Brain metastases are an increasingly frequent and serious clinical problem for cancer patients, especially those with advanced melanoma. Given the extensive tropism of neural stem/progenitor cells (NSPCs) for pathological areas in the central nervous system, we expanded investigations to determine whether NSPCs could also target multiple sites of brain metastases in a syngeneic experimental melanoma model. Using cytosine deaminase–expressing NSPCs (CD-NSPCs) and systemic 5-fluorocytosine (5-FC) pro-drug administration, we explored their potential as a cell-based targeted drug delivery system to disseminated brain metastases. Our results indicate a strong tropism of NSPCs for intracerebral melanoma metastases. Furthermore, in our therapeutic paradigm, animals with established melanoma brain metastasis received intracranial implantation of CD-NSPCs followed by systemic 5-FC treatment, resulting in a significant (71%) reduction in tumor burden. These data provide proof of principle for the use of NSPCs for targeted delivery of therapeutic gene products to melanoma brain metastases.

Keywords: brain metastases, cytosine deaminase, gene therapy, melanoma, neural progenitor cells, neural stem cells, tumor targeting

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3Abbreviations used are as follows: β-gal, β-galactosidase; CD, cytosine deaminase; CD-NSPC, cytosine deaminase–expressing NSPC; DAPI, 4',6-diamidino-2-phenylindole; DMEM, Dulbecco’s modified Eagle’s medium; 5-FC, 5-fluorocytosine; 5-FU, 5-fluorouracil; MSC, mesenchymal stem cell; NSPC, neural/stem progenitor cell; PBS, phosphate-buffered saline; X-gal, 5-bromo-4-chloro-3-indolyl β-d-galactoside.
Metastatic brain tumors are the most common brain tumor, with an annual incidence more than 10 times that of primary brain tumors (Hutter et al., 2003; Patchell, 2003), and malignant melanoma is the third most common cause of intracranial metastases (Saha et al., 1994). Each year, more than 100,000 patients in the United States are diagnosed with brain metastases, and although treatment for systemic disease has been improving, drug delivery to brain metastases remains ineffective. Although solitary metastasis can be surgically removed or treated with stereotactic radiosurgery, patients with multiple brain metastases have very limited therapeutic options (Westphal et al., 2003). Neural stem/progenitor cells (NSPCs) represent a promising new treatment strategy because of their inherent ability to migrate throughout the brain parenchyma and target tumor sites. We have previously reported that intracranially administered NSPCs target experimental orthotopic primary brain tumors and deliver therapeutic agents that successfully inhibit tumor growth (Aboody et al., 2000; Yip et al., 2003). In this study, we present evidence that NSPCs stably expressing cytosine deaminase (CD), which converts the pro-drug 5-fluorocytosine (5-FC) to the active chemotherapeutic agent 5-fluorouracil (5-FU), can effectively target melanoma metastases in the brain. Our results provide a prototype for the use of NSPCs as an effective vehicle for delivering exogenous therapeutic gene products to intracranial tumor metastases.

Materials and Methods

Cell Culture

Murine cell lines were established by retroviral transduction as previously described (Aboody et al., 2000). Neomycin or puromycin selection and subcloning were performed by limiting dilution (Aboody et al., 2000). The C17.2 clonal murine NSPC line constitutively expressing lacZ was provided by Evan Snyder (Burnham Institute, La Jolla, Calif.) (Martinez-Serrano and Snyder, 1999). The clonal C17.CD2 line was derived from C17.2 cells by retroviral transduction with an expression cassette for CD and puromycin resistance genes, and this cell line stably expresses both lacZ and CD (Aboody et al., 2000). Neomycin or puromycin selection and subcloning were performed by limiting dilution (Aboody et al., 2000). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, 5% horse serum, 1% glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin at 37°C in 5% CO2/95% air at 100% humidity. B16/F10 and C19 murine melanoma cells (American Type Culture Collection, Manassas, Va.) were maintained in DMEM supplemented with 10% fetal calf serum, 1% glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin under routine culture conditions. Cells for in vivo administration were harvested by trypsinization (0.05% trypsin, 0.5 mM EDTA; Gibco, Carlsbad, Calif.) and resuspended to desired concentration in phosphate-buffered saline (PBS) prior to in vivo administration (intracranial NSPCs, 5 × 10^5/μl, 2 μl per injection; intracarotid NSPCs, 1 × 10^5/μl, 5 μl per injection; and intracarotid B16/F10 melanoma, 0.2 × 10^5/μl, 5 μl per injection).

