Targeting Rat Brainstem Glioma Using Human Neural Stem Cells and Human Mesenchymal Stem Cells


Abstract

Purpose: Brainstem gliomas are usually inoperable and have a dismal prognosis. Based on the robust tropisms of neural stem cells (NSC) and mesenchymal stem cells (MSC) to brain tumors, we compared the tumor-tropic migratory capacities of these stem cells and evaluated the therapeutic potential of genetically engineered human NSCs encoding cytosine deaminase (CD) and IFNβ against brainstem gliomas.

Experimental Design: The directed migratory capacities of NSCs and MSCs to brainstem glioma (F98) were evaluated both in vitro and in vivo. The human NSCs (HB1.F3) and various human MSCs, such as bone marrow–derived MSCs (HM3.B10), adipose tissue–derived MSCs, and umbilical cord blood–derived MSCs, were tested. Human fibroblast cells (HFF-1) were used as the negative control. As a proof of concept, the bioactivity of HB1.F3-CD-IFNβ was analyzed with a cell viability assay, and animals with brainstem gliomas were injected with HB1.F3-CD-IFNβ cells followed by systemic 5-fluorocytosine treatment.

Results: In an in vitro modified Transwell migration assay and in vivo stem cell injection into established brainstem gliomas in rats, all the stem cells showed a significant migratory capacity compared with that of the control (P < 0.01). Histologic analysis showed a 59% reduction in tumor volume in the HB1.F3-CD-IFNβ-treated group (P < 0.05). Apoptotic cells were increased 2.33-fold in animals treated with HB1.F3-CD-IFNβ compared with the respective control groups (P < 0.01).

Conclusion: The brainstem glioma-tropic migratory capacities of MSCs from various sources were similar to those of NSCs. Genetically engineered NSCs show therapeutic efficacy against brainstem gliomas.

Brainstem gliomas account for 10% to 15% of childhood central nervous system tumors. Diffuse intrinsic pontine glioma is the most common of the brainstem gliomas, and it cannot be removed by surgery (1, 2). Despite technological advances in radiation therapy and chemotherapy, the prognosis is poor, with a mean survival of <1 year (1, 3). These disappointing results in the treatment of brainstem glioma have prompted numerous experimental trials in search of a new, effective treatment (4).

Recently, gene therapy using neural stem cells (NSC) as the vehicle for therapeutic agents has emerged as a promising treatment modality for malignant brain tumors. The strong inherent tumor-tropic properties of NSCs could make them an ideal vehicle for the delivery of therapeutic agents to the tumor bed. From the early 2000s, several preclinical trials of stem cell–based gene therapies have shown that NSCs can be effective tumor-specific delivery vehicles for transgenes to brain tumors (5–10).

However, the practical application of NSCs is limited by ethical and logistic problems related to their isolation and their potential immunogenicity because of the requirement for allogenic transplantation (11–16). Ideal stem cells should be autologous cells that can be harvested without difficulty from the patient, manipulated efficiently in vitro, and implanted back into the same patient (14). In this context, many efforts have been made to find alternatives to NSCs in stem cell therapy. Studies have confirmed that bone marrow–derived mesenchymal stem cells (BM-MSC) have all the properties of NSCs, such as an extensive migratory capacity and tropism for gliomas (15, 17–20). However, no quantitative comparison of the...
Translational Relevance

Generally, brainstem gliomas are inoperable and do not have an encouraging prognosis. These disappointing results in the treatment of brainstem gliomas have encouraged numerous experimental trials in the search for a novel treatment.

Recently, stem cell–based gene therapy has shown potential as a new treatment modality for malignant brain tumors because of the strong inherent tumor-tropic properties of stem cells. Not only neural stem cells but also mesenchymal stem cells can target brain tumors.

In this study, we quantitatively compared the tumor-tropic migratory capacities of neural stem cells and mesenchymal stem cells from various sources and qualitatively described the characteristics of migratory and nonmigratory stem cells. This is the first preclinical trial of a stem cell–based gene therapy directed against brainstem glioma in an animal model. Our results provide the rationale for further evaluation of this strategy for human brainstem gliomas.

