Lymphology 23 (1990) 4-14

QUANTITATION OF HUMAN MELANOMA, CARCINOMA AND SARCOMA TUMOR CELL ADHESION TO LYMPHATIC ENDOTHELIUM

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ABSTRACT

We have used an in vitro *adhesion assay to study the interaction of tumor cells with lymphatic endothelium, a dynamic event that leads to tumor metastasis* in vivo. *^{3}H-thymidine-labeled human tumor cells from: one primary Ewing sarcoma, two established melanoma cell lines, two colon and two breast carcinomas (one established line and one primary culture of each) were added to 24-well culture dishes containing confluent monolayers of bovine lymphatic endothelium. Radioactivity associated with either the cells in suspension or the attached cells was assessed and compared at frequent intervals up to 360 minutes. Generally, tumor cell attachment increased as a function of time reaching a plateau between 180 and 360 minutes. The modular media system described here facilitates the primary and secondary culture (or co-culture) of a variety of normal and transformed cells. Primary cultures with a rounded morphology (one breast and one colon carcinoma) showed the lowest preferential attachment for lymphatic endothelium. All established cell lines and the primary Ewing sarcoma cell line displayed a more fibroblastic morphology and achieved the highest adhesion profiles. There was a correlation between the malignancy and attachment potential for the melanoma and breast carcinoma cell lines. Collectively, these data show that established tumor cell lines with fibroblastic-like morphology exhibit more rapid adhesion than primary tumor cell cultures with more rounded morphologies. While this property may reflect* in vitro *selection and/or adaptation, it does correlate with the metastatic propensity for some human tumor cells.*

The major cause of cancer deaths is the formation of nearby or distant secondary tumors or metastases. This ability of tumor cells to form metastases within the circulation requires that they complete a complex, highly selective cascade of events, beginning with invasion into the surrounding vasculature, then survival within the harsh circulatory environment, arrest of emboli in capillary beds or attachment to the endothelial lining of vessels, extravasation from the microcirculation, and proliferation at the secondary site (for reviews, see 1-4). Many of the common human malignancies metastasize via the lymphatic circulation (5). In

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fact, the extent of involvement of the regional lymph nodes has become a parameter commonly used in the staging and prognosis of primary cancers (6).

Little is known about tumor cells' abilities to form lymphatic metastases. For example, it is uncertain whether metastasis to lymph nodes precedes blood-borne metastasis and whether tumor cells are altered comparably when passing through either the lymphatic or blood vessels. Also, the histologic organization of lymphatics, while similar to microvessels of the blood-vascular system, may require specialized mechanisms for metastasis formation. Nonetheless, many of the mechanisms involved in blood-borne metastasis are presumably similar since the barriers in the blood and lymphatic vessels are thought to be structurally similar (7). Although there is considerable evidence supporting the role of platelets in blood-borne metastases, which are absent from lymph, the fundamental mechanisms of attachment, degradation and motility through these vascular barriers are similar (8).

Each step in the metastatic cascade involves complex tumor cell-tumor cell and tumor cell-host cell interactions; yet, extremely little is known about tumor cell interactions with lymphatic components, especially the lymphatic endothelium. The vascular endothelial cell monolayer is one of the *in vitro* models currently being used to observe tumor cell/endothelial cell interactions (8-10, for review see 11). Others have shown (12,13) that tumor cells recognize and differentially bind to endothelial cells derived from the blood vasculature of different organs. Hence, it is thought that endothelium may be a recognition locus through which tumor cells 'home' to different sites. Malignant tumor cells exhibit higher attachment kinetics to 'target' organs than their benign counterparts (12,14). Since tumor cell adhesion to microvessel (capillary or post-capillary) endothelium is crucial for the extravasation of circulating metastases (9,15), the present study was designed to quantitatively evaluate the ability of human tumor cell lines of differing origin and malignant properties to adhere to lymphatic endothelium. We describe a simple and rapid method of measuring the attachment of human tumor cells to confluent monolayers of a recently established bovine mesenteric lymphatic duct endothelial cell line (16) using a novel modular media system in conjunction with an adaptation of previously described attachment assays (8-13). We found significant differences in both the adhesion rates and maxima between several human tumor cell lines and that these data correlate well with the metastatic activity and morphologic features, but not the tissue origin of the tumor cells.

