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In vitro quantification of melanoma tumor cell invasion

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In order to quantify the invasiveness of melanoma tumor cells *in vitro*, a modification of the amniotic basement membrane (BM) model, described by Liotta *et al.* (*Cancer Letters*, **11**, 141, 1980), was used in combination with radiolabeled tumor cells. B16-F10 metastatic murine melanoma cells and a derived clone (B16-F10L) were prelabeled with 0·1 μCi/ml of [^{14}C]thymidine for 20–24 h in serum-free medium at 37°C. Following incubation, fetal bovine serum was added to a concentration of 5 per cent, and the cells were allowed to grow to confluency for the next 24–28 h. The labeled cells were seeded onto amniotic membranes situated in Membrane Invasion Culture System (MICS) chambers at a density of $2{\cdot}5 \times 10^4$ per well. At various times points, radioactivity of tumor cells that completely traversed the membrane was determined using an under-the-membrane sampling method. The average percent invasion demonstrated by the B16-F10 line was 2·75 per cent, and 3·65 per cent exhibited by the B16-F10L cell line after 48–53 h *in vitro*. Since it was apparent that some variability in thickness existed among membrane samples, a morphological analysis was performed on five sectors of a three-inch-diameter sample from four different placentae. Differences and similarities in BM thickness within the same sector were noted by this technique and could possibly contribute to some variability observed in tumor cell invasion in this model. Another parameter examined was the proliferation of tumor cells in the upper and lower wells of the MICS chambers. By 48 h, approximately 32·1 per cent of the B16-F10 cell line as well as the clone had replicated in the upper wells associated with the BMs compared with a 32·9 per cent replication in the lower wells, which reaffirmed the viability of the tumor cells under experimental conditions and insured similarly replicating populations of cells. In order to quantify the invasiveness of radiolabeled tumor cells accurately through a biological membranous barrier, the proper concentration of cells must be used, tumor cell heterogeneity should be taken into consideration, the technique of sampling radiolabeled invasive cells should be critically analysed, and thickness of the membranous barrier should all be considered as possible important factors in the quantitative analyses.

Introduction

The study of metastatic melanoma has been an area of serious focus for clinical and basic science research. One of the most devastating problems with this disease is that it can remain undetected in many patients until it advances to incurable stages. Malignant melanoma has been linked to environmental conditions as a major

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causative factor, and a marked true increase in incidence has occurred in the last 15 years [3]. At the present time, new methodologies and chemotherapy offer only marginal benefits in the treatment of this invasive disease [9, 10, 20, 24, 35].

One of the prominent characteristics of malignant neoplasms, especially melanoma, is the capability to invade tissues. The association of tumor invasion and metastasis is a multi-referenced observation [for review see 5, 6, 18, 19, 25, 28, 33, 43]. The two processes that are thought to lead to metastases may involve (1) a population of tumor cells which become invasive by entering a carrier system in the body or (2) the attachment of cells to a substratum subsequently leading to penetration of the established area and formation of a tumor deposit or metastasis (4). To invade and metastasize, tumor cells must surmount established biological frameworks which exist in host tissues. Since the arrangement of the architectural components in the extracellular matrix (ECM) does not easily allow the intrusion of alien cells, specific matrix-degrading activity is required during certain steps of the multi-faceted invasion cascade [1, 2, 17].

To investigate the invasive characteristics and associated properties of tumor cells, numerous innovative models have been presented [for review, see 18]. One of the more popular *in vitro* techniques described is the amniotic BM model [15, 40]. This particular model offers a number of advantages, including: (1) a large quantity of material which can be isolated from each human placental sample; (2) a membrane and connective tissue stroma with an ideal composition for studying the degradative interactions of tumor cells with an ECM, which is identical to an *in vivo* environment; and (3) the ease of conducting morphological, immunohistochemical, and biochemical studies in this system.