Materials and Methods

Cells. Human NSCs (HB1.F3) were derived from a human fetal brain (ventricular zone) at 15 wk of gestation with an amphotropic, replication-incompetent retroviral vector containing v-myc (21–24). This is a well-established and well-characterized NSC line, which is multipotent, migratory, and nontumorigenic in vivo. The NSCs were cultured in DMEM (WelGene Biopharmaceuticals) supplemented with l-glutamine, 10% fetal bovine serum (FBS), and 1% antibiotic-antimycotic solution (Invitrogen).

The BM-MSCs (BM3.B10) were derived from human fetal spinal vertebrae bone marrow at 12 to 15 wk of gestation with an amphotropic, replication-incompetent retroviral vector containing v-myc (25). These cells differentiate into neural cell types and restore functional deficits in mice with intracerebral hemorrhage after brain transplantation. The BM-MSCs were suspended in α-MEM (WelGene Biopharmaceuticals) supplemented with l-glutamine, deoxyribonucleosides, ribonucleosides, 10% FBS, and 1% antibiotic-antimycotic solution.

Adipose tissue–derived MSCs (AT-MSC) were isolated from human fat tissue. Adipose tissues were obtained from the abdominal fat prepared for sellar floor reconstruction in patients who had undergone transsphenoidal surgery at Seoul National University Hospital. All eligible patients or their parents provided their written informed consent, and permission to isolate the MSCs from the fat tissues was given by the institutional review board of Seoul National University Hospital. The cells were cultured to passages 2 and 3. The AT-MSCs were suspended in MSC expansion medium (Chemicon) supplemented with 10% FBS and 1% antibiotic-antimycotic solution.

The human umbilical cord blood–derived MSCs (UC-MSC) were purchased from Medipost Biomedical Research Institute (Medipost). The UC-MSCs were suspended in α-MEM supplemented with l-glutamine, 110 mg/mL sodium pyruvate, 4,500 mg/L D-glucose, 10% FBS, and 1% antibiotic-antimycotic solution. All cells were incubated at 37°C in an incubator in a 5% CO2/95% air atmosphere.

Flow cytometric analysis. Whereas the NSCs and BM-MSCs are established cell lines, the AT-MSCs and UC-MSCs were primary cultured cells. Therefore, the primary cell lines were characterized further. AT-MSCs and UC-MSCs were cultured in control medium for 72 h before analysis. Flow cytometry was done using a FACSscan argon laser cytometer (Becton Dickinson). Briefly, the cells were harvested in 0.25% trypsin/EDTA and fixed for 30 min in ice-cold 2% formaldehyde. The fixed cells were washed in flow cytometry buffer (PBS, 2% FBS, 0.2% Tween 20) and incubated for 30 min in flow cytometry buffer containing fluorescein isothiocyanate–conjugated monoclonal antibodies directed against cluster of differentiation (CD) antigens (CD29, CD49d, CD105, CD34, and CD90; Chemicon) or phycoerythrin-conjugated monoclonal antibodies directed against CD antigens (CD34 and CD45; Chemicon).
**Kruskal-Wallis test.** Migratory capacity inferior to that of NSCs. UC-MSCs showed a medium compared with the migration of HFF-1 cells.

The directed migratory capacities of NSCs and MSCs toward F98 glioma cells were evaluated using a modified Transwell migration study. A, all stem cells showed significant directional migration toward F98-conditioned medium compared with the migration of HFF-1 cells. *, P<0.01, Kruskal-Wallis test. The migratory capacities of BM-MSC and AT-MSC did not differ significantly from that of NSCs. UC-MSCs showed a migratory capacity inferior to that of NSCs. **, P<0.05, Kruskal-Wallis test. B, representative photographs (magnification, ×200).

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**In vitro migration study.** The directed migratory capacities of NSCs and MSCs toward F98 glioma cells were evaluated using a modified Transwell migration study. F98 cells (50,000/0.5 mL) were incubated in serum-free medium for 24 h and placed in the lower well of Matrigel-coated (BD Biosciences) 10-mm tissue culture Transwell plates (8 μm; Nunc International). The NSCs and MSCs from various sources (50,000/0.5 mL) in serum-free medium were seeded in the upper wells of the Transwell plates. HFF-1 cells were used as the negative control for migrating cells. Serum-free medium served as negative control for trigger factors. After incubation for 6 h at 37 °C, the nonmigratory cells were removed from the upper side of the filter, and the filters were then stained with the Three-Step Stain Set (Richard-Allan Scientific) to quantify the migratory cells. All experiments were conducted in quadruplicate. The nuclei of the migratory cells were counted in five high-power fields (×200), and the values were expressed as means ± SE.

