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Pericytic-Like Angiotropism of Glioma and Melanoma Cells

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We have identified in malignant melanoma an angiotumoral complex in which tumor cells occupy a pericytic location along the endothelium of microvessels without evidence of intravasation. We have suggested that this pericytic-like angiotropism could be a marker of an extravascular migration of tumor cells along the abluminal surface of vessels. The extravascular migratory metastasis proposed for melanoma has close analogies with glioma migration. To compare our hypothesis of extravascular migration by melanoma with the migration of glioma cells, we have used the B16 murine melanoma cell line and the GL26 murine glioma cell line in an in vivo murine brain tumor model and in vitro using endothelial cells that have formed capillary-like structures and have been cocultivated with tumor cells. In the brain tumors, a clear progression of glioma and melanoma cells was observed along the abluminal surface of vessels, where they occupied a pericytic location along the periendothelial laminin. In vitro, time-lapse videomicroscopy recorded the migration of tumor cells toward endothelial tubules. After 24 hours, both the melanoma cells and the glioma cells were localized along the external surfaces of the vascular tubules, occupying a pericytic-like location. These similarities between glioma and melanoma support the hypothesis of an extravascular migration of melanoma cells, particularly along the abluminal surface of vessels.

Key Words: Melanoma—Glioma—Angiotumoral complex—Pericytic-like angiotropism—Extravascular migratory metastasis—Laminin.

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Our studies in human melanoma and in mouse melanoma models have identified an angiotumoral association termed the *angiotumoral complex*, characterized by an amorphous material juxtaposed between endothelial cells and tumor cells, which contains laminin (1–3). The ultrastructural observation of the angiotumoral complex in melanoma corresponds to what has been termed *angiotropic* melanoma, on which others and we have reported (4). The absence of any sign of intravasation and the pericytic location of tumor cells in this pericytic-like angiotropism have raised the question of the significance of such observations in the process of metastasis.

Given the role of specific laminins on angiogenesis, protease induction, migration, and metastasis (5–7), we have suggested another mechanism of tumor spread in which tumor cells migrate in a pericytic location along the abluminal surface of the endothelium or along other anatomical structures. We have termed this potential mechanism of migration extravascular migratory metastasis (EVMM), as distinct from intravascular dissemination (3,8). Such an extravascular migratory metastasis may be a mechanism by which some cells spread to nearby and even distant organs.

The extravascular migratory metastasis proposed for melanoma has strong analogies with the migration of neoplastic glial invasion of the nervous system. Indeed, it is known that invading glioma cells follow distinct anatomical structures within the central nervous system. Tumor cell dissemination occurs along tracts that contain extracellular matrix proteins such as the basement membranes of blood vessels or the glial limitans externa (9). Interestingly, the florid angiogenesis and the expression of matrix metalloproteinases observed in gliomas do not correlate with hematogenous tumor spread. The extent to which these tumors invade adjacent structures can be considerable. Glial tumor cells can rapidly migrate along these tracts, and single invasive glioma cells can be

found several centimeters away from the main mass of tumor. Extensive spread may result in the involvement of both hemispheres of the brain by the tumor, which was observed histologically in 47% of cases in a series of consecutive malignant gliomas (10). At its extreme, large portions of the brain are diffusely infiltrated by individual tumor cells with no distinct tumor mass (11). For migration along blood vessels, interactions between integrins on glioma cells and vascular extracellular matrix components such as collagens, laminin, and fibronectin are important (12). Among these molecules, much attention has been given to laminin, which has been shown to stimulate glioma cell migration in vitro (13,14).

To compare our hypothesis of extravascular migration for melanoma with the migration of glioma cells, we have undertaken experiments using the B16 murine melanoma cell line and the GL26 murine glioma cell line. These two cell lines have been used in an in vivo murine brain tumor model. We have focused on the relation of tumor cells to vessels using a polyclonal antibody directed against laminin. These two cell lines have also been tested in vitro using endothelial cells that have formed capillary-like structures and have been cocultivated with tumor cells on Matrigel, a model on which we have recently reported using human melanoma cell lines (15). In addition, we are reporting histopathologic findings from both human melanoma and human glioma specimens to illustrate the potential morphologic similarity of the angiotumoral association pattern in these two different forms of cancer.

