Quantitative in Vitro Assay for Tumor Cell Invasion through Extracellular Matrix or into Protein Gels

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ABSTRACT

A new quantitative method for the study of tumor cell invasion in vitro is presented. It is intended to facilitate the study of the mechanisms of invasion using an isolated basement membrane without the involvement of stromal structures or using defined protein gels. Cells are allowed to migrate through the pores of a Nuclepore polycarbonate filter into a protein gel on a nitrocellulose filter, or they may have to penetrate a cell-derived extracellular matrix (ECM) to reach the gel. Experiments with a nonmetastatic mouse lymphoma (Eb) and its two metastatic variants (ESb and ESb-MP) showed that the metastatic lines penetrated a Matrigel (a gel containing the components of a basement membrane) much better than the nonmetastatic cell line, but only the most metastatic line (ESb) was able to penetrate into a native collagen I gel. The presence of an ECM on the polycarbonate filter reduced the number of cells invading a fibrin gel, demonstrating that the dense, fibrillar structure of the cell-derived ECM was a barrier to the tumor cells. The metastatic lines penetrated the ECM to a 4- to 6-fold higher extent than the nonmetastatic cell line. It is concluded that in order to metastasize efficiently, the tumor cells must be able to penetrate many different kinds of barriers.

INTRODUCTION

The invasive capacity of tumor cells has attracted considerable interest in cancer research, since their invasion of blood vessels is considered to be a crucial step in metastasis. Distant metastases are believed to be formed largely from blood-borne tumor cells, which have been able to attach to and penetrate the vessel wall. Cells that fail to do this are believed to succumb in the circulation (1). Therefore, understanding the mechanisms of invasion may help the development of antimetastatic therapies.

Invasion is a complex process involving cell adhesion, motility, and the secretion of different classes of enzymes. This and the fact that mechanisms of invasion are very difficult to study in vivo call for the development of adequate in vitro methods.

Several in vitro invasion assays have been described, but thus far no single system has found widespread use. In particular, quantitative methods have proven difficult to develop. We present here a flexible system for the quantitative estimation of tumor cell penetration into protein gels of different composition, as well as through a cell-derived ECM, making it possible to study invasion through an isolated ECM without interference from stromal tissues. Thus, invasion through a complete, structured ECM can be investigated as well as invasion into a gel consisting of basement membrane components (Matrigel) or even gels containing single, defined ECM components. This would make the system suitable for investigating the mechanisms of invasion.

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1 To whom requests for reprints should be addressed. Supported by grants from Odd Fellows Svenska Storstolge and Deutsche Forschungsgemeinschaft (SFB 136).

2 The abbreviations used are: ECM, extracellular matrix; PBS, phosphate-buffered saline, pH 7.4, containing 8.0 g of NaCl, 0.2 g of KCl, 1.14 g of NaH2PO4, and 0.2 g of KH2PO4 per liter.

MATERIALS AND METHODS

Cells. The Eb murine T-cell lymphoma and the ESb variant line, exhibiting a highly increased metastatic potential (2), were propagated in suspension culture using RPMI 1640 supplemented with 10 mM N-2-(hydroxyethyl)-1-piperazine-N'-2-ethanesulfonic acid, 2 mM glutamine, 5% heat-inactivated fetal calf serum (all from Gibco, Paisley, Scotland), and (for ESb) 50 g/L mercaptoethanol. The adhesin ESb variant ESb-MP, which exhibits a delayed formation of metastases (3), was propagated in monolayer culture with mercaptoethanol-containing medium. The PF-HR9 cell line, a nontumorigenic murine teratocarcinoma differentiated to form an endodermal-like cell line (4), was cultured in Dulbecco’s minimal essential (Flow, Irvine, Scotland) supplemented with 10 mM N-2-(hydroxyethyl)-1-piperazine-N'-2-ethanesulfonic acid, 2 mM glutamine, and 10% fetal calf serum. Antibiotics were not used. Cultures were regularly screened for the presence of Mycoplasma.

