The Use of Interleukin 12-secreting Neural Stem Cells for the Treatment of Intracranial Glioma

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Abstract

Neural stem cells (NSCs) are capable of tracking migrating glioma cells. To exploit this tropism to generate an antitumor T-cell response, particularly against disseminating tumor pockets, we inoculated intracranial glioma-bearing mice with intracranial IL-12 (IL-12) producing NSCs. Intratumoral therapy with IL-12-secreting NSCs prolonged survival compared to treatment with nonsecretory NSCs or saline. NSCs demonstrated strong tropism for disseminating glioma, and IL-12-secreting NSC therapy was associated with enhanced T-cell infiltration in tumor microsatellites and long-term anti-tumor immunity. These results indicate that the use of tumor tracking NSCs represents a potent new therapeutic modality for glioma.

Introduction

Current therapy for intracranial glioma centers on surgical resection with adjuvant radio- or chemotherapy. Despite refinements in these techniques, tumor recurrence is common, and the prognosis for patients with malignant brain tumors remains poor (1). The failure of surgical resection, and other clinical and experimental therapies to effectively cure glioma is based partly on their inability to target disseminated tumor that has interspersed itself with normal brain parenchyma, often at great distance from the main tumor mass. This can consist of thin tumor extensions growing out from the core neoplasm deep into adjacent tissue or can comprise of independent microsatellites that have migrated away from the primary tumor. Surgical resection, even with the removal of a significant amount of peritumoral normal tissue, cannot eliminate these neoplastic reservoirs, which eventually serve as sources for recurrence. Because of their large number and varied locations, these tumor pockets also remain refractory to other conventional and experimental treatment strategies such as stereotactic radiosurgery or cellular and/or gene therapy strategies focused on delivering therapeutic genes or gene products directly into the tumor bed or postsurgical tumor cavity.

One possible method to target disseminated tumor islands is the use of NSCs, which can display intracranial migratory activity similar to that of glioma cells (2). NSCs have been shown to be capable of actively tracking migrating glioma cells within the brain (3) and have been used to deliver the therapeutic cytokine IL-4 to glioma in vivo with encouraging results (4). Therefore, the use of NSCs for the delivery of therapeutic gene products to migratory tumor islands may represent a viable modality for targeting these otherwise difficult to access neoplastic reservoirs. With the aim of additionally investigating this therapeutic strategy, we describe the use of IL-12-secreting NSCs for the treatment of intracranial glioma. We demonstrate that in C57Bl/6 mice bearing GL26 gliomas, intratumoral inoculation of IL-12-secreting NSCs significantly prolonged survival and resulted in long-term anti-tumor immunity. NSCs were generally found interspersed within the main tumor mass and were also present in small tumor islands detached from the primary tumor body. In addition, NSCs could be seen actively tracking outgrowths from the main tumor that extended deep into adjacent normal tissue. We also demonstrate that the prolonged survival seen in animals treated with IL-12-secreting NSCs was associated with significantly increased intratumoral CD4+ and CD8+ T-cell infiltration, particularly at the tumor/normal tissue boundary and in migrating tumor microsatellites.

Materials and Methods

Cell Culture and Virus. GL26 cells (murine glioma) and NIH-3T3 cells (murine fibroblasts) were grown in DMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum (Gemini Bioproducts, Calabasas, CA), 2 mM l-glutamine, 100 μg/ml penicillin, and 100 units/ml streptomycin (Life Technologies, Inc.). The recombinant replication-deficient adenoviral vectors bearing the genes for murine interleukin 12 (AdmIL-12) and β-galactosidase (AdLacZ) were described previously (5, 6).

NSC Harvest, Culture, and Differentiation. NSCs were harvested from the frontoparietal regions of day 15 fetal C57Bl/6 mice as described previously (7). The cells were grown in suspension in DMEM/F12 medium with B-27 supplement (Life Technologies, Inc.), 20 ng/ml murine epidermal growth factor, and 20 ng/ml human basic fibroblast growth factor (Peprotech, Rocky Hill, NJ). NSCs were differentiated in 24-well plates in DMEM/F12 medium supplemented with 1% fetal bovine serum (Gemini Biotechnologies), 1 μM retinoic acid, and 1 μM dibutyryl-cyclic AMP (Sigma, St. Louis, MO).

