METHODS

A Novel *In Vitro* System to Study Extravasated Tumor Cell-Induced Angiogenesis

Carlos J MALDONADO,[1] Katherine PALAZZOLO,[1] John D TAYLOR[1,2,3]

[1]Biological Sciences Department, Wayne State University, Detroit, [2]Karmanos Cancer Institute, Detroit, [3]Gershenson Radiation Oncology Center, Detroit, USA

Angiogenesis, the formation of new blood vessels from preexisting ones, is a fundamental stage in the metastatic pathway. For the primary tumor, this neovascularization provides nutrients and oxygen as well as a route by which metastatic tumor cells gain access to the circulatory system. Among these metastatic tumor cells, there are subgroups of cells that express an angiogenesis-inducing cells phenotype (AICs) as well as others that do not. Tumor cells not expressing the angiogenesis-inducing cells phenotype (non-AICs) invade new tissues and remain as dormant micrometastases unless they accompany AICs. Thus, either alone or with non-AICs, angiogenesis-inducing cells form rapidly growing, clinically detectable metastases. Much of the current research in this area is concentrated on the vascularization of primary tumors, but the regulation of angiogenesis by extravasating or invading tumor cells has not being extensively studied. We have developed a working model, which demonstrates that human metastatic prostate cancer cells (PC-3) appear to induce human vascular endothelial cells (HUVECs) to translocate across a Matrigel-coated 8 μm membrane. The parameters of this model (i.e. pore size, seeding-cell density, seeding times) were established using highly invasive murine melanoma cells (B16F10) seeded on murine microvascular endothelial cells (CD3). We have further modified our model in order to include a host compartment made of collagen gel, in order to mimic the *in vivo* site of metastases-induced angiogenesis. (Pathology Oncology Research Vol 4, No 3, 225–229, 1998)

Key words: blood vessels, collagen gel, extravasation, invasion, *in vitro*, metastasis, secondary angiogenesis

Introduction

During embryogenesis, two different mechanisms contribute to the formation of the vascular system: vasculogenesis, where in the yolk sac, prospective endothelial cells encapsulate blood islands, thereupon these capsules fuse and lead the formation and development of capillaries; and angiogenesis, the formation of microvasculature from preexisting vessels.[1] Angiogenesis occurs not only during embryonic development and post-natal growth, but also in adult life as well during wound healing and endometrial proliferation. In addition, it plays major roles in the progressive growth and metastasis of cancerous tumors.[2] The mechanisms, by which angiogenesis occur under normal physiologic conditions, can be distinguished from that occurring in solid tumors, by the lack of growth regulation. Under normal physiological conditions, angiogenesis is both temporally and spatially regulated.[3] In contrast, tumor induced-angiogenesis continues until the tumor is surgically removed, or until the host dies. In spite of their proliferative capacity, such tumor cells cannot expand their population beyond one or two cubic millimeters due to oxygen and nutrient deficiency, and the accumulation of both carbon dioxide and nitrogenous waste.[4] However, when neovascularization of the tumor mass occurs, rapid expansion and eventual metastasis of the cancer take place.[5] Tumor-induced angiogenesis produces an increased density of immature, highly permeable capillaries that have little basement membrane and fewer intercellular junctional complexes than normal capillaries.[6] Gaining access to the blood

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Correspondence: John D. TAYLOR, PhD; Department of Biological Sciences, 5047 Gullen Mall, Wayne State University, Detroit, MI 48202 USA; Tel: 1-313-577-6816, fax: 1-313-577-1093; E-mail: cheyenne@sun.science.wayne.edu

Abbreviations: AICs: angiogenesis-inducing cells; PC-3: human prostate adenocarcinoma cells, metastasis to vertebrae; HUVEC: human umbilical vein endothelial cells; B16F10: murine melanotic melanoma cells, high metastatic level; CD3: murine microvascular endothelial cells; bFGF: basic fibroblast growth factor.