**In vivo migration study.** Female Fisher 344 rats (Central Lab Animal) weighing 150 to 200 g were anesthetized with an i.m. injection of a solution of 20 mg/kg Zoletil (Virbac) and 10 mg/kg xylazine (Bayer Korea). The posterior cranial region was shaved and prepared in a sterile fashion. A midline scalp incision of ∼2 cm was made, and a small burr hole was created using a 22-gauge needle. To establish brainstem gliomas, F98 tumor cells were stereotactically implanted into the right brainstem, as previously described (26). The stereotactic coordinates were 1.4 mm to the right of the sagittal suture and 1 mm anterior to the bregma, at a depth of 2.5 mm from the dura mater. Each cell type (200,000 cells in 5 μL) was labeled for 30 min with chloromethylbenzamido-DiI (CM-DiI; Molecular Probes) before injection, according to the protocol of the manufacturer. Bromodeoxyuridin (BrdUrd; 10 μmol/L; BD Pharmingen) was added to the cultures of NSCs or MSCs for 72 h.

On day 21, the animals were perfused with 4% paraformaldehyde under deep anesthesia and killed. Their brains were harvested and immersed in 4% paraformaldehyde solution for 24 h. After fixation, the brains were immersed sequentially in 10%, 20%, and 30% sucrose solutions for dehydration, embedded in optimum cutting temperature compound (Tissue-Tek), and stored at -80°C.

To quantify the migratory capacity of the NSCs and the MSCs from various sources, the cells that had migrated to the brainstem glioma were counted. The brains were sectioned sagittally to 20 μm thickness across the whole extent of the tumor using a cryostat (Microm). We mounted the sections at intervals of ∼300 μm. Ten slides centered on the tissue sections that had strongly fluorescent DiI-positive cells were selected. The slides were stained with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories). The viable cells were quantified by BrdUrd staining. The tissue sections were washed twice with PBS. After incubation in permeabilizing buffer for 15 min, 0.3% H2O2 was added for 15 min to block any endogenous peroxidase activity. The tissue sections were incubated in 2 N HCl for 1 h to denature the nuclear DNA and were then washed thrice with PBS containing 1% normal goat serum. The sections were incubated overnight with the primary antibody. The sections were rinsed with PBS (0.05 mol/L, pH 7.4), treated for 30 min with 1% hydrogen peroxide in PBS to eliminate endogenous peroxidase activity and rinsed. .
with PBS. After rinsing, the secondary antibody, Alexa 488–conjugated goat anti-mouse IgG (1:100; Molecular Probes), was applied. The sections were mounted with antifading solution containing DAPI and observed under confocal and fluorescence microscopes (Zeiss).

The DiI- and BrdUrd-labeled cells were counted from images of high-power (×200) optical fields using a fluorescence microscope with Image-Pro Plus 4.5 software. Stem cell migration was calculated as follows:

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\frac{\text{Stem cells in the tumor bed}}{\text{C138}} \times 100
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To evaluate the differences between the migratory and nonmigratory cells, an immunohistochemical study was done with a stem cell–specific marker (nestin, 1:300; Chemicon), a neuronal marker (NeuN, 1:200; Chemicon), and a cell type–specific marker for astrocytes [glial fibrillary acidic protein (GFAP), 1:200; Chemicon]. All sections were treated for 30 min with a solution of normal goat serum to block nonspecific binding sites. The primary antibodies were diluted with PBS containing 1% normal goat serum. The sections were incubated overnight with the primary antibody. Sections were rinsed with PBS (0.05 mol/L, pH 7.4), treated for 30 min with 1% hydrogen peroxide in PBS to eliminate any endogenous peroxidase activity, and rinsed with PBS. The secondary antibody, Alexa 488–conjugated goat anti-mouse IgG (1:100), was then applied. The sections were stained with DAPI and were observed with confocal and fluorescence microscopy.

All animal studies were carried out at the animal facility of Seoul National University Hospital in accordance with national and institutional guidelines.