MATERIALS AND METHODS

Tumor cell lines and culture maintenance

The melanoma cell lines used in this study, A375P and A375M, were kindly provided by Dr. I.J. Fidler, The University of Texas, M.D. Anderson Cancer Center. The A375M cell line was developed from a soft agar subclone of a rare experimental lung metastasis formed in a nude mouse after injection with cells from the parental A375 human melanoma cell line (A375P; 17). The original A375 melanoma cell line was derived from a 54 year-old female and was established from minced solid tumor (18). The A375P cells had minimal metastatic activity, whereas A375M cells were highly metastatic in the nude mouse model.

The A375P and A375M cell lines were obtained, maintained, and assayed in Dulbecco's Modified Eagle's medium (DMEM; GIBCO) supplemented with 450mg/liter glucose (Sigma, grade III), 1.2g/liter sodium bicarbonate (MCB Reagents), 110mg/liter sodium pyruvate (Flow Laboratories), 1% Pen-Strep (Irvine Scientific: 10,000 units/ml penicillin and 10,000ug/ml streptomycin), 0.3g/liter glutamine (Irvine Scientific), and 10% fetal bovine serum (FBS; Irvine Scientific), at 37°C in a 5% CO_2 and 95% air humidified atmosphere.

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The human breast adenocarcinoma cell line, MCF-7, was established from a pleural effusion in a 69 year-old caucasian woman in 1973 (19). This well-characterized cell line has retained several characteristic phenotypes of differentiated mammary epithelium including: estrogen dependency, high metastatic potential, and the ability to form domes in tissue culture (20). The NRG breast carcinoma cells were derived from an infiltrating ductal carcinoma tumor removed from the breast of a 43 year-old Mexican-American woman with extensive vascular invasion of the dermal lymphatics. The fresh tumor tissue was processed (21) in 1986 and has not yet been established as a cell line.

The colon adenocarcinoma (grade III-IV) cell line, SW-480, was established (22) from a mince of a primary large bowel adenocarcinoma tumor. These highly differentiated cells produced carcinoembryonic antigen (CEA) and were tumorigenic in nude mice. Early passage ALN colon carcinoma cells were established (21) from forehead and abdominal metastatic lesions of a 67 year-old caucasian man with carcinoma of the sigmoid colon presenting simultaneously with liver metastasis.

The GDW Ewing sarcoma cell line, derived from a mince of extraosseous metastatic tissue taken from the lung of a 17 year-old caucasian female in 1987, was a gracious gift provided by the Cytogenetic Center, Phoenix, Arizona. This cell line had been in culture for approximately 3 months at the time this study began.

Tumor cells were cultured in M13 (23), M15, M19, or M33 *(see Table 1 for formulae)* and visually observed for proliferation. Generally, the medium with the least number of components that caused noticeable cell proliferation was used to culture each cell line. MCF-7, NRG, SW-480, ALN, and GDW were maintained and assayed with M19 medium in air-tight tissue culture ware and passaged by removing the cells from tissue culture plastic with 2mM EDTA in Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (PBS). Cells were counted using a hemacytometer.

Endothelial cell line and culture maintenance

Early passage endothelial cells from the bovine mesenteric lymphatic duct (16) were used for this study. Approximately 30% of these cells expressed Factor VIII antigen compared with 100% of bovine mesenteric artery or human umbilical vein endothelium. M33 medium, which is M19 supplemented with 10^{-8} estradiol *(Table 1)*, was used for maintenance of the cells and assays. Without the estradiol supplementation, these cells would grow slowly or not at all but would remain viable with normal morphology for >6 weeks with fresh media added weekly. For tissue culture passage, endothelial cells were incubated in 0.25% trypsin and 2mM EDTA in PBS at 37°C for 2-4 min. Bovine lymphatic endothelial monolayers were prepared by seeding $1x10^5$ cells into each well of several 24-well tissue culture plates. The plates were placed in airtight containers and incubated approximately 72 hours until the cell layers were confluent. To insure confluence, the medium above the monolayers was replaced daily for three additional days of culture. By the sixth day of growth, all wells were confluent as measured by direct visual observation using low power phase microscopy *(see Fig. 1A)*.

