Pericyte-Like Location of GFP-Tagged Melanoma Cells

Ex Vivo and In Vivo Studies of Extravascular Migratory Metastasis

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Previous studies have demonstrated that some tumor cells occupy a pericyte-like location in melanoma, forming angio-tumoral complexes. We hypothesized that these tumor cells are migrating along the abluminal surface of the endothelium, a mechanism termed “extravascular migratory metastasis.” In the present study, we have used human and murine melanoma cells that stably express enhanced green fluorescence protein (GFP) to examine, in an *ex vivo* co-culture model, melanoma cell interactions with vessels that have sprouted from rat aortic rings. We also used *in vivo* tumor growth on the chick chorioallantoic membrane (CAM) to observe the dissemination pathway of melanoma cells. In the *ex vivo* rat aorta system, we observed a pericyte-like location of tumor cells that were spreading along the vascular channels. For examination of the CAM *in vivo*, we have used the Lugassy preparation, allowing one to obtain striking images of the relationship between fluorescent GFP cells and microvessels. Melanoma cells were found cuffing the outside of vessels around the tumor. Tumor cells were observed along the vessels several centimeters from the tumor. Confocal microscopy and histopathology confirmed the pericyte-like location of tumor cells, without any observ-

able intravasation. The results indicate that melanoma cells can migrate along the abluminal surface of vessels. This study also demonstrates that these models can provide quantitation analysis that may prove useful in elucidating the molecular interactions involved in extravascular migratory metastasis. (*Am J Pathol* 2004, 164:1191–1198)

How metastases develop is poorly understood.¹ Tumor cells are thought to arrive in lymph nodes and other sites through the blood and lymphatic circulation where they will then extravasate. The concept of intravascular dissemination of cancer cells has been widely accepted as a central paradigm.^{2,3} In addition to this explanation however, other mechanisms may be operable. Tissue-based mechanisms of tumor migration may also contribute to the spread of cancer.^{4–8}

Although increased tumor angiogenesis has been generally associated with an increased incidence of distant metastasis,³ it has been demonstrated that tumor vascularity is not a marker of metastasis for melanoma.⁹ Many reports have demonstrated that cancer cells are located in the wall of tumor blood vessels, creating “mosaic” vessels.^{10–13} In the so-called mosaic vessels, both endothelial cells and tumor cells form the luminal surface. More recent observations have suggested that aggressive melanoma cells may generate vascular channels independent of tumor angiogenesis, a phenomenon characterized as “vasculogenic mimicry”^{14,15} in which some melanoma cells appear to acquire the capability to form blood channels in the absence of endothelial cells.

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Our studies have demonstrated a different form of melanoma-endothelial cell interaction, the "angio-tumoral complex," in which tumor cells occupy a pericyte-like location, ie, on the abluminal surface of the endothelium.^{16,17} Laminin has been localized in close proximity to these pericytic-localized tumor cells, raising the possibility that tumor cells are migrating along the abluminal surface of the endothelium, a mechanism termed extravascular migratory metastasis.^{18–21} The extravascular migratory metastasis (EVMM) proposed for melanoma has close analogies with glioma migration. Invading glioma cells follow distinct anatomical structures within the central nervous system. Tumor cell dissemination occurs along tracts, such as the basement membranes of blood vessels or the glial limitans externa, that contain extracellular matrix proteins.²² Interestingly, the florid angiogenesis, as well as the expressions of matrix metalloproteinases observed in gliomas, does not correlate with hematogenous tumor spread.²³ The extent to which these tumors invade adjacent structures can be considerable.²⁴ Using an *in vivo* murine brain tumor model, we have recently shown a clear progression of glioma and melanoma cells along the abluminal surface of vessels.²⁵ Furthermore, we have demonstrated melanoma angiotropism and migration along capillary-like structures *in vitro*.²⁶