 Pretreatment of Polycarbonate Filters. To allow cell attachment the Nuclepore polycarbonate filters (No. 110414 from Nuclepore, Pleasanton, CA) were treated as follows: filters were etched in a fresh solution of metallic sodium in absolute methanol (2 mg/ml) for 30 s, rinsed twice in methanol, dried, and placed on top of a drop of 25% glutaraldehyde (dissolved in water) for 1 h. After rinsing in distilled water, filters were placed on top of drops of a casein solution (10 mg/ml in PBS, pH 7.0) for 1.5 h and, after another rinse in distilled water, again treated with 25% glutaraldehyde for 30 min. Subsequently, filters were rinsed and incubated in distilled water overnight on a gyratory shaker adjusted to keep the filters in gentle, constant movement. After drying, filters were sterilized with 70% ethanol and used for invasion experiments with different gels.

 Filters used for culture with HR9 cells were etched, treated once with glutaraldehyde (see above), and incubated in water overnight. Subsequently, the dull sides of the filters were wetted with a fibrinogen solution (1 mg/ml in PBS; No. F-4883, from Sigma, St. Louis, MO) mixed 10:1 with thrombin (4 units/ml; No. T-6634 from Sigma), and the filters were placed on top of the wells in a 48-well tissue culture cluster (No. 3448 from Costar, Cambridge, MA) with the wet side down for 1 h, allowing polymerization of the gel. The wells have a diameter of 11 mm; thus the 13-mm filters were supported on the edges, allowing the formation of a gel covering one filter surface and filling the pores. The wells were partly filled with water to maintain humidity. After polymerization, the filters were air-dried, during which time the gel contracted, reopening the pores, and the gel-covered side of the filters was then more treated with 25% glutaraldehyde for 30 min. Rinsing and final incubation in water were carried out as described above.

 Invasion Assay with Collagen I Gel. Fifty g of a sterile preparation of native type I mouse tail collagen (1 mg/ml) in 0.1% acetic acid (5) were added to a 13-mm nitrocellulose filter with a pore size of 5 pm (No. AE98 from Schleicher and Schuell, Dassel, Federal Republic of Germany) placed in a 4-well tissue culture plate (No. 134673 from Nunc, Copenhagen, Denmark). The filters had been previously sterilized by 100 kilorads γ-irradiation using a 137Cs source. A pretreated polycarbonate filter (see above) was positioned on top of the nitrocellulose filter with the dull side up, and 0.5 ml of a 1% NH4OH solution was placed into the space between the wells to polymerize the gel. In this way, evaporating NH4 gas could neutralize the acetic acid in the collagen solution, initiating polymerization. The plate was sealed with Parafilm and allowed to stand for 1 h at room temperature, whereupon the NH4OH solution was removed and the plate was rinsed with 3 ml sterile water. Each well containing a filter was rinsed once and filled with 1 ml serum-containing medium, and the plate was incubated in...
10% CO₂ for 3 h to attain a neutral pH. The filters were then ready for use.

Invasion Assay with Fibrin Gel. A mixture of fibrin (5 mg/ml) and casein (10 mg/ml; No. 48005 from Serva, Heidelberg, Federal Republic of Germany) was dissolved in serum-containing medium by gentle stirring for 30 min, followed by centrifugation for 5 min in an Eppendorf centrifuge and sterilization by filtration through a 0.2-µm filter. This solution was mixed 10:1 with thrombin (4 units/ml, sterile), vigorously stirred for 30 s on a vortex mixer, and used within 5 min. Of this mixture, 30 µl were added to nitrocellulose filters in 4-well plates, and pretreated polycarbonate filters (see above) were rapidly placed on top of the gel mixture with the dull side up. Plates were sealed with Parafilm and were allowed to polymerize for 2 h at room temperature.