In Vitro Infection of NSC and 3T3 with Adenoviral Vectors. NSC and NIH-3T3 cells were infected with 100 multiplicity of infection of either AdmIL-12 or AdLacZ. NIH-3T3 cells were irradiated (5000 rads) to induce growth arrest. To ensure appropriate infection, neurospheres were disaggregated by vigorous pipetting before exposure to adenovirus. Forty-eight hours after infection, supernatant was collected and tested for IL-12 content using an ELISA (BD PharMingen, San Diego, CA), and cells were stained for β-galactosidase expression using an X-Gal substrate as per standard protocol. Before in vivo inoculation, all of the virally infected cells were washed three times with PBS (pH 7.4; ~50 ml/wash) to ensure that final inoculae were devoid of free viral particles.

Inoculation of Established Intracranial Gliomas with NSC. C57Bl/6 mice (6–8 weeks old; Harlan Sprague Dawley, Indianapolis, IN) were anesthetized with i.p. ketamine and xylazine, and stereotactically inoculated with 106 GL26 glioma cells in 2.5 μl of 1.2% methylcellulose/MEM in the right corpus striatum. For survival experiments, the animals were divided into five groups on day 2 after tumor implantation and treated with intratumoral inoculations of saline (5 μl; n = 9), 2 × 105 NSC-IL-12 (n = 10), NSC-LacZ (n = 9), NSC-mock (n = 9), or 3T3-IL-12 (n = 9) in 5 μl of serum and virus-free medium, injected directly into the established tumor using the same burr hole and stereotactic coordinates. Animals used for histological evaluation were treated similarly on day 7 after tumor implantation. To determine whether NSCs were capable of migrating across the brain toward a tumor in the opposite hemisphere, animals were inoculated with 2.5 × 105 NSC-LacZ in

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The abbreviations used are: IL, interleukin; NSC, neural stem cell; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; NSC-IL-12, IL-12-secreting NSC; NSC-LacZ, β-galactosidase expressing NSC; NSC-mock, mock-infected NSC; 3T3-IL-12, IL-12-expressing NIH-3T3.
the left corpus striatum contralateral to a 7-day established intracranial glioma. The stereotactic coordinates used for contralateral NSC inoculation were the mirror opposite of those used for initial tumor and ipsilateral NSC implantations. All of the animal use was performed in strict accordance with Animal Care and Use Committee guidelines in force at Cedars-Sinai Medical Center.

**Confirmation of in Vivo Cytokine Secretion.** Intracranial tumor tissue was harvested from GL26 glioma-bearing mice treated 1 week earlier with intratumoral inoculations of NSC-IL-12, 3T3-IL-12, or NSC-LacZ ($n = 3$ for each group). Tumors for each group were pooled separately, weighed, and then physically disrupted in medium using blunt dissection. Cellular debris was removed by centrifugation and the supernatant analyzed by an IL-12 ELISA. Cytokine quantities for each group were normalized to pooled tumor weight.

**Immunocyto- and Histochemistry.** Primary and differentiated NSCs were fixed with acetone/methanol and stained *in situ* using antinestin, anti-βIII tubulin, anti-CNPase, and anti-GFAP (1:100 each; Chemicon, Temecula, CA) primary antibodies, and a Cy3-conjugated secondary antimouse IgG antibody (1:400; Chemicon). The staining was visualized under a fluorescence microscope (Olympus, Melville, NY).

Flow cytometry was performed on intracranial tumor tissue harvested from GL26 glioma-bearing mice treated 1 week earlier with intratumoral inoculations of saline, NSC-IL-12, NSC-LacZ, NSC-mock, 3T3-IL-12, or nonsecretory 3T3 cells. Tumor tissue from each group was pooled separately and then physically disrupted in medium using blunt dissection. A single cell suspension was prepared by passing the disassociated tissue through syringe needles of

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Fig. 1. A, generation of neurospheres from primary fetal brain culture. After harvest and culture in growth factor supplemented medium (2–3 days), NSCs grew in spherical aggregates. B, neurospheres are comprised of neural progenitor cells expressing nestin. Nuclei are counterstained with 4',6-diamidino-2-phenylindole. NSCs replated in modified culture conditions were induced to differentiate into C, β-III tubulin expressing neurons, D, GFAP expressing astrocytes, and E, CNPase expressing oligodendrocytes. F, expression of β-galactosidase by NSCs infected *in vitro* with Ad-LacZ. (Magnification ×400 for B–E, and ×100x for A and F).
progressively decreasing bire. Viable cells from each group (2 × 10⁶) were stained at 4°C for 30 min with antitoxin CD4-FITC and antitoxin CD8-PE (1:12 each, clones H129.19 and 53–6.7, respectively; BD Pharmingen). Samples were then fixed with 1% paraformaldehyde before analysis on a FACStar II cell sorter. Flow cytometric gates for viable lymphocytes were established on the basis of forward and side scatter, and control stains from mouse spleen.