Here, we have used human and murine melanoma cells that stably express green fluorescent protein (GFP) to study the interaction of tumor cells with vessels over time. We have developed an *ex vivo* co-culture model system to study melanoma cell interactions with vessels that have sprouted from rat aortic rings. The rat aorta model is used for studying angiogenesis *ex vivo*, producing a microvascular network composed of branching endothelial channels surrounded by non-endothelial mesenchymal cells (pericytes), closely resembling the vessels formed during angiogenesis *in vivo*.^{27,28} We have also grown melanoma tumors on the surface of the chicken chorioallantoic membrane (CAM) to study tumor cell dissemination *in vivo*. In the *ex vivo* model systems, tumor cells are localized in a pericyte-like location and, in the CAM model, the tumor cells migrate several centimeters from the tumor along the outside of the vessels. Assays for quantifying the formation of the angio-tumoral complexes (melanoma cells in a pericyte-like location) have been designed, validated, and implemented.

Materials and Methods

GFP Melanoma Cells

C8161, a highly invasive and spontaneously metastatic human melanoma cell line,²⁹ was transfected with the enhanced green fluorescent protein (GFP) as previously described.³⁰ Cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (DME-F12, Gibco-BRL Life Technologies, Rockville, MD) supplemented with 5% fetal bovine serum (Invitrogen, New Zealand) and 1 μ g/ml puromycin (Gibco

BRL). The cells were cultured at 37°C in a humidified, 5% CO₂/95% air atmosphere.

B16F10 melanoma cells³¹ were used for GFP transfection. Briefly, gentamicin (Gibco BRL Life Technologies) sensitivity was determined to be 600 μ g/ml. GFP on the pRK5 plasmid and gentamicin resistance on the pcDNA III plasmid (Kenneth M. Yamada, National Institute of Dental and Craniofacial Research, National Institute of Health (NIDCR), NIH) were transfected into B16F10 cells by electroporation. Cells were incubated overnight in the presence of 3.3 mmol/L thymidine (Calbiochem, San Diego, CA). The cells were washed twice with Hanks' balanced salt solution (Gibco BRL Life Technologies) and grown for 7 hours in fresh medium. The cells were washed in electroporation buffer containing 20 mmol/L HEPES (pH 7.05), (Gibco BRL Life Technologies), 137 mmol/L NaCl, 5 mmol/L KCl, 0.7 mmol/L Na₂HPO₄, 6 mmol/L dextrose (Sigma, St. Louis, MO), and 1 mg/ml albumen (ICN Biomedicals, Inc., Aurora, OH). Cells were resuspended at 3×10^6 /ml in electroporation buffer, and 30 μ g of GFP plasmid and 3 μ g of the gentamicin resistance plasmid were added per 500 μ l. The cells were electroporated at 170 volts for approximately 5 seconds, suspended with a pipette, transferred to a plate containing medium with 0.5 mol/L n-butyric acid (pH 7.18) (Sigma, St. Louis, MO), and grown overnight. Fresh medium was added after washing with Hanks' balanced salt solution. After 24 hours, 600 μ g/ml gentamicin was added and treatment was continued for 4 weeks at which time the GFP-positive cells were selected by FACS (FAC-Star PLUS, Becton Dickinson, San Diego, CA) in the NIDCR, NIH, core facility. Cells producing GFP in the top 50% of the range were cultured further. Fluorescence levels were monitored with an Axiovert 135 microscope (Zeiss, Thornwood, NY). Selection was performed three times over 3 months to establish a "stable" GFP-producing cell line. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL Life Technologies), containing 10% fetal bovine serum (HyClone, Logan, UT), 100 U/ml penicillin, 100 μ g/ml streptomycin, and non-essential amino acids solution (Gibco BRL Life Technologies). The cells were maintained at 37°C in a humidified, 5% CO₂/95% air atmosphere.

