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Intracranial Tumor Cell Migration and the Development of Multiple Brain Metastases in Malignant Melanoma[1,2]

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Abstract

INTRODUCTION: A majority of patients with melanoma brain metastases develop multiple lesions, and these patients show particularly poor prognosis. To develop improved treatment strategies, detailed insights into the biology of melanoma brain metastases, and particularly the development of multiple lesions, are needed. The purpose of this preclinical investigation was to study melanoma cell migration within the brain after cell injection into a well-defined intracerebral site. *METHODS:* A-07, D-12, R-18, and U-25 human melanoma cells transfected with green fluorescent protein were injected stereotactically into the right cerebral hemisphere of nude mice. Moribund mice were killed and autopsied, and the brain was evaluated by fluorescence imaging or histological examination. *RESULTS:* Intracerebral inoculation of melanoma cells produced multiple lesions involving all regions of the brain, suggesting that the cells were able to migrate over substantial distances within the brain. Multiple modes of transport were identified, and all transport modes were observed in all four melanoma lines. Thus, the melanoma cells were passively transported via the flow of cerebrospinal fluid in the meninges and ventricles, they migrated actively along leptomeningeal and brain parenchymal blood vessels, and they migrated actively along the surfaces separating different brain compartments. *CONCLUSION:* Migration of melanoma cells after initial arrest, extravasation, and growth at a single location within the brain may contribute significantly to the development of multiple melanoma brain metastases.

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Introduction

Brain metastasis is a frequent and fatal complication of malignant melanoma, and 20% to 55% of patients with metastatic melanoma die as a result of their brain metastases [1–3]. Melanoma brain metastases are generally resistant to both radiation and chemotherapy, and furthermore, they are characteristically multifocal and are therefore difficult to treat with surgical resection [4]. Clinical studies have reported that approximately 60% of melanoma patients diagnosed with brain metastases develop multiple brain lesions, and these patients show particularly poor prognosis [5–7]. To develop improved treatment strategies for these patients, detailed insights into the biology of melanoma brain metastases, and particularly the development of multiple brain lesions, are highly needed.

Multiple brain metastases may arise from multiple tumor cells entering the brain from the arterial circulation at different times and at different locations within the brain. Alternatively, they may arise as a result of tumor cell migration within the brain after initial arrest, extravasation, and growth at a single location. Preclinical studies have demonstrated that melanoma cells can be highly motile within the brain and can migrate actively along the external surface of brain microvessels [8–10]. Carbonell et al. have suggested that this type of migration relies on β1 integrin-mediated tumor cell adhesion to the

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vascular basement membrane [9]. Furthermore, invasive growth of melanoma cells along brain microvessels has been observed in studies of patient biopsies and autopsy specimens [11,12]. These data suggest that migration of melanoma cells away from an original metastatic site within the brain may contribute to the development of multiple brain lesions in melanoma patients. However, most studies have detected melanoma cell migration across relatively short distances within the brain, and preclinical data are mainly derived from studies where tumor cells were injected into the arterial circulation, thus making it difficult to determine the origin of individual brain lesions.

In the study reported here, we injected human melanoma cells transfected with green fluorescent protein (GFP) into a well-defined site in the right cerebral hemisphere of nude mice by using a stereotactic device. Moribund mice were killed and autopsied, and melanoma cells within the brain were detected by GFP fluorescence imaging or by GFP immunohistochemistry. Multiple melanoma lesions involving brain regions far from the injection site were detected, and we identified multiple modes of melanoma cell transport within the brain.

Materials and Methods

Mice

Adult (8-10 weeks of age) female BALB/c *nu/nu* mice were used as host animals. The mice were bred at our institute and maintained under specific pathogen-free conditions at a temperature of 22°C to 24°C and a humidity of 30% to 50%. The animal experiments were approved by the Institutional Committee on Research Animal Care and were performed according to the Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Marketing, and Education (New York Academy of Sciences, New York, NY).