For immunohistochemistry, brains harvested from treated mice euthanized using CO₂ asphyxiation were frozen in dry ice, sectioned using a cryostat, mounted on slides, and allowed to air dry. Sections used for X-Gal detection were fixed with 0.05% glutaraldehyde, stained as per standard protocol, and counterstained with neutral red before mounting. For other stains, brain sections were fixed in acetone and stained using primary antibodies for CD4 and CD8 (1:100 each; clones H129.19 and 53–6.7 respectively; BD Pharmingen), a fibroblast-specific marker (1:100; clone ER-TR7; Cedarlane Laboratories, Hornby, Ontario, Canada), β-galactosidase protein (1:100; MAB1820; Chemicon), and IL-12 (1:50; H-306; Santa Cruz Biotechnology, Santa Cruz, CA). Secondary staining was then performed with the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Slides were developed with diaminobenzidine (Sigma) and counterstained with hematoxylin before final mounting.

Results

Neurospheres are Comprised of Progenitor Cells That Can Differentiate into Neurons, Astrocytes, and Oligodendrocytes. We isolated primary NSCs from fetal C57Bl/6 mice. These cells grew primarily as spherical aggregates (Fig. 1A) and were comprised mainly of cells that stained strongly for nestin, a marker of neural progenitor cells (Fig. 1B). We confirmed their pluripotency by in vitro differentiation in medium supplemented with serum, retinoic acid, and cyclic AMP. After 7 days of culture, cells with well-differentiated morphologies were visible, and these contained populations expressing markers specific for neurons (βIII-tubulin), astrocytes (GFAP), and oligodendrocytes (CNPase; Fig. 1, C–E, respectively).

In Vitro Expression of β-Galactosidase and Secretion of IL-12 by Infected NSC and NIH-3T3. To confirm biologically relevant secretion of cytokine, supernatant from NSC- and NIH-3T3-infected cells with AdmIL-12 was analyzed using ELISA. NSC and NIH-3T3 cells infected with AdmIL-12 were found to secrete 70 and 64.8 pg/mg cells per 48 h, respectively. In addition, NSCs infected with AdLacZ were stained in vitro with an X-Gal substrate and found to express high levels of β-galactosidase 48 h after viral infection (Fig. 1F). To determine the effect of our viral vectors on NSC viability, we followed adenoviral-infected NSCs in vitro and found them to maintain a viability in excess of 95% (as determined by trypan blue exclusion staining) up to 10 days after viral infection (data not shown).

Glioma-bearing Mice Treated with IL-12-secreting NSCs Demonstrate Prolonged Survival and Long-Term Immunity. To determine whether inoculation of NSCs into established gliomas affected a therapeutic benefit, we delivered NSC-IL-12, NSC-LacZ, NSC-mock, 3T3-IL-12, or normal saline into established intracranial gliomas in C57Bl/6 mice. NSC-IL-12-treated mice demonstrated significantly prolonged survival compared with saline and nonsecretory NSC-treated controls (P = 0.03, NSC-IL-12 versus NSC-mock; P = 0.01, NSC-IL-12 versus NSC-LacZ; P = 0.01, NSC-IL-12 versus saline; Log rank), with 30% of treated animals surviving beyond day 60 after tumor implantation (Fig. 2). However, 3T3-IL-12 therapy produced no long-term survivors and did not result in statistically enhanced survival compared with treatment with NSC-mock, NSC-LacZ, or saline. The observable difference in survival between NSC-IL-12-treated animals and 3T3-IL-12 therapy could, however, not be verified statistically (P = 0.08; Log rank). There were no detectable differences in survival between animals treated with NSC-mock, NSC-LacZ, or saline.

Three months after initial tumor implantation, NSC-IL-12-treated mice that survived long term (n = 3) were rechallenged intracranially with 2.5 × 10⁶ GL-26 cells. At the same time, naïve animals (n = 5) received similar GL26 implantations. All 5 of the control animals died within 30 days after implantation, whereas the NSC-IL-12-treated survivors survived beyond day 120 after the tumor rechallenge.