In the following experimental study we present a simple method for quantitating tumor cell invasion through amniotic membranes by the use of [^{14}C]thymidine labeling of metastatic murine melanoma cells (B16-F10 and B16-F10L). In addition, we show a morphological analysis of amnion thickness which statistically demonstrates the differences and similarities in several placental samples. We hope that the quantitative data presented serve as a foundation for future studies of inhibitors and enhancers of tumor cell invasion.

Materials and methods

Preparation of cell cultures

The B16-F10 metastatic melanoma cell line utilized in this study was a gift from Dr Garth Nicolson, M. D. Anderson Cancer Center, Houston, Texas, U.S.A. Passages 1–9 of this cell line (B16-F10) were used in the *in vitro* invasion assays. In addition, a cloned cell line was derived for experimental use by subcutaneously injecting 2×10^6 viable cells in single-cell suspension in 0·2 ml 1 : 1 DMEM/Ham's F12 into the thighs of female C57BL6 syngeneic mice. The derived cell line (B16-F10L) was specifically isolated from lung metastatic nodules, which formed within three weeks, and cloned according to previously established methods [26]. Passages 1–9 from the cloned cell line were also used in the *in vitro* invasion assays described below. The doubling time for the B16-F10 and B16-F10L cell lines was determined to be 18–20 h. The cells were plated in 100 mm tissue culture dishes (Nunc) with 1 : 1 Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F12 media (Gibco) and 5 per cent fetal bovine serum (FBS, Gibco) until they reached confluency. Growth curves were established prior to the invasion studies which represented the effects of various serum concentrations on cell growth, as shown in figure 2. Cell viability on

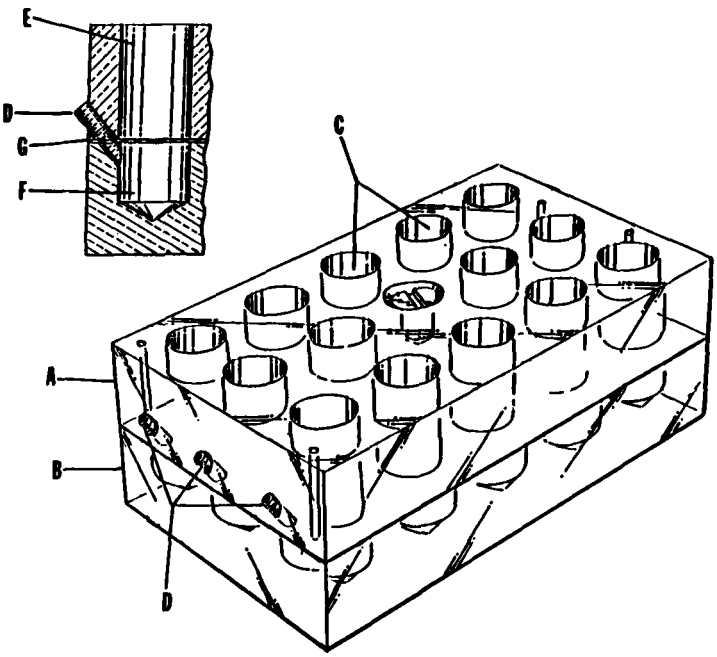

Figure 1. Membrane Invasion Culture System (MICS): (A) top plate, (B) bottom plate, (C) experimental wells, (D) sampling ports, (E) upper well, (F) lower well, (G) human amniotic basement membrane. Briefly, the human amniotic membrane is interposed between the top plate and bottom plate. Tumor cells are then seeded into the upper wells, attach to the BM, and are subsequently assayed at various time points in the bottom wells via the side sampling ports.

plastic was determined by the ability of the cells to exclude trypan blue. All cell cultures were determined to be free of Mycoplasma contamination with the use of a diphasic media test culture system (Mycotrim-TC, Hana Media, Inc.).

Utilization of a basement membrane model

Murine melanoma cells were seeded onto denuded amniotic BMs following the modifications of Liotta and co-workers' technique [16]. Briefly, fresh human placentae were obtained at birth, and in each case the amnion was dissected in the region of the umbilical cord. After several rinses in PBS, Fungi-Bact (Irvine Scientific) and PBS again, the amnion was trimmed to fit specially designed Membrane Invasion Culture System (MICS) chambers ([7] see figure 1). The amniotic epithelium was removed by treatment with freshly prepared 0·25 M ammonium hydroxide (NH_3OH) for 7 min at room temperature followed by extensive washing in PBS, which left a denuded BM with an underlying collagenous stroma. In this manner, the interaction of tumor cells with an ECM can be studied without the interference of host tissues or cells.