Invasion Assay with Extracellular Matrix. For production of ECM, polycarbonate filters pretreated for cell culture (see above) were seeded with HR9 cells in 16-mm tissue culture wells and, after confluence, transferred to new wells and cultured in medium supplemented with dextran (4% w/v; No. D-4133 from Sigma) and ascorbic acid (50 µg/ml). The medium was changed every second day. After 10 days, the filters were rinsed in sterile distilled water for 2 min. This treatment caused swelling and lysis of the cells, and the ECM was subsequently extracted by treatment for 1 min with 20 mM NH₄OH followed by rinsing with serum-containing medium or PBS with 10% serum. The filters were then incubated with medium in an incubator with 10% CO₂ overnight. ECM-coated filters were stored in PBS at 4°C. For experiments, the wet filters were placed in an empty tissue culture dish and dragged over the surface of the dish using a pair of forceps. This treatment removed cell debris adhering to the underside of the filter, as well as excess liquid. The moist filters were then immediately placed on a fibrin gel mixture as described above. The integrity of the ECM was tested by placing an ECM-coated filter on a moist nitrocellulose filter and adding a drop of dialyzed India ink on top of the sandwich. After 5 min the filters were separated, and any leakage could be seen as a stain on the lower filter.

Invasion Assay with Matrigel. Nitrocellulose filters were wetted on one side with a fibrin/casein/thrombin gel mixture (see above), placed in 4-well plates with the wet side down, and allowed to polymerize for 30 min in the Parafilm-sealed plates. Subsequently, plates were placed on an ice-cooled metal support, and 10 µl ice-cold Matrigel (No. 40234 from Collaborative Research, Inc., Lexington, MA) were applied to each filter using a precooled pipet. A pretreated polycarbonate filter was put on top of the gel drop (dull side up), and its position was adjusted to make sure that the gel filled the entire space between the filters. The plate was sealed with Parafilm and allowed to polymerize for 1 h at room temperature. It was necessary to use a fibrin gel to “saturate” the nitrocellulose filter, since otherwise some components of the fluid Matrigel would be absorbed by the dry filter, and others would remain on top of it.

Invasion Experiment. The principle of an invasion assay is depicted in Fig. 1. Monolayer cultures of ESB-MP cells were harvested by treatment with 0.02% EDTA; the cells were centrifuged, resuspended in complete medium, and counted in a hemocytometer. Suspension-grown ESB and ESB cells were also centrifuged and resuspended in fresh medium. One ml of a cell suspension containing 10⁶ cells/ml was placed in each well with an invasion gel and also into empty wells for assaying the cell number. After 20 h incubation (if not otherwise indicated) at 37°C, cells in these wells were counted, the medium was removed from the filter-containing wells, and the filters were fixed with 2.5% glutaraldehyde in PBS for 1 h or, in the case of Matrigel, overnight. Subsequently, the filters were carefully separated, and the upper (polycarbonate) filters were stained with Giemsa solution (No. 9204, Merck, Darmstadt, Federal Republic of Germany) for 30 s, briefly destained in 50% ethanol, rinsed in water, and air-dried. The lower (nitrocellulose) filters were placed in a staining basket, stained with hematoxylin (6), and finally embedded in Canada balsam on a slide together with the corresponding polycarbonate filter. Invading cells present on the lower nitrocellulose filter were counted under the microscope in 5 to 13 predetermined fields (covering 2-9% of the filter surface) using a ×10 objective. For counting cells attached to the upper polycarbonate filter a ×25 objective was used. The total number of cells present on each filter was calculated, and “invasion” was expressed as the ratio of the number of cells present on the lower filter to the total number of cells present on both filters. Adhesion was estimated by dividing the number of cells found on both filters with the cell number in the wells without filters, multiplying this quotient by 1.515. This factor corrects for the fact that the filter has a smaller diameter (13 mm) than the well (16 mm) and, consequently, only 66% of the cells added will come in contact with the filter.

As an alternative to this staining and counting procedure, radio-labeling the cells with 75Se-methionine was tested to assay cell numbers. It turned out, however, that radioactivity secreted from the labeled cells was bound to the fibrin gel in a manner that could not be corrected for; therefore the work with labeled cells was discontinued.

Adhesion Experiment. Adhesion of tumor cells to different gels was assayed in a hanging-drop microassay (7). Five µl of a fibrin gel, Matrigel, or rat collagen I gel were allowed to polymerize in the wells of a Terasaki plate (No. 653180; Greiner, Nürtingen, Federal Republic of Germany) as described. A 20-µl drop of a cell suspension containing 10⁴ cells/ml was placed in each well, and the plate was placed in an incubator at 37°C for 1 h. Subsequently, the plate was carefully inverted and allowed to stand for 30 min before the cells were counted under an inverted microscope. Cells adherent to the gel surface and nonadherent cells in the hanging drop were counted separately in each well. Adhesion was expressed as the number of adherent cells divided by the total number of cells in that well.