In vitro transfection of NSCs. The clonal HB1.F3-CD line was derived from the parental HB1.F3 cells, as previously described (10, 22, 23). An expression plasmid was constructed using the retroviral Fig.3. In vivo migration assay. A, F98 glioma cells were implanted into the brainstems of rats (T). One week later, CM-DiI–labeled and BrdUrd-labeled stem cells were injected into the right forebrains (I). After 2 wk, the migration of the stem cells to the tumor site was evaluated. B, all the stem cells showed conspicuously greater migratory capacity, measured as percentage migration, than that of the HFF-1 cells. *, P < 0.01, Kruskal-Wallis test. There was no significant difference in the cell numbers or percentages of migratory cells between the NSCs and MSCs. C, BrdUrd-labeled stem cells migrated across the brain parenchyma in the injected hemisphere (I) and populated the tumor bed (T) in the brainstem. Magnification, ×200.
pBabePuro backbone to include the *Escherichia coli* CD cDNA (1.5-kb fragment) transcribed from the long terminal repeat. The vectors were packaged by cotransduction into pA317 cells of the CD puro plasmid with the plasmid cDNA encoding MV12 envelope protein. The CD puro retroviral supernatant was used for multiple transductions of the HB1.F3 cells. The HB1.F3-CD cells were selected with 3 μg/mL puromycin for 4 wk. The clonal HB1.F3-CD-INFNβ line was derived from the parental HB1.F3-CD cells. An expression plasmid was constructed using the retroviral pLHCX backbone to include the human IFN cDNA (0.5-kb fragment) transcribed from the long terminal repeat. To produce retroviral vectors encoding human IFNβ, PA317 packaging cells were transfected with pLHC-INFNβ using Lipofectamine (Life Technologies-Bethesda Research Laboratories) and the viral producer cell clones were selected. The viral supernatants were collected from the viral producers, and the HB1.F3-CD cells were then incubated with the viral supernatants in the presence of polybrene (8 μg/mL). After incubation for 2 d in growth medium, the transduced cells were transfected with pLHC-INFNβ (Life Technologies-Bethesda Research Laboratories) and the viral producer cell clones were selected. The viral supernatants were collected from the viral producers, and the HB1.F3-CD cells were then incubated with the viral supernatants in the presence of polybrene (8 μg/mL) for 16 h. After incubation for 2 d in growth medium, the transduced cells (HB1.F3-CD-INFNβ) were selected with 5 μg/mL hygromycin for 4 wk.

Successful transduction of the HB1.F3-CD-INFNβ cells was confirmed by reverse transcription-PCR. The sense and antisense primers of each primer pair were designed to bind to different exons to avoid DNA contamination: CD (sense 5′-GAGTAGCCGCAGACCCACACCCGAGGG-GTGCAGTACATGGTATGCTGCTGCGTGC-3′, 550 bp ampiclon) and INFNβ (sense 5′-AAAGAAGCAGCAATTTTCAG-3′, 296 bp ampiclon). Total RNA was extracted with TRIzol reagent (Life Technologies-Bethesda Research Laboratories). Complementary DNA templates from each sample were prepared from 1 μg of total RNA primed with oligo(dT) primer (Pharmacia) using 400 units of Moloney murine leukemia virus reverse transcriptase (Promega), followed by 30 PCR amplification cycles (94°C for 30 s, annealing at 60°C for 60 s, and extension at 72°C for 90 s). Glyceraldehyde-3-phosphate dehydrogenase was used as the reaction standard: sense 5′-CATGCCAGACCTCCTGGCCT-3′, antisense 5′-CTAGGTTCACCGCCACCCGGG-3′, 450 bp ampiclon. Each PCR product (10 μL) was analyzed by 1.5% agarose gel electrophoresis. Authentic bands were determined by selective enzyme digestion.

**In vitro therapeutic efficacy of HB1.F3-CD-INFNβ.** To confirm the bioactivity of HB1.F3-CD-INFNβ, the cytotoxic effects of 5-fluorocytosine and 5-fluorouracil on HB1.F3-CD-INFNβ were analyzed with a cell viability assay. The therapeutic efficacy of HB1.F3-CD-INFNβ cells was tested in a migration assay. F98 cells (4,000 per well) were plated in 96-well plates (Corning, Inc.), and the experiments were done as follows: on day 1, HB1.F3 or HB1.F3-CD-INFNβ cells (8,000 per well) were added to the tumor cell cultures; on day 2, 5-fluorocytosine (100 μg/mL) and 5-fluorouracil (100 μg/mL) were added to the mixed cell cultures; and on day 6, cell viability was quantified with colorimetric assays using the Cell Counting Kit-8 (Dojindo Molecular Technologies). All experiments were carried out in triplicate. The determination of viability was based on the bioconversion of the tetrazolium compound, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8) into formazan, as determined by absorbance at 450 nm using a multiwell scanning spectrophotometer. Cell viability was expressed as the mean ± SE percentage relative to the viability of the control.