DMEM/Ham's F12 media and 5 per cent FBS were added to the upper and lower wells of the MICS chambers and incubated with the BM for at least 2 h prior to cell seeding at 37°C with 5 per cent CO_2 and 95 per cent air atmosphere in order to equilibrate the pH of the BMs following NH_3OH treatment. Subsequently, a predetermined concentration of cells from the murine melanoma cell lines (which was estimated with respect to cell number and nutrient dependence) was removed

from flasks with 2 mM EDTA in Ca^{2+}- and Mg^{2+}-free PBS, spun and resuspended in serum containing media prior to pipetting onto the membranes in MICS and placed in a humidified incubator. Prior to seeding the cells, all membranes were carefully examined for leakiness by: (1) macroscopic examination of discontinuities in the BM and (2) the use of colored density marker beads (Pharmacia) according to previously published methods [7].

As an additional control for passive diffusion of tumor cells through the BMs, normal Rat 1 fibroblasts and neural crest cells derived from chick neural tubes were seeded in the appropriate densities onto the membranes in lieu of melanoma cells, as previously published [8].

Assay of invasiveness

To determine cell invasion, the B16-F10 and B16-F10L cells were labeled with 0·1 μCi/ml of [^{14}C]thymidine (ICN) for 20–24 h in serum-free medium at 37°C. Following incubation, FBS was added and the cells were allowed to grow to near confluency for the next 24–48 h. The cells were initially plated at low density, so that they would be in log phase growth by the time they were harvested, and then seeded onto the amniotic BMs.

Prior to invasion studies, various concentrations of melanoma cells were seeded onto 24-well plastic dishes (Nunc), which were approximately the same size as the 15 mm diameter wells in the MICS chambers, and the most advantageous density was determined for the parental and cloned cell lines. Both the B16-F10 and B16-F10L cells were then seeded separately at a density of $2{\cdot}5 \times 10^4$ cells per well onto denuded BMs in MICS chambers. [^{14}C] thymidine at a level of 0·1 μCi/ml was shown to have negligible radiotoxic effect on the cells as determined by a comparison of growth curves of radiolabeled and unlabeled cells (data not shown).

The sampling technique preferred for this study consisted of collecting those cells which completely traversed the BMs by removing the media in the lower wells (1·1 ml) at 0, 5, 6, 12, 18, 24, 29, 52 and 53 h (for B16-F10) and 0, 6, 24 and 48 h (for B16-F10L) via the side ports in MICS chambers without piercing the BMs (figure 1). After each lower well was sampled, fresh media (1·1 ml) was replaced in the wells. In this manner, tumor cell invasion could be assessed repeatedly in the same 12 out of 14 wells per MICS chamber in each experiment over time. The cells in all the lower well samples were pelleted and then placed in scintillation vials. In order to examine the effects of gravity on the invasion experiments, in a few cases tumor cells were seeded only in the lower wells of the MICS chambers instead of the upper wells. Measurement of invasion could therefore be determined by assessing the radioactivity in the upper wells of the chambers. In all experiments, the collected cells were then lysed with 0·5 ml 1 N NaOH. Aliquots of 100 μl glacial acetic acid were added to each vial to prevent chemiluminescence, and 10 ml of ACS scintillation cocktail (Amersham) was added prior to radiolabel determination in a Searle 92 scintillation counter with a counting efficiency of 93·1 per cent.

