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Special section

Brain surface invasion and metastasis of murine malignant melanoma variants

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Abstract

Mouse B16 melanoma sublines were selected sequentially for their abilities to colonize brain meninges and leptomeninges of C57BL/6 mice. After 14 selections subline B16-B14b was established that formed significantly more brain tumor colonies than the parental B16 line. Examination of brains at various times after intravenous or intra-arterial injection of B16 cells by electron microscopy revealed that B14b melanoma cells lodged in small brain blood vessels, proliferated and invaded through vessel walls into brain parenchyma and also along small blood vessels at perivascular sites. Invasion into brain parenchyma was characterized by extension of melanoma cell filopodia resulting in fragmentation and sometimes enfulgment of glial and neural cells.

Analysis of cell surface proteins of B16 melanoma sublines revealed increased exposure of a M_r ~90000 glycoprotein on the high brain-colonizing cells. Antibodies against the M_r ~90000 glycoprotein reacted with a variety of human melanoma cell lines and with some fetal and adult tissues, indicating that this melanoma-associated component is not species-, tumor- or tissue-specific. The glycoprotein could be a cell surface receptor important in the survival and growth properties of melanoma cells in brain microenvironments.

Introduction

Preferential metastasis of malignant melanoma to such distant secondary sites as brain and other organs is believed to be the result of several highly selective, sequential steps (1–3). The malignant tumor cells capable of metastasis to brain possess unique properties that are probably important in the metastatic process (4–9). In addition, the unique environment of the brain is undoubtedly important in determining implantation, survival, and growth of circulating malignant cells (9–12).

In malignant melanoma, the majority of patients with advanced disease have some evidence of brain or leptomeningeal metastases (13–15) and, at autopsy, these are found in more than 70–90% of patients (16, 17). When metastasis occurs at these sites, palliation is possible, but effective treatments are rarely achieved (17, 19, 20).

There are few models to study the process of melanoma metastasis to brain and/or leptomeninges and to develop new therapeutic strategies (4, 6, 21–23). We recently reported the development of an animal model for brain meningeal metastasis that uses in vivo selected sublines of B16 melanoma of low metastatic potential in C57BL/6 mice. We selected the subline B16-F1 for the ability of blood-borne cells to implant, survive and grow at brain surface sites. After 14 in vivo selections for brain tumor colonization, subline B16-B14b was established, and it showed dramatically increased preference for tumor formation in brain meninges

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and subsequent invasion of brain parenchyma than the parental B16-F1 melanoma line (6, 9, 22).

We have used the melanoma subline B16-B14b to study the modes of brain tumor colonization in syngeneic hosts by injecting tumor cells into the circulation via three routes: tail vein (i.v.), left ventricle intracardiac (i.c.), or left common carotid artery (i.a.) (22). Using these three routes of tumor cell administration, we noted the formation of slightly different distributions of brain and meningeal tumors. The predominating tumor structural types were intravascular, nodular, or diffuse-infiltrative. The former was the most common type found after i.v. injection of tumor cells, although at later times of tumor development the other types were found also. Intracardiac or intra-arterial injection of B16-B14b cells resulted in the formation of mainly nodular or diffuse-infiltrative types of tumors (9, 22).

The cell surface properties of metastatic tumor cells have been shown to be of important in certain steps of the metastatic cascade (1–3, 10, 23). Examination of the cell surface proteins and glycoproteins of B16 melanoma cells has revealed differences in the amounts or displays of particular components (4, 6, 7). We have been interested in a cell surface glycoprotein of M_r ~90000 that is expressed at higher levels or is more exposed on B16 sublines, such as B16-B14b, that have high brain and meningeal colonization properties (6, 7, 24).

Materials and methods

Animals

Barrier-raised 4- to 6-week-old female C57BL/6 mice, which were certified to be free of Sendai virus, were obtained from Charles River (Wilmington, MA). The mice were shipped in filtrated cages and were quarantined for 2 weeks before use. They were fed normal chow and chlorine-free spring water ad libitum and their weights were monitored weekly. All animals were maintained according to the guidelines established by the National Institutes of Health and The University of Texas System Cancer Center. For each experiment the animals were matched for sex, age (6–8 weeks), and weight (25–30 g) (25).