Tumor cell adhesion assay

In order to quantitate the ability of various tumor cell types to attach to lymphatic endothelium, we used a modification of the adhesion assay previously described by Nicolson and co-workers (8,24). Once they had reached 30% to 40% confluency, tumor cells were radiolabeled with 5μCi/ml of ^{3}H-thymidine (ICN; 72Ci/mmol). After one cell doubling (i.e., confluence=60% to 80%) the radiolabel was removed, the cells were rinsed twice with PBS, and then detached with a solution of 2mM EDTA in PBS for 5-15 min at 37°C. After adding 2 volumes of serum-containing medium, cells were centrifuged at 500xg for 5 min at room temperature. The tumor cell

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Table 1
Formulations for Media M13, M15, M19, and M33[1]

INGREDIENT	STOCK SOLUTION	FINAL WEIGHT PER LITER	VOLUME PER LITER
	Base medium M13		
L-15 medium modified[2]			1-L pkg
Hydrocortisone	10^{-5} M	3.60 mg	1.00 ml
Insulin, bovine pancreas	1.00%	10.00 mg	1.00 ml
Transferrin	1.00%	10.00 mg	1.00 ml
Glutamine	2.92%	292.00 mg	10.00 ml
Antibiotics[3]			10.00 ml
	Additions for Medium M15		
DETOXIFICATION REAGENTS			
Sodium selenite	10^{-5} M	0.80 mg	1.00 ml
Glutathione, reduced	0.30%	15.00 mg	5.00 ml
Catalase (11,000 U/mg)	0.50%	5.00 mg	1.00 ml
Methyl cellulose 15 CPS	2.00%	2.00 gm	100.00 ml
Polyvinylpyrrolidone-360	5.00%	1.00 gm	20.00 ml
GROWTH REAGENTS			
2-Mercaptoethanol	10^{-5} M	0.80 mg	1.00 ml
Orotic acid	0.30%	15.00 mg	5.00 ml
DL-Orinthine	0.30%	15.00 mg	5.00 ml
	Addition for Medium M19		
Whole bovine pituitary extract[4]	20.00%		2.50 ml
	Addition for Medium M33		
Estradiol	10^{-5} M	0.003 mg	1.00 ml

[1]Just prior to use all media were supplemented with 5% fetal bovine serum (Irvine Scientific).
[2]L-15 medium (American Biorganics) was modified by reduction of NaCl from 0.8% to 0.644% and by adding 20mM HEPES and 6.93mM Trizma Base buffers.
[3]100X Pen-Strep (Irvine Scientific).
[4]Crude salt bovine pituitary extracts were prepared by the methods described by Peehl and Ham (29). Note: Unless otherwise specified, all reagents were purchased from Sigma (St. Louis, Missouri). Osmolarity is 300mOsm for M13 and 322mOsm for M19. Methyl cellulose and polyvinylpyrrolidone-360 are added aseptically to all other ingredients after batch filter sterilization.

pellet was resuspended in 10-15ml medium and the cell concentration was determined using a hemacytometer. Medium was added to adjust the cell concentration to 10^5/ml. The medium was replaced in the 24-well plates with 0.5ml of the appropriate medium to grow each cell line. The assay was initiated by seeding $1x10^4$ tumor cells onto each endothelial monolayer.