Cell Lines

Intracerebral tumors were initiated from the A-07, D-12, R-18, and U-25 human melanoma cell lines [13]. When these lines were established, large stocks of cells were frozen and stored in liquid nitrogen. Cells derived directly from the frozen stocks were constitutively transfected with GFP by lipofection using the pEGFP-N1 plasmid (Clontech Laboraories, Mountain View, CA) as described elsewhere [14]. Frozen stocks of cell clones showing high and stable GFP expression were established, and the GFP transfected cell cultures used in the experiments described here were initiated from these frozen stocks. Monolayer cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air and subcultured twice a week in RPMI 1640 (25 mM HEPES and L-glutamine) medium. The medium was supplemented with 13% bovine calf serum; 250 µg/ml of penicillin; 50 µg/ml of streptomycin; and 700 µg/ml (A-07), 900 µg/ml (D-12), 2200 µg/ml (R-18), or 1200 µg/ml (U-25) of genetecin. However, genetecin selection may not have been necessary because we have revealed that the cells show high GFP expression even when grown for 3 months in medium without genetecin. By initiating new cell cultures from the frozen stocks at regular short intervals, we ensured that the experiments were not influenced by changes in the characteristics of the cell lines that may be induced during long-term culture *in vitro*.

Anesthesia

Intracerebral injection of tumor cells was carried out with anesthetized mice. Fentanyl citrate (Janssen Pharmaceutica, Beerse, Belgium), fluanisone (Janssen Pharmaceutica), and midazolam (Hoffmann-La Roche, Basel, Switzerland) were administered intraperitoneally in doses of 0.63 mg/kg, 20 mg/kg, and 10 mg/kg, respectively. The body core temperature of the mice was maintained at 37°C to 38°C by using a heating pad.

Intracerebral Tumor Cell Inoculation

The mice were fixed in a stereotactic apparatus (Model 900; Kopf Instruments, Tujunga, CA) for inoculation of tumor cells into the right cerebral hemisphere. The injection point was 2 mm anterior to the coronal and 1 mm lateral to the sagittal suture lines (Figure 1). The immediate mouse mortality and the development of neurological signs shortly after cell inoculation have been shown to be low by using this injection point [15]. A 100-µl Hamilton syringe with a 26-gauge needle was used to inject 3.0×10^3 tumor cells suspended in 6 µl of Ca^{2+}-free and Mg^{2+}-free Hanks' balanced salt solution at a depth of 3.0 mm below the skull. To minimize tumor cell reflux, the cells were injected slowly, and the needle was left in place for 2 minutes before it was retracted slowly, as suggested by Fidler [15]. Twenty mice of each

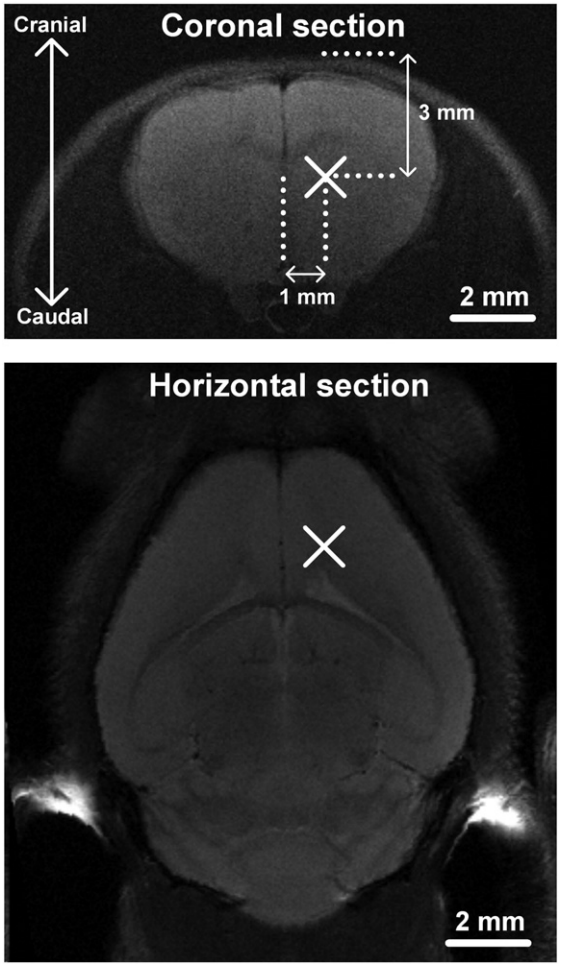

Figure 1. T_2-weighted magnetic resonance (MR) images of the mouse brain illustrating the cell inoculation site (cross) located 1 mm lateral to the sagittal suture lines and 3 mm caudal to the skull. The images show a coronal (top) and a horizontal section (bottom), and were recorded by using a 7.05-T Bruker small-animal MR scanner and a fast spin echo pulse sequence with a repetition time of 2200 milliseconds and an echo time of 36 milliseconds.