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stream (intravasation) allows cancer cells to travel through the circulatory system to distant sites where they may successfully attach to and exit from the blood vessel (extravasation) and invade the surrounding tissue. In their new location, the metastases will grow, vascularize and start the cycle again. Much of the current angiogenesis research is concentrated on the vascularization of primary tumors, but the regulation of angiogenesis by extravasating or invading tumor cells has not being extensively studied. It is the successful neovascularization of these metastases what confers the lethality of most solid-tumor cancers. No model system exists to study neovascularization of metastases generated *via* extravasating angiogenesis-inducing cells (AICs). We have developed a working model, which demonstrates that human cancer cells (PC-3) appear to induce human vascular endothelial cells (HUVECs) to translocate across a Matrigel-coated 8 μm membrane. We have further modified our model in order to include a host compartment made of collagen gel, in order to mimic the *in vivo* site of metastases-induced angiogenesis.

Materials and Methods

Cell Lines

Normal human umbilical vein endothelial cells (HUVEC, ATTC CRL 1730, Rockville, MD), were maintained in 90% F-12 medium (Gibco, Grand Island, NY) + 10% fetal bovine serum (FBS, Sigma, St. Louis, MO) + 100 μg/ml heparin (Sigma) + 30 μg/ml endothelial cell growth supplement (ECGS, Sigma). Prostate adenocarcinoma cell line PC-3 (ATCC CRL 1435) was maintained in RPMI medium 1640 (Gibco) supplemented with 10% FBS. Murine melanotic melanoma (B16F10, kindly provided by Dr. Clement Diglio) were maintained in 90% minimum essential medium (MEM, Gibco) + 10% fetal bovine serum (FBS, Sigma, St. Louis, MO). Murine lung microvascular endothelial cells (CD3, kindly provided by Dr. Clement Diglio) were maintained in 80% Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 20% FBS (Sigma). Cells were incubated in a humidified atmosphere at 37°C with 5% CO_2 (Forma Scientific, Marietta, OH) and the media was changed every 48 hours. All cells were passaged with a mixture of EDTA and Trypsin (Gibco) and used at low passage number (≤15). All media were supplemented with a mixture of 200 IU/ml penicillin (Gibco) and 200-μm/ml streptomycin (Gibco).

Tumor Cell Invasion System

Regular Matrigel Invasion Chambers (Collaborative Biomedical Products, Bedford, MA) were rehydrated for 2 hr prior to the start of the experiment with serum free F-12 medium, at 37°C. HUVEC were removed from the flasks with Trypsin-EDTA (Gibco) and seeded on the Matrigel Invasion Chamber for 12 hr at ~ 400,000 cells/chamber. The prostate adenocarcinoma cell lines PC-3 were seeded at ~ 25,000 for 24, 48 and 72 hours after the HUVEC reached confluency. They were seeded so that all time intervals ended at the same time. After the periods were concluded, the upper surface of the membrane (Matrigel-coated surface) was swabbed with a cotton tip applicator. Cells in the host compartment, were then washed with Trypsin-EDTA (Gibco) for ~ 5 min, then centrifuged, resuspended in serum-free F-12 medium and plated on glass coverslips. Plated cells were then reacted (standard protocol) with antibodies against Factor VIII realted antigen (von Willebrand factor (vWF), kindly provided by Dr. Clement Diglio). Briefly, plated cells were fixed with cold methanol for 5 minutes and air-dried. After washing with phosphate-buffered saline, plated cells were incubated with rabbit antiserum to human factor VIII antigen (Behring Diagnostics, La Jolla, California) (1:4 dilution) for 30 minutes. Coverslips were then washed three times with phosphate-buffered saline and incubated for 30 minutes with fluorescein-conjugated goat antirabbit IgG (Santa Cruz Biotechnology, Santa Cruz, California) using a 1:10 dilution. Collagen gels were prepared as described by Elsdale and Bard.7 A polystyrene sleeve (1.5 cm height X 0.9 cm inside diameter) was inserted around the membrane part of the plastic insert at about 0.5 cm. This created a gel plug, which allowed rapid and complete perfusion of the system by the fixative as well as the removal of the membrane/gel compartment while keeping contact between the membrane/matrix intact. The host compartment was fixed with a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde (Electron Microscopy Sciences, Ft. Washington, PA). A secondary fixation with 1% osmium tetroxide (Electron Microscopy Sciences, Ft. Washington, PA) followed.

