Role of Laminin in the Attachment and Metastasis of Murine Tumor Cells


ABSTRACT

We have studied the attachment of two murine metastatic cell lines and of a transformed, nonmetastatic sarcoma cell line to type IV (basement membrane) collagen. The metastatic cells attached preferentially to type IV collagen, whereas the nonmetastatic cells attached best to type I collagen. Laminin increased both the rate and the number of metastatic cells attaching to type IV collagen, while fibronectin had no effect. Antibodies to laminin prevented the attachment of metastatic cells to type IV collagen, while antibodies to fibronectin prevented the attachment of the nonmetastatic cells. The number of pulmonary metastases which formed after i.v. injection of cells into C57BL mice was used to measure the metastatic propensity of these cell lines. A subpopulation of the metastatic cells selected for by their ability to attach to type IV collagen in the presence of laminin produced more metastases than did unattached cells or cells attached with fibronectin. In addition, incubation of metastatic cells with antibody to laminin prior to injection into mice markedly reduced the number of lung metastases. These data suggest that laminin promotes the attachment of metastatic tumor cells to basement membrane during the metastatic process.

INTRODUCTION

Previous studies have demonstrated that a large degree of specificity exists in the interaction of cells with their extracellular collagenous matrices (12, 13, 20). In culture, some cell types adhere preferentially to the type of collagen with which they are normally associated in vivo (12). This interaction is mediated by glycoproteins which bind the cells to the collagen. Most cells of mesenchymal origin, such as fibroblasts, myoblasts, and smooth muscle cells, utilize fibronectin to bind to collagen (4, 12). Cells which abut on a basement membrane use laminin to attach to type IV (basement membrane) collagen (25). Chondrocytes, which are associated with type II collagen in cartilage (18), use still another glycoprotein, chondronectin, to bind to this cartilage-specific collagen (8, 9). Thus, the interaction of cells with their extracellular matrix involves specific proteins which recognize both the cell surface and the collagenous matrix. Presumably, these interactions are important in embryogenesis and in repair mechanisms. Abnormalities in these interactions could underlie certain pathological conditions.

Many investigators have speculated that the malignancy of a tumor may be related to alterations in the synthesis and degradation of matrix components or to alterations in the interactions between the tumor cells and the extracellular matrix (2, 5, 19). For example, many transformed fibroblasts either cease fibronectin synthesis or have greatly reduced amounts on the cell surface (10, 27, 31). Similarly, it has been observed that, after transformation, rat kidney cells lack both laminin and fibronectin (7).

A few studies have been carried out on the attachment of tumor cells to extracellular matrices. Fibronectin has been shown to bind melanoma cells to the subendothelial matrix prepared from cultured endothelial cells (21, 22), while the basement membrane of blood vessels offers an even better substrate (24). Highly metastatic tumor cells have been shown to adhere to subendothelium basement membrane more avidly than do cells with a lesser metastatic potential (24). It has been suggested that sarcomas use fibronectin for attachment and carcinomas use laminin, although this was not demonstrated directly (29).

In previous work (19), it was shown that metastatic cells bind preferentially to type IV over type I collagen, but these studies did not identify which, if any, attachment protein was involved. Here, we have studied the attachment of 2 tumorigenic and metastatic cell lines, the BL6 from B16 melanoma (6) and the PM2 from the PMT fibrosarcoma (15), to type IV basement membrane collagen. A tumorigenic but nonmetastatic cell line, the C3H fibrosarcoma, was also studied. The data indicate that the metastatic cell lines attached preferentially to type IV collagen using laminin while the nonmetastatic cell line did not utilize laminin. A role for laminin in the metastasis of these cells to lung was also demonstrated.

MATERIALS AND METHODS

Cell Lines. BL6 melanoma cells were kindly supplied by Dr. Ian Hart, Frederick Cancer Research Institute-National Cancer Institute, Frederick, Md. The BL6 murine melanoma cells were selected from B16-F10 melanoma cells based on their ability to invade the murine bladder wall in vivo (16). PM2 tumor cells are derived from a pulmonary metastasis of the T241 fibrosarcoma (14, 17).

C3H tumor cells were derived from C3H mouse adult connective tissue cells which had spontaneously transformed in culture (28) and were cultured from a nonmetastatic encapsulated fibrosarcoma that arose when the transformed cells were transplanted into a syngeneic host.

