Relationship between Cellular Procoagulant Activity and Metastatic Capacity of B16 Mouse Melanoma Variants

Linda C. Gilbert and Stuart G. Gordon

Department of Medicine, University of Colorado Health Sciences Center, Denver, Colorado 80262

ABSTRACT

The metastatic process is a complex sequence of steps that may involve coagulation and the presence of fibrin. F1 (low incidence of lung colonization) and F10 (high incidence of lung colonization) variants of the B16 mouse melanoma were used to examine the relationship between the level of cellular procoagulant activity and their metastatic potential. Cell suspensions were prepared from cultures of B16-F1 and B16-F10 cell lines. Aliquots (0.2 ml) containing 50,000 cells were assayed for procoagulant activity in recalcified citrated rat plasma and for metastatic capacity by tail vein injection followed by counting of melanotic lung tumor colonies 17 days later. In one series of experiments, procoagulant activity and metastatic capacity were determined at 1, 2, 3, and 4 days after plating. The data showed an almost parallel decrease in both characteristics as the culture density increased. To examine the correlation between cellular procoagulant activity and the metastatic capacity of the B16 variants in two different series of experiments, regression analysis of the level of procoagulant activity and the number of lung tumor colonies gave correlation coefficients of 0.9 (n = 15) and 0.79 (n = 8). These results suggest that fibrin formation resulting from cellular procoagulant activity may play a role in the metastatic process.

INTRODUCTION

It is generally accepted that cancer kills its host because it invades normal tissue and metastasizes to secondary sites where further invasion occurs. The metastatic process is a complex sequence of steps; different steps may be of greater relative importance in the metastasis of different cancers. This process begins with the invasion by a primary tumor into the blood or lymphatic system followed by shedding of malignant cells into the circulation. These circulating malignant cells aggregate with blood cells (usually platelets) and become lodged in the small capillaries of an organ, such as the lung, liver, or kidney. After becoming attached to the vascular endothelium, the malignant cells invade the epithelium into the underlying tissue and develop into a secondary tumor.

Lodgement and invasion at the secondary site appear to be critical steps in metastasis. Although Fidler and Hart have shown that there are organ-specific characteristics of the host that influence the site of metastases, metastatic capacity is thought to be primarily a function of tumor cell characteristics. Some of these characteristics include cell surface antigens, adhesive strength of cells to their growth substrate, buoyant density, and production of enzymes that may participate in the metastatic process, such as enzymes capable of inducing fibrin formation and lysis.

It has also been suggested that some fibrin on the surface of a tumor cell may protect it from the host immune system. In addition, deposition of fibrin on tumor cells may facilitate the aggregation of cancer and blood cells (usually platelets) which could promote the arrest of these aggregates in small capillaries and facilitate the initial phase of their adhesion to the capillary endothelium. Defibrination or anticoagulant therapy with warfarin or heparin reduces metastasis and/or tumor growth.

The apparent role of fibrin in the malignant process has led to the search for procoagulants in malignant tissue and clarification of their role in the malignant and metastatic processes. Studies from this laboratory have resulted in the identification, purification, and partial characterization of a proteolytic enzyme that promotes blood coagulation through the direct activation of Factor X in extracts of malignant tissue and transformed cells and is distinct from tissue factor. Recent studies evaluated the level and characteristics of the procoagulant activity of intact normal and transformed or malignant cells by using a Sonoclot coagulation analyzer; the results have confirmed previous data obtained with a Fibrometer on soluble enzyme preparations.

In this paper, we have examined the relationship between cell-associated procoagulant activity levels and the metastatic capacity of i.v.-injected mouse melanoma B16-F1 and B16-F10 cells. There was a strong positive correlation between procoagulant activity levels and the number of both B16-F1 and B16-F10 melanotic lung tumor colonies, suggesting that fibrin formation may be involved in the metastatic process.