Light Microscopy

Per Nikon of America, on using a Nikon, Optiphot-2 (100 x with ND-2 filter) fluorescence microscope. The area of the field of view using the 10x objective can be calculated as follows. Diameter (D) of field of view when using the 10x objective:

$$\frac{\text{20 mm (eyepiece aperture)}}{\text{10X[1.25 (mag. coefficient)]}} \Rightarrow \text{D = 1.6 mm radius (r) of field of view = 0.8 mm}$$

Area (a = $\Pi \times r^2$) of field of view ⇨ $\Pi \times (0.8)^2$ ⇨ 2.01 mm^2.
Area (cs) of glass coverslip ⇨ 324 mm^2

Nine areas (a) or "spots" equidistant to each other, where selected so that the largest possible surface area of the coverslip was covered Total cells within the nine spots were counted and averaged, obtaining number of cells/spot. Total spots on entire coverslip area ⇨ (324 mm^2 / 2.01 mm^2) = 161.19 spots.

Transmission Electron Microscopy (TEM)

Standard TEM procedure was used. Briefly, membranes were fixed in 2% glutaraldehyde (Electron Microscopy Sciences, Ft. Washington, PA) at 4°C overnight. A secondary fixation with 1% osmium tetroxide (Electron Microscopy Sciences, Ft. Washington, PA) followed. The membranes were then dehydrated in ethanol and embedded in Spurr's mixture. Thin-sections obtained on a Reichert-Jung ultramicrotome were mounted on uncoated copper grids (Ted Pella) and counterstained with a saturated solution of uranyl acetate in 50% ethanol and Reynolds lead citrate. Sections were examined with a Philips 201 electron microscope at 80 kV.

Results

Model System

Our working model system consists of three major compartments separated by a Matrigel-coated 8 μm membrane *(Figure 1)*. The upper chamber contains growth media for the cellular compartment. Adding the endothelial cell medium first while the HUVECs were being seeded and then switching to the cancer cell medium while the PC-3 cells were being seeded, produced the best results for both cell types. The cellular compartment incorporates a monolayer of human vascular endothelium, and human metastatic tumor cells seeded on top of the monolayer. The host compartment provided a three-dimensional substratum where both HUVECs and PC-3 cells invaded through *(Figure 4)*.

Transmission Electron Microscopy (TEM)

TEM examination of the precursor model showed the interactions between metastatic tumor cells and microvascular endothelial cells. Highly invasive B16F10 tumor cells invaded through the CD3 microvascular endothelial cells, 12 hours after the tumor cells were seeded *(Figure 2)*. Several layers of CD3 cells were observed while debugging the logistics of the model system. This was corrected by seeding different quantities of endothelial cells and establishing a cell density that produced a monolayer. At later time intervals (i.e. 18, 24 hours) B16F10 cells had migrated through the CD3 cell monolayer, the Matrigel and the membrane. It was also shown that placing an angiogenic factor (i.e. bFGF or FGF1) in the bottom chamber, increased the number of tumor cells that invaded through the compartments, during the different time intervals (data not shown).

Light Microscopy

Immunofluorescent microscopy analysis showed that HUVECs were intensively labeled for Factor VIII related antigen. PC-3 cells showed some fluorescence due to background "noise" *(Figure 3)*. By reacting the cell population in the host compartment with antibodies against a vascular endothelial cell-specific marker, we were able to discriminate between HUVECs and PC-3 cells. *Table 1* shows the total number of cells (extrapolated) per time interval. These numbers suggest that invading PC-3 cells, appear to induce HUVECs to translocate across the membrane. As the time intervals were increased, so did the number of translocated HUVECs. The total number of translocated cells suggests a ratio of approximately six

