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NeuroImage 37 (2007) S18 – S26

Novel method for visualizing and modeling the spatial distribution of neural stem cells within intracranial glioma

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Received 21 January 2007; revised 20 March 2007; accepted 26 March 2007
Available online 13 May 2007

Neural stem cells (NSCs) hold great promise for glioma therapy due to their inherent tumor-tropic properties, enabling them to deliver therapeutic agents directly to invasive tumor sites. In the present study, we visualized and quantitatively analyzed the spatial distribution of tumor-tropic NSCs in a mouse model of orthotopic glioma in order to predict the therapeutic efficacy of a representative NSC-based glioma therapy. U251.eGFP human glioma was established in the brain of athymic mice, followed by stereotactic injection of CM-DiI-labeled human NSCs posterior-lateral to the tumor site. Confocal microscopy, three-dimensional modeling and mathematical algorithms were used to visualize and characterize the spatial distribution of NSCs throughout the tumor. The pattern of NSC distribution showed a gradient with higher densities toward the centroid of the tumor mass. We estimate that NSC-mediated therapy would eradicate 70–90% of the primary tumor mass and the majority of invasive tumor foci. Our method may serve as a model for optimizing the efficacy of NSC-based glioma therapy.
Published by Elsevier Inc.

Keywords: Brain tumor; Confocal imaging; Gene delivery; Glioma therapy; Neural stem cells; Tumor targeting; Three-dimensional modeling

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Available online on ScienceDirect (www.sciencedirect.com).

1053-8119/$ - see front matter. Published by Elsevier Inc.
doi:10.1016/j.neuroimage.2007.03.076

Introduction

Despite advances in neuro-oncological therapies, the median survival of patients with malignant glioma is less than 1 year from diagnosis (Jubelirer, 1996; Surawicz et al., 1998). The high degree of glioma cell invasion and infiltration into normal brain tissue limits treatments of glioma by resection, irradiation, chemotherapy, or gene therapy. In order to increase efficacy, novel glioma therapies must be specific and selective in targeting invasive tumor micro-foci.

Neural stem cells (NSCs) hold great promise as an emerging alternative or adjunct glioma therapy because of their inherent tumor-tropic and migratory properties (Aboody et al., 2000; Benedetti et al., 2000; Ehtesham et al., 2002b; Lee et al., 2003; Kim et al., 2006). NSCs can potentially be used as delivery vehicles to target and disseminate therapeutic gene products to invasive glioma cells. Significant therapeutic efficacy, as measured by reduction in tumor burden and/or increase in long-term survival, has been demonstrated in mouse models using NSCs engineered to express cytosine deaminase (CD) (Aboody et al., 2000; Kim et al., 2006), interleukin-12 (IL-12) (Ehtesham et al., 2002b), interleukin-4 (IL-4) (Benedetti et al., 2000), platelet factor 4 (PF4) (Lee et al., 2003), tumor necrosis factor-related apoptosis inducing ligand (TRAIL) (Ehtesham et al., 2002a; Shah et al., 2005), PEX (Kim et al., 2005), and herpes simplex virus type I thymidine kinase (HSV-TK) (Uhl et al., 2005). However, quantitative studies of NSC distribution throughout the tumor have not been adequately addressed and are necessary for optimizing this promising therapeutic strategy.

Two issues must be addressed to advance NSC-mediated therapy for brain tumors towards patient trials. First, although it has

been shown *in vitro* that the NSC-tumor tropism may involve the stromal cell-derived factor-1 (SDF-1)/CXCR4, stem cell factor (SCF)/c-Kit, hepatocyte growth factor (HGF)/c-Met, vascular endothelial growth factor (VEGF)/VEGFR and macrophage chemotactic protein-1 (MCP-1)/CCR2 ligand/receptor axes (Ehtesham et al., 2004; Sun et al., 2004; Zhang et al., 2004; Heese et al., 2005; Lesniak, 2006), the *in vivo* biological mechanisms responsible for NSC-tumor tropism must be more clearly elucidated. Analyzing the spatial distribution of NSCs throughout the tumor is important for a better understanding of *in vivo* biological effects of these cytokines. Second, the efficacy of NSC-mediated glioma therapy utilizing various transgenes must be more accurately estimated (Zhang et al., 2004). Quantifying the pattern of NSC distribution will provide estimates for the percentages of glioma cells that could be affected by specific NSC therapies. Such analyses will be facilitated by our newly developed methods described herein.

Our current understanding of the distribution of NSCs within brain tumors in experimental animals is limited to visualizing NSCs using contrast agents in magnetic resonance imaging (MRI) or photon flux in bioluminescence imaging (Zhang et al., 2004; Shah et al., 2005). Bioluminescence imaging does not provide the cellular-level resolution (micrometer-scale) that is needed to precisely quantify distribution (Weissleder, 2002). Recent work in high-resolution, *in-vivo* MR imaging of stem cells labeled with iron oxide nanoparticles has shown great promise for visualizing cell distribution, but the quantitative characterization of this distribution has been limited (Hinds et al., 2003; Anderson et al., 2005; Arbab et al., 2006; Heyn et al., 2006; Tallheden et al., 2006). In addition, while mathematical approaches have been shown for cell growth and tumor invasion *in vitro* (Dunn et al., 2006; Frieboes et al., 2006), there are no existing mathematical approaches to characterizing the pattern of distribution of NSCs at glioma sites *in vivo*.