Statistics. Results were evaluated by the Kruskal-Wallis test with correction for multiple comparisons according to the method of Dunn.

RESULTS

Adhesion of Cells to the Upper Filter. The cells must adhere to the upper surface of the polycarbonate filter before they are able to penetrate into a gel. These filters are rather hydrophobic and therefore do not normally allow cell attachment. With the etching/fixing procedure described here, 20–60% of the cells (depending on cell line and filter batch) attached to the filter. Each set of experiments (i.e., each table or figure) was performed using filters from the same batch. This filter treatment was, however, not sufficient for ECM coating; the ECM tended to detach after extraction, and the rather laborious procedure of glutaraldehyde fixing a dried gel layer had to be used to

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produce a filter surface to which the ECM was firmly attached. It was necessary to perform the surface treatment on only one side of the filter, since otherwise some invading cells would attach to the underside of the upper filter instead of invading the underlying gel.

Invasion into a Fibrin Gel. Eb and ESb cells penetrated the fibrin/casein gel to roughly the same extent, while ESb-MP cells were several times more active (Table 1). Eb and ESb cells present within the gel were mostly round and lacked processes. The ESb-MP cells, in contrast, frequently had a spread-out morphology with several processes (Fig. 2), which is most probably due to the fact that the ESb-MP cells adhere better to a fibrin gel than cells from the other two lines (Table 2). The pore size of the polycarbonate filters used does not seem to be a critical factor for invasion, since ESb-MP was more invasive than Eb or ESb with both 5- and 8-µm filters (Tables 1 and 3).

Table 1 Penetration of tumor cells into protein gels

<table>
<thead>
<tr>
<th>Cells</th>
<th>Fibrin gel (%)</th>
<th>Collagen I gel (%)</th>
<th>Matrigel (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Invasion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eb</td>
<td>8.3 ± 1.0</td>
<td>1.7 ± 0.3</td>
<td>4.7 ± 0.6*</td>
</tr>
<tr>
<td>ESb</td>
<td>6.5 ± 0.9</td>
<td>5.3 ± 1.1*</td>
<td>29.3 ± 1.8#</td>
</tr>
<tr>
<td>ESb-MP</td>
<td>35.5 ± 1.4*</td>
<td>1.1 ± 0.2</td>
<td>32.1 ± 2.6</td>
</tr>
<tr>
<td>Adhesion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eb</td>
<td>44.7 ± 5.3</td>
<td>36.6 ± 4.6</td>
<td>49.0 ± 7.3</td>
</tr>
<tr>
<td>ESb</td>
<td>46.3 ± 5.8</td>
<td>40.4 ± 6.4</td>
<td>38.2 ± 8.9</td>
</tr>
<tr>
<td>ESb-MP</td>
<td>43.1 ± 5.5</td>
<td>69.2 ± 6.6</td>
<td>40.6 ± 5.6</td>
</tr>
</tbody>
</table>

* Significantly less than Matrigel invasion by ESb and ESb-MP (P < 0.001) and fibrin gel invasion by Eb (P < 0.05).
# Significantly more than collagen gel invasion by Eb and ESb-MP (P < 0.02 and 0.001, respectively).
+ Significantly more than fibrin gel invasion by ESb (P < 0.0001).
* Significantly more than fibrin gel invasion by Eb and ESb (P < 0.0001).

It was necessary to perform the surface treatment on only one side of the filter, since otherwise some invading cells would attach to the underside of the upper filter instead of invading the underlying gel.

Invasion into a Collagen I Gel. The rapidly disseminating ESb tumor cells were able to invade the native collagen I gel to a significantly higher extent than the nonmetastatic Eb or the slowly metastasizing ESb-MP cells (Table 1). In this case, adhesion to the gel was not parallel to invasion, since ESb-MP cells adhered much better than Eb cells (Table 2) although their ability to invade was identical (Table 1). The kinetics for the invasion of ESb and ESb-MP cells into the gel were similar, reaching a plateau after approximately 20 h (Fig. 3).