All cells were routinely cultured in Roswell Park Memorial Institute Medium 1640 supplemented with 10% fetal bovine serum, glutamine, penicillin (10 units/ml), and streptomycin (10 μg/ml).

Preparation of Substrate, Antibody, and Attachment Factors. Type I collagen was prepared from lathyritic rat skin (1), and type IV collagen (23) and laminin (26) were prepared from the EHS tumor. Fibronectin was a gift from Dr. H. K. Kleinman (National Institute of Dental Research). Purified antibodies to laminin were prepared as described previously (3).

Attachment Assay. The attachment assay was adapted from that described by Klebe (11). Where indicated, the cells were preincubated with Eagle’s minimum essential medium plus cycloheximide (25 μg/ml) for 4 hr to inhibit protein synthesis. This level of cycloheximide inhibits protein synthesis by more than 90% but does not affect the viability of the cells during the period of the attachment assay. The cells, usually 2 × 10⁶, were added in 0.9 ml Eagle’s minimum essential medium, supplemented with bovine serum albumin (200 μg/ml), to collagen-
coated 35-mm bacteriological Petri dishes. After incubation, the "unattached" cells were removed, and the "attached" cells were harvested from the assay dishes using 0.05% trypsin/0.1% EDTA and counted with an electronic particle counter. The data obtained represent the average of measurements made on 3 dishes which varied by less than 10%. Each experiment was repeated 3 times.

Assay for Metastatic Potential. The "attached," "unattached," and parent cells of all 3 tumor cell lines were assayed for their ability to colonize and grow in the lungs of inoculated animals. In these studies, the cells were detached from the dish using a sterile rubber scraper and then suspended in Dulbecco's modified Eagle's medium without serum. The attached cells from at least 4 Petri dishes were pooled and then aspirated through a 22-gauge needle to disaggregate cell clumps. A portion of these cells was assayed twice for viability by their ability to exclude trypan blue. Suspensions of viable cells (2 x 10^6) in a volume of 0.1 ml were injected into the tail vein of syngeneic, pathogen-free, 6-week-old recipient male mice. Twenty-five days later, the mice were sacrificed, and their lungs were removed, filled with Bouin's fixative, and then immersed in the same fixative. Growing tumors were identified and counted in separated lung lobes with the aid of a dissecting lens (x 10). Their identification as tumor metastases was confirmed in representative histological studies on sections from each tissue group.

In Vivo Tumor Cell Retention. The retention of radioactivity in the lungs was studied after tail vein injection of labeled tumor cells. BL6 tumor cells were incubated in the log phase of cell growth with [3H]thymidine for 24 hr at a concentration of 1 mCi/ml. A portion of the cells was separated into "attached" and "unattached" cells as outlined above. The labeled tumor cells were injected into the mouse tail vein and, at various times after injection, the mice were sacrificed, and their lungs were excised. The excised lungs were completely flame oxidized, and the tritium-labeled water that formed was assayed for radioactivity.

Complement-dependent Antibody Cytotoxicity. In some studies, antibodies to laminin or to fibronectin were injected along with the cells to test the in vivo roles of laminin and fibronectin. Complement-mediated cytotoxicity tests were performed on the cells to assess possible toxic effects of the antisera. Tumor cells were washed and suspended in serum-free Dulbecco's modified Eagle's medium. The cells were diluted in the same medium and inoculated in Costar microtiter plates to yield a concentration of 10^5 cells/well in a volume of 100 µl. Ten wells received rabbit complete complement plus dilutions of anti-laminin antiserum (1/5, 1/10, 1/50, 1/100, 1/500). After incubation for 3 hr at 37°C, the number of viable (trypan blue stain exclusion) cells in each well was determined.

RESULTS

We first measured the rate at which the BL6, PM2, and C3H cells attached to type I or type IV collagen substrates. The metastatic BL6 and PM2 cells attached more rapidly to type IV than to type I collagen-coated dishes, while the nonmetastatic C3H cells attached preferentially to type I collagen-coated dishes (Chart 1).