Cells

The murine melanoma subline B16-B14b was used. Tumor cells were maintained in vitro in Falcon (Falcon Plastic) tissue culture plates in Dulbecco-modified Eagle's medium (DME) without antibiotics and containing 5% fetal bovine serum and 1% non-essential amino acids. Cells were grown in a humidified gas mixture of 5% CO_2, 95% air at 37 °C and were harvested by overlaying the cells with calcium-magnesium-free phosphate-buffer solution (PBS) containing 2 mM EDTA. The harvested, suspended single cells were washed twice by centrifugation and stored at 0 °C until use. Tumor cells were used within ten passages from frozen stocks to eliminate phenotypic drift (6, 24).

Experimental metastasis assays

B16 cells were grown to subconfluency and harvested as described above. Animals were placed under mild anesthesia by Metofane inhalation, and they were injected i.v. or i.a. with the singly suspended tumor cells (10^5 cells/0.2 ml) in serum-free DME. For the i.a. injections the left common carotid artery was carefully separated from the vagus nerve and ligated with thread. Within 30 s after ligation, a 0.2 ml tumor cell suspension was injected i.a. using a 27 gauge needle. Each animal's brain and other major organs were removed at various times, fixed in Bouin's or buffered formalin solution, and prepared for histologic examination by standard methods. The brains of some animals were fixed in situ in buffered formalin and decalcified with 5% formic acid for 1 week (9, 22).

Electron microscopy

B16-B14b cells (10^5 cells/0.2 ml of inoculum) were injected i.a. into the sex-, age- and size-matched groups of mice. Three to 14 days later, the animals were placed under slight Metofane anesthesia and injected i.v. with 0.1 ml of heparin solution (100 unit/0.1 ml). Two to 3 min later, the animals were perfused i.c. via the left ventricle to inferior caval vein with Ringer's solution for 2 min, followed by perfusion with 1% glutaraldehyde in cacodylate buffer, pH 7.34, using the same route for an additional 2 min. As soon as possible after perfusion, the brains were removed and placed in

cold (4 °C) modified Karnovsky's fixative for 1.2 to 1.5 h. Then the brains were carefully washed in cold 0.1 M cacodylate buffer three times for 10 min each, and the tissue pieces were post-fixed in 2% osmium tetraoxide in the same cacodylate buffer for 1.0 to 1.2 h at 4 °C. After being rinsed again in cacodylate buffer, the tissue fragments were stained in situ with 4% uranyl acetate in 50% ethanol, solution for 5 min at room temperature, dehydrated in a graded series of ethanol, and embedded in Spur resin. Thin and ultrathin sections were cut on a Reichert OMU-O ultramicrotone using a diamond knife. The sections were stained with Azur and Methylane Blue, and ultrathin sections with 2% uranyl acetate and lead citrate. The former were observed in an Olympus Vanox research microscope, and the latter were observed in a Hitachi Model HU-12 transmission electron microscope (9).

Purification of B16 glycoproteins and anti-gp90

B16-B14b cells (1×10^8) were harvested as described above and washed three times by centrifugation and resuspension in 0.25 M sucrose, 0.05 mM calcium chloride, 10 μM phenylmethylsulfonylfluoride, 10 mM Tris-HCl, pH 7.2. The final cell pellet was lysed in 2 ml of the same buffer containing, in addition, 0.5% Nonidet P-40 at 4 °C. After 5 min, nuclei were removed by centrifugation for 5 min in a microfuge (Beckman Instruments, Model B) at 4 °C. The cell lysate was applied to a 10 ml column of Affi-Gel-10-wheat germ agglutinin equilibrated with 0.1% Nonidet P-40, 120 mM sodium chloride, and 50 mM Tris-HCl, pH 7.3, and allowed to incubate for 30 min at 4 °C (26). The void volume of the column (~9 ml) was removed and repassed through the column at 4 °C. Unbound protein was removed with buffer until the absorbance at 280 nm was similar to the rinsing buffer, whereupon the same buffer containing 0.1 M N-acetyl-D-glucosamine was used to elute the bound glycoproteins. Peak fractions were pooled and dialyzed against PBS. The average yield was approximately 0.5 mg protein from 10^8 cells (26).