In Vitro Tumor Lysis Assays and Detection of Cytosine Deaminase Bioactivity

For in vitro assays, B16/F10 (2 × 10^5) or C19 (7 × 10^5) murine melanoma cells were plated onto 10-cm culture dishes (day 0). On day 2, murine NSPCs (C17.2) or cytosine deaminase–expressing NSPCs (CD-NSPCs) (C17. CD2) were added to create NSPC–tumor cell ratios of 1:2, 1:4 (for B16/F10 targets), and 1:14 (for C19 targets). On day 3, 5-FC (0, 250, or 500 μg/ml) was added to the cell cultures. Control dishes were (a) cocultures with no 5-FC and (b) tumor cells alone with 5-FC. On day 6, plates were fixed and histochemically stained for 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) to visualize NSPCs and counterstained with neutral red.

Tumor Formation, NSPC Injections, and Treatment

An in vivo model for melanoma metastasis to brain was established as described (Fidler et al., 1999; Lin et al., 2001). Briefly, mice were anesthetized with xylazine-ketamine, and the left carotid artery was exposed. The external carotid artery was ligated, and a 30G needle was inserted into the lumen of the common carotid artery, enabling selective flow of injected cell suspension into the internal carotid artery, which supplies blood to brain parenchyma. One hundred thousand B16/F10 cells, resuspended in 5 μl of sterile PBS, were injected slowly into the left internal carotid artery and chased with 40 μl of PBS. The hole in the artery was closed with a 10-0 suture under the dissecting microscope to prevent bleeding and restore blood circulation. The mice were sacrificed after predetermined periods or if they appeared moribund (usually within three weeks). Brains were removed and fixed, and 10-μm sections were stained with hematoxylin-eosin. Planimetric sizes of tumors were measured on sections obtained at 200-μm intervals along the rostral-caudal axis of the brain.

Eight days after intracarotid injection of tumor cells, the mice for stem cell–tracking studies were anesthetized, and NSPCs (stably transfected with the β-galactosidase reporter gene or cytosine deaminase) were injected into the left carotid artery (1 × 10^5/μl, volume injected = 5 μl) and chased with 20 μl of PBS. Mice were sacrificed three days after the injection of NSPCs, and the brains were processed for immunohistochemistry for β-galactosidase to determine the distribution of the NSPCs in relation to the tumor sites.

In the therapeutic paradigm, eight days after intracarotid tumor injection, mice were placed in a stereotactic frame (David Kopf Instruments, Tujunga, Calif.) and received intracranial injections of CD-NSPCs rostral and caudal to bregma (5 × 10^5/μl, 2 μl each injection). Coordinates were 1 mm frontal and 4 mm caudal to the bregma, 1 mm lateral from the midline, and 2.5 mm deep. Injections were performed over the course of 5
min, and the needle was left in the brain parenchyma for an additional 2 min and then gradually removed over 3 min. Control mice received injections of NSPCs without CD expression. Three days later (allowing time for migration of NSPCs to tumor sites), mice received daily i.p. injections of 5-FC (500 mg/kg) for 8 days. One group of control mice received tumor only, with no treatment (control group 1), and a second control group received NSPCs (no CD gene) with i.p. injections of pro-drug 5-FC for eight days (control group 2). All mice were sacrificed at the end of the eight-day treatment regimens. Four mice were in each experimental group.

Histochemical and Immunocytochemical Analysis

Cryostat sections were processed for X-gal histochemistry, as previously described (Turner and Cepko, 1987). Briefly, sections were stained for Escherichia coli β-galactosidase (β-gal) activity to identify foreign transgene (lacZ)-expressing NSPCs (staining blue) and then counterstained with neutral red to detect distinctively dark-red, elongated tumor cells. Adjacent sections were prepared for dual-filter immunofluorescence by using a rabbit polyclonal anti-β-gal antibody (Chemicon, Temecula, Calif.) detected with a Texas Red–conjugated secondary antibody to rabbit immunoglobulin G (1:1000; Vector Laboratories, Burlingame, Calif.) to identify lacZ-expressing cells as red and 4′,6-diamidino-2-phenylindole (DAPI)–stained nuclei in blue. Sections were counterstained with hematoxylin or DAPI after the final PBS rinse. Slides stained for X-gal–hematoxylin were mounted with Crystal Mount (Electron Microscopy Sciences, Hatfield, Pa.), and slides stained for β-gal immunofluorescence–DAPI were mounted with Fluorescent Mounting Medium (Dako, Carpinteria, Calif.) and examined by either light or fluorescence microscopy.