Tumor cell attachment was measured for 10-360min in quadruplicate for each time point. To terminate the experiment at each time point, medium from the appropriate well was removed and placed into a scintillation vial labeled

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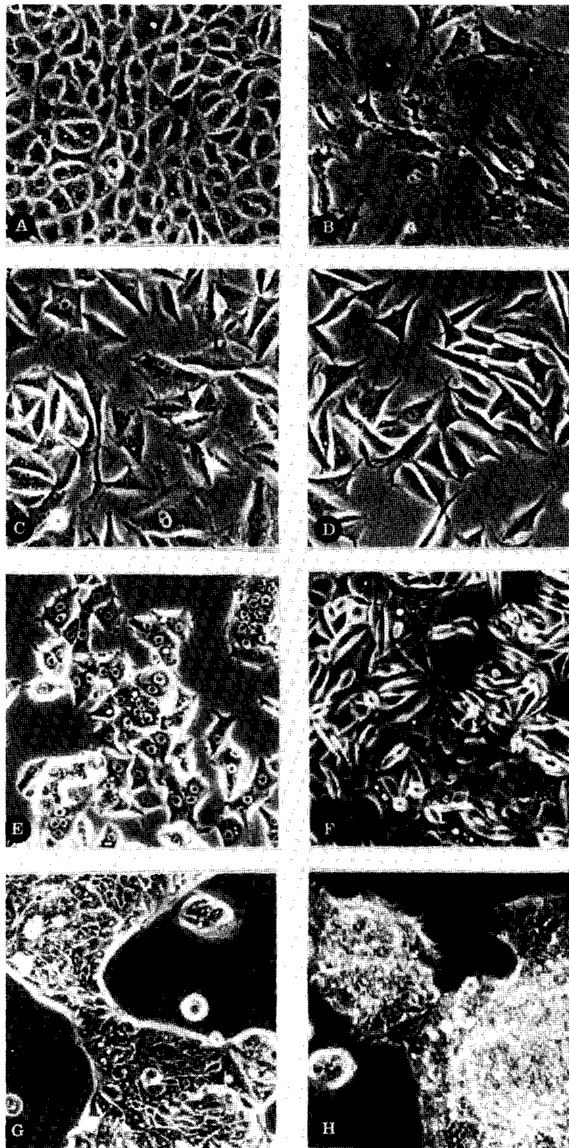

*Fig. 1. Phase contrast micrographs of cell lines. **A**: Bovine lymphatic endothelial cells, passage 5; **B**: GDW Ewing sarcoma cells, passage 4; **C**: A375P human melanoma, passage >200; **D**: A375M human melanoma, passage >200; **E**: SW-480 human colon adenocarcinoma, passage >200; **F**: MCF-7, human breast adenocarcinoma, passage 256; **G**: ALN colon carcinoma, passage 15; **H**: NRG human breast carcinoma, passage 17. All photographs were taken at an original magnification of x250.*

'Unattached'. The well was then gently rinsed with 0.5ml of PBS to remove all unattached and weakly attached cells and this rinse added into the 'Unattached' vial. To remove the attached tumor cells, 0.5ml of 0.25% trypsin/2mM EDTA in PBS was then added to each well. After incubation at 37° for at least 15 min, the cell harvest was removed and transferred to a second scintillation vial labeled 'Attached'. This step was followed by a rinse with 0.5ml of fresh medium added to the 'Attached' vial. In the end, each experimental well yielded two scintillation

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Table 2
Analysis of Variance in Tumor Cell Attachment Profiles

Cell Line	SW480	A375M	MCF7	A375P	GDW	ALN	NRG
Analysis of Variance	———	———					
			———	———			
				———	———		
					———	———	
							———

Comparison of all tumor cell adhesion profiles using Tukey's Honestly Significant Difference Test. Analysis of variance showing highest to lowest adhesion potential of all tumor cell lines tested on lymphatic endothelial monolayers. Cell lines that share common horizontal lines did not have significant variance over the entire length of the assay to be considered different from each other. Cell lines on differing horizontal lines represent statistically significant ($p < 0.05$) difference between entire adhesion profile curves.

