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A COMPARATIVE STUDY ON IN VITRO INVASION RATES BY MELANOMA CELLS IN THE HUMAN AMNIOTIC BASEMENT MEMBRANE MODEL VERSUS IN VIVO TUMOR NODULE FORMATION IN C57BL6 MICE

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Abstract

Invasion by murine B16-F10 melanoma cells was studied using the human amniotic basement membrane (HABM) assay. B16-F10 cells were collected after a single passage through the amnion and grown to near confluency. The cycle of plating, passaging, collecting, and culturing B16-F10 cells was repeated five times. The invasion rate for B16-F10 cells remained relatively unchanged after six passages through the amnion. Injection of first-passage B16-F10 cells into C57BL6 mice resulted in 29 lung tumors per animal whereas sixth-passage cells resulted in 300+ lung tumors. While there exists no correlation of the number of cells penetrating the amnion with colonization number, lung colonization appears correlated with increased number of passages through the amnion.

KEY WORDS: Metastasis, B16-F10 cells, Invasion Rates, Human Amniotic Basement Membrane Model, Invasion, Mice, Lung, Tumor, Cancer, Amnion

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Introduction

Metastatic melanoma is usually a highly lethal human cancer. Great strides in melanoma therapy have been made in the last decade, but these gains in therapy are mainly due to earlier diagnosis of the disease and hence initiating treatment at an earlier stage when the disease is more responsive to treatment. The complete clinical elimination of melanoma, and for cancer in general, will require inhibition of the metastatic process. Insight into the metastatic process can be gained by the study of tumor cell invasion, a subset of metastasis. Invasion may be defined as the ability of cancer cells to break down and travel through underlying basement membrane and connective tissue. The study of tumor cell invasion is complicated by the need for a model system that provides for an accurate measure of tumor invasion and an ability to recover invasive cells for further study. There are various in vitro invasion assay systems which meet to varying degrees the above two prerequisites. These assays include endothelial cells (Kramer et al., 1980), blood vessels (Jones, 1982), cartilagenous matrices (Pauli and Kuettner, 1982), embryo organ cultures (Mareel, 1982), human amnion (Liotta et al., 1980a), and nuclepore filters (Tullberg and Burger, 1985). Additional in vitro invasion assay systems may be found in the review by Russo et al. (1982). The human amniotic basement membrane (HABM) assay (Liotta et al., 1980a) was utilized in this study to determine melanoma cell invasion rates. A Membrane Invasion Culture System (MICS) chamber, developed by Gehlsen et al. (1984) and originally presented for quantification by Hendrix et al. (1985), was used in this study. The MICS chamber allows for quantification of invasion and for recovery of invasive cells for subsequent study.

The murine B16-F10 melanoma cell line was used to study invasiveness. Murine B16-F10 cells are highly metastatic and metastasize primarily to the lungs. Successive in vivo selection of the B16-F1 cell line to the B16-F10 cell line is paralleled by an increase in their invasive capability as witnessed by an increase in the number of nodules in murine lungs (Fidler, 1973; 1975).

This study investigated in vitro invasion rates for B16-F10 cells for each of six passages

through the amnion. The in vivo metastatic ability of B16-F10 cells was studied by comparing the number of lung tumor nodules formed in mice injected with B16-F10 cells that passed through the membrane once with those cells that passed through the membrane six times.

Materials and Methods

Routine Cell Culture

Murine B16-F10 melanoma cells (DCT Tumor Repository, NCI-Frederick Cancer Research Facility, Frederick, Maryland 21701) were cultured in T75 flasks containing Dulbecco's Modified Eagles Medium (DMEM) plus 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin-fungizone (PSF). Cell cultures were refed every three days and were split before becoming confluent. The length of trypsinization with VMF trypsin (Worthington Diagnostic Systems, Inc., Freehold, New Jersey) was kept to two minutes or less in order to reduce the effect on the cells' metastatic potential. Cells were incubated at 37°C in 5% CO_2 and 95% air. All other parameters for tissue culture were routine.

Human Amniotic Basement Membrane Assay

Fresh human placentas were obtained immediately following normal term delivery. The amnion, which consists of an epithelium, a basement membrane, and a collagenous stroma, was removed from the underlying chorion and placed in an embroidery ring (Persky et al., 1984). The amnion was then rinsed in distilled water to remove blood from its surface. The epithelium was removed by adding 0.25M ammonium hydroxide at room temperature and agitating constantly for seven minutes. The amnion was rinsed with distilled water until the pH of the amnion reached 7.4 and then immersed in phosphate buffered saline (PBS). The amnion was placed in a Sterilguard Hood (Baker Co., Sanford, Maine), washed 2X each side with PBS (pH 7.4), treated with 6.7% fungizone, washed 2X with PBS, and placed in a Membrane Invasion Culture System (MICS) chamber. Bottom wells contained DMEM plus 10% FBS and 1% PSF. Top wells were washed with 6.7% fungizone, washed with and then filled with PBS (pH 7.4) and placed in the incubator for 30 min. In order to access epithelial removal by the ammonium hydroxide treatment at this step of the protocol, selected intact and denuded amnions were processed for scanning electron microscopy. Tissue was fixed overnight in cacodylate buffered aldehydes (3% glutaraldehyde, 3% paraformaldehyde), post-fixed in 2% osmium tetroxide buffered in 1.0 N cacodylate buffer, rinsed in 1.0 N cacodylate buffer, dehydrated in ascending concentrations of acetone, critical point dried with CO_2 (Denton DCT-1), mounted on specimen stubs with copper tape, coated with gold-palladium (Hummer VI), and observed with a scanning electron microscope (ISI SX30).