The number of cells traversing the BM was quantified by assessing the radioactivity associated with cells in the media below the BMs (lower wells) compared to the radioactivity of cells seeded onto the membranes (upper wells). Thus, a percentage of cells completely penetrating the membrane was obtained. For statistical analysis of those lower well aliquots which were collected via the side sampling ports, initially per cent invasion was compared to the number of cells seeded in the upper wells for the earliest time point sampled (0 h). And, cells

collected in the same lower well aliquots during subsequent time points were simply summed with the invasion data collected during previous time periods. In several experiments, total counts were recovered from each well by assaying the radioactivity associated with the media from the upper well, the membrane, and the lower well (separately and collectively). In addition, to demonstrate that radioactivity was not being lost indiscriminately, a direct relationship between radiolabeled tumor cells and radioactive c.p.m. was determined prior to experimental invasion assays by serially diluting [^{14}C]thymidine labeled cells, lysing them, and counting the radioactivity (data not shown).

Proof of cell viability was obtained by the trypan blue exclusion test performed on aliquots taken from the upper and lower wells of the MICS chambers, in addition to estimating DNA replication with cesium chloride gradients, as previously described [23].

Quantification of membrane thickness

In order to evaluate the possible variation in thickness of several amniotic BMs, an analysis of the membranes was performed on 3 in diameter samples of four different amnions, each divided into five distinct sectors. Briefly, the membrane samples were immersed in a modified Karnovsky's fixative [14] with 0·1 per cent picric acid, buffered in 0·1 M cacodylate buffer, overnight at 4°C. The specimens were them postfixed in 2 per cent osmium tetroxide for 1 h on ice in the dark, serially dehydrated in ethanol, and finally embedded in Spurr resin. Cross-sections of BMs were subsequently cut 1 μm thick with glass knives on a Bausch and Lomb MT2-B ultramicrotome and stained with toluidine blue solution. BM thickness was analysed with a Zeiss Photomicroscope I and an optical micrometer. Measurements were recorded randomly at four different locations per membrane. Linear regression and correlation [39] were performed with the use of STAT PACK (v4.1) on a DEC10 computer. Correlation of membrane thickness was analysed using a one-way analysis of variance and least significant difference.

Results

Prior to conducting any invasion assays, it was important to verify that the serum concentration used for the cells was indeed appropriate. Specifically, serum concentrations that allow for optimal growth were established with growth curves over a 120 h period (figure 2) for both cell lines, which indicated 5 per cent FBS to be an appropriate concentration. This concentration was not varied during the course of the experiments. The next critical consideration prior to quantifying tumor cell invasion was to determine the appropriate number of cells to seed within the 15 mm diameter wells of each MICS chamber, which contain approximately 1·1 ml of media in the upper and lower wells. A representative growth curve for the B16-F10 and B16-F10L cells is shown in figure 3. The data indicate that $2{\cdot}5–5 \times 10^4$ cells per well should be utilized in these experiments so that the cells would be viable throughout an extended period in culture. It is important to note that the serum concentration used for the cells was previously determined and not varied during the course of the experiments.

Neither the neural crest cells nor the Rat 1 fibroblasts penetrated the BM in detectable amounts. And when tumor cells were initially placed in the lower wells of the MICS chambers and the radioactivity assessed after 24 h in the upper wells, it was estimated that 0·12 per cent of the population of cells penetrated the BM against gravity.