Each affinity-purified glycoprotein fraction was further purified by preparative sodium dodecyl sulfate (SDS) polyacrylamide slab-gel electrophoresis (26). The gel was washed thoroughly, and the M_r ~90000 band was cut out (~1 ml of gel containing ~38 μg of protein), emulsified with 1 ml of complete Freund's adjuvant, and injected intradermally into New Zealand white rabbits. After boosting the rabbits with an intragranuloma injection of the same antigen preparation and then boosting each rabbit with an i.v. injection of about 20 μg of antigen, sera were collected for testing against B16 cells in a ^{51}Cr-release cytotoxicity assay (25). The anti-gp90 prepared by this procedure specifically immunoprecipitated gp90 solubilized from B16 cells (26).

The relative amounts of gp90 epressed on various murine and human tumors were assessed with an ELISA assay (Hy-BRL-Screen, Bethesda Research Laboratories). Using biotinylated goat anti-rabbit immunoglobulin instead of biotinylated goat anti-mouse immunoglobin (26). Whole cells were used as antigen, and each cell line was tested in three separate assays.

Immunoperoxidase staining of tissues

For the tissue and cell localization studies, an immunoperoxidase staining kit (Vectastain ABC Kit, Vector Labs) was used with various dilutions of anti-gp90 (26). The peroxidase substrate was 3-amino-9-ethylcarbazole. Sides were counterstained with Moyer's hematoxylin-eosin.

Results

Experimental brain metastasis

Intravenous tail injection of B16-B14b cells resulted in tumor colonization in brain meninges, lung, and thoracic cavity, and occasionally in ovary and kidney (22). Since the brain meningeal tumors formed after injection i.v. of B16-B14b cells were quite small (<1 mm in diameter) and not very pigmented, histologic examination for positive identification of tumor colonies was required. The initial tumors formed in the brain meninges were mainly of the intravascular type, but these tumors proliferated, invaded blood vessel walls, and also infiltrated into brain parenchyma along blood vessels (22). Intra-arterial injection of B16-B14b cells produced more tumor colonies in brain meninges than did i.v. injection and tumor growths were noted in the cervical or submandibular regions at the