MATERIALS AND METHODS

Samples of Human Melanoma and Human Glioma

Three primary angiotropic human cutaneous melanomas with tumor thicknesses of 3.21 mm, 5.0 mm, and 0.68 mm as well as three human brain gliomas were retrieved from the dermatopathology and surgical pathology files, respectively, at the George Washington University Medical Center, Washington, DC. The specimens were fixed in 10% formalin, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin for pathologic observation.

Cell Lines

B16-F10 melanoma cells and GL26 murine glioma cells were obtained from the Division of Cancer Treatment Tumor Repository (National Cancer Institute, Frederick, MD). One human melanoma cell line from a cutaneous metastasis (15) also has been used in the in vitro dynamic assay. The cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum, 1% L-glutamine, and 1% penicillin-streptomycin. The immortalized human microvascular endothelial cell line HMEC-1 (16) was cultured in MCDB 131 media containing 10% fetal bovine serum (Hyclone, Logan, UT), 2 mM of L-glutamine, 1% penicillin-streptomycin, 1 μg/mL of hydrocortisone (Becton Dickinson, Bedford, MA), and 10 ng/mL of human recombinant epidermal growth factor (hEGF; Becton Dickinson). All cell lines were maintained in humidified incubators gassed with 5% carbon dioxide.

Culture monolayers were harvested with trypsin, counted, and resuspended in the complete medium before in vivo and in vitro assays.

Murine Brain Tumor Model

As described previously for stereotactic intracranial injections of tumor cells (17), the surgical site was shaved and prepared with 70% ethyl alcohol iodine-containing solution. After a midline incision, a 2-mm burr hole centered 2 mm posterior to the coronal suture and 2 mm lateral to the sagittal suture was made. Animals were then placed in a stereotactic frame, and the tumor cells (5-μL total volume) were delivered by a 26-gauge needle inserted to a depth of 3 mm. The needle was removed, the site was irrigated with sterile 0.9% sodium chloride solution, and the skin was closed. In total, 1,000 cells from the murine glioma and 100 cells from the B16 murine melanoma cell line were injected intracranially into mouse brains utilizing stereotaxy on day 0. Mice were killed on days 10, 15, and 20 after tumor implantation (five mice for each time point and tumor type). The specimens were fixed in 10% formalin, embedded in paraffin, and sectioned at 4 μm. The sections were deparaffinized and incubated with protease (0.01 g of Sigma P8038 type XXIV per 10 mL of phosphate-buffered saline [PBS]; Sigma, St. Louis, MO) for 8 minutes. The sections were then incubated with the primary antibody against laminin (rabbit polyclonal antibody at 1:2,000 dilution; DAKO, Copenhagen, Denmark) for 1 hour. After the sections were rinsed with PBS, incubation with biotinylated goat–anti-rabbit IgG took place, followed by the avidin biotin complex anti-phosphate (ABC-AP) detection system (Vector Laboratories, Burlingame, CA). The immunoreactions were visualized by incubation with diaminobenzidine or fast red chromogen (BioGenex, San Ramon, CA) and counterstained with Gill's hematoxylin.

In Vitro Dynamic Assay Using Capillary-Like Structures Cocultivated With Tumor Cells

Plates (96 wells) were coated with 70 μL of the matrix Matrigel (18) (10 mg/mL; Collaborative Biomedical Products, Bedford, MA) and incubated at 37°C for 30 minutes to promote gelling. Then, 12,000 human endo-

thelial cells (HMEC-1) were added to each well on day 0. The endothelial cells cultured on Matrigel are known to form connected tubules representing capillary-like structures within 24 hours (day 1) (19). These tubules have been described as possessing a lumen surrounded by endothelial cells attached to one another by junctional complexes. The cells possess Weibel-Palade bodies and factor VIII–related antigen (20). In total, 3,000 B16 melanoma cells and 3,000 GL26 glioma cells were plated on these newly formed vascular tubules at day 1 (5 wells for each tumor type). One human melanoma cell line was used as well. The same experiment was processed with tumor cells preincubated for 1 hour with the fluorescent vital stain Calcein, AM (Molecular Probes, Eugene, OR) (5 wells for each tumor type). As a control, tumor cells were plated on Matrigel alone (5 wells for each tumor type). Cells were observed with an inverted microscope with and without fluorescence microscopy after 24 hours.

Time-lapse (transmitted light, phase contrast, fluorescence) microscopy of cell movement was digitalized at the rate of one picture every 20 minutes, and measurements of velocity were taken every 10 minutes for a period of 24 hours. Digitalization and measurements were performed with MetaMorph software (universal imaging).