In Vivo Transplanted NSC and 3T3 Remain Viable and Secrete Significant Levels of Cytokine. To ascertain whether our transplanted 3T3 and NSCs survived in vivo for a sufficient length of time to allow a therapeutic effect to ensue, we harvested and stained sections from tumor-bearing brains treated 18 days earlier with intratumoral inoculations of 3T3-IL-12, NSC-IL-12, or NSC-LacZ. Immunohistochemistry was performed for a fibroblast specific marker, β-galactosidase protein, and IL-12 cytokine. Tumors treated 18 days earlier with 3T3-IL-12 demonstrated positive staining for a fibroblast-specific marker, indicating the presence of fibroblasts within the tumor (Fig. 3A). NSC-LacZ- or NSC-IL-12-treated brains were devoid of any fibroblast staining (not shown and Fig. 3C, respectively). Tumors inoculated with NSC-LacZ demonstrated numerous β-galactosidase-positive cellular clusters within the tumor mass and in tumor outgrowths (Fig. 3B) confirming the presence of NSC-LacZ in these sections. We were also able to detect IL-12-positive cells within 3T3-IL-12- and NSC-IL-12-treated tumors (not shown and Fig. 3D, respectively) 18 days after inoculation. These results indicate that both transplanted 3T3 and NSCs survived in significant quantity within treated tumors and continued to produce the specific gene products they had been engineered to express.

To quantify the amount of cytokine secreted in vivo, tumors from glioma-bearing mice treated with NSC-IL-12, 3T3-IL-12, and NSC-LacZ were harvested, processed, and analyzed for IL-12 content using ELISA. Tissue from both NSC-IL-12- and 3T3-IL-12-treated tumors and continued to produce the specific gene products they had been engineered to express.

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NSC Migrate Extensively through the Tumor Mass and Are Strongly Tropic for Disseminating Tumor Cells. To determine whether intratumorally inoculated NSCs demonstrated tropism for disseminating glioma cells, brains from NSC-LacZ-treated mice were harvested and stained with an X-Gal substrate to detect the presence of β-galactosidase-expressing NSCs. NSC-LacZ cells were readily identifiable, dispersed within treated tumors, and were clearly visible tracking glioma cells as they migrated away from the main tumor.
mass. We were able to detect several distinct patterns of tumor spread and found NSCs tracking migrating glioma cells in each case. These included thin outgrowths of tumor deep into adjacent normal brain (Fig. 4, A and B), direct extension of the tumor mass into adjacent tissue (Fig. 4C), migration of glioma cells along established white matter tracts (Fig. 4D), and dissemination of solitary tumor pockets at considerable distance from the primary tumor bed (Fig. 4, E and F).

NSCs inoculated into the corpus striatum contralateral to the site of tumor implantation did not randomly disperse into adjacent normal tissue (Fig. 4G, left panel) nor could they be seen migrating to any distant nontumorous region of the brain. However, some NSCs were visible tracking directly across the brain into the immediate vicinity of the tumor (Fig. 4G, center panel) and into the tumor itself (Fig. 4G, right panel). We also found that NSCs inoculated into nontumor-bearing brains did not randomly dissipate into adjacent tissue or to the contralateral hemisphere (Fig. 4H). These results indicate that NSCs exhibit strong, specific tropism for intracranial tumor.

Tumors Treated with IL-12-secreting NSCs Demonstrate Enhanced CD4+ and CD8+ T-Cell Infiltration. On the basis of the known ability of IL-12 to promote antitumor cell-mediated immunity, we wished to assess whether the survival benefit observed with NSC-IL-12 therapy was associated with enhanced intratumoral T-cell infiltration. Tumors from NSC-IL-12-treated animals demonstrated robust infiltration with CD4+ and CD8+ T cells (Fig. 5A, left panel; Fig. 5D). 3T3-IL-12 therapy also resulted in increased intratumoral T-cell infiltration (Fig. 5A, center panel; Fig. 5B), which was significantly higher than the immune cell infiltration seen in GL-26 tumors inoculated with nonsecretory 3T3 (data not shown).

In addition to enhancing T-cell infiltration within the tumor parenchyma, NSC-IL-12 therapy also resulted in the accumulation of nu-
merous CD4+ and CD8+ T-cell aggregates along the tumor/normal tissue boundary, and in tumor outgrowths and microsatellites (Fig. 5D; Fig. 5E, left panel), which was not seen in 3T3-IL-12-treated tumors (Fig. 5B; and Fig. 5E, right panel).

T-cell infiltration, as determined by flow cytometry and immunohistochemistry, in NSC-LacZ (Fig. 5A, right panel; Fig. 5C), NSC-mock (data not shown), and saline-treated tumors (data not shown) was low and comparably negligible.