Murine B16-F10 melanoma cells were harvested from log phase growth. After removing the PBS from the top wells B16-F10 cells were pipetted onto the membrane at a concentration of $1x10^5$ cells per well in DMEM containing 10% FBS and 1% PSF. The MICS chambers were incubated at 37°C in 5% CO_2 and 95% air. After 3 days the top wells were emptied and rinsed with PBS. The MICS chamber was then opened and the amnion removed intact. Fluid in each of the bottom wells was agitated with a pipette. Samples were pipetted from the bottom wells to determine viability (trypan blue exclusion method) and to count the number of invasive cells (standard hemocytometer method). Evaluation of each well was done in triplicate to determine both viability and the number of invading cells. The percent invasion rate was defined as the number of cells per ml of fluid in the bottom well of a MICS chamber divided by $1x10^5$ (number of cells plated per ml into the top well). The remaining fluid in the bottom wells was then harvested and placed into a T-75 flask or a 24 well tissue culture plate containing DMEM plus 10% FBS and 1% PSF. Flasks and plates were placed in the incubator at 37°C in 5% CO_2 and 95% air. B16-F10 cells were then grown to near confluency, harvested, counted, and reseeded at $1x10^5$ cells per well into a MICS chamber. Amnions from the MICS chambers were fixed in 10% buffered normal formalin for light microscopic studies.

In Vivo Assay

Female C57BL6 mice (Cumberland View Farms, Clinton, Tennessee) were used to check the in vivo metastatic capability of the B16-F10 cells. Complete methodologies on this lung metastasis assay have been published (Fidler, 1978; Ostrowski et al., 1986). Two nearly confluent T-75 flasks of B16-F10 cells, that had undergone one passage through the human amniotic basement membrane, were suspended in PBS and adjusted to 500,000 cells per ml. Twenty 75 day old mice were then injected using the lateral tail vein with 0.2ml of PBS containing 100,000 B16-F10 cells. Any mice that were not completely and successfully injected were discarded. Mice were then killed after 15 days. The lungs were placed in PBS and the metastatic nodules were immediately counted under a stereo dissecting microscope at 6.4x magnification. In addition to the lungs, a gross examination was made of all thoracic and abdominal viscera, thoracic and abdominal body walls, brain, inguinal nodes, axillary nodes, back, shoulder, and subcutaneous regions. The entire injection procedure was repeated on seventeen mice with B16-F10 cells that underwent five additional passages through the HABM with the exception that mice were one day older.

Electron Microscopy

The murine B16-F10 melanoma cell line was grown to near confluency in DMEM plus 10% FBS and 1% PSF in T75 flasks. Cell pellets were made from the cell line before the first amniotic passage. B16-F10 cell pellets were also made after the first and sixth passage through the amnion. All cell pellets were processed for routine transmission electron microscopy and examined with a Hitachi H-600 transmission electron microscope. Lung biopsies from passage one and passage six injected mice were also processed for routine transmission electron microscopy.

Results

Figure 1 outlines the experimental procedure. Cell viability ($\geq$ 96%) was determined by trypan blue exclusion tests immediately before plating

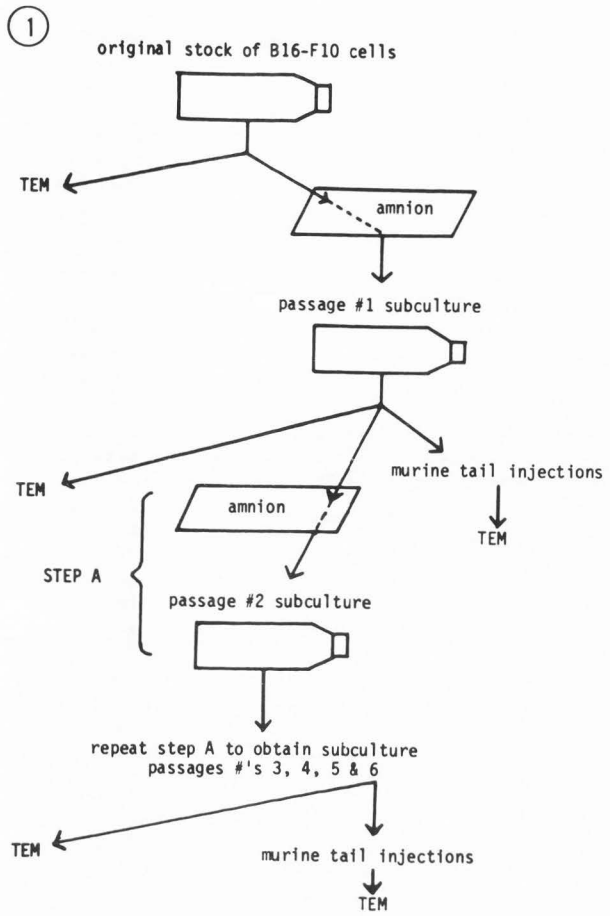

Fig. 1. Schematic diagram of the experimental protocol.

TABLE 1. B16-F10 invasion rates for different amniotic passages

Amnion Passage No	Percent Invasion Rate
1	1.7±.6[a] n=3
2	2.8±.4 n=3
3	2.4±.2 n=3
4	8.3±.9[b] n=6
5	2.0±.5 n=6
6	2.0±.3 n=6

[a] standard error
[b] invasion rate significantly different from 1,2,3,5,&6 ($p<0.01$) according to statistical comparisons between individual invasion rates based on the least significant difference (Steel and Torrie, 1960).

