An in Vitro Quantitative Assay for Tumor Cell Invasion

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ABSTRACT

An in vitro quantitative assay of tumor cell invasion is described. The assay measures the rate at which [\(^{125}\)I]iododeoxyuridine-labeled tumor cells migrate through the chorioallantoic membrane of developing chicken embryos.

Invasion chambers were prepared from amber latex cylinders, chorioallantoic membrane, and thread ligatures. The chambers were placed in glass vials to rest on discs of photographic sponge immersed in tissue culture medium. Labeled tumor cells were added to the chamber, and the vial was incubated at 37°C. At various time points individual chambers were monitored to determine the number of viable, labeled cells that had passed through the membrane and were present in the medium, on the glass vial, or in the sponge.

The application of the assay may be of use in delineating the mechanisms of tumor cell invasion.

INTRODUCTION

Malignant neoplasms are characterized by their ability to invade a host's tissues and then spread to and grow in distant organs. Despite the fundamental importance of invasion in the pathogenesis of tumor dissemination, relatively little is known about the mechanisms underlying this process. Although many studies have been performed on interactions between malignant cells and a variety of normal tissues (1-3, 10, 12, 14-17), these have been mainly qualitative rather than quantitative in nature.

Few attempts have been made to examine the phenomenon of invasion in a quantitative manner. Mutual invasion in cocultivations of normal fibroblasts and sarcoma cells grown in vitro has been estimated quantitatively (1). Despite good correlations between the in vitro and in vivo behavior of sarcoma cells, the biological relevance of this model is doubtful. An alternative assay measuring the invasive capacities of a variety of tumor cell lines, as determined from in vitro invasion studies, which used \(^{51}\)Cr release from labeled fragments of embryonic chick heart and mesonephros, have been described (12). Histological evidence of invasion was not associated with changes in \(^{51}\)Cr release as compared to the control cultures. \(^{51}\)Cr, however, is not firmly bound to cells, and thus its release cannot be equated with cell loss or death (4). This major disadvantage can be overcome with the use of tumor cells labeled in vitro with \(^{125}\)IIdUrd. This analog of thymidine is incorporated exclusively into the DNA of proliferating cells and is released only after cell DNA breaks down following cell death.

Several questions about the process of tumor cell invasion remain unanswered. Is the process solely dependent on the properties of tumor cells or is it modified by host factors? Are all tumor cells within a malignant population equally invasive or is the phenomenon due to a specialized invasive subpopulation? A suitable technique permitting the quantitative analysis of tumor cell invasion in vitro would be of considerable help in the clarification of these similar problems.

This report describes an in vitro quantitative assay of tumor cell invasion that measures the rate at which \(^{125}\)IIdUrd-labeled cells pass through the chorioallantoic membrane of chicken embryos.

MATERIALS AND METHODS

Eggs. Fertile chicken eggs (Gaines Hatchery, Frederick, Md.) were incubated for 14 to 16 days in a humidified chamber at 37°C.

Tumor Cell Lines. The B16 melanoma syngeneic to the C57BL/6 mouse (5), the UV-induced 2237 fibrosarcoma (8) syngeneic to the C3H/HeN- mouse, a hamster sarcoma virus-transformed hamster sarcoma line (provided by Dr. G. H. Poste, Roswell Park Memorial Institute, Buffalo, N. Y.), and NIH/3T3 fibroblasts (provided by Dr. J. Ihle) were maintained on plastic in CMEM. Supplementation of media and growing conditions have been detailed elsewhere (6). The cells were prelabeled with \(^{125}\)IIdUrd as previously described (4).

Preparation of Invasion Chambers. The invasion chamber consists of a latex rubber cylinder, a piece of chorioallantoic membrane, and a thread ligature. The chamber is placed in a glass vial containing a disc of photographic sponge and CMEM.