vials: one contained the radiolabeled tumor cells that had not attached, and the other contained the tumor cells that had attached to the endothelial monolayer. Each vial contained an equal amount of tumor cell medium to insure uniform quenching between the two groups. In order to dissolve the cells and completely release the radiolabeled DNA, 100ul of TS-1 tissue solubilizer (Research Products International Corporation) was added to each scintillation vial. Aliquots of 0.1ml glacial acetic acid (EMS) were added to each vial to retard chemiluminescence, and 10ml of Ecoscint scintillation cocktail (National Diagnostics) was added prior to radiolabel quantitation in a Searle 92 scintillation counter.

Background counts were determined as above from wells that were not seeded with tumor cells. Total counts (100% attachment) were obtained by adding a 100ul aliquot of $1x10^4$ radiolabeled tumor cells directly to a scintillation vial which contained a background sample (0.5ml of media plus the cells from an endothelial monolayer).

Statistical analysis

The fraction of attached cells was calculated for each experimental well by the following equation:

$$\% \text{ ATTACHMENT} = \frac{\text{cpm in 'Attached' fraction}}{\text{cpm in 'Attached' + cpm in 'Unattached' fractions}} \times 100$$

Although the total and background counts were determined in quadruplicate for each experiment, they were not used in the calculations. Any experiment that had significant background counts (≥45cpm) or greater than 5% error in cell delivery was eliminated from the study.

The mean ± S.D. % attachment was calculated for each time point. The change in average percent attachment over time for each cell line was graphed and analyzed for variance using Tukey's Honestly Significant Difference Test *(Table 2)*. Average maximum rates of adhesion were obtained by calculating the greatest slope observed in the average percentage attachment vs. time graphs and reported below. For all cell lines, the maximum rate of adhesion occurred within the first 10-15 min of the assay.

RESULTS

Phase contrast micrographs of all cell lines used in this study are shown in *Fig. 1*. The bovine lymphatic endothelial cells appeared as a "cobblestone" of close-

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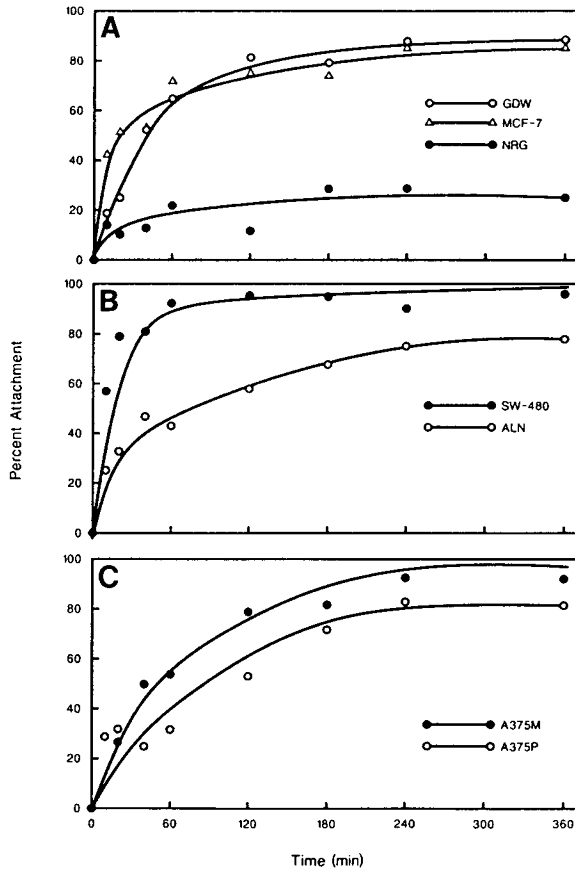

Fig. 2. Adhesion of 7 human tumor cell lines to lymphatic endothelium. Tumor cells radiolabeled with ^{3}H-thymidine were seeded onto confluent lymphatic endothelial monolayers (10^4 cells/well). The percentage attachment was determined by the following equation:

$$\% \text{ ATTACHMENT} = \frac{100 \times \text{cpm in 'Attached' fraction}}{\text{cpm in 'Attached' + cpm in 'Unattached' fractions}}$$