MATERIALS AND METHODS

Animals. Six-week-old C57BL/6 mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. For each experiment, all mice were sex and age matched.

Cell Lines. The F1 (low incidence of lung colonization) and F10 (high incidence of lung colonization) variants of the B16 mouse melanoma were obtained from 2 sources: Dr. lan R. Hart, Cancer Biology Program, National Cancer Institute, Frederick Cancer Research Center, Frederick, Md., and the National Cancer Institute Tumor Bank, E G & G Mason Research Institute, Worcester, Mass. Frozen stocks of the cell lines were prepared after receipt. Cultures from these stocks were maintained for no longer than 60 days in continuous culture; usually, they were started from the frozen stocks just prior to each experiment. The B16-F1 and B16-F10 cell lines were grown as described previously.
or 24 hr before harvesting. Since cell density appeared to affect vein injections of 0.2 ml of the cell suspensions through a 27-gauge needle and killed 17 days later. The number of melanotic lung tumor colonies were too low for the differences to be statistically significant (p < 0.05). The metastatic capacity also showed a decreasing trend; however, the numbers of lung tumor colonies were too low for the differences to be statistically significant.

In the B16-F10 experiment, the procoagulant activity decreased over the 4-day period from a CR of 21.3 ± 0.76 on Day 1 to a CR of 3.75 ± 0.21 on Day 4. The differences in procoagulant activity and melanotic lung tumor colonies between Days 1 and 2, 3 and 4, and 1 and 4 were all statistically significant (p < 0.02). Only the differences between Days 2 and 3 were not statistically significant (p > 0.05).

Correlation of Procoagulant Activity and Metastasis Capacity. The previous experiments suggested that there is a relationship between cellular procoagulant activity, metastatic capacity, and growth density of the cell cultures. This was determined by harvesting B16 cells 1, 2, 3, and 4 days after plating and measuring the procoagulant activity and metastatic capacity of 0.2-ml cell suspension samples containing 50,000 viable cells.

### RESULTS

**Effect of Cell Density on Metastatic Capacity and Procoagulant Activity.** Preliminary experiments suggested that both metastatic capacity and cell-associated procoagulant activity might be influenced by the density of the cell cultures at the time they were used to prepare cell suspensions. In order to test whether metastatic capacity and procoagulant activity were related to cell density, B16-F1 and B16-F10 cells were plated (Day 0) and then harvested on Days 1, 2, 3, and 4 in 2 experiments. The metastatic capacities and cell-associated procoagulant activities of the cells were determined for each day (Table 1). As the culture density increased, there was an overall downward trend in both metastatic capacity and cell-associated procoagulant activity.

In the B16-F1 experiment, the differences in the levels of procoagulant activity between consecutive days and between Days 1 [CR 15.5 ± 0.64 (S.E.)] and 4 (CR 8.00 ± 0.46) were statistically significant (p < 0.05). The metastatic capacity also showed a decreasing trend; however, the numbers of lung tumor colonies were too low for the differences to be statistically significant.

In the B16-F10 experiment, the procoagulant activity decreased over the 4-day period from a CR of 21.3 ± 0.76 on Day 1 to a CR of 3.75 ± 0.21 on Day 4. The differences in procoagulant activity and melanotic lung tumor colonies between Days 1 and 2, 3 and 4, and 1 and 4 were all statistically significant (p < 0.02). Only the differences between Days 2 and 3 were not statistically significant (p > 0.05).