The closed ends were cut off 2-inch-high amber latex
rubber bulbs (Scientific Products, Columbia, Md.) to yield open-ended cylinders that were then washed, rinsed, and autoclaved. Glass vials (25 × 80 mm; Amersham/Searle Corp., Arlington Heights, Ill.) were washed, rinsed in distilled water, and autoclaved. Cellulose photographic sponge (Samigon Division/Agraph Corp., Carlstadt, N. J.) was cut into cylinders (2 mm high) with a diameter of 1.5 cm. These discs were prepared and sterilized according to the method of Leighton (10). Briefly, the sponges were boiled 3 times in distilled water for a total of 90 min before immersion for 15 min each in acetone, ether, and ethanol. They were then boiled 3 times in distilled water for a further 90 min and then autoclaved.

All procedures were then performed in a laminar-flow hood. A sterile photographic sponge was added to each autoclaved glass vial. The fertile eggs, which had previously been candled to determine fertility, were washed in 70% ethyl alcohol prior to being cracked. The embryo and its surrounding membranes were deposited in a sterile 100-mm plastic Petri dish. The chorioallantoic membrane was gently washed with 10 ml CMEM. A small nick was made in the chorioallantoic membrane, and the rimmed end of the rubber bulb was inserted into the allantoic cavity. A thread ligature was then placed around the rim and tied tightly to capture the stretched chorioallantoic membrane across the open mouth of the rubber cylinder with the ectoderm on the outside. The bulb and attached chorioallantoic membrane were placed into a glass vial containing a photographic sponge cylinder and 5 ml CMEM (Fig. 1; Chart 1).

**Assay for Tumor Cell Invasion.** [125I]IdUrd-labeled tumor cells were harvested by a 1-min trypsinization (0.25% trypsin-0.02% EDTA solution), washed twice in Hanks’ balanced salt solution to remove all nonbound radiolabel, and resuspended in CMEM. The number of single, viable tumor cells was determined and adjusted to 2 × 10^6 cells/ml CMEM. Tumor cell suspension (0.5 ml of a total of 1 × 10^6 cells) was added to each chamber. The vial was capped loosely and incubated in a humidified atmosphere at 37° (5% CO₂).

At various times after the addition of the tumor cells, individual invasion chambers were monitored to determine the number of tumor cells that had passed through the chorioallantoic membrane. Prior to radioactive monitoring and immediately after removal from the glass vial, 0.5 ml of India ink was added to each invasion chamber to ascertain the integrity of the chorioallantoic membrane. Only chambers retaining the India ink for at least 10 min were assayed, whereas those that showed signs of leakage were discarded.

Intact invasion chambers were placed in a fresh glass vial, and the radioactivity contained within was monitored in a γ counter (Model 1185; Searle Analytic, Des Moines, III.). The corresponding original vial, which contained the sponge disc and incubating CMEM, was also monitored for activity. The sponge was then removed and washed for 24 hr in 2 changes of methanol (to fix the cells) before both it and the methanol washes were counted. The incubating medium was filtered through a 0.45-μm filter held in a Swinnex 25-mm filter holder (Millipore Corp., Bedford, Mass.); the containing vial was washed twice with Dulbecco’s phosphate-buffered saline, and these washes were filtered through the same filter and added to the filtered medium. The filtrate, the filter, and the washed incubating vial were all counted separately. The totals of these counts plus those from the methanol washes and the sponge were checked to see that they closely approximated those counts originally obtained for the vial with the invasion chamber removed. Radioactivity in the cell-free filtrate and the methanol washes was taken to represent free [125I] or dead cells that had not attached to the sponge. Radioactivity in the filters and sponges was considered to represent viable cells that had moved through the chorioallantoic membrane and were either suspended in the incubating CMEM or were attached to the sponge, and radioactivity obtained from the washed glass vial represented tumor cells that, having invaded through the chorioallantoic membrane, had not attached to the sponge. Radioactivity in the filters and sponges was considered to represent viable cells that had moved through the chorioallantoic membrane and were either suspended in the incubating CMEM or were attached to the sponge, and radioactivity obtained from the washed glass vial represented tumor cells that, having invaded through the chorioallantoic membrane, plated onto the glass vial. The number of cells invading through the chorioallantoic membrane in a given time period was calculated from the total cells (cpm) added and the cpm associated with either live or dead cells within and without the invasion chamber.