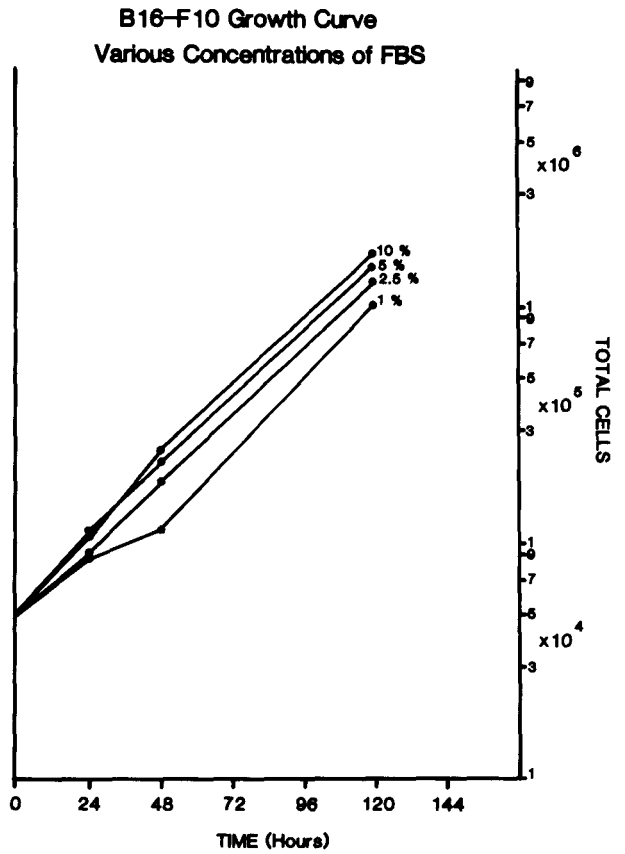

Figure 2. Growth curve representative of B16-F10 cells and B16-F10 clone grown in the presence of various concentrations of FBS in 1 : 1 DMEM/Ham's F12 media. These data were determined by counting the viable cells, grown on 60 mm tissue culture dishes, with a Coulter counter. Five per cent was selected as the appropriate concentration of FBS to be used in the MICS chambers.

Assessment of radioactivity associated with the radiolabeled tumor cells was performed prior to the analysis of invasion data. These results showed a linear relationship between the c.p.m. and labeled cells such that measurement of radiolabel would be reliably associated with cellular material and not with nonspecific counts in the media. The correlation coefficient for this preliminary data was evaluated as $r = 0{\cdot}9833$ with a P-value $< 0{\cdot}001$ (data not shown). In addition, when total radioactivity was measured in several experimental wells (consisting of media from the upper well, the BM and media from the lower well), total c.p.m. were associated primarily with the BM and the media in the lower wells.

The invasion profiles expressed by the B16-F10 and B16-F10L cells reflect values from the side sampling port method. Percent invasion for the B16-F10 cell line, sampled at 0 through 53 h, was plotted as the average value for each time point with standard error bars (figure 4). $N = 12$ for each point averaged in the three separate experiments performed. The highest invasion achieved with this heterogenous cell line after 53 h was 2·75 per cent. Quantification of the cloned line B16-F10L through the human amnion showed a comparable, although slightly higher

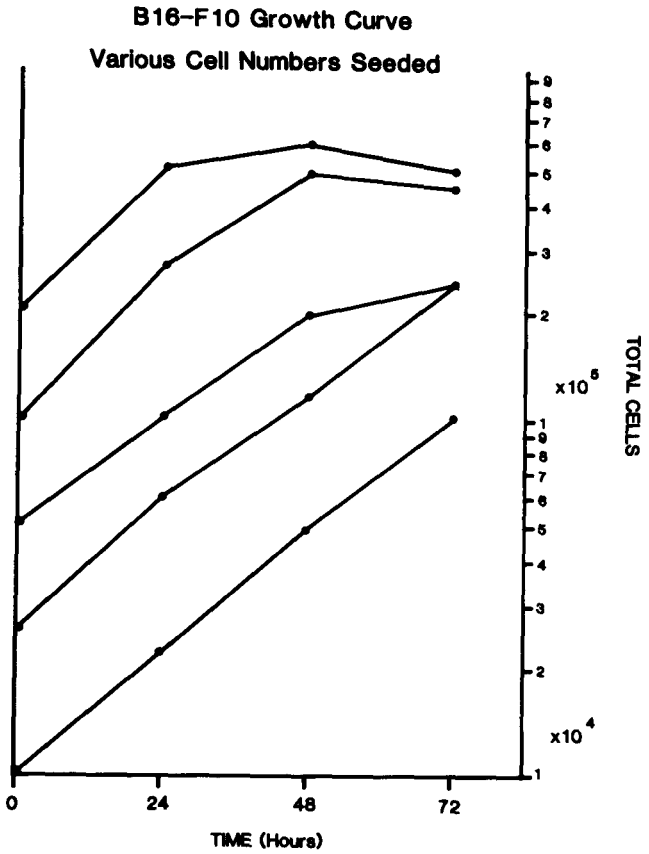

Figure 3. Typical growth curves of various concentrations of B16-F10 cells and clone seeded on plastic multi-well dishes (wells approximately the same size as the MICS chamber wells). Number of cells is plotted versus time. Cell number was determined by use of a Coulter counter. These data indicate that seeding 25 000 cells per well allows high viability over time in culture.

average invasion of 3·65 per cent by 48 h (figure 5) with $n = 7$ for each point averaged with standard error bars.