Invasion into a Matrigel. When invasion into a Matrigel was tested, a clear-cut difference between metastatic and nonmetastatic cell lines was observed; ESb and ESb-MP cells were significantly more invasive than Eb cells (Table 1), but there was no difference between the rapidly metastasizing ESb and the more slowly disseminating ESb-MP cell lines. A correlation between malignancy and invasion into a Matrigel has previously been reported for several cell lines (8). With Matrigel, adhesion was parallel to invasion; Eb cells had the lowest value with both parameters (Tables 1 and 2).

Invasion into an ECM. Under the culture conditions used, the HR 9 cells secrete a continuous, highly structured ECM several µm thick (9). The extracted ECM can be visualized by staining with Coomassie blue; it appears as a continuous sheet virtually free from holes but with some variation in thickness (Fig. 4). The extracted ECM was partly covered by cell debris from the HR 9 cells, but any attempt to remove this debris resulted in damage to the ECM. The use of 8-µm polycarbonate filters sometimes resulted in a leaky ECM, but with filters with a pore size of 5 µm the ECM was regularly impermeable to colloidal carbon. As can be seen in Table 3, the highly metastatic ESb cells invaded the ECM to a 6-fold higher degree than the nonmetastatic Eb line, while the ESb-MP cells were 4-fold more invasive.

DISCUSSION

The invasive properties of malignant tumor cells have been attributed a central role in the process of metastasis, and the penetration of the blood vessel wall has received particular interest since metastasis is considered to occur mainly via the circulatory system. A considerable number of systems have been devised for the in vitro study of invasion (10, 11), but no single system is as yet in general use. Every system has its drawbacks, and the investigator must choose the system most appropriate for the study of a certain aspect of the complex process of metastasis. To invade a blood vessel wall, the tumor cell has to penetrate the vascular endothelium and the basement membrane to reach the parenchymal tissue. There are several in vitro methods...
methods for studying the penetration of a layer of endothelial cells, but only one assay for the quantitative determination of tumor cell invasion through a basement membrane has been described (12), using denuded human amnion membrane. This method is hampered by the fact that in addition to the basement membrane, the cells must penetrate approximately 0.5 mm of stromal tissue before they are detected on the other side of the barrier, a distance that is far too great for a relevant model of a small blood vessel. To overcome this obstacle, Migatti et al. (11) used [125]iododeoxyuridine-labeled cells to assay cells present within the amnion. It is not clear, however, whether cells that have penetrated the ECM but have failed to invade the stromal layer are detected by this method. That cells able to penetrate an ECM may not readily invade a collagensous stroma is indicated by the behavior of EB versus ESb-MP cells (see below). Furthermore, metabolic [125]iododeoxyuridine labeling of cells is hazardous, since radiotoxicity at dose levels normally used for labeling can severely impair the in vitro proliferation and s.c. transplantability of the cells and probably their metastatic properties as well (13).

The invasive behavior of EB and ESb cells has previously been investigated in vitro using lung tissue (2), brain tissue (15), and monolayers of vascular endothelial cells (15, 16) with the consistent finding that the metastatic ESb cells exhibit a more invasive behavior than the nonmetastatic EB cells. Here we show that ESb cells are also superior in invading an isolated fibrin gel system. Polycarbonate filters with pore sizes of 5 μm with or without ECM were used. "Adhesion" and "Invasion" are defined as in Table 1. Values represent the mean ± SE of at least nine filters collected from at least three different experiments.

Table 1: ECM penetration by tumor cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>Fibrin gel (%)</th>
<th>Collagen I gel (%)</th>
<th>Matrigel (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EB</td>
<td>48.8 ± 1.2</td>
<td>4.9 ± 1.1*</td>
<td>35.6 ± 1.8*</td>
</tr>
<tr>
<td>ESb</td>
<td>19.6 ± 3.4</td>
<td>44.0 ± 3.2</td>
<td>61.9 ± 4.2</td>
</tr>
<tr>
<td>ESb-MP</td>
<td>68.7 ± 1.5*</td>
<td>28.1 ± 2.5</td>
<td>56.1 ± 2.4</td>
</tr>
</tbody>
</table>

*Significantly less than ESb or ESb-MP (P < 0.0001 and 0.0005, respectively).
#Significantly less than ESb or ESb-MP (P < 0.0001 and 0.0002, respectively).