**RESULTS**

Data from several representative experiments with the B16 melanoma are shown in Chart 2. Few labeled tumor cells traversed the chorioallantoic membrane from 1 to 48 hr, but by 72 hr there was a sharp increase in the number of cells found on the distal side of the membrane. Even so, the number of cells that crossed the membrane within 72 hr was only a small proportion (approximately 1%) of the total cells added to the chorioallantoic membrane. The phenomenon of tumor cell invasion appears to be an active process or is at least associated with tumor cell viability, since tumor cells heated to 80° for 10 min prior to addition to the
which previously had been incubated with tumor cells for 72 hr, were stretched onto lens paper for support and then counted associated with the filter and washed sponge did indeed represent tumor cells. To rule out the possibility that cpm on sponges and alcohol-rinsed sponges is taken to represent invasive capacity. In fact, the calculated number of 3T3 cells found on the distal side of the chorioallantoic membrane equaled that of dead tumor cells, and these figures appear to be associated with neoplastic cells since \[^{125}\text{I}]\text{IdUrd}\)-labeled NIH/3T3 fibroblasts did not exhibit an invasive capacity in vitro. In the individual experiments was observed in the absolute number of cells that moved through the chorioallantoic membrane. This may be due to variation in chorioallantoic membrane thickness caused by different stages of embryo development between separate batches of eggs. In addition, differences in levels of radiolabeling of the cells may lead to differences in final calculated cell numbers. Most work done during the development of this assay was carried out with the B16 melanoma, but 1 experiment with hamster sarcoma virus-transformed hamster sarcoma cells and 3 experiments with UV-induced 2237 fibrosarcoma are shown in Chart 3. The curves for the 3 tumor lines are different, which suggests that the tumor cells may have different capabilities for in vitro invasion. Some variation in the individual experiments was observed in the absolute number of cells that moved through the chorioallantoic membrane. This may be due to variation in chorioallantoic membrane thickness caused by different stages of embryo development between separate batches of eggs. In addition, differences in levels of radiolabeling of the cells may lead to differences in final calculated cell numbers.

In our assay radioactivity associated with Millipore filters and alcohol-rinsed sponges is taken to represent invasive cells. To rule out the possibility that cpm on sponges and filters were due to residual contamination by free radiolabel, we added free \[^{125}\text{I}]\text{IdUrd}\) to the chambers. At 1, 24, 48, and 72 hr later, chambers were terminated and processed as described above. As shown in Table 2, the final cpm from the filters and sponges were of an insignificant level (average, 76 cpm), notwithstanding the extremely high cpm obtained on the distal side of the chorioallantoic membrane (average, 130,629 cpm). This demonstrates that final counts associated with the filter and washed sponge did indeed represent tumor cells.

**Histology.** Representative chorioallantoic membranes, which previously had been incubated with tumor cells for 72 hr, were stretched onto lens paper for support and then fixed, sectioned, and stained. The stretching of the chorioallantoic membrane during the assay caused a marked reduction in its thickness but, despite some deterioration in tissue architecture, the membrane appeared to maintain its integrity, a finding corroborated by its ability to retain India ink. The majority of tumor cells were not adherent to the surface of the chorioallantoic membrane (Fig. 2) or, if they had been, were attached insufficiently to withstand the trauma of fixation and histological preparation. In some fields it was possible to find tumor cells that, presumably having migrated through the chorioallantoic membrane, had not attached to the photographic sponge or detached into the CMEM but had remained attached to the distal side of the membrane. At the light microscopic level, it was not possible to detect evidence of damage to the chorioallantoic membrane caused by the migrating cells, as might have been expected from the observed functional integrity of the membrane.

**DISCUSSION**

Our studies show that it is possible to assay quantitatively the invasive capacities of tumor cells in vitro. Although the chorioallantoic membrane of the embryonated chicken egg has been used by a number of investigators (1, 3, 13, 15) to study tumor cell invasion, none of these studies has been wholly quantitative in nature. In many of these investigations, the invasive tumor cells infiltrated and expanded into the loosely arranged stroma of the chorioallantoic membrane mesoderm; this, however, was not a feature of our study. It may be that the in vitro situation, coupled with the stretching of the chorioallantoic membrane across the bulb lumen, abolishes the loose and diffuse nature of the mesoderm, thus denying invading tumor cells access to this previously more penetrable tissue.