Addition of laminin (1 µg/ml) to the assay medium increased the attachment of the BL6 and the PM2 cells to type IV collagen (Chart 2) but had no effect on the attachment of C3H cells (Chart 3). The addition of purified fibronectin (5 µg/ml) markedly stimulated the attachment of the C3H cells (Chart 3). Serum did not stimulate the attachment of the BL6 (Chart 4) or PM2 cells (data not shown) to type IV collagen, suggesting that fibronectin or another serum factor was not an attachment protein for these cells.

We next tested whether the adherence of the cells in the absence of added attachment factors was due to endogenous synthesis of these proteins. In the presence of cycloheximide, laminin stimulated the attachment of metastatic cell lines to basement membrane collagen in a time (Chart 4)- and concentration (Chart 5) dependent manner. Laminin at 1 µg/ml caused maximal attachment for BL6 and PM2 cells, while levels as high as 10 µg/ml had no effect on C3H cell attachment (Chart 5). The C3H cell line, when assayed in the presence of cycloheximide, used fibronectin for attachment (data not shown).

We also measured the attachment of BL6, PM2, and C3H cells in the presence of affinity-purified anti-laminin and anti-

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Chart 1. Time course for the attachment of BL6, PM2, and C3H cells to type I and type IV collagen-coated dishes in the absence of added factors. Dishes (35 mm) were coated with 10 µg of substrate. At the indicated times, the unattached cells were removed and counted, and the attached cells were removed with 0.05% trypsin/0.1% EDTA and counted electronically. Each point represents the mean of triplicate experiments.

Chart 2. Time course for the attachment of PM2 and BL6 cells with and without added laminin. Laminin was added at the beginning of the assay at a concentration of 1 µg/ml. The substrate was type IV collagen. Each point represents the mean of triplicate assays.

Chart 3. Time course for the attachment of tumorigenic but nonmetastatic C3H cells with and without added laminin and fibronectin. Fibronectin was added at the beginning of the assay at a concentration of 5 µg/ml. Laminin was added at the beginning of the assay at a concentration of 1 µg/ml. Type IV collagen was used as a substrate. Assays were carried out in triplicate.

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Laminin-mediated Selection of Metastatic Cells

Chart 4. Time course for the attachment of BL6 melanoma cells after a 4-hr preincubation in cycloheximide (25 μg/ml). + C, preincubated in cycloheximide and assay medium (Eagle's minimum essential medium) contained cycloheximide; + Serum, addition of 10% fetal bovine serum at the onset of the assay; + Laminin, laminin added at the beginning of the assay at a concentration of 1 μg/ml. Type IV collagen was the substrate. Each value is the mean of triplicate assays.

Chart 5. Dose-response curve for laminin-mediated attachment of PM2, BL6, and C3H cells. + C, assays carried out in the presence of cycloheximide (25 μg/ml) after 4 hr of preincubation in cycloheximide. Each value represents the mean of triplicate assays.

Chart 6. Effect of affinity-purified anti-fibronectin antibody (100 μg protein per ml initial concentration) at various dilutions on the attachment of PM2, BL6, and C3H cells to type IV collagen. The antibody was added at the onset of the attachment assay. Values are the mean of triplicate assays.

Chart 7. Effect of affinity-purified anti-laminin antibody (100 μg protein per ml initial concentration) at various dilutions on the attachment of PM2, BL6, and C3H cells to type IV collagen. The antibody was added at the onset of the attachment assay. Each value represents the mean of triplicate assays.

Table 1

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Anti-laminin antibody</th>
<th>Laminin selected</th>
<th>Fibronectin selected</th>
</tr>
</thead>
<tbody>
<tr>
<td>PM2</td>
<td>Untreated</td>
<td>Treated</td>
<td>Attached</td>
</tr>
<tr>
<td>BL6</td>
<td>90 ± 16</td>
<td>5 ± 3</td>
<td>102 ± 21</td>
</tr>
<tr>
<td>C3H</td>
<td>110 ± 20</td>
<td>16 ± 5</td>
<td>160 ± 32</td>
</tr>
</tbody>
</table>

* Mean ± S.E.
we show that metastatic and nonmetastatic cells use different attachment proteins to interact with different collagens. Laminin, dronectin, or laminin (12). Previously, it was shown that certain cells utilize various attachment proteins such as fibronectin, chondro-.

membranes is probably attachment. Recent studies have shown that the basement membrane collagen is resistant to attack by the animal collagenases that degrade interstitial collagens (16). Degradation of basement membrane collagen involves a separate type IV collagenase. Metastatic tumor cells have been shown to be high producers of this specific type IV collagenase (16). Metastasizing tumor cells traverse organ boundaries and capillary walls to initiate colonies in distant sites. In the process, they encounter basement membranes which are continuous sheets of extracellular matrices which lie beneath epithelial and endothelial cells (14).