RESULTS

Human Cutaneous Melanoma and Human Brain Glioma

In all three melanoma and three glioma specimens from human patients, a similar pattern of pericytic-like angiotropism was observed. Cells from both melanoma and glioma were observed to cuff the external surfaces of vessels, without any evidence of intravasation (Fig. 1) (1–4). This pericytic-like angiotropism of tumor cells was much more frequent in glioma. The cuffing of microvessels was present at the invasive front of the tumor or at some distance (within 1–2 mm) from the tumor mass and thus was not simple entrapment of vessels by tumor. This similar angiotropic pattern of human glioma and melanoma suggests a possible extravascular migration of melanoma cells along vessels.

Murine Brain Tumor Model

We next examined the kinetics of melanoma and glioma tumor cell spread in a murine brain tumor model, where the tumor cells are injected directly into the brain. In the sections of brain tumors, both B16 and glioma cells were observed along the choroid plexus and along the abluminal side of vessels, where they occupied a pericytic location. A clear progression along these structures was noted from the 10th day to the 20th day after tumor cell injection. Similar findings were observed with both tumor cell types, but melanoma cells were progressing more rapidly than glioma cells (Table 1). On day 10, both melanoma and glioma cells were observed along the choroid plexus and along the adjacent vessels, exhibiting a pericytic-like angiotropism in a pattern analogous to the angiotumoral complex (Fig. 2A). On day 15, larger numbers of tumor cells were observed around vessels, extending into the brain parenchyma (Fig. 2B). Pericytic-like angiotropism of glioma and melanoma cells was noted between the area next to the choroid plexus and the tumor mass (Fig. 2C) in a pattern analogous to the observations in human tumors (Fig. 1). On day 20, tumor masses had developed in the brain parenchyma (Fig. 2D) in four mice injected with B16 melanoma cells versus two mice injected with GL26 glioma cells (Table 1). There was prominent immunostaining for laminin between tumor cells and both the choroid plexus (data not shown) and vascular endothelium (Fig. 2A and D). Laminin formed a lattice around the vessels, with projections into the tumor mass (Fig. 2D). These data suggest the

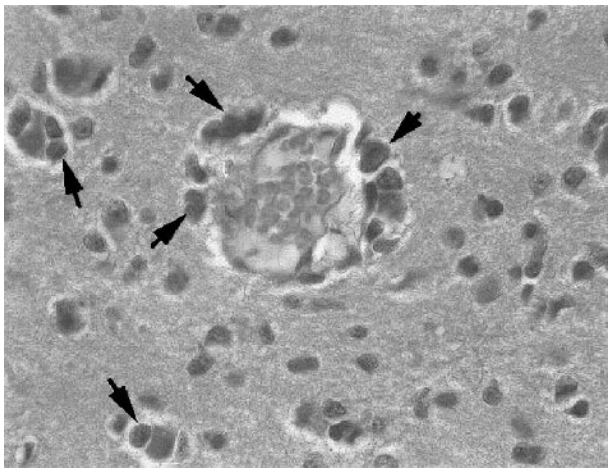

FIG. 1. Human brain glioma. Angiotropic tumor cells occupying a pericytic location around small vessels are indicated by arrows.

TABLE 1. *Murine brain tumor model: number of mice injected with tumor cells, day of sacrifice, and anatomical location of tumor cells*

	Day 10	Day 15	Day 20
Mice injected with B16 melanoma cells (5 mice in each group)			
Choroid plexus	5*	5	5
Adjacent vessels	4	5	5
Vessels in the brain parenchyma	0	3	5
Brain tumor masses	0	1	4
Mice injected with GL261 glioma cells (5 mice in each group)			
Choroid plexus	5*	5	5
Adjacent vessels	4	5	5
Vessels in the brain parenchyma	0	2	5
Brain tumor masses	0	0	2

* Number of mice.