The successful development of this assay required the introduction of many modifications before arriving at the described procedure. Originally, the chorioallantoic membrane was stretched across the end of a plastic test tube (13 x 100 mm; Falcon Plastics, Oxnard, Calif.), but the unyielding nature of the plastic resulted in a number of slight disruptions in the integrity of the membrane at the site of the ligature. Substituting the soft, yielding rubber bulb for the plastic tube reduced this problem to insignificance. Resting the invasion chambers on the floor of the glass vial resulted in extremely variable numbers of tumor cells recorded as being on the distal side of the membrane. The photographic sponge was included in the assay to give tumor cells a matrix for attachment (10). It was not possible to detect cells in the sponge by histological examination, but this is not surprising in view of the large size of the sponge and the relatively small number of tumor cells suspected of being attached to it.

We postulate that the addition of methanol to the sponge causes viable tumor cells to contract and attach more firmly to the sponge fibers, and this hypothesis appears to be supported by the results obtained when free \[^{125}\text{I}]\text{IdUrd}\) is added to the chambers. Despite a cpm value fully 10 times that normally found on the distal side of the chorioallantoic membrane, the residual contamination of the sponge remains at markedly low levels compared with the counts.

![Chart 2. Migration of B16 melanoma cells through the chorioallantoic membrane (CAM).](chart2.png)
Table 1
Calculation and data processing of the invasion through chorioallantoic membrane of \[^{125}\text{I}ldUrd\]-labeled B16 melanoma cells

<table>
<thead>
<tr>
<th>Chamber components</th>
<th>1 hr</th>
<th>24 hr</th>
<th>48 hr</th>
<th>72 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chamber</td>
<td>403,121(^\text{b})</td>
<td>321,635</td>
<td>342,951</td>
<td>374,098</td>
</tr>
<tr>
<td>Sponge + medium</td>
<td>38</td>
<td>511</td>
<td>2,923</td>
<td>7,260</td>
</tr>
<tr>
<td>Filter</td>
<td>27</td>
<td>449</td>
<td>2,268</td>
<td>4,597</td>
</tr>
<tr>
<td>Vial</td>
<td>8</td>
<td>24</td>
<td>55</td>
<td>1,060</td>
</tr>
<tr>
<td>Methanol washes</td>
<td>4</td>
<td>7</td>
<td>13</td>
<td>28</td>
</tr>
<tr>
<td>Sponge</td>
<td>0</td>
<td>21</td>
<td>130</td>
<td>343</td>
</tr>
<tr>
<td>Viable cells(^c)</td>
<td>12</td>
<td>52</td>
<td>198</td>
<td>1,431</td>
</tr>
<tr>
<td>Estimated no. of cells(^d)</td>
<td>35</td>
<td>151</td>
<td>574</td>
<td>4,150</td>
</tr>
</tbody>
</table>

\(^{a}\) 1 \times 10^6 \[^{125}\text{I}ldUrd\]-labeled B16 cells were added into each chamber.

\(^{b}\) Mean of triplicate samples; variation from the mean did not exceed 10%.

\(^{c}\) Tumor cells on Millipore filter, adherent to the vial and sponge.

\(^{d}\) Calculated cpm/tumor cell.

Table 2
Data processing of invasion chambers incubated with free \[^{125}\text{I}ldUrd\]

<table>
<thead>
<tr>
<th>Chamber components</th>
<th>1 hr</th>
<th>24 hr</th>
<th>48 hr</th>
<th>72 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chamber</td>
<td>215,760(^\text{A})</td>
<td>63,310</td>
<td>52,815</td>
<td>19,940</td>
</tr>
<tr>
<td>Sponge + medium</td>
<td>15,270</td>
<td>157,350</td>
<td>170,418</td>
<td>190,200</td>
</tr>
<tr>
<td>Filter</td>
<td>13,195</td>
<td>137,930</td>
<td>155,950</td>
<td>179,478</td>
</tr>
<tr>
<td>Vial</td>
<td>22</td>
<td>76</td>
<td>80</td>
<td>84</td>
</tr>
<tr>
<td>Combined washes</td>
<td>615</td>
<td>6,943</td>
<td>8,694</td>
<td>5,902</td>
</tr>
<tr>
<td>Sponge(^c)</td>
<td>7</td>
<td>12</td>
<td>10</td>
<td>12</td>
</tr>
</tbody>
</table>

\(^{a}\) \[^{125}\text{I}ldUrd\] was placed into each chamber at a rate of 250,000 cpm.