The initial interaction between metastatic cells and basement membranes is probably attachment. Recent studies have shown that many cells do not bind directly to collagen but utilize various attachment proteins such as fibronectin, chondro-nectin, or laminin (12). Previously, it was shown that certain metastatic cells attached more rapidly to type IV than to type I collagen while nonmetastatic cells preferred type I (19). Here we show that metastatic and nonmetastatic cells use different attachment proteins to interact with different collagens. Laminin stimulated the attachment of the metastatic cells to type IV collagen, while fibronectin stimulated the attachment of non-

<table>
<thead>
<tr>
<th>Dilution of anti-laminin antisera</th>
<th>% of nonviable tumor cells (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complement alone</td>
<td>15</td>
</tr>
<tr>
<td>Complement + anti-laminin (1/1500)</td>
<td>20</td>
</tr>
<tr>
<td>Complement + anti-laminin (1/100)</td>
<td>19</td>
</tr>
<tr>
<td>Complement + anti-laminin (1/8)</td>
<td>25</td>
</tr>
<tr>
<td>Complement + anti-laminin (1/2)</td>
<td>40</td>
</tr>
</tbody>
</table>

a Dilution of anti-laminin used in the experiment described in Table 1.

Table 3

<table>
<thead>
<tr>
<th>BL6 subpopulation</th>
<th>Cpm [3H]thymidine x 10^-3/lung</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adherent^a</td>
<td>31</td>
</tr>
<tr>
<td>Nondifferentiated</td>
<td>4</td>
</tr>
<tr>
<td>Parent (unselected)</td>
<td>22</td>
</tr>
</tbody>
</table>

a Cells attached to type IV collagen in the presence of laminin.

related to antiseria toxicity since at the concentrations used, 1:100 dilution, an increase in toxicity of only 3% over complement alone was noted (Table 2). Two replicate experiments using preimmune sera treatment did not reduce metastasis formation (data not shown).

We next examined the retention in the lungs and metastatic potential of cells which attached or did not attach by laminin. The retention of i.v.-injected [3H]thymidine-labeled tumor cells in the lungs is shown in Table 3. At all time points, cells which attached to laminin in vivo remained longer in the lungs.

DISCUSSION

Basement membranes contain a dense zone rich in a unique collagen (type IV) plus other basement membrane-specific proteins including the glycoprotein, laminin. We and others have shown that the basement membrane collagen is resistant to attack by the animal collagenases that degrade interstitial collagens (16). Degradation of basement membrane collagen involves a separate type IV collagenase. Metastatic tumor cells have been shown to be high producers of this specific type IV collagenase (16). Metastasizing tumor cells traverse organ boundaries and capillary walls to initiate colonies in distant sites. In the process, they encounter basement membranes which are continuous sheets of extracellular matrices which lie beneath epithelial and endothelial cells (14).

The initial interaction between metastatic cells and basement membranes is probably attachment. Recent studies have shown that many cells do not bind directly to collagen but utilize various attachment proteins such as fibronectin, chondro-nectin, or laminin (12). Previously, it was shown that certain metastatic cells attached more rapidly to type IV than to type I collagen while nonmetastatic cells preferred type I (19). Here we show that metastatic and nonmetastatic cells use different attachment proteins to interact with different collagens. Laminin stimulated the attachment of the metastatic cells to type IV collagen, while fibronectin stimulated the attachment of non-

metastatic tumor cells to both type I and type IV collagen. Both the rate of attachment and the percentage of the cells that attached were increased in the presence of the active factors. Serum stimulated the attachment of the nonmetastatic cells but not the metastatic cells. This is expected, since serum contains fibronectin but little or no laminin (25). The tumor cells attached slowly to collagens in the absence of added attachment factors. Such attachment was dependent on the synthesis of attachment proteins by the cells. Furthermore, antibody to laminin blocked the attachment of the metastatic cells, and antibody to fibronectin blocked the attachment of the nonmetastatic cells. Thus, at least for the cells studied here, metastatic murine melanoma and sarcoma cells preferentially utilize laminin rather than fibronectin to bind to type IV collagen.