Invasion after 20 h incubation in serum-containing medium using the fibrin gel system. Adhesion and invasion are defined as in Table 1. Values represent the mean ± SE of at least nine filters collected from at least three different experiments.

Table 2: Adhesion of tumor cells to protein gels

<table>
<thead>
<tr>
<th>Cells</th>
<th>Fibrin gel</th>
<th>Collagen I gel</th>
<th>Matrigel</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESb-MP</td>
<td>30.9 ± 5.3</td>
<td>72.8 ± 5.4</td>
<td>52.1 ± 1.6</td>
</tr>
<tr>
<td>ESb</td>
<td>56.8 ± 5.6</td>
<td>58.6 ± 4.1</td>
<td>62.1 ± 1.6</td>
</tr>
<tr>
<td>EB</td>
<td>51.2 ± 5.2</td>
<td>51.2 ± 5.2</td>
<td>66.6 ± 2.5</td>
</tr>
</tbody>
</table>

Values represent the mean ± SE of at least nine filters collected from at least three different experiments.

*Significantly more than ECM invasion by EB (P < 0.001).
#Significantly more than ECM invasion by EB (P < 0.02).

By comparing the invasion into a fibrin gel versus a Matrigel (Table 1), it can be seen that invasion of the highly malignant ESb cells into a Matrigel is 4-fold higher than that into a fibrin gel. On the other hand, for the nonmetastatic EB cells, invasion is halved, and no difference is noted for the ESb-MP cells. This may reflect a greater ability of ESb cells to penetrate the Matrigel, or a haptotactic effect by some basement membrane component(s). This question is currently under investigation using gels containing defined basement membrane components.

There is an inherent difficulty in the study of ECM invasion. There seems to be no effective method to assay the integrity of the very membranes used in invasion experiments. Nevertheless, we assume that the cells present on the lower filter have actively penetrated the ECM since: (a) the staining and surveying of membranes from the same batch demonstrated an essentially faultless ECM; (b) the ECM was impermeable to colloidal carbon; (c) ESb cells penetrated the ECM to a 6-fold higher extent than EB cells, but both were equally effective in invading a fibrin gel. In Table 3, "ECM invasion" has been calculated assuming that cells able to penetrate the ECM are able to invade a fibrin gel as well. If it is presupposed, however, that cells penetrate ECM and fibrin gels by different mechanisms, the cells present on the lower filter have been selected by two criteria, and the reduction in fibrin gel invasion caused by an ECM should be calculated. In this case, EB and ESb-MP invasion into the fibrin gel is reduced by approximately 90%, and ESb invasion is reduced by 50%. Thus, ESb cells are more invasive than EB cells under all circumstances, while the invasive capacity of the ESb-MP cells is more ambiguous. It should be pointed out that this arrangement is just one of several possible; another gel or a gel-free system like the one used by Albini et al. (8) could be used.

The invasive behavior of EB and ESb cells has previously been investigated in vitro using lung tissue (2), brain tissue (15), and monolayers of vascular endothelial cells (15, 16) with the consistent finding that the metastatic ESb cells exhibit a more invasive behavior than the nonmetastatic EB cells. Here we show that ESb cells are also superior in invading an isolated ECM as well as a Matrigel or a collagen I gel; it seems that the metastatic phenotype of the ESb cells enables them to penetrate many different kinds of tissue barriers. It should be noted that although there is a considerable difference in the malignant
behavior of ESb and ESb-MP cells in vivo (3), there is no significant difference in their invasion of an ECM or a Matrigel. There is, however, a substantial difference in the invasion of a collagen I gel, and this suggests that the rapid dissemination of ESb cells in vivo is due to a capacity to invade tissue stroma as well as basement membranes.

In conclusion, we hope that this model will prove useful for the study of the mechanisms of basement membrane invasion by tumor cells.

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