cells (7, 8, 18), host blood-clotting mechanisms (12, 24), host platelet activity (13), or endothelial cell wall properties (22). We found that, when tumor cells were mixed with low concentrations of glasswool-filtered lymphocytes, multim tumor cell lymphocyte clumps resulted, promoting secondary tumor growth formation following i.v. inoculation. High lymphocyte to tumor cell ratios, while resulting in clumping, lead to a significant inhibition of subsequent metastases (7, 8).

In this study we have investigated the in vitro tumor cell-lymphocyte clumping phenomenon utilizing tumor cells from line F1 or line F10 and the non-glass adherent lymphocytes obtained from syngeneic normal mice or mice immunized to the B16 melanoma. The results demonstrated that the resultant clump size could be influenced by both the tumor cell line or the lymphocytes. Lymphocyte to tumor cell binding can be rapid and take place in the absence of serum (19). Cell surface glycosyltransferases, as well as cell surface acceptors, could influence the degree of cell to cell adhesion. Boseman et al. (1) measured the activity levels of surface glycosyltransferase acceptor reaction in the melanoma lines and found line F10 to have higher levels of enzyme activity than B16 cells from line F1. The high levels of surface enzymes in cells of line F10 could explain their increased tendency for heterotypic clumping. Such clumping could occur between tumor cells and other host cells. Recently, Gasic et al. (13) reported that platelet aggregation contributed directly to the outcome of experimental metastasis. A direct correlation between tumor cell platelet aggregation in vitro and the outcome of metastasis was noted in their study when our B16 cell lines were utilized. Cells from line F8 (high metastases) aggregated platelets in vitro while cells from line F1 (low metastases) did not. This finding suggested that platelet aggregation by tumor cells is a critical event in the process of metastasis (13), correlating with our earlier studies of the importance of tumor cell embolic size to the outcome of experimental metastasis (5).

In this study different patterns of tumor cell distribution and fate were observed with the various lines. These studies utilized cells prelabeled in vitro with [125I]lUdR and then injected i.v. into normal syngeneic mice. At various intervals after injection mice were killed, and their lungs and blood monitored for radioactivity. As in our earlier studies (4, 9, 10), we found that most tumor emboli were trapped by the lungs immediately after injection with a few cells passing through and recirculating. It appeared that the increasing metastatic capability of the tumor cells was directly related to the degree of their initial arrest in the pulmonary capillary bed. In addition, the survival rate of tumor cells from lines producing high numbers of metastases was appreciably increased. Initial arrest of tumor cells in a capillary bed may indicate unique surface properties allowing for such arrest (1). These studies demonstrate the importance of tumor cell characteristics to its fate and survival. Furthermore, the B16 melanoma lines may offer an approach to investigate in detail such properties of malignant cells.

REFERENCES

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