Nevus Cells

Nevus cells (NC) were used as control. Isolation and culture of NC was performed as described.³² Briefly, the tissue was incubated overnight in HAM-F10 medium containing 0.6 U/ml 1 dispase and 0.05 U/ml collagenase, supplemented with 200 U/ml penicillin G, 0.2 mg/ml streptomycin sulfate, 0.2 mg/ml kanamycin sulfate, and 25 μ g/ml gentamicin. The detached cells were washed, seeded in culture flasks at the density of 10^5 cells/ml and incubated for 48 hours with 0.1 mg/ml geneticin in HAM-F10 medium, supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin sulfate, 2% Ultrosor-G, 16 nm phorbol 12-myristate 13-acetate, and 0.1 mm isobutyl methylxanthine. NC were cultured for a maximum of 15 passages.

Ex Vivo Assay

Rat aortic ring cultures were established as previously described.^{27,28} Briefly, aortas were obtained from 4- to 5-week-old Sprague Dawley rats. The aortic vessel was cross-sectioned at 1-mm intervals with a scalpel. Each resulting vascular ring was transferred into the center of a well (96-well plate) that has 30 μ l Matrigel added to it. Additional Matrigel was added on top of the ring to secure it in place. Aortic rings were cultivated for 5 to 7 days until they were surrounded by a meshwork of vascular sprouts.

To obtain a cohesive tumor cell aggregate containing the same number of cells, 10,000 GFP melanoma cells and 10,000 NC were centrifuged, and the pellet was suspended in 5 μ l of Matrigel. Each of these tumor aggregates was delicately inoculated on the periphery of each well containing a ring beginning to sprout vessels (4 to 5 days), at a distance of 2 mm from and at the same depth as the aortic ring. Tumor aggregates were also cultured in the absence of aortic rings as controls. Isolated cultures of rat aortic rings were also studied as controls. Aortic rings and tumor aggregates were cultured together for up to 1 week, and were examined daily using an inverted fluorescence microscope. Photographs of living cultures were taken. After 48 hours, the percentage of melanoma cells in a pericyte-like location along the endothelial network was counted. Between the aggregate and the ring, three fields containing a total of approximately 100 tumor cells or NC were randomly selected. The total number of fluorescent tumor cells and the number of fluorescent tumor cells in a pericyte-like location were quantified. The maximum migrated distance between the aggregate and melanoma cells or NC along vascular sprouts was also measured. The co-culture assay was performed in triplicate and each experiment was repeated three times. NC were used as control. NC were pigmented in culture and therefore were easily recognized by conventional microscopy in co-culture with aortic rings.

In Vivo Chick Chorioallantoic Membrane Assay

In addition, we used a modified chorioallantoic membrane assay.³³ Briefly, fertilized white leghorn eggs were incubated in an humidified, 37°C incubator for a total of 20 days. At day 10, a small hole was drilled on the top of the egg above the area of greatest vasculature, causing the CAM to detach from the shell membrane. GFP melanoma cells (10^5 B16F10 and 10^6 C8161), in a volume of 50 μ l of complete corresponding cell culture media plus 50 μ l of Matrigel, were inoculated onto the dropped CAM with a pipette tip (five eggs for each tumor type). The holes were sealed with tape and the eggs were returned to the incubator. Ten days after inoculation (day 20), eggs were carefully opened. Tumor growth on the CAM was obtained by measuring tumor volume ($l \times w \times h$) with a caliper.

For examination of the CAM, we have developed a new, simple, and novel preparation never used before,