Discussion

The inevitability of tumor recurrence after surgical resection and standard adjuvant therapy remains an unfortunate reality for the vast majority of patients with malignant glioma. The refractoriness of these neoplasms to treatment possibly stems from several independent mechanisms. This may include the documented ability of gliomas to exert immunosuppressive effects at both the systemic (8) and local intratumoral (9) levels. In addition, malignant gliomas are highly invasive in nature (10), which provides them with the ability to infiltrate deep into normal tissue, thereby establishing microscopic reservoirs from which regrowth can occur after surgical resection. The utilization of IL-12-secreting NSCs represents a novel method aimed at targeting both these phenomena. IL-12 is a potent tumoricidal cytokine (11) with demonstrated efficacy against intracranial glioma (5, 12). NSCs, with their extensive migratory and tumor tracking characteristics (3), are capable of delivering this therapeutic protein to neoplastic pockets separate from the main tumor mass.

The inoculation of NSCs engineered to secrete IL-12 resulted in significant prolongation of survival in intracranial glioma-bearing mice. To determine the potential role of NSC migration in this result, we compared the survival benefit offered by NSC IL-12 secretion to that conferred by IL-12 secretion by nonmigratory NIH-3T3 cells, which produce similar levels of cytokine to NSC-IL-12 in vivo and in vitro. 3T3-IL-12 treatment did not result in statistically significant survival compared with saline and nonsecretory NSC inoculated controls. In contrast, NSC-IL-12 therapy was able to demonstrate a statistically significant enhancement in survival compared with NSC-LacZ, NSC-mock, and saline treated controls, with 30% of NSC-IL-12 treated animals surviving beyond 60 days after tumor implantation. NSC-IL-12-treated long-term survivors were also immune to intracranial tumor rechallenge. This may be a direct consequence of
the ability of NSCs to target migrating tumor cells, as confirmed by our observation that NSCs exhibit strong tropism for disseminating tumor in vivo. Of significance was our inability to detect any survival benefit with the use of NSC therapy alone, as described previously (4). Both NSC-mock and NSC-LacZ-treated animals demonstrated survival similar to saline inoculated controls. This represents a significant divergence from the results forwarded by Benedetti et al. (4), in which intratumoral delivery of noncytokine-secreting NSCs also resulted in survival prolongation of tumors with CD4+ and CD8+ T-cell infiltration, with immunohistologically positive cells interspersed in tumor tissue. Aboody et al. (3) described previously the tropism of an immortalized NSC line for intracranial glioma-bearing brains. Our results confirm that primary, autologous, nontransformed NSCs are also capable of demonstrating potent migratory activity in vivo, a finding that was not reported by Benedetti et al. (4) in their primary NSC treatment model. Of significance was our finding that NSC inoculated into normal corpus striatum contralateral to an existing tumor did not randomly disperse throughout the brain. Rather, these cells exhibited specific migration to the tumor in the opposite hemisphere, indicating that our observed dissemination of NSC was not a random phenomenon, but
was in fact a process resulting from a strong, specific tropism of NSCs for intracranial glioma. The underlying basis of this tropism is unclear and may be the result of a chemotactic gradient resulting from the production of chemokines from tumor cells that are actively infiltrating normal tissue.

We have shown previously that in situ IL-12 gene transfer in intracranial glioma results in potent Th-1-mediated antitumor cytotoxic immunity (5). On the basis of this experience, we wished to determine whether the survival benefit associated with NSC-IL-12 therapy was accompanied by increased intratumoral CD4$^+$ and CD8$^+$ T-cell infiltration. Both NSC-IL-12- and 3T3-IL-12-treated tumors demonstrated robust T-cell infiltration. However, in contrast to 3T3-IL-12-treated tumors, NSC-IL-12 inoculated brains also demonstrated large, numerous CD4$^+$ and CD8$^+$ T-cell aggregates along their tumor margins and in migrating tumor microsatellites. Because NSCs also concentrate along the tumor/normal tissue boundary while aligning themselves with tumor cells that have begun to migrate, these lymphocytic aggregates may represent a chemotactic colocalization of T cells with marginating and migrating IL-12 producing NSCs. The survival benefit observed with NSC-IL-12 therapy may, therefore, be a consequence of IL-12-induced taxis of tumoricidal T cells to disseminating tumor pockets being tracked by migrating NSC-IL-12 cells.

The current focus of experimental NSC therapeutics is primarily based around their use in the treatment of neurodegenerative disorders and stroke. We have demonstrated that combining the tumoricidal potency of IL-12 with the extensive tumor tracking capability of NSCs results in a synergistic therapeutic benefit. This additionally extends the scope of NSC therapy to include their use as vehicles for protein delivery to in vivo glioma, and, therefore, represents a promising new treatment modality for malignant brain tumors.

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References

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