By the 48 h time point, new DNA replication was estimated for the B16-F10L clone and cell line, and it was determined that approximately 32·1 per cent of the cells had replicated in the upper wells of the MICS chambers compared with 32·9 per cent replication in the lower wells. These findings thus emphasize the viability of the tumor cells under experimental conditions, as well as their similarity in proliferative ability above and below the BM.

Since it was apparent macroscopically that some variability in thickness existed among placental samples, an in-depth morphological analysis of five independent sectors from 3 in diameter areas of four amnions was performed (table). Morphological measurement of the cross-sections of human amnion showed differences in thickness within the same membrane sample. Overall, the BM thickness ranged from 0·02 to 0·08 mm for these samples examined. Only one membrane sample was consistent in thickness throughout the five sectors.

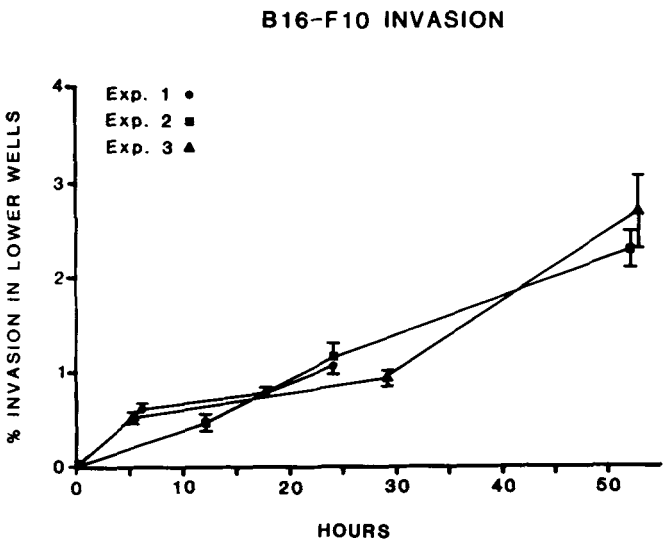

Figure 4. Per cent invasion of B16-F10 cells labeled with [^{14}C]thymidine which completely traversed the BM over time in MICS chambers. The number of cells that penetrated the membranes was quantified accordingly:

$$\frac{\text{post-invasion cell associated radioactivity (lower wells)}}{\text{pre-invasion cell associated radioactivity (upper wells)}}$$

The data points were plotted as the mean value ($n=12$) for each time point (with standard error bars) derived from three representative experiments of B16-F10 invasion through the BMs at 0 through 53 h.

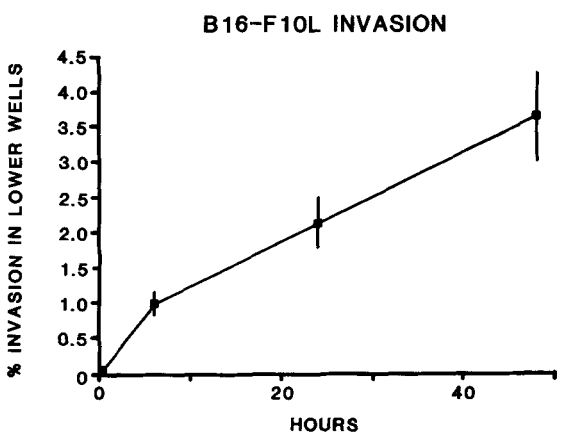

Figure 5. Per cent invasion of B16-F10L cells labeled with [^{14}C]thymidine which were collected in the lower wells of the MICS chambers over time. The data were plotted as the mean value for each time point ($n=7$), ranging from 0 to 48 h, with standard error bars. By 48 h, approximately 3·65 per cent tumor cell invasion through the BMs has occurred.