which we hereby term the Lugassy preparation. We have used fluorescence microscopy to examine the fresh CAM after tumor development, ie, before any fixation or sectioning. This preparation has allowed us to obtain striking images of the relationship between fluorescent GFP cells and microvessels. Indeed, since the CAM is a thin membranous structure, the vessels are generally arrayed throughout the two-dimensional anatomy of the CAM. If the CAM is carefully spread on the flat surface of a petri dish or glass slide, it is possible to observe with acceptable focus the vascular network of the CAM. It is even possible to observe on the fresh CAM fluid and cellular movement within these vessels. Using GFP fluorescent tumor cells, this simple technique presents a great opportunity to observe the dynamic relationships of tumor cells with vessels. The tumor containing area, as well as the remaining complete CAM, were removed, and placed in a petri dish with 1 PBS to maintain moisture. The CAM was cut in several parts to obtain flat observable specimens. These specimens were directly observed under a fluorescent microscope. We have counted the number of vessels cuffed by the fluorescent melanoma cells in five consecutive fields around the tumor mass. The maximum distance between fluorescent tumor cells in a pericyte-like location on the CAM and the tumor was measured. Using the same preparation, some areas next to the tumor were also observed using a confocal laser-scanning microscope. These areas were directly mounted on glass slides using ProLong Mounting Media (Molecular Probes). After dissection, tumor and not tumor areas were fixed in 10% formalin for histopathological observation and immunohistochemistry with melanoma markers (S100 Protein, HMB45, and Mart1). The experiment was repeated twice. 2.0×10^6 NC were inoculated onto the CAM as control.

Confocal Laser-Scanning Microscopy

A Bio-Rad MRC 1024 confocal laser-scanning microscope (Hercules, CA) equipped with a krypton-argon laser and an Olympus IX-70 inverted microscope (Melville, NY) was used to visualize GFP fluorescence (488 nm laser line excitation; 522/35 emission filter) and transmitted-light images (transmitted light detector). Optical sections ($Z = 1 \mu$ m) of confocal epifluorescence images and a non-confocal, transmitted-light image were acquired simultaneously³⁴ using a 20 $\times$ objective lens ($NA = 0.7$) with LaserSharp v3.2 software. Adobe Photoshop v6.0 software with Bio-Rad plug-ins was subsequently used to merge GFP and transmitted-light images.

Statistical Analysis

Student's *t*-test ($P < 0.05$) was used to compare the differences between the means of the melanoma cells and the melanocytes used in the *ex vivo* and *in vivo* models.

Figure 1. Maximum migrated distance in millimeters of melanocytic cells toward vessels sprouting from rat aortic rings after 24 hours. The results represent the mean $\pm$ standards errors of three independent experiments. The average maximum migrated distance for the human C8161 melanoma cells was significantly more than for the B16F10 melanoma cells ($P = 0.0032$, one-tailed F test), and also significantly more than for the nevus cells ($P = 0.028$). The average maximum migrated distance for the nevus cells was significantly more than for the B16 melanoma cells ($P = 0.011$).

Results

Co-Culture of Rat Aortic Rings and Melanoma Cell Aggregates

We first examined the co-culture of tumor cells with vessels sprouting from rat aortic rings. Twenty-four hours after aggregate inoculation, the C8161 human melanoma cells migrated preferentially in the direction of the ring aorta, demonstrating angiotropism. In contrast, B16F10 melanoma cells exhibited very little migration toward the sprouting vessels at this time point. The average maximum migrated distance toward vessels for the human C8161 melanoma cells (0.69 mm) was significantly more than for the murine B16 melanoma cells (0.12 mm), as shown in Figure 1. Interestingly, endothelial cells migrated toward both the C8161 and B16F10 tumor aggregates, demonstrating tumorotropism (Figure 2,A and B). By 48 hours, C8161 melanoma cells continued to migrate toward the ring along the vascular sprouts in a pericyte-

Figure 2. Co-culture of melanoma cells with sprouting rat aortic rings. **A, C, and E** demonstrate conventional microscopy, and **B, D, and F** show corresponding fields with fluorescent microscopy. **A–B:** Rat aortic ring 24 hours after the inoculation of a murine GFP B16F10 melanoma cell aggregate. Note an endothelial cell (black arrow in **A**, not fluorescent in **B**), having sprouted from the aortic ring, and which is migrating toward the GFP tumor aggregate (ag), demonstrating tumorotropism. Bar, 25 μ m. **C–D:** Rat aortic ring 24 hours after the inoculation of human GFP C8161 melanoma cell aggregate. Note the progression of fluorescent C8161 melanoma cells from the aggregate (ag) toward the ring (r), along the vascular sprouts (black arrows in **C**). Bar, 100 μ m. **E–F:** Rat aortic ring 48 hours after the inoculation of a murine GFP B16F10 melanoma cell aggregate. GFP melanoma cell (black arrow in **A**, fluorescent in **B**) migrating from the tumor aggregate in the direction of the ring, along a vascular sprout (red arrow in **A**). Bar, 25 μ m.