**In vivo therapeutic efficacy of HB1.F3-CD-INFNβ.** Animals were stereotactically implanted with F98 cells (50,000 in 3 μL PBS) in the right brainstem as described above. One week after tumor cell implantation, the animals were randomized into three groups: group 1, treated with an intratumor injection of PBS (5 μL, n = 10); group 2, treated with an intratumor injection of 200,000 HB1.F3 cells in 5 μL of PBS (n = 10); group 3, treated with intratumor injection of 200,000 HB1.F3-CD-INFNβ cells in 5 μL of PBS (n = 10). In this study, the ratio of stem cells/tumor cells was 4:1. HB1.F3 and HB1.F3-CD-INFNβ were labeled with the CM-DiI cell tracker before injection. One week after PBS, HB1.F3, or HB1.F3-CD-INFNβ injection, all groups received i.p. injections of 5-fluorocytosine (500 mg/kg) twice a day for 14 d. After the last 5-fluorocytosine treatment, the animals were perfused and their brains were harvested and processed as described above. The brains were sectioned coronally, and the tissue was stained with DAPI or H&E using standard protocols. The intratumoral distribution of CM-Dil-labeled NSCs was assessed by fluorescence microscopy. The tumor volumes were estimated using the formula for ellipsoids and expressed as means ± SE, as described previously (27). Immunohistochemistry was done with the Vectastain Elite ABC kit (Vector Laboratories). The brain sections were fixed in cold acetone. The primary antibodies included one directed against cleaved caspase-3 (1:100; Cell Signaling Technology) to detect apoptosis. The sections were counterstained with hematoxylin, and negative control slides were established by omitting the primary antibody. The apoptotic indices were defined as the percentage of positively stained cells per 100 nuclei from five randomly selected high-power fields.

**Statistical analysis.** All the values were calculated as means ± SE or were expressed as a percentage of the control ± SE. Differences in stem cell migration, tumor volume, and apoptosis index was tested with Kruskal-Wallis test with post hoc analysis. Difference in tumor cell viability was determined using the Mann-Whitney U test. Values of *P* < 0.05 were considered significant.

**Results.**

*Flow cytometric analysis of AT-MSCs and UC-MSCs.* Fluorescence-activated cell sorting analysis of AT-MSCs and UC-MSCs showed that the AT-MSCs and UC-MSCs expressed MSC-specific cell type markers, including CD29, CD49d, and CD105, but did not express cell type-specific markers for hematopoietic stem cells, including CD34, CD45, and CD90 (Fig. 1).

**Migratory ability of stem cells in vitro.** Using a Transwell migration assay, the *in vitro* migratory capacities of NSCs and MSCs were compared. All stem cells showed significant directional migration toward F98-conditioned medium compared with HFF-1 cells (25.6 ± 3.7, *P* < 0.01; Fig. 2), whereas very few cells migrated toward the serum-free medium (17.5-20). The mean number of migratory cells was highest for the NSCs (142.4 ± 22.8). The migratory capacities of the MSCs, including the BM-MSC (96.1 ± 12.7) and AT-MSC (107.6 ± 15.2), were not statistically different from that of the NSCs. However, the migratory capacity of the UC-MSCs (57.4 ± 5.7) was inferior to that of the NSCs (*P* < 0.05; Fig. 2).

**Migratory capacities of stem cells in vivo and differences between migratory and nonmigratory stem cells.** To compare the migratory capacities of NSCs and MSCs toward brainstem glioma *in vivo*, F98 glioma cells were implanted into the brainstems of rats. One week later, labeled stem cells were implanted into the right forebrains. Two weeks after labeled stem cell implantation, the migration of the stem cells to the tumor sites were examined. Stem cells migrated across the brain parenchyma and populated the tumor bed. In contrast, the HFF-1 cells showed no targeted migration and dispersed randomly around the implanted site. The percentage of migratory NSCs was 29.7 ± 5.4%. The percentages of migratory BM-MSCs, AT-MSCs, and UC-MSCs were 22.2 ± 3.1%, 29.0 ± 5.4%, and 19.7 ± 4.0%, respectively. In terms of the percentages of migratory cells, all stem cells showed markedly greater migratory capacities than that of HFF-1 cells (6.6 ± 1.2%, *P* < 0.01; Fig. 3). There were no statistical differences between NSCs and MSCs in their cell numbers or percentages of migratory cells.