Data are presented without error bars for ease of presentation but the standard errors were less than 6.6% for all average data points (n=4 to 8 for each averaged data point). Comparison of adhesion profiles in A: for GDW Ewing sarcoma and MCF-7 and NRG breast carcinomas; B: for ALN and SW-480 colon carcinomas; and C: for A375P and A375M melanoma cell lines.

ly packed, evenly spaced monolayer of cells which is typical of many endothelial cell lines *(Fig. 1A)*. Both melanoma cell lines demonstrated a more fibroblast-type morphology, as did the Ewing sarcoma, SW-480 colon carcinoma, and the MCF-7 breast carcinoma cell lines *(Fig. 1B-G)*. When grown on tissue culture plastic, the ALN colon carcinoma and the NRG breast carcinoma cells had formed dense colonies of rounded cells that piled up on each other rather than spread onto adjacent cell-free areas *(Fig. 1H and I)*. Cells within these colonies were tightly adherent to each other and to the tissue culture plastic, and it was difficult to obtain single-cell suspensions or detach the cells from the culture dish. The morphology of

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the GDW Ewing sarcoma cells was more fibroblastic than rounded. This was somewhat atypical since these cells had been cultured *in vitro* for only a very short time.

The average maximum rates of adhesion (cells/min) for each tumor cell line are listed here (from greatest to least): SW-480, 570 cells/min; MCF-7, 424 cells/min; A375M, 297 cells/min; A375P, 280 cells/min; ALN, 252 cells/min; GDW, 187 cells/min; NRG, 141 cells/min. Maximum rates for all lines occurred within the first 10 min of assay time. Individual values did not vary more than 6% from the mean for any cell line (n=4 to 8).

Large variability in the kinetics of adhesion was observed for 1 Ewing sarcoma, 2 colon carcinoma, and 2 breast carcinoma cell lines *(Fig. 2A and B)*. The SW-480 and MCF-7 cell lines demonstrated the highest maximal adhesion levels and adhesion rates *(Table 2 and Fig. 2)*.

The adhesion profiles of melanoma cell lines, A375P, and A375M are compared in *Fig. 2C*. There was a small but consistent and significant pattern, whereby the A375M cells were more adherent than the less malignant A375P. For example, at one hour, 57.6% of the A375M cells attached to the monolayers as compared to only 30.2% of the A375P cells.

DISCUSSION

Although tumor cell-vascular endothelial cell interactions have been measured using a variety of tumor cell types and assays (8-13; for reviews, see 3,4,11), corresponding studies with lymphatic endothelial cells have not been performed because of the difficulty of obtaining and culturing such cells. Also, problems were associated with the presumed incompatibility of culture conditions required by endothelial vs. tumor cell lines. Using a series of increasingly supplemented media, we found that: (a) the determination of primary and secondary culture conditions is facilitated for numerous normal and neoplastic cells, (b) the culture conditions required for rapid growth and proliferation need not be particularly different for normal and neoplastic cell cultures, and (c) long-term or temporary cocultures are possible, thereby facilitating the study of normal cell-tumor cell interactions. Here we describe a simple and rapid quantitative method to measure the ability of human tumor cells to attach to confluent monolayers of lymphatic endothelium.