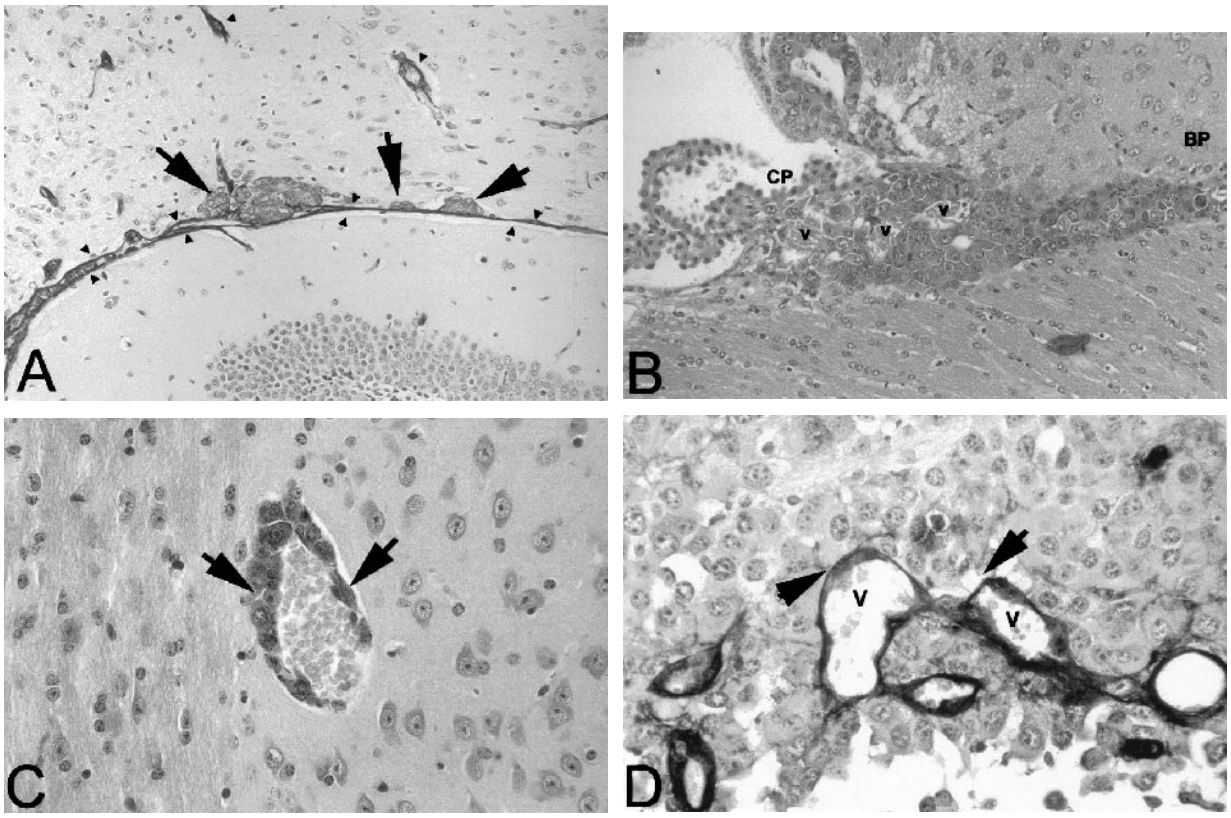

FIG. 2. Murine brain tumor model. (**A**) Murine brain glioma at day 10 stained with an antibody directed against laminin. Glioma cells (*arrows*) exhibit a pericytic location along a microvessel close to the choroid plexus. Note the periendothelial laminin (*arrowheads*) along the tumor pathway. (**B**) B16 brain melanoma at day 15. Note the progression of melanoma cells along the choroid plexus (CP) and around vessels (V) extending into the brain parenchyma (BP). (**C**) B16 brain melanoma at day 15. Arrows indicate angiotropic tumor cells occupying a pericytic location around a small vessel in the brain parenchyma. (**D**) B16 brain melanoma at day 20 stained with an antibody directed against laminin. Laminin forms a lattice about vessels (V) with projections into the tumor mass (*arrows*).

migration of both glioma and melanoma cells along the choroid plexus and the abluminal surface of vessels.

In Vitro Assay Using Capillary-Like Structures Cocultivated With Tumor Cells

We next compared the ability of glioma and melanoma cells to attach and migrate along newly formed vessel-like structures in vitro. On day 1, GL26 glioma cells, B16 melanoma cells, and human melanoma cells were plated on the new vascular tubules formed by the endothelial cells. After 1 hour, both the tumor cells plated on the capillary-like structures and the tumor cells plated on Matrigel alone were still round and randomly attached on Matrigel throughout the well (Fig. 3A). After 6 hours, the tumor cells had migrated toward the endothelial tubules, and 50% were elongated along the tubules (Fig. 3B). After 24 hours, both the glioma cells and the melanoma cells were spreading along the external surfaces of the vascular tubules, occupying a pericytic-like location (Figs. 3C and 4). The tumor cells on Matrigel alone were generally dispersed with some nests of partially connected cells, especially the B16 melanoma cells.