Analysis of human amnion thickness.

Membrane number	Sector	Mean ($n=4$)	s.e.	LSD multiple range (<5 per cent)
125	1	0·054	0·009	
	2	0·048	0·004	2 3 1 4
	3	0·049	0·004	——— ———
	4	0·078	0·007	
	5	N.O.	N.O.	
126	1	0·020	0·002	
	2	0·059	0·001	1 4 3 2
	3	0·043	0·002	— — —
	4	0·035	0·002	—
	5	N.O.	N.O.	
127	1	0·077	0·002	
	2	0·073	0·011	4 5 2 1 3
	3	0·081	0·007	
	4	0·071	0·003	
	5	0·072	0·005	
129	1	0·080	0·000	
	2	0·060	0·002	4 5 2 3 1
	3	0·064	0·002	——— — —
	4	0·060	0·000	
	5	0·060	0·000	

N.O. = No observations.

Statistical analysis of human amnion thickness was performed on five sectors from 3 in diameter areas from four separate amnions. Thickness of cross-sections of BMs was measured with an optical micrometer placed within a light microscope. Means and standard error (s.e) were calculated for each membrane sector. Least significant difference (LSD) analysis determined which sectors from any one membrane varied significantly (P 0·05) from each other. Sectors which are statistically similar are underlined. Sectors which are different from each other are denoted by different levels of underlining. Sectors in membrane 127 are statistically of the same thickness, whereas all sectors are different thicknesses in membrane 126. Membranes 125 and 129 have two and three areas of statistically significant thicknesses, respectively.

Discussion

A simple method has been presented for quantifying the invasiveness of murine melanoma tumor cells through a biological membranous barrier (human amnion) in an invasion chamber. From this study we conclude that the following variables should be carefully monitored in each tumor cell invasion study with *in vitro* invasion chambers: (1) the proper concentration of cells seeded in each chamber well must be determined from cell kinetics studies, nutrient requirements, and spatial diameter for growth in culture under specific experimental parameters; (2) an appropriate, reliable and consistent sampling technique should be used throughout the course of the invasion assays; (3) tumor cell viability should be assessed during the *in vitro* invasion with the use of a dye technique in combination with measuring new DNA produced, and (4) if the thickness of the biological membranous barrier varies for each experiment it should be measured and considered an additional factor in quantifying tumor cell invasion.

The data presented in this study support an efficient and reliable assay for tumor cell invasion based on the following observations: (1) melanoma tumor cells penetrate the BM, but fibroblasts and neural crest cells do not, (2) the morphological data previously published by our laboratory show these cells attached to the BM, penetrating the BM, and within the underlying stroma (12), and (3) these radiolabeled cells can be quantified and collected after one invasion and reintroduced to the BM, which produces a higher invasive profile than the original one (data not shown).

Previous investigators [11, 32] have presented the quantitative analysis of radiolabeled tumor cells in other invasion models, such as the chick chorioallantoic membrane and the perfused canine vein system. However, these *in vitro* models are more complex to work with than the human amnion. Studies based on the human amniotic membrane have focused primarily on the invasive and degradative processes involved in tumor cell interaction with an ECM, since it is considered to be one of the more discernible (measurable) aspects of the invasion process. These studies have provided helpful information pertaining to the morphological and mechanical aspects of cell invasion [16, 18, 34, 37, 40], as well as to the underlying mechanisms involved in this intricate cascade of events. It is highly probable that the murine melanoma tumor cells used in this study produced specific collagenases [19, 36, 44] in addition to plasminogen activator [21, 28, 36], a serine protease, to traverse the BM, rather than to rely on simple haptotaxic migration [15, 22]. It has also been shown that protein synthesis rather than DNA replication is necessary for tumor cells to invade the BM *in vitro* [41].