\(^{b}\) Mean of triplicate samples, with variation from the mean not exceeding 10%.

\(^{c}\) Photographic sponge rinsed twice with methanol for 24 hr.

Chart 3. Migration of hamster sarcoma and UV 2237 fibrosarcoma tumor cells through the chorioallantoic membrane (CAM). Points, averages of 2 to 3 individual values.

obtained when labeled tumor cells are added to the invasion chamber. Nonetheless, the assay almost certainly underestimates the number of cells that have migrated through the chorioallantoic membrane, since some cells are attached to the distal side of the membrane (Fig. 2) and some cells are probably detached from the sponge during handling and are counted with the methanol washes. In this assay tumor cells were introduced onto the endodermal surface of the chorioallantoic membrane. The initial attachment of tumor cells to the ectodermal surface (3) may differ. The possibility that tumor cell invasion through the chorioallantoic membrane can be affected by the orientation of the membrane is currently under investigation.

Checking the integrity of the membrane with India ink eliminated the possibility that false results could be obtained as a result of tumor cells moving through preexistent holes in the chorioallantoic membrane. However, with this technique we cannot rule out the likelihood that invading tumor cells, possibly by the release of proteolytic enzymes, disrupt the integrity of the chorioallantoic membrane during the process of invasion. In this case the invasive capacity of the cell line would again be underestimated by our assay.

Bearing in mind these reservations, we feel that the assay system described offers considerable possibilities for the investigation of the multiple factors that are important in tumor cell invasion. The use of synthetic filter membranes allows for studies of tumor cell locomotion. In contrast, the invasion of tumor cells through the chorioallantoic membrane is probably far more complex and more closely approximates the conditions that prevail in vivo. The effects of tumor cell locomotion, different host cell populations,
protease inhibitors, serum components, and tumor cell products on invasion could all be investigated by the addition of various factors to 1 side or the other of the chorioallantoic membrane. If the labeling efficiencies of different tumor cell lines were comparable and if they were to be assayed on chorioallantoic membrane at a similar stage of development, then the invasive capacities of these cell lines could be compared. In preliminary experiments (data not shown), cloned tumor lines of a fibrosarcoma syngeneic to C3H mice (9) were tested for invasion in vitro. Cell lines that were found to be metastatic in vivo invaded the chorioallantoic membrane at higher rates than did cell lines that were not metastatic in vivo. After a 72-hr incubation only a small fraction of added tumor cells had moved through the thin chorioallantoic membrane. Whether this is a random phenomenon or whether it represents the presence of a small subpopulation of cells with an enhanced ability for invasion and metastasis (5, 7, 11) remains to be determined. The application of the invasion assay could be of fundamental importance in delineating these and other aspects of tumor cell invasion.

ACKNOWLEDGMENTS

We would like to thank Sheila Schweighofer for technical assistance.

REFERENCES

Fig. 1. Preparation of the invasion chamber. A, chorioallantoic membrane of 14-day-old chicken embryo; B, chorioallantoic membrane is draped over the end of rubber cylinder; C, thread ligature traps the chorioallantoic membrane around the end of rubber cylinder; D, trimming attached chorioallantoic membrane.
Fig. 2. Histological sections of the chorioallantoic membrane and B16 melanoma tumor cells. A and B, B16 cells in close proximity to the chorioallantoic membrane 24 hr after addition to the chorioallantoic membrane. A, × 100; B, × 400. C and D, 48-hr incubation period. Note tumor cell attachment to the endodermal surface of the chorioallantoic membrane and the beginning of penetration. C, × 100; D, × 400. E, 72-hr incubation period. The chorioallantoic membrane is still intact and tumor cells are attached to both the endodermal and ectodermal sides of the membrane. × 400.
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