Figure 3. Co-culture of melanocytic cells with rat aortic rings after 48 hours. **A:** Percentage of tumor cells in a pericytic location. The results represent the mean $\pm$ standards errors of three independent experiments. The average percentage of melanoma cells in a pericytic location was significantly more for C8161 cells than for B16F10 cells ($P = 0.0038$, one-tailed F test). The average percentage of cells was significantly more for C8161 melanoma cells than for the nevus cells ($P = 0.0003$). The average percentage of cells was significantly more for B16F10 melanoma cells than for the nevus cells ($P = 0.0011$). **B:** Maximum migrated distance in millimeters between the tumor aggregate and the ring along vascular sprouts. The results represent the mean $\pm$ standards errors of three independent experiments. The average maximum migrated distance for the human C8161 melanoma cells was significantly more than for the nevus cells ($P = 0.001$). The average maximum migrated distance for the human C8161 melanoma cells was reproducibly more than for the murine B16F10 melanoma cells, but the difference was not statistically significant ($P = 0.062$ one-tailed F test). The average maximum migrated distance for the B16F10 melanoma cells was reproducibly more than for the nevus cells, but the difference was not statistically significant ($P = 0.053$).

like location (Figure 2, C and D), and the B16F10 melanoma cells also now demonstrated such migration (Figure 2, E and F). The nevus cells also migrated toward the endothelial tubules (Figure 1). Once along the tubules, they also migrated in a pericytic location (not shown).

Forty-eight hours after aggregate inoculation, the average percentage of melanoma cells in a pericytic location was significantly more for C8161 cells (96%), as well as for the B16F10 cells (85%), than for the nevus cells (67%). The average percentage of the human C8161 melanoma cells in a pericytic location was significantly more than for the B16 melanoma cell line (Figure 3A). The average maximum migrated distance for the human C8161 melanoma cells (1.67 mm) was significantly more than for the nevus cells (0.55 mm). The average maximum migrated distance for the human C8161 melanoma cells was reproducibly more than for the murine B16F10 melanoma cells, but the difference was not statistically significant ($P = 0.062$). The average

maximum migrated distance for the murine B16F10 melanoma cells was reproducibly more than for the nevus cells, but the difference was not statistically significant ($P = 0.053$, Figure 3B).

The murine melanoma cells growing from B16F10 aggregates plated on Matrigel in the absence of aortic rings exhibited rounded or elongated shapes, with some nests of partially connected cells. The human melanoma cells growing from C8161 aggregates plated on Matrigel in the absence of aortic rings became elongated and connected, forming a network. NC also gave rise to a partial network (not shown).

Tumor Growth and Spread on the Chick Chorioallantoic Membrane Assay

We next analyzed the spreading of tumor cells from inoculates grown on the CAM. At day-20, B16F10, and C8161 melanoma cells developed highly vascularized, solid tumors at the inoculation site. Nevus cells did not produce tumors on the CAM.

The average tumor volume in mm^3 was 72 for the human C8161 melanoma tumor (Figure 4A), and 130 for the B16F10 melanoma tumors, which was significantly more than for the C8161 melanoma tumors ($P < 0.05$, data not shown). A pericyte-like location of the melanoma cells was observed directly on the fresh CAM by the Lugassy preparation. This preparation allowed the observation of fluid and cellular movement inside vessels, showing that the GFP tumor cells observed around the vessels were clearly not part of the circulating cells. Fluorescent tumor cells completely cuffed vessels around the tumor perimeter (Figure 4, B–D and Figure 5). The average number of vessels cuffed by the fluorescent melanoma cells around the tumor mass was 14 for the human C8161 melanoma cells, and 10 for the B16F10 melanoma tumors, which was not a significant difference ($P > 0.05$, data not shown). Histopathology and immunohistochemistry with the melanoma markers (S100 Protein, HMB45, and Mart1) has confirmed the pericyte-like location of tumor cells and absence of tumor cells in the vessel lumen (Figure 4B). Observation of the same preparation under confocal laser-scanning microscopy allowed the observation of fluorescent tumor cells along the external surface of the vascular plexus, as shown in Figure 5.