Differences in the migratory and nonmigratory stem cells were also observed. With fluorescence microscopy, expression of the stem cell marker nestin was observed at the injection site.
but was not observed in the tumor bed. The expression of the astrocyte marker GFAP was scanty or not observed at the injection site but was observed in the tumor bed. The expression of the neuronal marker NeuN was found at the injection site or in the tumor bed (Fig. 4). These different patterns were similar in the NSCs and MSCs.

**In vitro therapeutic efficacy of HB1.F3-CD-IFNβ.** The expression of the CD-IFNβ transcript was confirmed by reverse transcription-PCR. The CD-IFNβ transcript was only expressed in the HB1.F3-CD-IFNβ cells and not in the parental HB1.F3 cells (Fig. 5A). To confirm the cytotoxic effects of the HB1.F3-CD-IFNβ cells, cell viability studies were done in a coculture system. After 5-fluorouracil treatment, the viability of the F98 cells did not differ when cocultured with HB1.F3 or HB1. F3-CD-IFNβ cells. After 5-fluorocytosine treatment, the F98 cells cocultured with the HB1.F3-CD-IFNβ cells decreased by 45.5% compared with cells cocultured with HB1.F3 cells \( (P < 0.05; \text{Fig. 5B}) \). These data show significant growth inhibition in the F98 cells induced by HB1.F3-CD-IFNβ cells. Without treatment, F98 cells cocultured with HB1.F3 or HB1.F3-CD-IFNβ cells showed no growth inhibition. This indicates that HB1.F3-CD-IFNβ cells can convert sufficient amounts of 5-fluorocytosine to 5-fluorouracil to effectively kill F98 cells *in vitro*.

**In vivo therapeutic efficacy of HB1.F3-CD-IFNβ.** To assess the therapeutic efficacy of HB1.F3-CD-IFNβ cells in an animal model of brainstem glioma, we injected CM-Dil-labeled HB1.F3-CD-IFNβ cells into tumor-bearing animals. The distribution of HB1.F3-CD-IFNβ cells and the tumor volumes were...
determined in harvested brain tissue after the last 5-fluorocytosine treatment. Labeled HB1.F3-CD-IFN$\beta$ cells were found predominantly at the border between the tumor and the normal parenchyma. The survival rates of the animals were 70% (7 of 10) in the PBS-treated group, 90% (9 of 10) in the HB1.F3-treated group, and 100% (10 of 10) in the HB1.F3-CD-IFN$\beta$–treated group. The average tumor volumes in the surviving animals in the PBS-treated, HB1.F3-treated, and HB1.F3-CD-IFN$\beta$–treated groups were 126.6 ± 17.5, 101.7 ± 10.7, and 51.8 ± 12.1 mm$^3$, respectively. The reduction in tumor size in the HB1.F3-treated animals was 19.7% compared with the tumor size in the PBS-treated control. In contrast, the reduction in tumor size in the HB1.F3-CD-IFN$\beta$–treated animals was 59.1%, significantly different from that in the PBS-treated control ($P$ < 0.05; Fig. 6A and B). We next sought to determine the biological action of CD-IFN$\beta$ on the gliomas. Histologic analysis revealed a significant increase of 2.33-fold in apoptotic cells in the animals treated with HB1.F3-CD-IFN$\beta$ relative to those in the various control groups ($P$ < 0.01; Fig. 6C and D). The HB1.F3 group showed a 1.27-fold increase in apoptotic cells relative to that in the control group, which is not statistically significant.

Discussion

In the present study, we quantitatively compared the tumor-tropic migratory capacities of NSCs and MSCs from various sources and qualitatively described the characteristics of migratory and nonmigratory stem cells. This is the first preclinical trial of stem cell–based gene therapy directed against brainstem glioma in an animal model.