Experiments using fluorescent tumor cells have confirmed the pericytic-like location of the tumor cells (data not shown). Time-lapse videomicroscopy visualized the migration of tumor cells toward endothelial tubules and the permanent movement of tumor cells along the capillary-like structures (Fig. 3). These data suggest that tumor cells in vitro may mimic their ability to migrate along vessel structures.

DISCUSSION

The mechanism of tumor cell migration may involve numerous components, including manifold cellular and stromal interactions. Some of these interactions may simply be physical in nature. For example, anatomical structures often oriented by mechanical influences such as tension may provide contact guidance and paths of least resistance for cellular migration (21,22). Recent studies have suggested efficient cellular and molecular plasticity in tumor cell migration strategies (23). Glioma cells are

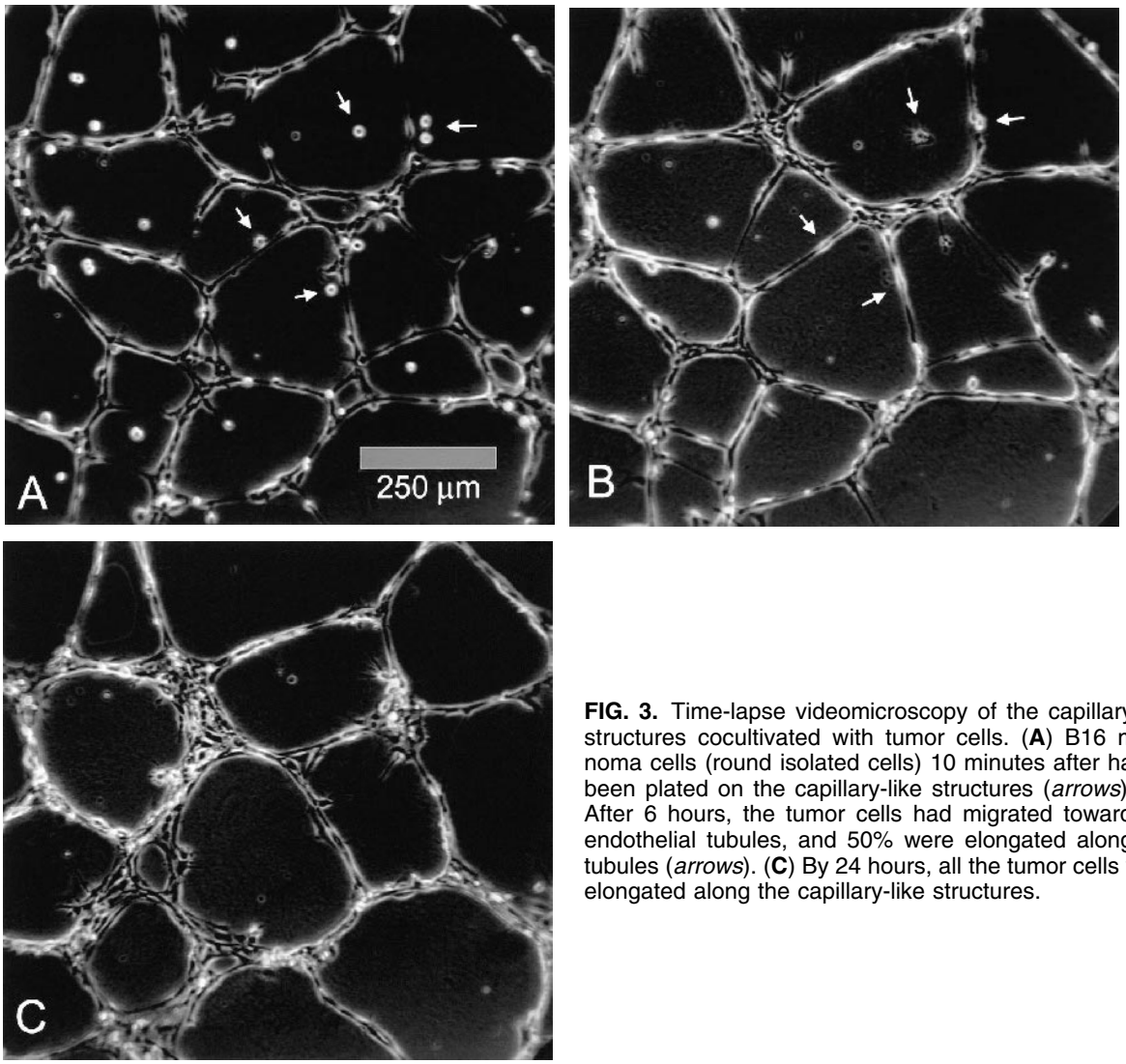

FIG. 3. Time-lapse videomicroscopy of the capillary-like structures cocultivated with tumor cells. (**A**) B16 melanoma cells (round isolated cells) 10 minutes after having been plated on the capillary-like structures (*arrows*). (**B**) After 6 hours, the tumor cells had migrated toward the endothelial tubules, and 50% were elongated along the tubules (*arrows*). (**C**) By 24 hours, all the tumor cells were elongated along the capillary-like structures.

known to migrate a considerable extent along the basement membranes of blood vessels, and this migration does not correlate with hematogenous tumor spread (9,12). In the current study, melanoma cells demonstrate the same morphologic and dynamic pattern as glioma cells in relation to vessels in vivo or capillary-like structures in vitro. These analogies between glioma and melanoma cells suggest a similar mechanism of migration (i.e., migration along the external surface of vessels) as proposed in the EVMM hypothesis (8).

Other anatomical structures are known to be involved in tumor migration, for example, the glial limitans externa in glioma (9) or nerves and epidermal appendages in melanoma (24). This kind of migration through or within the mesenchyme has 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, cells occupying the dorsal portion of the neural tube