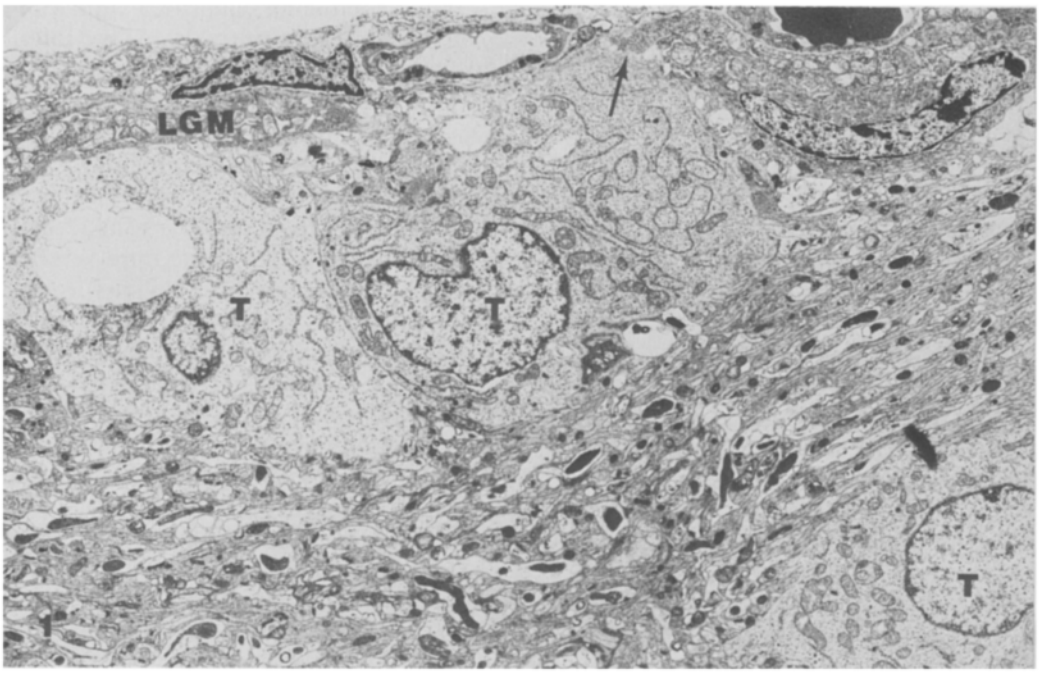

Fig. 1. An invading B16-B14b melanoma cell in mouse cerebrum. Tumor cells (T) are seen below the limiting glial membrane (LGM); one of these appears to be invading an astrocyte (arrow), while another has invaded deeper in the cerebral cortex (× 3290).

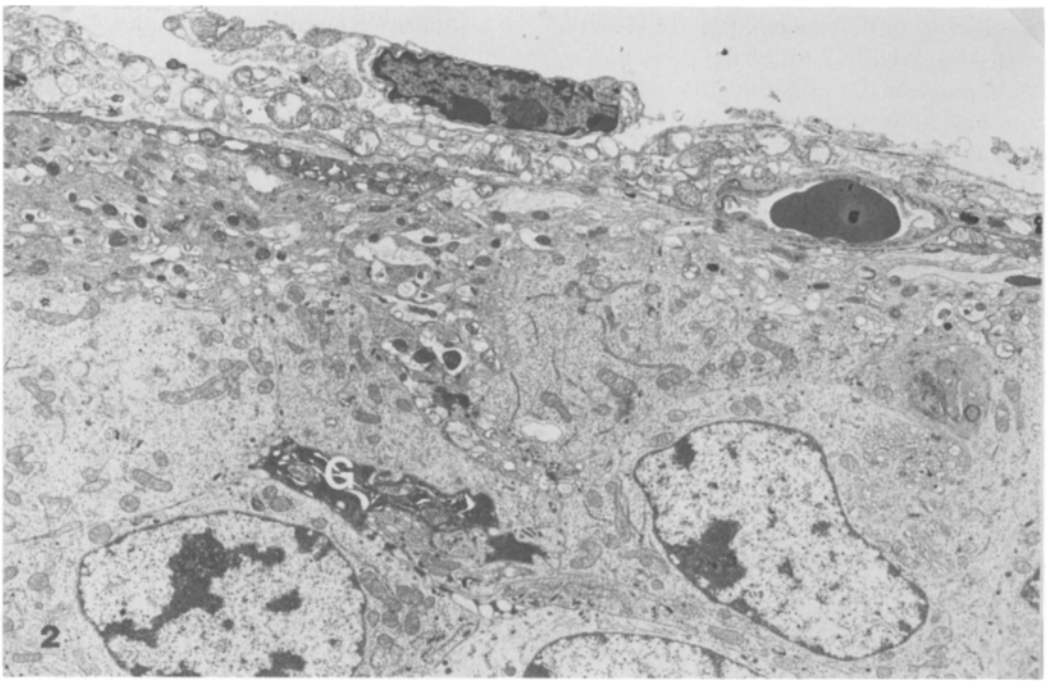

Fig. 2. Nodular tumor formation by B16-B14b tumor cells in the granular layer of the cerebral cortex. Degenerative glial cell (G) is adjacent to tumor cells. × 3850.

injection site. Intra-arterial injections resulted also in the formation of nodular tumor colonies in the brain meninges, with invasion of the dura mater (22).

Leptomeningeal and cerebral invasion

Tumors formed in the leptomeninges and brain parenchyma after injection i.a. of B16-B14b melanoma cells, and they eventually invaded into the cerebral cortex directly or along small blood vessels. B16-B14b cell invasion began at an area between the limiting glial membrane and the cerebral surface basal lamina (Fig. 1). With time expansive nodular tumors formed in the brain parenchyma, and the melanoma cells invaded glial and nerve cell tissue (Fig. 2). The invading B16-B14b tumor cells produced numerous elongated cytoplasmic protrusions or filopodia that often penetrated at the zones of brain parenchymal invasion separating and fragmenting glial and neural cells, sometimes engulfing them (Fig. 3A). Tumor cell interactions with neural cells were found to lead to the formation of tumor cell cytoplasmic processes in areas of synapses and synaptic vesicles (Fig. 3B).