In the present study we used an orthotopic mouse model of human glioma, high-resolution fluorescent confocal microscopy, and three-dimensional (3D) modeling to develop a novel algorithm that allows the following analyses: (1) estimation of primary tumor boundary and centroid; (2) identification of cancer cells and NSCs in the tumor; (3) determination of coordinates and 3D distribution of NSCs; (4) estimation of tumor volume that would be eradicated by NSCs (using a cell killing radius) with a defined intra-tumor and surrounding micro-foci distribution. Our findings demonstrate that a single administration of NSC-mediated therapy for glioma would affect the vast majority (70–90%) of tumor cells within the primary mass, as well as at surrounding infiltrative tumor foci. Given that the pattern of cellular distribution is important for nearly all cell-based delivery strategies, our new methods provide a quantitative framework for understanding the therapeutic potential of a wide variety of targeted treatment systems.

Materials and methods

Intracranial implantation of glioma cells

U251 human glioma cells were transfected with pCI-*eGFP-neo* expression vector using Lipofectamine 2000 (Invitrogen) to express enhanced Green Fluorescent Protein (eGFP). U251.eGFP cells were grown in DMEM + 10% fetal calf serum (FCS) + 1% penicillin/streptromycin/fungizone (GIBCO) at 37 °C in a humidified atmosphere of 10% CO_2. Cells were prepared for injection into the brains of experimental mice (athymic female *nu*/*nu*, 7–8 weeks old) as follows: the culture medium was removed and cells were washed in 5 ml of sterile PBS. The cells were then detached by trypsinization, collected by centrifugation, rinsed in PBS, and re-suspended in PBS at a density of 4×10^4 cells/μl. Mice were anesthetized using ketamine/xylazine. Using a stereotactic apparatus, a burr hole was drilled into the skull and U251.eGFP cells (8×10^4 cells in 2 μl PBS) were injected into the right frontal lobe 2 mm lateral, 0.5 mm anterior to bregma and 2.5 mm deep from the surface of the dura mater with a 30-gauge 5-μl Hamilton syringe. Injections were performed over 2–3 min—the needle was drawn slowly from the 2.5-mm depth upward in 0.25-mm increments, with 0.5 μl cell suspension injected at each increment (Fig. 1a).

Intracranial injection of neural stem cells

Seven days (Group 1, Animal A) or fourteen days (Group 2, Animal B) after intracranial implantation of U251.eGFP glioma cells, NSCs (HB1.F3.C1—a human fetal stem cell line immortalized with the v-*myc* gene (Kim, 2004)) were labeled with CM-DiI and injected intracranially in a posterior-lateral position with regard to the glioma injection site. Three ml of 1 μg/ml CM-DiI solution was added to NSC pellets (prepared as described for glioma cells) and the cell pellet was re-suspended by gentle trituration. Cells were incubated for 5 min at 37 °C and then incubated on ice for 15 min, followed by rinsing and re-suspension in PBS. A new burr hole was drilled into the skull and CM-DiI NSCs (2×10^5 cells in 2 μl PBS) were injected into the right frontal lobe 2.75 mm lateral, 0.5 mm posterior to bregma and 2.5 mm deep from the surface of the dura mater with a 30-gauge 5-μl Hamilton syringe.

Harvesting and processing of tissue

Five days following the injection of NSCs, both Group 1 and Group 2 animals were euthanized by CO_2 asphyxiation. Mice were perfused transcardially with ice-cold PBS, pH 7.4 (~20 ml of PBS/mouse), followed by perfusion of ice-cold 4% (w/v) paraformaldehyde (PFA) in PBS (~20 ml). Brains were harvested and post-fixed in 4% PFA/PBS for 48 h at 4 °C. Brains were then sectioned horizontally at 100-μm thickness on a vibratome (Pelco, Vibratome 1000). Sections containing tumor were identified using a Nikon Eclipse TE 2000U inverted epifluorescence microscope and mounted on slides in Prolong-Gold confocal mounting solution (Molecular Probes) containing 70 μg/ml of DAPI (Sigma) for visualization of cell nuclei.

Confocal microscopy

Mounted sections were imaged using a Zeiss LSM 510 confocal microscope (Carl Zeiss Microimaging Inc., Thornwood, NY). Optical sections were obtained using the following objectives: 1.25×/0.035 NA Plan-Neofluar, 2.5×/0.12 NA Fluar, 20×/0.5 NA Plan-Neofluar, and 63×/1.4 NA oil immersion Plan-Apochromat at a resolution of 1024 × 1024 pixels. Low-power objectives (1.25×, 2.5×) showing brain anatomy were used primarily for alignment of physical sections. At 20×, the location of the tumor was identified in the eGFP channel (detection of glioma) and overlaid with CM-DiI (detection of NSCs) and 2-photon DAPI channels. The centroid of the tumor, as visually identified, was centered in the 20× field. The primary magnification of interest was 20× because this allowed for both cellular-level

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