We found the maximum percentage of attached cells and the maximum rates of adhesion for the SW-480 and MCF-7 cell lines to be significantly higher than those for the ALN and NRG cell lines. ALN and NRG cells had a rounded morphology *(Fig. 1)* and had been cultured for only 15 to 17 passages at the time the study began. In contrast, the SW-480, MCF-7, as well as the A375P and A375M were more fibroblastic and had all been in culture for >200 passages. Thus, it seemed that time in culture and cell morphology might predict the interaction of tumor cells with lymphatic endothelium. The BDW Ewing sarcoma tumor cells, which had a fibroblast-like morphology and had only been cultured *in vitro* for 4 passages, were exceptional in that they attached to the endothelial cell layer more rapidly and completely than most of the other primary cell cultures. Presumably residing only in the blood vasculature *in situ*, these sarcoma cells exhibited an adhesion maximum comparable to the established cell lines and, in contrast, a maximum rate of adhesion comparable to other primary cell lines tested. Collectively, these data suggest different adhesion mechanisms may be involved for each cell line and that tumor cell morphology may be a determinant of lymphatic endothelial cell adhesion potential. This includes the possibility that the cells possess carcinoma-associated or sarcoma-associated components which recognize host/lymphatic endothelial components (25).

The maximum rates of adhesion of the melanoma cell lines correlated with their previously reported metastatic and invasive potentials. The A375M and A375P cells have long been of interest as

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a model to study the metastatic process, since the A375M cells produce approximately 10 times more experimental lung metastases in nude mice, and, using an *in vitro* invasion model, invade basement membranes approximately 5 times faster than the A375P cells. Melanoma cells often aggressively invade lymphatic vessels proximal to the primary site and rapidly spread to distant sites via the lymphatics; yet, whether invasion has occurred at the time of biopsy cannot be reliably predicted at this time. We have recently discovered that a third human melanoma cell line, C8161 (27), has a significantly greater metastatic and invasive capacity than that of the A375M cell lines (26). Preliminary studies with these cells in our assay have demonstrated an attachment profile and maximum rate of adhesion that is slightly greater but statistically equivalent to that of the highly metastatic A375M cells. Collectively, these data suggest a strong correlation between the metastatic and invasive potentials of human melanoma cells and their maximum rate of adhesion to lymphatic endothelium. By using this assay to measure maximum rate of adhesion of tumor cells to lymphatic endothelium, it may be possible to predict the metastatic potential of a primary tumor before the appearance of secondary metastases.

We approached this study with the following hypotheses: first, tumor cell types known to metastasize via lymphatics, such as melanoma and breast carcinoma, should show a greater ability to attach to lymphatic endothelium than tumor cell types that are rarely associated with the lymphatics, such as Ewing sarcoma. The ability to attach to lymphatic endothelium probably parallels differential recognition mechanisms similar to those for organ 'homing' by blood-borne metastatic cells. And, third, these 'homing' mechanisms involve the expression of cell surface proteins and/or receptors. Our data are equivocal for proving any of these hypotheses. Instead, the data suggest an alternative hypothesis: that tumor cells with fibroblastic morphology and/or high metastatic potential are most adaptive to rapid adhesion to lymphatic endothelium. This may be influenced by the fibroblast's cytoskeletal/transmembrane interactions with the lymphatic endothelial cells, which would allow more surface area attachment compared with tumor cells displaying a rounded morphology.

Although methods used to detach and collect tumor cells remove plasma membrane components involved in attachment, previous studies have shown that tumor cells can rapidly (10-30 min) regenerate plasma membrane-bound proteins when incubated in serum-containing medium after collection (28). Perhaps the tumor cell lines which demonstrated the lowest rates of adhesion may have greater difficulty regenerating membrane-bound proteins needed for adhesion. In general, it is likely that the higher rates of adhesion were rather due to an increased propensity of tumor cells to penetrate the endothelial cell layers and attach to the underlying extracellular matrix as has been previously noted (8,11,25). Another reason may be that the bovine lymphatic endothelial cells are sufficiently different from human lymphatic endothelium such that some cells can distinguish and, therefore, not bind. Moreover, it is highly likely that endothelial linings isolated from different places will be as different as vascular endothelium from different organs. Until human lymphatic endothelial cells from multiple sources are isolated and characterized these questions will remain unanswered.

Nonetheless, the data presented here confirm the expected difference between tumor types for adhesion properties. The data also suggest that this rapid, quantitative approach, with modifications and improvements, may be practical for the evaluation of tumor malignancy from primary explants. This, of course, would be very useful in planning therapeutic follow-up.

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