Results and Discussion

We previously demonstrated the directed migration of C17.2 NSPCs to B16/F10 melanoma conditioned media in a modified Boyden chamber assay, suggesting the presence of chemotactic factors produced by melanoma cells (Brown et al., 2003). In a recent study, we showed an increased migration of C17.2 NSPCs toward tissue extracts from surgical specimens of human brain metastases derived from lung and breast cancer (Schmidt et al., 2005), which have the highest frequency of metastasizing to the brain (Hutter et al., 2003; Patchell, 2003). The high in vitro chemotactic responses of NSPCs to tumors that had metastasized to the brain prompted us to investigate the potential use of NSPCs to deliver the CD pro-drug activating enzyme to disseminated melanoma metastases in the brain. With systemic administration of the pro-drug 5-FC, which crosses the blood–brain barrier, we hypothesized that localized production of the chemotherapeutic 5-FU specifically at the tumor sites would lead to an antitumor effect.

In Vitro Tumor Lysis Assays and Detection of Cytosine Deaminase Activity

We investigated the in vitro cytotoxicity of CD-NSPCs in the presence of 5-FC when cocultured with B16/F10 or C-19 melanoma cells. A robust tumor cell–killing effect was observed after three days in the presence of CD-NSPCs and 5-FC (Fig. 1E, F, H, I, K, and L), but not when 5-FC was omitted from the medium (Fig. 1D, G, and J). In addition, the NSPCs (no CD) displayed no significant cytotoxic effect in the absence of 5-FC (Fig. 1A) or in its presence (Fig. 1B and C). This indicates that CD-NSPCs converted sufficient amounts of 5-FC to 5-FU during the three-day culture period to kill the majority of tumor cells effectively. Of note, the CD-NSPCs displayed a robust cytotoxic effect on melanoma cells, even when they were present at only 1:14 ratio in the tumor coculture. That relatively small ratios of CD-NSPCs to tumor cells can exert an oncolytic effect has important implications for in vivo treatment of melanoma brain metastases.

Tumor Formation, NSPC Injections, and Treatment

We used a syngeneic experimental mouse model for melanoma metastases to the brain and injected melanoma cells (B16/F10) into the internal carotid artery of immunocompetent C57BL/6J mice (Fidler et al., 1999; Lin et al., 2001). Multiple large and small metastatic infiltrates of highly pigmented melanoma cells were observed within two to three weeks in the brains of these mice (Fig. 2). These tumors displayed aggressive growth that, left untreated, led to death of mice within 20 to 23 days.

We used this in vivo model to first test the ability of NSPCs to localize to established B16/F10 melanoma brain metastases after intracarotid or intracranial administration. NSPCs were found colocalized with multiple brain metastases whether injected intra-arterially (Fig. 3A) or intracranially (Fig. 3B). NSPCs were concentrated in areas of the brain infiltrated by tumor cells (Fig. 3B) and absent from brain areas devoid of tumor (Fig. 3C). Cellular and molecular mechanisms of this exquisite melanoma-targeting behavior of NSPCs are most likely multifactorial and may include the CXCR4–SDF-1 (Ehtesham et al., 2004), the c-Kit–SCF (Sun et al., 2004), and the Flk-1–VEGF (Schmidt et al., 2005) signaling axes. Indeed, melanoma cells produce SDF-1, SCF, and VEGF, which are powerful chemotactic agents for NSPCs (Lefevre et al., 2004; Streit and Detmar, 2003; Sun et al., 2004; Vermi et al., 2003).

The experimental mouse model was also used to demonstrate proof of principle for the in vivo therapeutic efficacy of NSPCs genetically engineered to stably express the pro-drug activating enzyme CD to selectively kill tumor cells in the presence of systemically administered 5-FC (Fig. 4A). As noted earlier, CD converts the nontoxic 5-FC to the cytotoxic drug 5-FU, a chemotherapeutic agent in its own right with selective toxicity to dividing cells (Fig. 4B). 5-FU and its toxic metabolites readily enter the surrounding tumor cells by
nonfacilitated diffusion, thereby giving CD a significant tumor-toxic “bystander” effect (Aboody et al., 2000; Huber et al., 1994). NSPCs, therefore, would facilitate a selective localization of 5-FU chemotherapy directly to the location of the tumor cells.

Mice that received intracarotid injections of $1 \times 10^5$ B16/F10 melanoma cells developed large tumors in the left hemisphere of the brain (representative sections are shown in Fig. 4C), but mice that received melanoma cells and were subsequently treated with CD-NSPCs and 5-FC displayed significantly smaller tumors (Fig. 4D). Quantitative analysis of tumor volumes showed 71% and 69% less tumor burden in mice that had received melanoma cells and were subsequently treated with CD-NSPCs and 5-FC when compared to control group 1 (tumor only, no treatment) or control group 2 (tumor + NSPCs + 5-FC), respectively (Fig. 4E; mean ± SD; Student’s t-test, n = 4).