TABLE 2. Number of murine lung nodules from passage one B16-F10 cells

Mouse number:	1	2	3	4	5	6	7
Number of lung metastases at 6.4x	43	24	49	28	19	17	23

Mean ± standard deviation = 29.0 ± 12.3

TABLE 3. Number of murine lung nodules from passage six B16-F10 cells

Mouse number:	1	2-11
Number of lung metastases at 6.4x:	>300 (707 actually counted)	>300

the cells on the amnion. The initial in vitro invasion rate for B16-F10 cells was 1.7%. The invasion rate was 2.0% after five additional successive passages through the amnions (Table 1).

Passage 1 and passage 6 B16-F10 cells were 95.7% and 94.7% viable, respectively, before injection into the murine tail veins. The number of B16-F10 lung tumor nodules from amnion passages one and six are listed in Tables 2 and 3 respectively. All mice that were completely and successfully injected with B16-F10 cells developed lung nodules. The changes in nodule number and color between passage one cells and passage six cells were apparent at the gross level. Passage one nodules were melanotic (Fig. 2) while passage six nodules were mostly amelanotic. The size of the lung nodules within individual lobes varied (Fig. 2). Counting the amelanotic nodules was difficult due to their large number and amelanotic appearance. Therefore only one set of lungs, which was representative of the passage six tail-injection regime, was meticulously counted. The set of lungs containing amelanotic nodules was

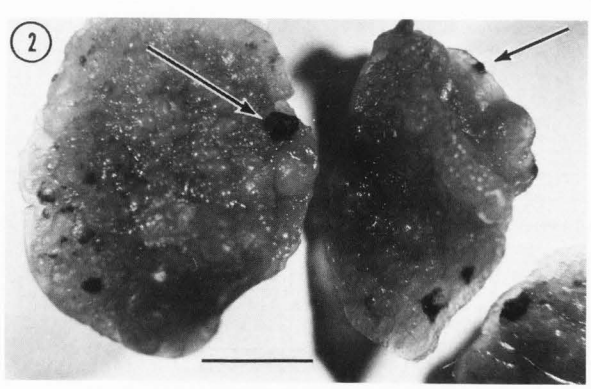

Fig. 2. Lungs from a C57BL6 mouse injected 15 days earlier with $1x10^5$ B16-F10 cells. The B16-F10 cells had previously been passed through the amnion once. Melanotic nodules of variable size are discernable (arrows). Bar = 1mm.

arbitrarily chosen from the eleven sets of lungs available for evaluation (Table 3).

Gross pathological examination of all animals only identified one potential extrapulmonary metastatic nodule. This nodule was present subcutaneously on the dorsal scapular surface of the right forelimb of a passage-one animal. The nodule had the same coloration as that animal's pulmonary nodules. Positive diagnosis of the melanotic-like nodule at the electron microscopic level was not done.

Transmission electron microscopy of B16-F10 cells before the initial plating onto the amnion revealed stage III premelanosomes. Similarly, electron microscopic evaluation of passage one and passage six B16-F10 cells demonstrated stage III premelanosomes (Figure 3). Lung biopsies of melanotic nodules (passage one and passage six) and amelanotic nodules (passage six) were also positively identified as melanoma by the presence of stage III premelanosomes.

Scanning electron microscopy of intact amnions revealed a monolayer of epithelial cells (Figure 4). In contrast, scanning electron microscopy of ammonium hydroxide treated amnions showed the absence of epithelial cells (Figure 5). Preformed channels through which tumor cells could passively migrate were not observed.

Discussion

The purpose of this study is to test the invasiveness of B16-F10 cells using the human amniotic basement membrane model. This study also determines if invasiveness is increased with repetitive cell passages through the amnion and if repetitive cell passages through the amnion have subsequent effects on tumor nodule formation in the murine lung. It is paramount to work with cells that are invasive when studying invasion. Two murine cell lines were originally selected for this investigation due to their reported invasive capability through type IV collagen, the collagen type found in basement membranes (Liotta et al., 1980b). These two murine cell lines, B16-F10 and B16-BL6, were evaluated *in vitro* and *in vivo* (unpublished data). Although B16-BL6 cells have a greater level of type IV collagen-degrading activity than B16-F10 cells (Liotta et al. 1980b), and although collagenase may be the critical enzyme in B16-F10 cell invasion through the human amnion (Persky et al., 1986) the B16-F10 cell line was chosen for this study as the preferred cell line because of better invasion rates in the HABM model.

In this study the B16-F10 cells were determined to be invasive by their invasion through the amnion and were determined to be tumorigenic by the *in vivo* lung nodules. Additionally, extrapulmonary metastases were rare as only one potential extrapulmonary nodule was noted in all 17 autopsied animals. This observation of low extrapulmonary metastasis accompanying high lung colonization is in agreement with Fidler (1973, 1975).

A function of the amnion is to select from the functionally heterogeneous, phenotypically homogeneous B16-F10 population a subpopulation with differing invasive capabilites. The amnion served to enrich the fraction of invasive subpopulations present in the heterogeneous parent B16-F10 tumor stock. Similar enrichment methodologies have been used by Hart and Fidler (1978) and Poste et al. (1980) to enhance tissue invasive capabilities. Recently, Steeg and coworkers (1985) demonstrated the selective capability of the human amnion to the point of identifying six specific mRNAs from metastatic cell lines whose translation was quantitatively increased when compared to the RNA from a benign clone.