Our results show that the tumor-tropic migratory capacities of MSCs were statistically similar to that of NSCs. This is the first preclinical trial of stem cell–based gene therapy directed against brainstem glioma in an animal model.

According to the previously suggested mechanism of tumor tropism (28), it seems that migratory capacity is mediated by the tumor cell itself or the surrounding tissue. The kind of stem/progenitor cell is a less important issue in defining tumor tropism. We postulate that tumor-tropic migration is a common feature of stem cells, regardless of their origin. The similar tropic capacities of MSCs of various origins including bone marrow, adipose tissue, and umbilical cord blood observed in our study support this hypothesis.

MSCs have recently been derived from various sources, including bone marrow, adipose tissue, and umbilical cord blood. Several studies have shown the tumor tropism and antitumor effects of genetically engineered BM-MSCs in animal models of intracranial glioma (15, 18, 19). AT-MSCs are reported to show broad multipotency with differentiation into a number of cell lineages, including adipocytic, osteocytic, and chondrocytic lineages, like BM-MSCs (29). The applicability of human AT-MSCs as cellular vehicles for targeted cancer chemotherapy has been reported for colon cancer (30). The easy and repeatable access to subcutaneous adipose tissue, the simple isolation procedure, and the greater numbers (~500-fold) of fresh MSCs derived from equivalent amounts of fat than can be derived from bone marrow are clear advantages in using AT-MSCs over BM-MSCs (31). Isolated AT-MSCs can also be easily cryopreserved (31). Although the inferior osteogenic and chondrogenic effects of AT-MSCs compared with those of BM-MSCs might limit their applicability in regenerative medicine (32, 33), this might not be a problem when used as a cellular vector system for gene therapy in the neurooncological field. Recently, umbilical cord blood has been used as an alternative source of MSCs (34). The potential advantages of UC-MSC compared with adult BM-MSCs include their ready availability from storage, the ease and low cost of harvesting, the ability to select them according to the human leukocyte antigen type required, the reduced risk of the transmission of
transplant-related infections, and their better immunologic tolerability (35, 36). A possible disadvantage of UC-MSCs is the finite number of cells that can be harvested (37). Our data suggest that not only BM-MSC but also AT-MSCs and UC-MSCs have satisfactory tumor-tropic properties and may be ideal substitutes for NSCs in stem cell–based gene therapies directed against brainstem glioma.

Despite their robust tumor tropism, stem cells do not always move toward the target site. Around 30% of all stem cells migrated to the target glioma in our study. This incomplete migration of stem cells can be explained in several ways: technical errors related to the quantification of migratory cells (localization of cells at the injection site and the diffuse distribution of cells within the tumor bed), failure of cell adaptation in a new microenvironment, the great distance between the injection site (forebrain) and the tumor site (brainstem), or poor survival rate of the human stem cells grafted into nonimmunosuppressed rat brain. Considering the invasiveness of glioma, attempts to enhance the tumor-tropic migratory capacity of stem cells and to combine therapeutic genes more effectively are interesting issues for future studies. A recent study reported that cell migration modulators, such as transmembrane protein 18, can enhance the tumor tropism of NSCs (38).

The migratory cells and nonmigratory cells differently expressed a stem cell marker and a differentiation marker. The “migratory” cells in the tumor bed no longer expressed markers of stem/progenitor cells but expressed a marker of glial differentiation, regardless of their origin, which is consistent with previous observations (38, 39). Contrary to this finding, the “nonmigratory” cells at the injection site were nestin positive and GFAP negative. This may simply suggest that only astrocytic precursors migrate to the tumor. However, when we examined the stem cells inoculated into the tumor, they only expressed the glial differentiation marker, regardless of their location in the tumor, in either the core or periphery (data not shown). Therefore, the differences between the migratory and nonmigratory cells are thought to be caused by the microenvironment.