The Lugassy preparation permitted one to follow the dissemination pathway of melanoma cells along vessels at the single cell level on the complete CAM. Single B16F10 and C8161 tumor cells were regularly observed spreading along vessels several centimeters away from the tumor (Figure 4, E and F), as well as small tumor masses. The average maximum migrated distance for the human C8161 melanoma cells (2.6 cm) was not significantly different from for the murine B16F10 melanoma cells (2.7 cm), $P > 0.05$. No evidence of vessel damage or tumor intravasation was observed.

Figure 4. GFP melanoma cells on the chick chorioallantoic membrane 10 days after inoculation. **C** demonstrates conventional microscopy associated with fluorescence, **E** demonstrates conventional microscopy, and **D** and **F** corresponding fields with fluorescent microscopy. **A:** C8161 human melanoma tumor. The tumor measures 1.1 centimeters in diameter. The darkened vascular areas correspond to tumor extension along vessels (**arrows**), as shown at higher magnification under fluorescent microscopy (**C** and **D**). **Bar**, 2.5 mm. **B:** CAM inoculated with B16F10 murine melanoma cells, 3 mm from the main tumor. Immunostaining using the HMB45 melanoma marker. Note the pericyte-like location of the stained B16F10 melanoma cells around vessels, along the abluminal surface of the endothelium (**arrows**). Note the absence of tumor cells in the vessel lumen. **Bar**, 25 μ m. **C–D:** Lugassy preparation showing C8161 human melanoma cells (fluorescent in **C** and **D**, **green arrows**) spreading along vessels (**red arrow** in **C**). **Bars:** **C**, 25 μ m; **D**, 50 μ m. **E–F:** Lugassy preparation showing a GFP C8161 human melanoma cell (**green arrows** in **E** and **F**) along a vessel (**red arrow** in **E**), 2.8 cm distance from the tumor. **Bar**, 50 μ m.

Discussion

The present study strongly suggests that melanoma cells can migrate along vessels. In addition, it is possible to obtain quantifiable results (percentage of cells in a pericyte-like location, average maximum migrated distance) in the described models. Using co-culture of tumor cells and aortic rings, the migration of melanoma cells along vessels sprouting from the ring was observed. In the CAM assay, tumor cells cuffed vessels and were observed spreading along vessels several centimeters away from the tumor mass. Using both murine B16F10 and human C8161 melanoma cells, a tropism was characterized between the tumor cells and the endothelial cells leading to a pericyte-like location of tumor cells along the abluminal surface of vascular channels. It is important to note that the delayed pericyte-like location of the B16F10 melanoma cells in the aortic ring model re-

sulted from tumorotropism of endothelial cells, while the pericyte-like location of C8161 resulted from both angiotropism of tumor cells and tumorotropism of endothelial cells. In a previous study using capillary-like tubules *in vitro*, tumorotropism of endothelial cells toward invasive DU45 prostate tumor cells was observed,²⁶ as well as tumorotropism toward some melanoma cell lines (unpublished observations). The observation that different melanoma cells exhibit different levels of angiotropism may be relevant *in vivo*. Because of the mesenchymal, neural crest origin of melanocytes, it is not surprising that melanocytes from benign nevi, as well as melanoma cells, are spreading along the endothelial tubules, as we already observed in co-culture with capillary-like cultures *in vitro*.²⁶ However, *in vivo*, normal melanocytes, as well as melanocytes from benign nevi, are not in direct contact with vascular basement membranes.¹⁷ In the present

in vivo experiment, nevus cells did not form tumors on the CAM.