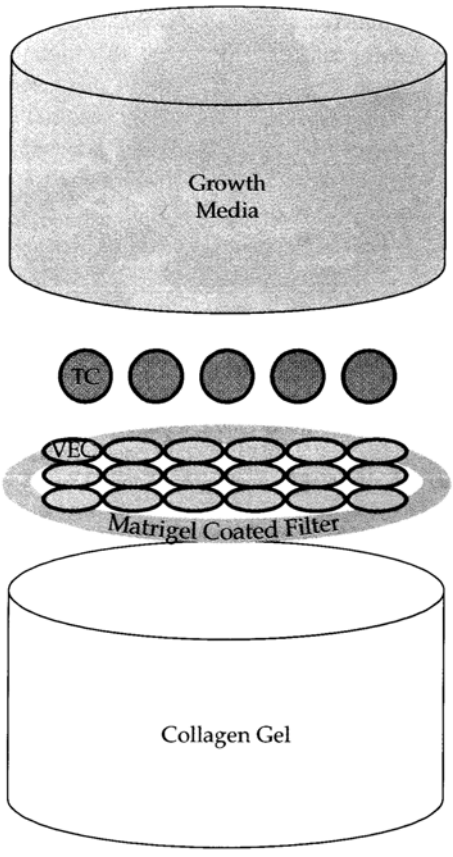

Figure 1. *Our model system consists of three major compartments separated by a Matrigel-coated 8 μm membrane. The upper chamber contains the growth media for the cellular compartment. The latter consists of vascular endothelium (VEC) and metastatic tumor cells (TC). The host compartment is composed of extracted collagen gel.*

Table 1. **Total translocated cells across an 8-μm Matrigel-coated membrane**

	24 hr	*48 hr*	*72 hr*
HUVECs only (control)	0	0	4
Translocated HUVECs	1,254	1,952	3,063
Translocated PC-3	7,344	12,734	17,409

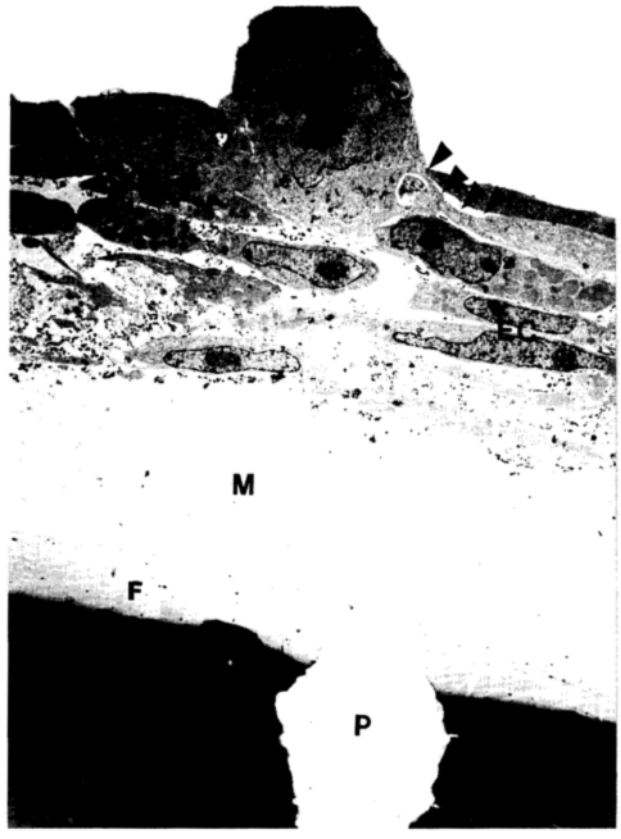

Figure 2. Transmission Electron Microscopy (TEM) of a cross section of our model system. Highly invasive B16F10 (TC), invades through (arrows) vascular endothelial cells CD3 (EC), 12 hours after the TC were seeded. The Matrigel (M) coating can be seen intact at this early time interval. Image also shows the role of the 8 μm membrane (filter, F; pore, P) as it provides support for the components of the upper compartments. (x3000).

PC-3 cells for each translocating HUVEC. This ratio was maintained throughout the time intervals. These experiments were conducted in quadruplets. *Figure 4* shows how the membrane/matrix interface remain intact after its removal from the system. The top of the membrane was swabbed to remove the cells that had not migrated through after 24 hours. A clean, porous upper surface of the membrane (M) can be seen. Further down the plane of the image (away from the viewer), is the collagen gel (Co) containing a HUVECs and PC-3 cell-cluster (c). The cells are connected to one another and appear to be forming a continuos network.