Some of the critical factors necessary to produce consistent and reliable invasion data in the human amniotic model, which have been overlooked in preceding studies, are presented here. The determination of proper cell density seeded in invasion chambers is an important consideration in these experimental assays. Previous work with cell crowding and nutrient depletion in melanoma cell cultures has reported the effects of these unfavorable conditions, which result in dimunition of cell size, loss of reproductive functions, and rapid cell death [38]. Other studies which tried to quantify tumor cell invasion through an amnion reported comparable invasion with B16-F10 cells over time [27, 29]; however, the statistical variance was quite large.

Two different methods of collecting invasive cells in the lower wells over time were used in preparation for this study. Initially, triplicate upper wells were depleted of media, a pipette pierced the center of the BM stretched across each well, and then the sampler triturated the media in the lower well and aspirated the sample. These wells were then terminated and could not be used subsequently. With this method, a great deal of variability existed in the radioactive counts associated with triplicate wells for the same cell line and the same time point. We then postulated that additional tumor cells were possibly being collected in some samples with the pipette by trapping the cells from the upper portion of the BM and carrying them through to the lower well. So, an alternative sampling technique was then tried, and later adopted, which consisted of sampling the lower wells via side ports in the MICS chambers, as shown in figure 1. This method allowed for unobstructive sampling of invasive cells in the lower wells over time. In fact, this method is preferred over the previous one since contamination of cells attached to the upper BM surface is no longer a concern, and the invasive propensity of cells placed into each experimental well can be determined by sampling repeatedly from the same lower wells at each time point. Thus, a more reliable analysis of the heterogeneity involved in tumor cell

invasion can be performed by examining the invasive profiles produced by the same $2{\cdot}5 \times 10^4$ cells in each experimental well over time. This side-port method definitely provides a more reliable method for collecting invasive tumor cells than pooling invasion data collected via the pipette-puncture method.

Our data indicate two similar invasive profiles demonstrated with the B16-F10 and B16-F10L cells in this assay. Since the B16-F10L line was cloned from the B16-F10, it is interesting that comparable invasion profiles could be produced with these two cell lines. These data, in general, appear to reflect the metastatic heterogeneity expressed by tumor cells, which has certainly been shown previously [31, 32]. However, the ability to study such heterogeneity expressed by established cell lines and selected clones can be performed easily with our quantitative assay, specifically due to the advantageous side-sampling technique.

Since it was apparent that some variability existed in the biological BMs used in each MICS experimental assay, the thickness of the BM was taken into consideration in four experimental assays. Statistical analysis shows that differences do exist in the thickness within the same amnion and between amnions. Only one amnion showed consistent morphological measurements of thickness throughout the five sectors examined. The actual role these differences and similarities in BM thickness play in tumor cell invasion is still unexplainable. Suffice it to say that further investigation is required before any conclusions can be drawn.

At the current time, this assay appears to quantify the invasive activity of the more aggressive cells seeded onto the BMs, since viable cells still remain in the upper wells of the invasion chambers even after two days, again indicating a heterogenous propensity to invade. Vital work has been devoted to the metastatic heterogeneity which exists in B16 clonal tumor cell subpopulations [25, 30, 31]. Another study which focused on the dynamic heterogeneity of B16 melanoma variants suggested that the majority of cells in the F1 and F10 lines are essentially nonmetastatic [13]. And, the greater metastatic propensity of the F10 line is possibly attributable to a higher turnover of metastatic variants within the population.

An important goal for establishing the experimental parameters of the MICS assay is to utilize this technique as a chemosensitivity screen for patient-derived cell lines *in vitro*. However, additional work is in progress to correlate this *in vitro* study with *in vivo* metastasis. Previous work has shown that selective invasion of B16 sublines *in vitro* through bladder wall or veins results in significantly increased ability to form spontaneous and experimental metastases *in vivo* [32]. Moreover, the usefulness of human tumor cell lines in the study of chemosensitivity has been demonstrated [24, 42]. It is our intention to combine and extend these studies to evaluate the invasive capability of sensitive and drug-resistant cell lines *in vitro* with the MICS assay and most importantly to correlate these data with *in vivo* interactions in a substantial animal model.

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