A common route of B16-B14b tumor cell invasion into brain parenchyma was along blood vessels. Perivascular migration of tumor cells in cerebral veins between the leptomeninges and cerebral cortex was observed (Fig. 4). The melanoma cells appeared to be proliferating and migrating along these vessels while remaining attached to the endothelial basal lamina. At some sites, the tumor cells seemed to push aside perivascular astrocytes and invade the basal lamina and vascular wall, causing the blood vessel to constrict (Fig. 4).

Cell surface glycoproteins and brain metastasis

Analysis of cell surface proteins and glycoproteins of B16 melanoma cells by lactoperoxidase-^{125}I-iodination and SDS polyacrylamide gel electrophoresis indicated that the exposure of an M_r ~90000 protein is increased on the more metastatic B16 cells (4, 6). Staining the M_r ~90000 component (gp90) with various ^{125}I-labeled lectins after SDS polyacrylamide gel electrophoresis separation revealed that gp90 is glycoprotein that binds *Ricinus communis* agglutinin I and wheat germ agglutinin but not *Lens culinaris* or peanut agglutinins. Removal of sialic acid by mild acid hydrolysis of gp90 resulted in loss of wheat germ agglutinin-binding sites, indicating that gp90 is a sialoglycoprotein (7).

We have purified gp90 by Affi-Gel-10-wheat germ agglutinin affinity chromatography and preparative electrophoresis on SDS polyacrylamide gels (26). The purified gp90 was used to make polyclonal rabbit antibodies against gp90. These antibodies have been used, in turn, to examine the quantities and distributions of gp90 on tumor and normal cell lines and tissues of fetal and adult origin. Examination of various B16 melanoma cell lines by immunoprecipitation and ELISA assays indicated that gp90 is expressed on melanoma cells in the following relative amounts from highest to lowest: B16-B14b > SK-MEL-23 > B16-B15b > B16-O13 > B16-F10, B16-F1. Analysis of several human melanoma cell lines indicated that gp90 is expressed in the following relative amounts from highest to lowest: SK-MEL-23 > SK-MEL-93, DX6 > Hs294 > SK-MEL-40 > SK-MEL-75, SK-MEL-10, SK-MEL-93, DX1, SK-MEL-93, DX3, Hs852T, Hs939, Hs695T.

Since Nishio et al. (27) reported that a melanoma-associated oncofetal antigen of M_r ~90000 is expressed on melanoma cells as well as on some carcinoma, myeloma, leukemia cells, and on fetal fibroblasts, we examined the relative amounts and tissue distributions of gp90 using peroxidase-labeled antibodies. Fetal mouse tissues showed little reactivity with anti-gp90 until the 19th day of development, when gut epithelium stained with the anti-gp90 reagents. We found that gp90 (or cross-reacting antigens), although not commonly expressed in adult tissues, was expressed in the pancreas, pituitary, lacrimal, and sebaceous glands, and on individual cells in the adrenal medulla, stomach, and small-intestine epithelium, and in rare cells in the kidney and ovary (Table 1).

Discussion

The formation of brain meningeal and leptomeningeal metastases by B16-B14b melanoma cells occurs via blood-borne tumor cell implantation in the small vessels of the meninges, leptomeninges, and probably the dura mater (22). Tumor growth occurs at the sites of implantation and