The murine B16-F10 *in vitro* invasion rate is essentially unchanged in five of the six passages. The one exception, passage number four, was significantly higher than the other five invasion rates ($p>0.01$). The reason for the higher invasion rate for passage four is unknown. It is difficult to understand why the rate of invasion does not significantly change with passages since the amnion is reported to select a population of cells with higher metastatic potential. The lack of an increase in invasion rate might suggest that invasion is a random event and that it is by chance that after six passages a population with high metastatic potential is observed. This suggestion is countered by observations that metastasis is a selective rather than a random process (Talmadge and Fidler, 1982). Additional studies will be needed to clarify the relationship between amniotic invasion and tumor colony formation *in vivo*.

The injection of first passage and sixth passage B16-F10 cells into the lateral tail veins of C57B16 mice resulted in a >10 fold increase in lung tumors (29 ± 12 to >300). In actuality, the increase may be >24 fold if all of the passage six mice had as many tumors as was meticulously counted for the one passage six animal. It is an established procedure to stop counting murine lung nodules at 300 since further counting is difficult if not technically impossible (Fidler, 1978). Together, this *in vitro* - *in vivo* tandem protocol showed the B16-F10 cells to be both invasive and metastatic. Increased metastasis with passage indicates that while there exists no correlation of the number of cells penetrating the membrane with colonization number, the increase in specific activity for colonization appears correlated with increased number of passages through the membrane. In contrast, other studies have noted a positive correlation of increased invasion rates with increased metastasis (Brunson et al., 1978; Poste et al., 1980; Tullberg and Burger, 1985; Terranova et al., 1986). It is tempting to speculate why some studies show a correlation in invasive rate to that of metastasis. There are at least two important aspects that must not be overlooked, however, in trying to interpret the varying results. The first point is that most invasion rates are low. Studies have indicated invasion rates of B16 cells to be less than 5% (Hendrix et al., 1985; Persky et al., 1986). Other studies indicate the survival rate of cells to form secondary growths to be approximately 1% or less (Fisher and Fisher, 1967; Fidler, 1970; Weiss, 1980). Methodologies to determine changes in

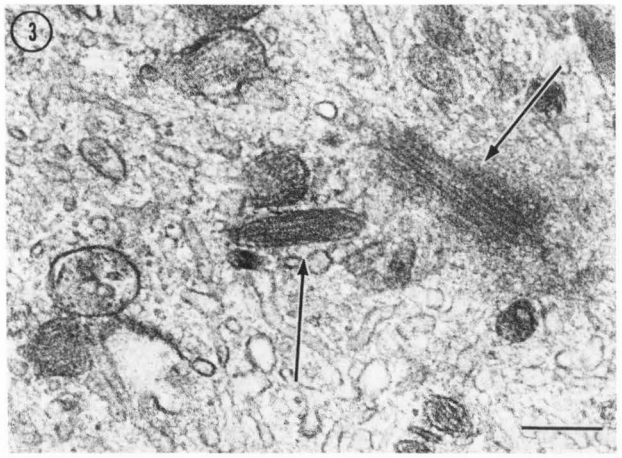

Fig. 3. Transmission electron micrograph of stage III premelanosomes (arrows). The premelanosomes are from a melanotic lung nodule taken from a passage one mouse. Bar = 0.2 μm.

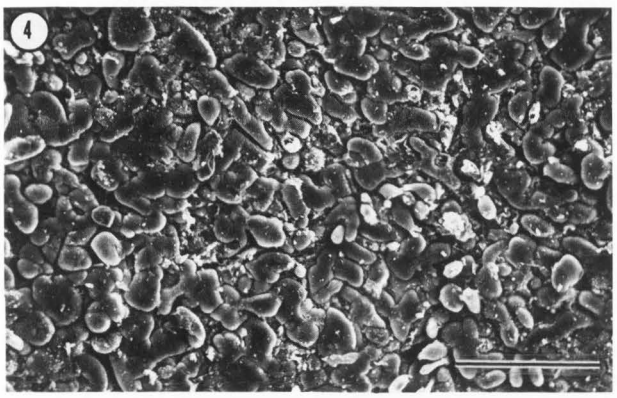

Fig. 4. Scanning electron micrograph of an intact amnion. The epithelial cell layer is complete. Bar = 1 mm.

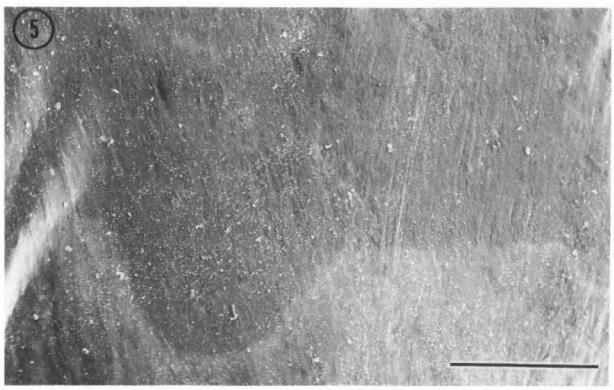

Fig. 5. Scanning electron micrograph of a denuded amnion. Treatment with ammonium hydroxide has removed the epithelial cell layer. Bar = 1 mm.

these small invasive cell populations may not adequately discriminate the de facto condition. The second point is that all in vitro tumor models developed to date do not perfectly reflect the in vivo condition and as tumor models may represent different components of the metastatic process. Although it is necessary to dissect the metastatic process into its various components in order to better understand metastasis, conceptualization of the entire metastatic process must not be lost in evaluating individual components, such as invasion. Although the amnion assay of Liotta and coworkers (1980a), the canine vein assay of Poste et al. (1980), the nuclepore filter assay of Tullberg and Burger (1985), and the reconstituted membrane assay of Terranova et al. (1986) evaluate invasiveness through a biological or artificial membrane barrier, there may be little correlation between the models. For example, tumor cell substrate for these four assays could be epithelium/denuded basement membrane/connective tissue stroma, endothelium/connective tissue, filter paper, and laminin/type IV collagen respectively. Since cell attachment is a required step in the process of invasion, all four invasive assays may largely reflect differences in substrates for cell attachment. For example, it has been recently demonstrated that monoclonal antibodies which interfere with cellular adhesion to laminin and which prevent adherence of B16 cells to tissue culture dishes reduce B16 experimental lung metastases (Vollmers et al., 1984).