This finding is in contrast to the attachment studies of other investigators. Nicolson et al. (22) reported that melanoma cells utilize fibronectin to attach to subendothelial matrix produced by cultured endothelial cells. Vlodavsky and Gospodarowicz (29) reported that tumors of fibroblast origin (sarcomas) utilize fibronectin for attachment whereas tumors of epithelial origin (carcinomas) utilize laminin. The latter attachment studies were performed on subendothelial matrix from cultured cells. The present report differs from these previous studies in that it utilizes purified mouse basement membrane (type IV) collagen as a substrate. Present attachment studies in our laboratory have shown for both murine and human tumor cells the attachment factor utilized is unrelated to the histological type of origin. However, it is certainly not justifiable to conclude that the present findings represent a constant difference distinguishing metastatic from nonmetastatic cells.

We further tested the metastatic activity of cells separated on the basis of attachment mediated by laminin or fibronectin. The C3H sarcoma cells, irrespective of added attachment proteins, remained nonmetastatic. The cells that attached, mediated by laminin, from the metastatic sarcoma and melanoma lines were retained in the lungs longer and were more metastatic than either the parent cells or those that did not bind rapidly, mediated by laminin. Thus, an affinity for laminin appears to correlate with metastatic activity. It is likely that laminin is also necessary for metastasis since fewer lung tumors occurred when metastatic cells were injected after preincubation with antibody to laminin. The present selection experiments were performed using tumor cells attached for 120 min. It is possible that further selection of metastatic tumor cells can be achieved by using shorter attachment time periods.

The attachment factor utilized by the tumor cell in vivo may be derived either from the host or from the tumor cell itself. At present, it is not possible to conclude from our studies whether the endogenous or exogenous attachment factor is more important to the biology of metastases.

The mechanism of the increased metastatic potential of the laminin-attached tumor cells was partially demonstrated by experiments in which (a) tumor cells were injected along with laminin antibodies and (b) the retention of labeled tumor cells in the lung was measured. The presence of laminin antibody markedly reduced the number of lung metastases with both metastatic cell lines (Table 1). This effect was not mediated by direct cytotoxicity because, as shown in Table 2, anti-laminin antibodies in the presence of complement did not cause tumor

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cell lysis. However, it cannot be ruled out that anti-laminin antibodies stimulated tumor cell killing in vivo by a cell-mediated immunity mechanism involving macrophages or natural killer cells. Laminin-attached tumor cells also showed a much greater retention in the lung compared to nonattached cells (Table 3). The injected cells were matched in viability and were both injected as a suspension of single cells. Consequently, the difference in retention was not due to a difference in viability or in the clumping of the 2 cell populations injected. Nevertheless, it is possible that the laminin-attached population once reacted with blood components formed clumps in vivo and were rapidly cleared. Alternatively, the unattached cells may have been in a nonproliferative state or may be more sensitive to host immune cell killing.

Interestingly, BL6 melanoma and PM2 sarcoma cells which had been exposed to fibronectin and selected for attaching and nonattaching cells produced fewer lung metastases. The reduction in the number of lung metastases may be due to a reduced retention of the cells in the lung. Alternatively, it is known that exogenous fibronectin alters the phenotype of tumor cells, chondrocytes, liver cells, and myoblasts suppressing certain characteristic activities (reviewed in Refs. 12 and 31). It is possible that fibronectin suppresses one or more properties of the cells required for metastases. It is likely that culture of tumor cells in serum containing fibronectin may give a selective advantage to nontumorigenic cells.

It has been shown in vivo by Warren and Vales (30) and Poste and Fidler (24) that, in large vessels, tumor cells bind preferentially to regions of exposed basement membrane. In vivo, circulating tumor cells may utilize laminin to bind to capillary or venule basement membrane. Tumor cells which utilize laminin preferentially may have a selective advantage in forming metastases.

REFERENCES

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