### Table 1

<table>
<thead>
<tr>
<th>Time (days) after plating</th>
<th>No. of cells/ flask</th>
<th>Normalized CR</th>
<th>Lung tumor colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.2 x 10⁴</td>
<td>15.5 ± 0.64</td>
<td>2.5</td>
</tr>
<tr>
<td>2</td>
<td>6.0 x 10⁵</td>
<td>13.0 ± 0.40</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>1.9 x 10⁶</td>
<td>9.77 ± 0.13</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>4.0 x 10⁷</td>
<td>8.00 ± 0.46</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>8.5 x 10⁴</td>
<td>21.3 ± 0.76</td>
<td>85</td>
</tr>
<tr>
<td>2</td>
<td>8.4 x 10⁵</td>
<td>7.87 ± 0.20</td>
<td>23</td>
</tr>
<tr>
<td>3</td>
<td>3.1 x 10⁶</td>
<td>5.69 ± 0.52</td>
<td>17.5</td>
</tr>
<tr>
<td>4</td>
<td>1.5 x 10⁷</td>
<td>3.75 ± 0.21</td>
<td>7.5</td>
</tr>
</tbody>
</table>

**a** Mean ± S.E.  
**b** Numbers in parentheses, number of observations.
correlation between the level of cell-associated procoagulant activity and the metastatic capacity for both the B16-F1 and B16-F10 cell lines. In 2 series of experiments using B16 variant cells from 2 different sources, a suspension of both variant cell lines was prepared, portions of the suspension were injected into the tail veins of C57BL/6 mice to determine metastatic capacity, and another portion of the same cell suspension was assayed for procoagulant activity.

The results of the experiments performed with B16-F1 and B16-F10 cells from both sources are presented in Chart 1. Each data point represents the average of between 2 and 4 values for procoagulant activity and the median of 4 to 5 values for melanotic lung tumor colonies that were obtained from aliquots of the same cell suspension sample. The equations for the regression lines (Table 2) show that there was a linear relationship (r = 0.90 for Experiment 1; r = 0.79 for Experiment 2) between the level of cell-associated procoagulant activity and the ability of both the B16-F1 and B16-F10 cells to form melanotic lung tumor colonies. There was considerable overlap between the data for the B16-F1 and B16-F10 cells. For example, the procoagulant activity levels of the cells in Experiment 1 ranged from 5.25 to 20.8 for the B16-F1 line and from 8.2 to 41.1 for the B16-F10 line. Similarly, the metastatic capacities ranged from 0 to 8.0 melanotic lung tumor colonies for the B16-F1 line and from 1.0 to 29.0 for the B16-F10 line.

The slopes of the regression lines of the B16-F1 and B16-F10 data from each experiment were statistically analyzed to determine whether or not they represent the same line. The slopes of the regression line for the B16-F1 cell data and for the B16-F10 cell data within each experiment, representing cells from the same source, were not statistically different (p > 0.05). However, interexperimental comparison of the regression lines for the B16-F1 data and for the B16-F10 data showed a significant difference (p < 0.02) between the regression lines from the first series of experiments compared to the same cell line data from the second series of experiments, suggesting that there were differences between the B16 variant cells from the 2 sources.

**DISCUSSION**

The ability of circulating tumor cells to form emboli and arrest and to grow in secondary sites to form metastases may be dependent on the expression of a procoagulant activity. This activity may result in the formation of tumor cell clumps held together by fibrin strands. Alternatively, the tumor cell emboli may consist of tumor cells and platelets with or without fibrin. Both of these alternatives would be possible for tumor cells that possess a procoagulant activity, since they could form fibrin or aggregate platelets through the generation of thrombin (49). It has been reported that tumors with the ability to aggregate platelets usually produce lung metastases (19). Liotta et al. (33) have shown that circulating tumor cell clumps produce significantly greater numbers of metastases than did similar numbers of single tumor cells.

Procoagulants from a variety of tumors and transformed cells have been examined. Three general types of procoagulant activity have been described: (a) a tissue-factor-like activity (9, 37); (b) a procoagulant associated with plasma membrane vesicles (13); and (c) a procoagulant capable of direct activation of Factor X (21). This latter type of procoagulant was recently shown to be a cysteine protease (20). This procoagulant activity has been identified in the mouse Lewis lung carcinoma (10, 39), in several transformed hamster fibroblast cell lines (24), and in extracts from a number of different malignant human tissues (21, 22). Previously, it has been shown that a B16-F11 variant of the melanoma cell line releases a procoagulant with the characteristics of cancer procoagulant (23) and the ability to directly activate Factor X.