It is worth noting that passage one lung nodules were essentially melanotic whereas passage six lung nodules were mainly amelanotic. This change in phenotypic appearance from melanotic to amelanotic may be related to increased metastatic potential. It should be noted, however, that there is no unequivocal definitive correlation between degree of pigmentation and metastatic potential (Fidler et al., 1981). There is a positive correlation between successive passages through the amnion and metastatic potential as seen by log increases in the number of lung colonies with amniotic passages one and six cells.

Transmission electron microscopy (TEM) was used to verify that the original stock of B16-F10 cells plus the invasive cells of amnion passages one and six were melanoma. In addition, TEM positively identified lung nodules as melanoma. The definitive diagnosis of melanoma was made by finding stage III premelanosomes, which are the best ultrastructral marker for melanoma (Mackay and Osborne, 1978).

Ideally, experiments to determine the effects of successive cell passages through the amnion would eliminate the tissue culture phase of the protocol. Tissue culture was needed in this study to increase the number of cells available for seeding onto the amnion. The selective capability of the amnion to discriminate more invasive cells might be compromised by the ability of these cells to attach to flasks and grow in tissue culture. Perhaps the subpopulation of cells that pass through the amnion has a minor subpopulation that will attach and grow in culture. Perhaps the

selective capability of the amnion is thus being diluted by increasing the number of cells via tissue culture techniques (in vitro) rather than in vivo techniques. Work by Fidler (1973) suggests that this is not the case, however, as in vitro culture did not reduce the relative metastatic abilities of his B16 tumor lines. It is theoretically possible to avoid increasing the stock of passage one cells without injecting cells into syngenic mice (in vivo method) or by growing them in tissue culture (in vitro method). However, such a protocol would require a gigantic "industrial size" set up for passage one cells since only approximately 2% of the plated cells would be expected to pass through the amnion.

In summary, the HABM model in conjunction with the MICS chamber provided a very satisfactory method for quantification of invasion and retrieval of invading cells. The in vivo lung data were interpreted as showing that the HABM selected a more tumorigenic subpopulation of cells. However, this selection of a more tumorigenic cell subpopulation in vivo was not correlated to increased invasion rates in vitro. Lung nodules from passage six cells were mainly amelanotic while lung nodules from passage one cells were melanotic, thereby demonstrating heterogeneity in the original B16 stock. We concluded that the use of an in vivo host, such as C57BL6 mice, is needed to demonstrate that the in vitro HABM assay has selected a special subpopulation of cells.

Acknowledgements

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Discussion with Reviewers

L.A. Repesh: After reviewing several published papers using the same assay with B16F10 cells (5×10^4 - 1×10^5 cells in 0.2 ml per animal via the tail vein), the mean number of pulmonary nodules that develop is significantly higher (e.g. Gehlsen KR, Hendrix MJC (1986). Cancer Letters, 30: 207-212; 225 ± 37.2 metastatic colonies) than what the authors report here (29.0 ± 12.3 metastatic colonies for first passage B16F10 cells). Please comment.

M.J.C. Hendrix: Regarding the in vivo results, the reported number of lung mets. from p - 1 B16F10 cells is unacceptably low, according to several published results. This number should fall within a 150 - 250 met. range per animal. However, the reported low number of mets. would be acceptable for the metastatic potential of B16F1 cells. Please comment.

Authors: These reviewer statements are extremely important as they challenge a fundamental aspect of the study. Questions that could be derived from the statements would include the following: Why were there so few metastatic lung nodules? Were too few tumor cells injected? Were the tail vein injections properly administered? Were the C57/BL6 mice virally infected? Did the authors receive the correct murine cell line or perhaps the murine B16F1 cell line by mistake? Answers to these questions follow. Upon receiving the cells from the DCT Tumor Repository, the shipped cells were immediately characterized by transmission electron microscopy as being melanoma due to the presence of stage III premelanosomes. Stage III premelanosomes provide definitive diagnosis for melanoma (text reference #15). The cells were also shown to be tumorigenic via subcutaneous injections and metastatic via tail vein injections (unpublished data). Only after having been shown to be melanotic, tumorigenic, and metastatic were the shipped cells accepted into the authors' laboratory as B16F10 cells. Why there were so few lung mets. is puzzling. A previous in vivo study by Ostrowski et al. (text reference #17) with the same DCT cell shipment showed a range of 30 to 127 lung mets in six of seven similar experiments. There is, therefore, strong evidence that the shipped cells routinely produce lung mets. below the 150-250 range. In order to further address the criticism of too low a number of lung mets., A.S. Bajkowski (Loyola University of Chicago, Department of Biochemistry and Biophysics) recently ordered B16F10 cells (#8547, passage unknown, medium MEM with 10% FCS, date frozen 4/16/85) and B16F1 cells (#G00031, passage unknown, medium MEM with 10% FCS, date frozen 8/1/85) from NIH. Although there was approximately a five fold difference in the number of lung mets. obtained from tail vein injections of B16F1 and B16F10 cells (unpublished data), the overall number of lung mets. for both cell lines was drastically reduced from values in the literature.