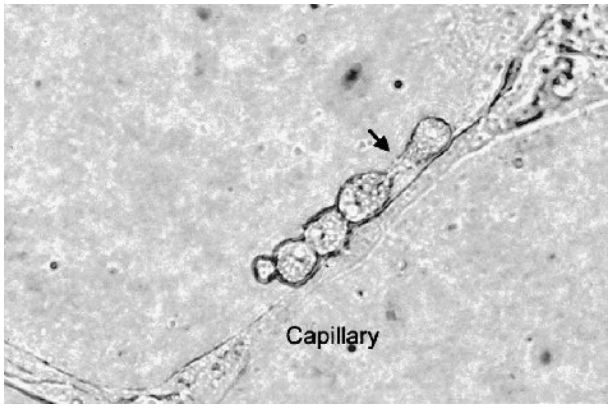

FIG. 4. Human melanoma cells plated in the presence of the capillary-like structures after 24 hours. All tumor cells are localized along the external surface of the vascular tubules, occupying a pericytic-like location in a pattern analogous to the angiotumoral complex. Note the spreading of the melanoma cells along the tubule (*arrow*).

disrupt their cadherin-mediated cell-to-cell contacts, acquire motile properties, and embark on an extensive migration through the embryo to reach their ultimate phenotype-specific sites (25). In general, neural crest cells appear to migrate without the guidance of distant physical or chemical cues; rather, they respond to heterogeneity in the extracellular matrix, which provides their migration substrate. The neural crest expresses cell surface receptors that permit interaction with the extracellular matrix and may also modify the matrix by secretion of proteases (26). Laminin is one of the master extracellular matrix molecules in the control of neural crest cell movement (25). The identification of the precise laminin involved is of fundamental importance. A recent study utilizing gene microarray analysis identified in human glial tumors an overexpression of α4 chain–containing laminins, a major blood vessel basement membrane component. Interestingly, the authors suggested that overexpression of laminin-8, a blood vessel laminin isoform comprising α4, β1, and γ1 chains, may be predictive of glioma recurrence (27). In a preliminary study using immunohistochemistry on cryostat sections of human melanoma, we have observed expression of the laminin β2 chain in an angiocentric pattern around microvessels with immunostaining extending into the surrounding tumor mass (28).

In conclusion, the similarities observed between glioma and melanoma in vivo and in vitro support the hypothesis of an extravascular migration of melanoma cells, particularly along the abluminal surfaces of vessels. Understanding the molecular basis of extravascular migratory metastasis may be of critical importance for the identification and evaluation of new therapeutic approaches to melanoma. □

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