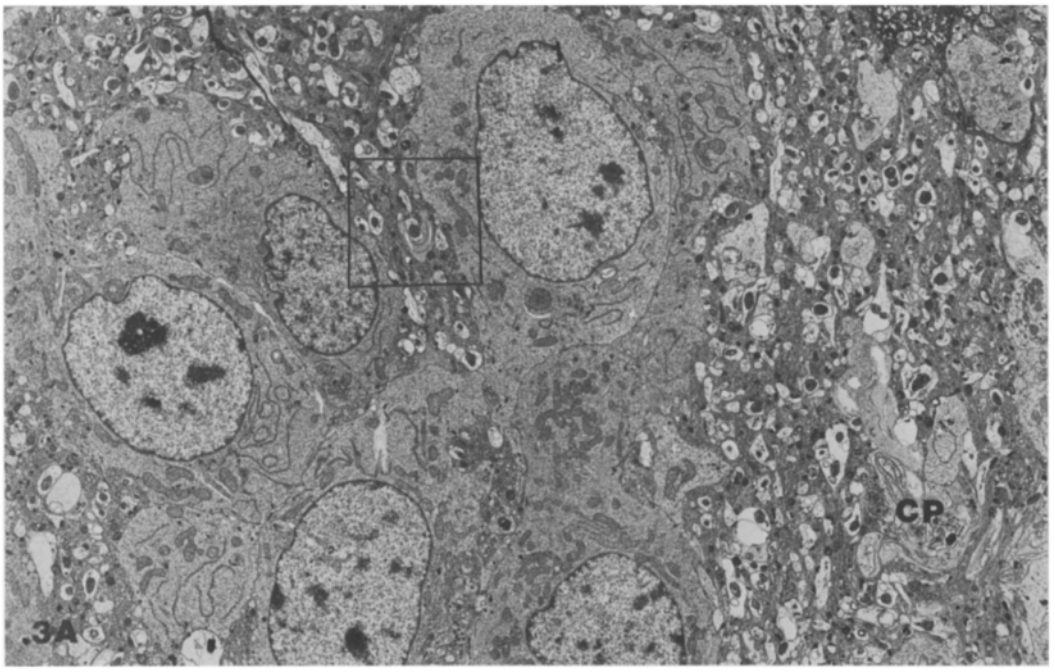

Fig. 3A. Direct invasion and migration of B16-B14b tumor cells into brain parenchyma. Elongated cytoplasmic protrusions (CP) can be seen between nerve elements (× 3290).

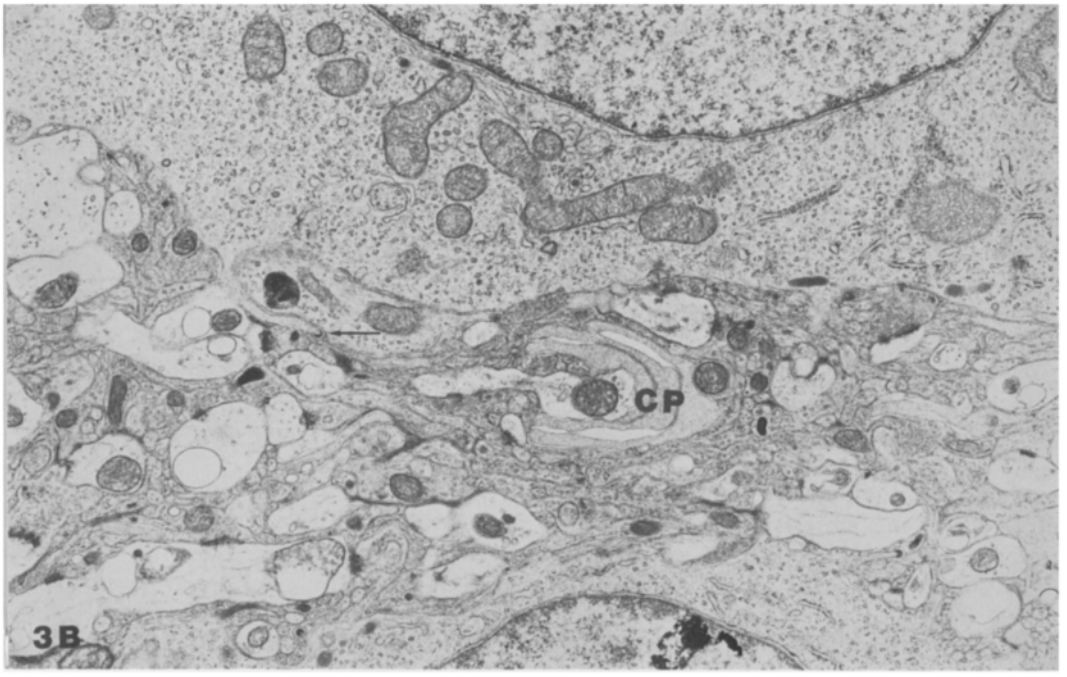

Fig. 3B. Higher magnification shows tumor cell cytoplasmic protrusions (CP) separating nerve elements. Seen at the tips of some of the melanoma cell protrusions are virus-like particles (small arrow) (× 13230).