It is highly unlikely that three independent investigative laboratories (B. Persky, L.E. Ostrowski and R.M Schultz, and A.S. Bajkowski) following similar protocols injected too few cells. It is also unlikely that the tail vein injections were improper since the personnel making the injections each have a minimum of two years of tail vein injections.

Although the three laboratories and Loyola's Animal Research Facility have had no problems with the health of mice, viral contamination cannot be ruled out. Mice held in conventional animal quarters, such as Loyola's animal quarters, routinely contract virus but have subclinical manifestations. Clinical manifestations were not seen in the animal quarters or in the laboratories.

We have no way of determining if we were accidently sent the B16F1 cell line instead of the B16F10 cell line. It is unlikely, but theoretically possible.

B. Forslind: You do not express overtly that the passage through a basement membrane selects for tumor cells with high capacity for metastasis. Would you agree with such a generalized opinion?

Authors: Yes. We had hoped to see not only an increase in lung mets., but also an increase in the invasion rate in the HABM assay. The HABM assay is used to evaluate the properties of invasion. Invasion is a subset of the larger process of metastasis. Therefore, explaining the in vivo metastatic process solely by the in vitro HABM assay data would be unjustified. The title of our paper was carefully chosen to reflect a comparison of in vitro and in vivo data. It is of interest to note that a recently published paper (Gehlsen KR, Hendrix MJC (1986). Cancer Letters, 30: 207-212.) demonstrated no significant difference in the invasion profiles of B16F1 and B16F10 cells in the MICS in vitro. In addition, the metastatic potentials of both the B16F1 and B16F10 cell lines were confirmed in vivo to be unequivocally different. The results of Gehlsen and Hendrix thus agree with our results in that cells with different metastatic potential in vivo do not have different invasive profiles in vitro when using the HABM assay with the MICS apparatus.

Reviewer IV: Is it possible to show micrographs of tumor cells trapped in the amnion, i.e. in the process of migrating through the amnion by either histological section or TEM.
Authors: Please see Figure 6.

Reviewer IV: Perhaps the authors could enlighten us about the structures seen in the denuded amnion. A histological section could verify the absence of epithelium and will be more informative to the reader than the existing micrograph.
Authors: Please see Fig. 7 for a higher magnification of the denuded amnion. The absence of amniotic epithelium is also seen in the histological section illustrated in Fig. 6.

Reviewer IV: A photographic comparison of melanotic nodules from passage one and passage six in terms of size and number of nodules would be helpful. Granted the passage six nodules are mainly amelanotic they are easily visualized by fixation of the tissues in Bouin's fixative and has been used extensively by I.J. Fidler.
L.A. Repesh: A figure demonstrating the gross appearance of passage six cells should have been included for comparison to passage one cells.
Authors: Please see Figure 8.

Reviewer IV: Does TEM of amelanotic nodule also show premelanosomes? If so, how much when compared with the stock or with the melanotic nodules.
Authors: Premelanosomes are observed by TEM in amelanotic nodules. However, unlike both the original stock of B16F10 cells and the various melanotic nodules, there were fewer stage III and stage IV (fully melanized) premelanosomes. There appeared to be more abnormal stage I and stage II premelanosome development in the amelanotic nodules. Text reference #15 provides morphological descriptions of amelanotic and melanotic tumors with regard to tumor diagnosis.

B. Forslind: Are there any data to suggest that the specific cell line used in your work has a collagenase that is purely specific for the type IV collagen of the basement membrane or does it have a broader spectrum of capacities? Further is there in your work any suggestion that the amount of collagenase produced by the tumor cells is correlated to the invasive capacity of the tumor cells?
Authors: Liotta et al. (text reference #14) have shown the B16F10 cell line to have a collagenase that is highly specific for type IV collagen, i.e. basement membrane collagen, and is different from collagenase that cleaves collagen types I,II, and III.

We have not measured the amount of collagenase produced by tumor cells. However, Liotta et al. (text reference #14) have identified type IV collagenase activity in B16F10 cells and have determined that the amount of collagenase activity is directly correlated to the invasive capacity of various tumor cell lines. Thus Liotta's study of enzymatic degradation of basement membrane collagen showed the following cell lines to have increasing type IV collagenase activity: B16F1, B16F10, B16BL6, and PMT.

L.A. Repesh: In what manner were the membranes examined for leakiness before the addition of the cells to the upper wells?
Authors: Membranes were initially examined for tears or rips while in the embroidery ring and again when being inserted into the MICS chamber. Finally, leakiness of individual wells was evaluated by reviewing the standard error values for each invasion rate (see text Table I). The standard error rates suggest that individual wells did not leak. As for determining if an entire amnion was leaky, the statistical comparison between individual invasion rates based on least significant difference values suggests that amnions from passages 1,2,3,5, and 6 are significantly different ($p<0.01$) than the amnion of passage 4. Therefore the entire amnion #4 might have been leaky. However, leaky here should mean a "thinner" amnion membrane rather than a damaged amnion membrane (hole, rip, tear). B16F10 cells have been known to invade more quickly through thinner amnion membranes. Amnion thickness is discussed in the following reviewer question.