Discussion

Angiogenic metastases are responsible for the high death-rate of cancer.[8,9,10] Understanding the biology of these metastases is crucial for the appropriate design and development of successful anti-metastatic therapies. Solid tumors contain subgroups of cells that express an angiogenesis-inducing cell phenotype (AICs) as well as cells that do not.[11] Tumor cells not expressing the cell phenotype (non-AICs) invade new tissues and remain as dormant micrometastases unless they accompany AICs. Thus, either alone or with non-AICs, angiogenesis-inducing cells form rapidly growing, clinically detectable metastases. From a clinical perspective, this secondary angiogenesis, resulting from the presence of AICs, allows secondary tumors to proliferate and subsequently undergo secondary metastasis.[12] At the cellular level, when the tumor reaches a critical mass, AICs release angiogenic factors which induce the onset of neovascularization allowing perfusion of the tumor mass by malformed, leaky capillaries that originate from pre-existing capillary beds

Figure 3. Positively labeled HUVECs (H) have joined initiating the formation of a continuous capillary-like structure. These cells were removed from the host compartment and reacted with antibodies against Factor VIII related antigen. An unlabeled PC-3 cell (P) can be seen at the top right corner. (x400).

Figure 4. Removed membrane (M) showing collagen gel (Co) containing a cluster (c) of cells. The "swabbed" upper surface of the membrane (M) can be distinguished from the host compartment (Co). The cells (c) appear to be interconnected forming a contiguous network (x200).

and venules.[13] Angiogenesis-inducing cells released from the primary tumor into the bloodstream extravasate from the hematogenous environment and invade new tissues producing clinically detectable metastases.[11] Therefore, the origin of the secondary tumor is different from that of the primary tumor. The successful growth of the metastases requires perfusion of the tumor mass similar but different to that of the primary tumor. The question arises as to which mechanisms are used. Our results suggest that extravasated prostate tumor cells induce angiogenesis by recruiting vascular endothelial cells from the capillary it extravasated from. The mechanisms involved in the post-extravasation, angiogenic phase of the metastatic process, needs to be dissected and the stages susceptible to intervention be identified. Our *in vitro* model provides both, a novel tool and approach for such task. It mimics *in vivo* extravasating, human, tumor cell-induced angiogenesis. Although our *in vitro* model system is an extension of previous techniques[14,15] that have utilized invasion chambers, it is unique. In the past, our laboratory developed a precursor to our current model using a Modified Boyden chamber.[16,17] This precursor used metastatic variants of murine melanoma tumors (B16F1 and B16F10) seeded on murine microvascular endothelial cells (CD3). The CD3 cells were seeded on Matrigel coated membrane. As the tumor cells invaded through the endothelial cell layer and the coated membrane *(Figure 2)*, they remained attached to the bottom side of the membrane or sank to bottom of the well. The major change from previous systems is the creation of a host compartment using a collagen gel, which now can be manipulated in order to resemble the morphological and physiological states *in vivo* during various controlled states. The various compartments in our model correlate with the microvascular environment found *in vivo (Figure 1)*. The growth media compartment correlates with the hematogenous environment within capillary beds. The endothelial cell lining of these beds is reproduced by our vascular endothelium monolayer and the Matrigel coat provides the role of the basement membrane. The host compartment mimics the environment encountered by invading tumor cells as they extravasate into distant sites. Interactions between invading tumor cells and the vascular endothelium can be examined at the ultrastructural level using standard methods in electron microcopy *(Figure 2)*. Vascular endothelium appears to follow invading tumor cells as they translocate through the Matrigel and the 8 μm membrane into the host compartment *(Figure 4)*. Moreover, the host compartment tolerates normal laboratory handling allowing various methods of analysis. Our model provides a novel tool in which questions relating to therapy can be asked. For example, screening novel drugs or combinations of them, which either inhibit or induce angiogenesis can be examined as well as establishing a standard tumoral vascularity profile for various tumor-forming cancers. This will expand our understanding of the events that occur during and after the extravasation step of metastasis along with facilitating therapy and prognosis of secondary tumors resulting from angiogenesis-inducing cells.

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