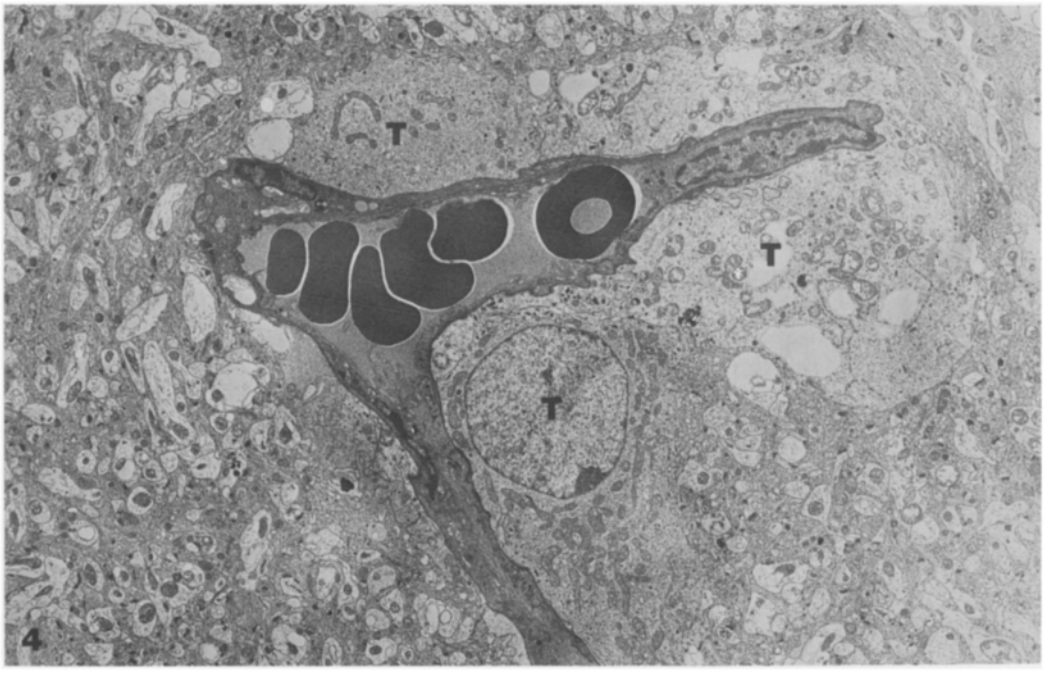

Fig. 4. Perivascular migration of B16-B14b melanoma cells. Tumor cells (T) are seen along cerebral capillary in perivascular space (× 3290).

Table 1. Histochemical detection of melanoma-associated oncofetal antigen gp90 in normal fetal and adult mouse tissues.

Tissue	Anti-gp90 reactivity	No anti-gp90 reactivity
Fetal	none	9.5 days age, all tissues
	none	10 days age, all tissues
	none	14 days age, all tissues
	none	17 days age, all tissues
	gut epithelium	19 days age, all tissues except gut
Adult	adrenal medulla*	adrenal cortex
	small intestine*	colon
	stomach*	esophagus
	sebaceous gland	salivary gland
	lacrimal gland	skin
	pituitary	thyroid, parathyroid
	liver*	lung, trachea, bronchi
	kidney*	ureter, urinary bladder
	ovary*	testes, uterus, aveal tract
	pancreas	mammary gland, lymph nodes, retina, cornea, lens, sclera, skeletal muscle, heart, veins, arteries, brain, spinal cord, peripheral nerves

*Individual specialized cells in organ-reactive tissues (26).

is followed by direct invasion into underlying dura mater and brain parenchyma and along cerebral blood vessels (9). Although other researchers have proposed a role for macrophages and other host-invasive cells in brain tumor invasion (28), we have not noted the association of large numbers of host inflammatory cells with invading melanoma cells (9, 22). This may be because B16-B14b cells are relatively refractory to host-mediated antitumor responses, such as macrophage-mediated tumor cell cytolysis (25). We did note, however, that tumor foci growing in the dura mater were accompanied occasionally by some immunocyte infiltration and development of granulomatous tissue (22).