L.A. Repesh: Have you considered variations in the thickness of the amniotic membrane as a factor for the similar invasive profiles of B16F10 tumor cell variants?
Authors: We are well aware of variations in the thickness of the human amnion. L.A. Liotta, who is credited with developing the amnion assay (text reference #13), continues to stress that one of the problems with the amnion assay is variability from amnion to amnion (Welch DR (1986). Discussion of the suitability, availability, and requirements for in vivo and in vitro models of metastasis. In: Welch DR, Bhuyan BK, Liotta LA (eds.) Progress in Clinical and Biological Research. Volume 212, Cancer Metastasis Experimental and Clinical Strategies, p. 134. New York: Alan R. Liss, Inc.). Two papers which demonstrate and discuss both intra-amniotic and inter-amniotic variations in thickness are text reference #10 and Persky B, Grganto DM (1987). Diffusion of dextrans and microspheres in the human amniotic basement membrane model. Clin. Expl. Metast., in press.

L.A. Repesh: Have you considered that the similarity in the invasive profiles of first and sixth passage B16F10 melanoma cells could be attributed to incompatability interactions between human amnion and murine tumor cell antigens?
Authors: Numerous immunological studies have demonstrated invasion to be dependent, in part, on tumor cell antigenicity. Since the amnion has been treated with NaOH, the amniotic epthelium has been removed and fibroblasts in the collagenous stroma have been killed. Thus the amnion is essentially an acellular collagenous stroma with an overlying basement membrane. To the best of our knowledge, there has been no evidence of incompatability interactions in the HABM assay literature between human amnions and murine tumor cells.

L.A. Repesh: How did you determine that 1×10^5 cells was an appropriate number of cells to seed in the upper wells of the MICS chamber? Was nutrient depletion and cell crowding considered?

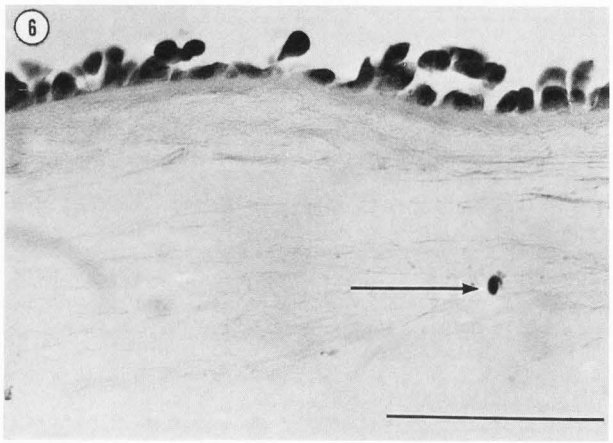
Authors: We have previously plated 2 x 10^3 to 1 x 10^6 B16F10 cells per ml in the MICS chamber. From data collected from cell viability, which served as a guide to nutrient depletion and cell crowding, the cell plating number of 2 x 10^4 to 1 x 10^5 was determined to be optimum. This range of cells is supported by the work of Hendrix et al. (text reference #10) where 2.5 - 5 x 10^4 cells/ml was determined to be optimum for plating.

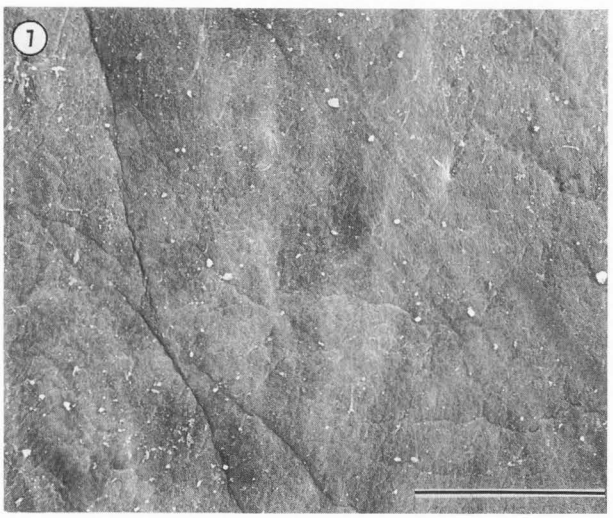
M.J.C. Hendrix: It is difficult for CO_2 to diffuse adequately from the incubator to the lower wells over a 3 day period. Was this controlled for in any way?

Authors: The ability of CO_2 to diffuse into the lower wells is important. One way to "control" the CO_2 level in the lower wells was to maintain a 5% CO_2 level in the VWR 1810 automataic incubator. The water pan was emptied and refilled every 7-14 days to help stabalize the CO_2 concentration. The incubator is checked yearly with a fyrite tester to determine the accuracy of the automatic CO_2 level. It would be possible to further enhance CO_2 diffusion by (1) redesigning the MICS chambers or (2) using a pump to circulate the media from the bottom wells to the 5% CO_2 atmosphere of the incubator. Ron Misiorowski (University of Arizona, Department of Anatomy) has discussed such modificataions with us (personal communication).

L.A. Repesh: In what passage were the melanoma cells that were used?

Authors: The passage of the B16F10 cells as received from the DCT Tumor Repository was unknown. The cells used for passage 1 were from a frozen stock in our laboratory dated 11-30-84. The same stock of cells had been previously shown by our labortory to be tumorigenic (subcutaneous injections), metastatic (tail vein injections), and melanotic (TEM identification of stage III premelanosomes).