We have shown in these experiments that there is an overall decrease in both the level of cell-associated procoagulant activity and the ability of the B16-F1 and B16-F10 variants to produce experimental lung metastases as the culture density increases. Since all samples were processed in an identical manner and since there was no apparent effect of the harvesting procedure on the procoagulant activity of cultured malignant cells (23), this phenomenon may be due to increased cell density in the cultures. The almost parallel behavior of the 2 characteristics suggests that there may be a relationship between procoagulant activity and metastatic capacity.

To further investigate this relationship, we have performed a series of experiments to examine the correlation between cell-associated procoagulant activity and the ability of the B16-F1 and B16-F10 cell lines to form melanotic lung tumor colonies after i.v. injection. We found a strong positive correlation between these 2 tumor cell characteristics in which the data for both cell lines represent portions of the same regression line. The B16-F10 cell line was derived from the B16-F1 cell line by 9 successive passages in which lung tumor colonies were isolated after i.v. injection. Since the increased metastatic capacity of cell lines derived in this manner has been shown to be a selective, rather than an adaptive process (34, 42), the B16-F10 cell population is enriched for a segment of the B16-F1 cell population that is more capable of producing lung tumor colonies after i.v. injection.

The overlap in the data for the B16-F1 and B16-F10 cell lines in Chart 1 is the result of the culture density effect on cell-associated procoagulant activity level and metastatic capacity. For instance, B16-F1 cultures of low density (Day 2 after plating) express higher levels of enzyme activity and are more metastatic than some B16-F10 cultures of medium density (Day 3) and all B16-F10 cultures of high density (Day 4).

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* S. Gordon and B. Lewis, unpublished observation.
The difference in the regression lines for the B16-F1 and B16-F10 cell lines from the 2 sources is most likely due to subtle selection pressures that have resulted in slightly different tumor cell characteristics. Even cloned sublines of tumor cells exhibit heterogeneity in their metastatic capacity (17, 40).

Our results are in agreement with the previously mentioned studies suggesting a role for fibrin formation in metastasis and with the reported relationship between procoagulant activity and the formation of a fibrin-gel investment by the guinea pig line 10 hepatocarcinoma (12). They also agree with recent studies by Colucci et al., in which they showed that vitamin K depletion decreased cellular Factor X activator activity and metastatic capacity of Lewis lung carcinoma cells to a similar extent in a spontaneous metastasizing murine animal model. This was in contrast to the negative correlation between tissue factor activity and metastatic capacity of murine fibrosarcoma variants after vitamin K depletion (9).

The metastatic process appears to be a complex sequence of events that depend on various properties and functions of the tumor cell. This paper suggests that procoagulant activity, and therefore the ability of the tumor cell to form fibrin, is an important requirement for one of the steps in the metastatic process after the tumor cell has entered the circulatory system.

REFERENCES


Table 2

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Source 1</th>
<th>Source 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>B16-F1</td>
<td>y = 1.26x + 9.11 r = 0.80 (6)</td>
<td>y = 2.10x + 10.3 r = 0.78 (4)</td>
</tr>
<tr>
<td>B16-F10</td>
<td>y = 1.07x + 9.84 r = 0.90 (9)</td>
<td>y = 1.69x + 17.9 r = 0.66 (4)</td>
</tr>
<tr>
<td>B16-F1 + B16-F10</td>
<td>y = 1.98x + 9.63 r = 0.90 (15)</td>
<td>y = 2.37x + 12.6 r = 0.79 (8)</td>
</tr>
</tbody>
</table>

6 Numbers in parentheses, number of data points.

8 B16-F1 and B16-F10 cells were obtained from 2 sources, as explained in "Materials and Methods."

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