We have observed a pericyte-like location and the migration of the fluorescent melanoma cells along the endothelial tubules in the aortic ring models from the growing tips to the explanted ring tissue. Previously, we showed melanoma cells attaching to capillary-like tubules *in vitro*.²⁶ The advantage of the *ex vivo* model is that it allows the observation of a directional migration of the tumor cells toward the aortic ring.

The fundamental question of this work is the relevance of these observations to human melanoma. Any result obtained *in vitro* or *ex vivo* may not appear directly applicable to the human clinical problem. However, the *in vivo* model using tumor growth on the chick chorioallantoic membrane is a reliable *in vivo* model of tumor invasion, and the Lugassy preparation for the observation of the CAM demonstrated the dissemination of tumor cells along vessels leading away from the tumor. While tumor cells close to the tumor inoculate were completely cuffing some vessels, further from the tumor, melanoma cells were observed as smaller groups of cells along the outside of the vessels. At distances several centimeters from the tumor, isolated fluorescent tumor cells, as well as small tumor masses, were observed along the vessels. Histopathologically, the pericyte-like location of tumor cells was comparable to the angio-tumoral complex described in human melanoma biopsy specimens.^{16,17} The vascular wall was intact and the tumor cells occupied a pericyte-like location along the abluminal surface of the vascular surface, without any observation of intravasation of tumor cells.

Angiotropism (more precisely, pericyte-like location) of melanoma cells has recently been suggested as a potential prognostic factor in melanoma.³⁵ The fact that a pericyte-like location is rarely reported³⁶ may result from a problem of sampling around the tumor mass. In addition, it may be difficult, without a specific marker of angio-tumoral association, to distinguish tumor cells associated with the abluminal surface of vessels inside the tumor mass from vessels entrapped within the tumor. Furthermore, if an observation has not heretofore been thought to be important or has not been explicitly searched for, it may simply have been unconsciously overlooked. In fact, in addition to our former observations, we have just described a series of 36 human primary melanomas demonstrating such angiotropism.³⁷

The EVMM proposed for melanoma has strong analogies with the migration of neoplastic glial invasion of the

Figure 5. Confocal optical sections of C8161 melanoma cells growing on a chick chorio-allantoic membrane. Using the "Lugassy" technique, some areas next to the tumor were also observed using a confocal laser-scanning microscope. **A:** Image projection of 21 1- μ m thick optical sections showing the localization of GFP. Fluorescent GFP tumor cells are seen spreading on the surface of the CAM. Note the small fluorescent tumor mass on the left part of the field. **B:** Transmitted-light image acquired in the same location as (**A**) using a Bio-Rad transmitted light detector. The dark network indicated with arrows corresponds with a vascular plexus. **C:** Merged image comprised of (**A**) and (**B**). Note the fluorescent tumor cells (**A**) are along the external surface of the vascular plexus (**B**). The extraluminal localization of the melanoma cells was confirmed by conventional histology (Figure 4B). Scale marker, microns.

nervous system, as already discussed.²⁵ This kind of migration through or within the mesenchyme has also striking analogies with the migration occurring during embryogenesis, particularly the migration of the neural crest cells (precursors of melanocytes). Once differentiated to become neural crest, these cells acquire motile properties, and embark on an extensive migration through the embryo to reach their ultimate phenotype-specific sites.³⁸ Metastasizing cells may acquire motile properties, and embark on an extensive continuous migration through the body, ie, EVMM, to reach their ultimate specific secondary sites.

In conclusion, these studies strongly suggest that some melanoma cells are able to migrate along the outside of vessels in a pericyte-like location, according to the proposed extravascular migratory metastasis mechanism. Given the possibility to obtain quantifiable results, the *ex vivo* and *in vivo* models used in the present study may prove useful in elucidating the molecular interactions involved in EVMM, with the goal of identifying and testing new therapeutic approaches for controlling melanoma progression.

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