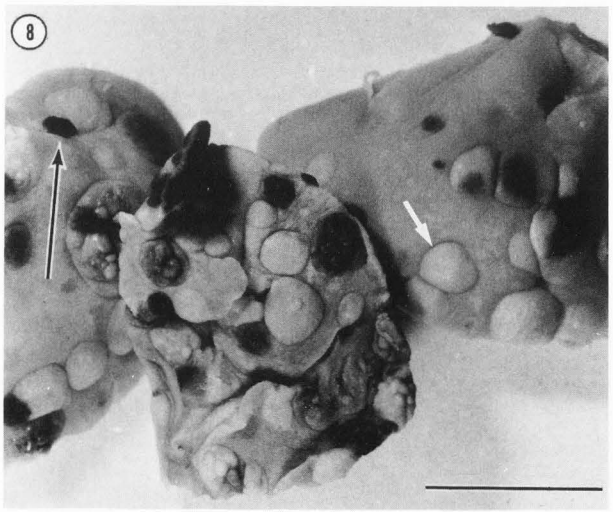


Fig. 6. This light micrograph illustrates B16F10 cells on the basement membrane and a B16F10 cell (arrow) in the process of migrating through the collagenous stroma of the amnion. Note that the amniotic epithelium is absent due to the NaOH treatment. Hematoxylin and Eosin. Bar = 10 mm.

Fig. 7. This SEM micrograph reveals an intact continuous basement membrane surface. The epithelium has been completely removed. The thin avascular stroma of the amnion is not visible as it lies below the basement membrane. Bar = 5 mm.

Fig. 8. These lungs from a C57/BL6 mouse were injected 15 days earlier with 1 x 10^5 B16F10 cells. The B16F10 cells had previously been passed through the amnion six times. Amelanotic (white arrow) and melanotic (black arrow) nodules of variable size are discernable. Unlike the fresh lung tissue illustrated in Fig. 2, this lung tissue was immersed in Bouin's fixative immediately after the lung nodules were counted. Tumor nodules, especially amelanotic, were more easily visualized after being preserved in Bouin's fixative. Bar = 1 mm.

M.J.C. Hendrix: Even though the authors were extremely honest in the presentation of data regarding the presence of amelanotic nodules in p - 6 injected mice, counting only one set of lungs does not render statistically relevant data for evaluation.
L.A. Repesh: Only one pair of lungs from animals injected with sixth passage B16F10 were "meticulously" quantitated. All the lungs should have been counted for statistical analysis to be more relevant.
Authors: Fidler (text reference #5), Ostrowski (text reference #17) and ourselves have found it difficult, if not impossible, to accurately identify individual melanotic/amelanotic tumors in excess of 300 mets per lung. After a point counting tumor mets. in the lung becomes subjective because of the vast merging together of various mets. The message of Tables II and III (see text) is that the mean and standard deviation of lung nodules for passage 1 (29.0 ± 12.3) and for passage 6 (>300 in all animals) is greater than a log difference. Therefore there is a significant difference in the number of mets. between the two groups.

M.J.C. Hendrix: Percent invasion cannot be accurately quantitated the way it is outlined in this paper. It does not take into consideration the rate of cell division in the upper wells and in the lower wells of the MICS chambers. After 3 days, some cell division must have occurred.
Authors: In this study the percent invasion rate was defined as the number of cells per ml of fluid in the bottom well of a MICS chamber divided by 1×10^5 (number of cells plated per ml into the top well). Comparison of invasion rates could be made since the definition of precent invasion was applied equally to all parameters of the experiments. The important message of our study is that the invasion profile of passage 1 and passage 6 cells remained relatively unchanged. After the completion of our study, a study was published demonstrating no significant difference in the invasion profiles of B16F1 and B16F10 cells in the MICS appartus in vitro (Gehlsen KR, Hendrix MJC (1986). Cancer Letters, 30: 207-212.). The results of these two papers compliment each other in that low and high metastatic cells, as determined in vivo, pass through the amnion with appearantly similar invasive profiles.

Cell division is known to occur in the upper and lower wells (text reference #10) and in the amnion membrane itself (Mignatti P, Robbins E, Rifkin DB (1986). Tumor Invasion through the Human Amniotic Membrane: Requirment for a Proteinase Cascade. Cell 47: 487-498.). However, the rate of cell division is reduced in the amnion as compared to the upper and lower wells.

L.A. Repesh: Was it verified that the serum concentration used in the amnion assay was optimal for cell growth?
Authors: We have cultured murine melanoma cells in the MICS chamber for 72 hours in 0%, 2%, 5%, acid inactivated 5%, and 10% fetal calf serum (FCS). Cells grew best in 10% FCS. We now utilize the MICS assay with 10% FCS unless serum must be deleted, as in proteolytic enzyme investigations (text reference #20). Hendirx et al. (text reference #10) have published growth curves for different concentrations of serum (1%, 2.5%, 5%, and 10%) for use in the MICS chamber and have shown 10% FCS as the optimum serum concentration for cell growth. However, Hendrix often used 5% FCS in the MICS chamber as the optimum serum concentration permitting both cell growth and cell invasion (personal communication).

L.A. Repesh: What is the total volume of the upper and lower wells of the MICS chamber?
Authors: The upper and lower wells in our particular MICS chambers are routinely filled with 1.0 and 1.1-1.2 ml of fluid respectively.

L.A. Repesh: Since the MICS chambers have side ports for sampling, why were the membranes pierced? Could this procedure have allowed for contamination of the lower wells with cells from corresponding upper wells? Other studies using this technique have shown great variability in cell counts from well to well.
Authors: There are various models of the MICS apparatus. All MICS chambers used in this study did not have side ports. At no time were membranes pierced in order to sample the contents of the lower wells. Contamination of the lower well is known to occur with the "pipette-puncture method" (unpublished data and text reference #10). We have modified the MICS chambers since the completion of this experiment. Access ports are on the bottom of the chamber rather than the side.

M.J.C. Hendrix: How long did it take for the cells that invaded the amnion to come to confluency?
Authors: Cells that invaded the amnion were not grown to confluency. Instead cells were grown to near confluency in order to optimize conditions for logrithmic growth. The mean number of days for growing cells to near confluency from one passage to the next was 19.6 days.