The parenchyma surrounding the tumor in mice receiving the CD-NSPC-mediated local chemotherapy appeared normal.

These studies provide proof of concept that NSPCs can target disseminated micromelanoma and macromelanoma metastases in the brain and that tumor burden can be reduced significantly with this NSPC pro-drug.
therapy. This expands potential applications shown by previous studies using NSPCs as delivery vehicles to target primary, infiltrative glioma (Aboody et al., 2000), to include systemic primary tumor metastases to the brain. However, tumor size reduction, as assessed by histopathology, may not necessarily correlate with increased survival. Future investigations should include long-term survival studies to demonstrate therapeutic efficacy. Studies quantifying NSPC distribution and time course will also be necessary to identify conditions that maximize therapeutic benefit.

This CD-NSPC/5-FC system resulted in significantly reduced tumor burden in the described intracranial tumor metastatic animal model. Other candidate therapeutic gene products to metastatic brain tumor sites, as well as primary brain tumors (e.g., glioblastoma), may include cell differentiation-promoting agents, cell-cycle modulators, apoptosis-promoting agents, antiangiogen...
esis factors, and agents enhancing antitumor immunity. Other sources of stem cells, including fetal and adult allogeneic brain NSPCs and bone marrow–derived mesenchymal stem cells (MSCs), have been used for experimental brain tumor studies, especially glioma therapy in animal models (summarized in Table 1).

Furthermore, stem cells expressing various oncolytic payloads might be applicable to melanoma metastases in other organs as well. A recent study with adult human MSCs carrying the interferon-β gene significantly reduced the metastatic melanoma burden in lungs of mice and resulted in longer survival times (Studeny et al., 2004). Similarly, human MSCs have been used to deliver interferon-β to human intracranial glioma in a mouse model, which resulted in extended survival time of glioma-bearing mice (Nakamizo et al., 2005). Future research studies will likely include determination of factors mediating migratory tropism of stem cells to tumors and defining their applicability to other cancers. Effective treatment paradigms need to be established through quantification of time course and stem cell distribution in the context of both primary and metastatic tumors.

Currently, there are no treatment options for patients with multiple brain metastases. Whole-brain irradiation is not very effective and has many negative side effects. Chemotherapy is insufficient because delivery to the brain is problematic, and multiple metastases occur at later stages in the disease, generally after several cycles of chemotherapy regimens and induced drug resistance.

Because patients with central nervous system melanoma metastases have very limited therapeutic options, further exploration of targeted therapeutic gene delivery via stem cells is of importance. The most effective ap-

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<th>Table 1. Promising stem cell–based experimental therapies for brain tumors</th>
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<tr>
<td><strong>Stem/Progenitor Cells (Source)</strong></td>
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<tr>
<td>---------------------------------</td>
</tr>
<tr>
<td>C17.2 (mouse)</td>
</tr>
<tr>
<td>C57.npr.IL-4 (mouse)</td>
</tr>
<tr>
<td>ST14A.IL-4.3 (rat)</td>
</tr>
<tr>
<td>C17.2 (mouse)</td>
</tr>
<tr>
<td>NSPC-IL-12 (mouse)</td>
</tr>
<tr>
<td>NSPC-TRAIL (mouse)</td>
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<tr>
<td>ST14A (rat)</td>
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<td>C17.CD2 (mouse)</td>
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<tr>
<td>MDNCC (adult human)</td>
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<td>MSCs (rat)</td>
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<td>MSCs (human)</td>
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<td>C17.CD2 (mouse)</td>
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**Abbreviations:** 5-FC, 5-fluorocytosine; 5-FU, 5-fluorouracil; CD, cytosine deaminase; HSV, herpes simplex virus; IFN-β, interferon-β; IL, interleukin; MDNCC, marrow-derived neural-competent cells; MSCs, mesenchymal stem cells; NSPC, neural/stem progenitor cell; PF4, platelet factor 4; TRAIL, tumor necrosis factor–related apoptosis-inducing ligand.
approaches for successful treatment of melanoma brain metastasis are likely to come from a combination of existing treatment strategies (surgery, chemotherapy, and radiation therapy) and small-molecule-based pharmacologic interventions (Bagnato et al., 2004), immunotherapy (Dudley and Rosenberg, 2003), and targeted NSPC-mediated delivery of therapeutic gene products and bioactive agents to sites of tumors metastases.

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References

Aboody et al.: Neural stem/progenitor cells target brain metastases


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