At the invasive edges of B16-B14b tumors, many elongated cytoplasmic protrusions were found that separated and fragmented normal tissue cells, such as nerve elements, glial cells, and endothelial cells. The tissue-destructive effects of B16-B14b cells may be mediated by release of melanoma-degrading enzymes, such as heparanase (29, 30), collagenase (31, 32) and cathepsin B (33, 34), which are believed to be also responsible for destruction of the basal

lamina of blood vessels (1–3). We found that some B16-B14b cells migrated along the blood vessels' basal lamina and appeared to displace the perivascular astrocytes. This could reflect the melanoma cells' strong preference for adhering to endothelial basal lamina (5, 35). The formation of cytoplasmic protrusions on B16-B14b cells seemed to be tissue-dependent. Protrusions were extensive at the leading edges of the melanoma cells invading brain parenchyma, but not many were found on B16 cells in dura mater. This difference may be related to tumor microenvironment and the local release of substances from nervous elements (11, 12).

One notable characteristic of brain-colonizing B16 melanoma cells is the expression of an M_r ~90000 molecular weight glycoprotein that we have called gp90. We thought at first that gp90 might play a role in metastasis to brain meninges as a cell surface molecule involved in implantation in the blood vessels of brain meninges. Experiments to demonstrate this role for gp90, however, were inconclusive. We attempted to coat brain-colonizing B16 melanoma cells with anti-gp90 $(Fab')_2$ antibody fragments before the cells were injected i.v. into mice, but this did not result in a reduction of brain tumor colonies (26). In other systems we have been able to inhibit experimental metastasis completely by blocking organ cell adhesion sites with specific $F(ab')_2$ antibody fragments (36, 37). Thus, gp90 may not be a cell surface(s) molecule involved in organ preference of implantation. Since we found that gp90 or cross-reacting molecules are present in a number of adult murine tissues (Table 1), we reasoned that gp90 might be more important in the survival and growth characteristics of B16 melanoma cells after their brain implantation.

Malignant cells capable of dissemination to distant sites must successfully invade and implant at these sites, but their failure to respond to local concentrations of hormones and growth factors could limit their growth and the formation of detectable metastases. Recently we demonstrated that the proliferation of organ-colonizing B16 melanoma cells is stimulated or inhibited by soluble factors released from specific organ tissues (38). Thus, we hypothesized that gp90 might be a cell surface receptor for hormones or growth factors.

A cell surface receptor found on most tumor cells similar to gp90 in molecular weight and other characteristics is the transferrin receptor, the major serum-iron transport system for most mammalian cells (39–41). The transferrin receptor has been identified as an M_r ~180000 cell surface glycoprotein composed of two disulfide-linked chains of M_r ~90000 (42, 43). Transferrin receptors have been found on human malignant melanoma cells (44), as well as on a variety of normal tissues (45). Since gp90 and the transferrin receptor have similar molecular weights, disulfide-linked subunits, pI and bind to similar lectins, it is likely that gp90 is a transferrin or transferrin-like receptor that functions in melanoma cells by transporting transferrin or transferrin-like molecules into cells where they are required for cell growth. Since the melanoma cells that colonize brain meninges and leptomeninges have higher amounts of gp90, these may be sites where tissue transferrin levels are particularly low. Melanoma cells that implant and invade into brain meninges, leptomeninges and brain parenchyma might, therefore, have to express higher amounts of transferrin receptors in order to respond to low transferrin levels and proliferate in these tissues (24).

To escape dormancy and proliferate at metastatic sites, malignant cells must be capable of responding to their organ and stromal environments. Although such tumor cell responses may not be 'normal', they could mimic certain normal cell responses that occur during cellular differentiation and development (24). Since almost all gene products of malignant cells are probably identical or nearly identical to those that control or are involved in normal cellular differentiation, growth, and development, it is likely that most malignancy-associated properties are also expressed during certain stages of normal development (10, 11).

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