Next, we studied the antitumor effects of genetically engineered human NSCs directed against brainstem glioma. As a proof of concept, we tested the potential of the cellular vector using HB1.F3 cells as the representative stem cells, which were engineered to secrete the prodrug activating enzyme CD and IFNβ. Our aim of adopting fusion gene (CD and IFNβ) was to maximize the antitumor effect. The therapeutic actions of CD and IFNβ are different; thus, we could expect a synergic effect. CD acts as a prodrug activating enzyme (10) and IFNβ can enhance the antiangiogenic effect and the immune response (15). In addition, we would like to prepare safety barrier, such as the survival rate of the animals after stem cell transplantation, is critical for protection from potential tumorigenesis of therapeutic stem cells. The suicidal tendency of CD can protect the host brain tissue by killing stem cells, as well as tumor cells. Any uncontrolled cell division of HB1.F3-CD-IFNβ cells during antitumor therapies will likely be kept under control by administering the 5-fluorocytosine, which leads to the killing of dividing cells, including HB1.F3-CD-IFNβ cells, which may undergo cell division. If necessary, 5-fluorocytosine could be given repeatedly to eradicate any residual HB1.F3-CD-IFNβ cells. Histologic analysis showed a significant reduction in the tumor volume in the treated group. The apoptotic index of the tumor cells was also elevated in the treated group. When a spatial relationship between the stem cells and the apoptotic cells was sought using a fluorescent microscope, the apoptosis of tumor cells predominantly occurred around the implanted stem cells (data was not shown), suggesting the therapeutic effect of genetically engineered stem cells. This study provides a rationale for the further evaluation of stem cell–based gene therapies for brainstem glioma, which might be the first candidate cancer for stem cell–based gene therapy because of its inoperability and dismal prognosis. Using a more effective therapeutic gene, these stem cells could provide a practical antitumor modulatory effect for this inoperable tumor.

There may be some limitation to our study. Firstly, the NSCs and BM-MSCs were established cell lines, whereas the AT-MSCs and UC-MSCs were primary cells. This discrepancy may interfere with the study as a confounding factor. However, there was no clear difference in the tumor-tropic migration of the cell lines and the primary cells. Secondly, we could only show cellular differentiation in a qualitative way. A laser-captured microdissection technique might be useful in effectively quantifying cellular differentiation. Finally, no long-term outcome, such as the survival rate of the animals after stem cell–based gene therapy, was examined. However, our short-term results provide some clues: 3 of 10 rats in the PBS-treated group

The migratory cells and nonmigratory cells differently expressed a stem cell marker and a differentiation marker. The “migratory” cells in the tumor bed no longer expressed markers of stem/progenitor cells but expressed a marker of glial differentiation, regardless of their origin, which is consistent with previous observations (38, 39). Contrary to this finding, the “nonmigratory” cells at the injection site were nestin positive and GFAP negative. This may simply suggest that only astrocytic precursors migrate to the tumor. However, when we examined the stem cells inoculated into the tumor, they only expressed the glial differentiation marker, regardless of their location in the tumor, in either the core or periphery (data not shown). Therefore, the differences between the migratory and nonmigratory cells are thought to be caused by the microenvironment.

Next, we studied the antitumor effects of genetically engineered human NSCs directed against brainstem glioma. As a proof of concept, we tested the potential of the cellular vector using HB1.F3 cells as the representative stem cells, which were engineered to secrete the prodrug activating enzyme CD and IFNβ. Our aim of adopting fusion gene (CD and IFNβ) was to maximize the antitumor effect. The therapeutic actions of CD and IFNβ are different; thus, we could expect a synergic effect. CD acts as a prodrug activating enzyme (10) and IFNβ can enhance the antiangiogenic effect and the immune response (15). In addition, we would like to prepare safety barrier for protection from potential tumorigenesis of therapeutic stem cells. The suicidal tendency of CD can protect the host brain tissue by killing stem cells, as well as tumor cells. Any uncontrolled cell division of HB1.F3-CD-IFNβ cells during antitumor therapies will likely be kept under control by administering the 5-fluorocytosine, which leads to the killing of dividing cells, including HB1.F3-CD-IFNβ cells, which may undergo cell division. If necessary, 5-fluorocytosine could be given repeatedly to eradicate any residual HB1.F3-CD-IFNβ cells. Histologic analysis showed a significant reduction in the tumor volume in the treated group. The apoptotic index of the tumor cells was also elevated in the treated group. When a spatial relationship between the stem cells and the apoptotic cells was sought using a fluorescent microscope, the apoptosis of tumor cells predominantly occurred around the implanted stem cells (data was not shown), suggesting the therapeutic effect of genetically engineered stem cells. This study provides a rationale for the further evaluation of stem cell–based gene therapies for brainstem glioma, which might be the first candidate cancer for stem cell–based gene therapy because of its inoperability and dismal prognosis. Using a more effective therapeutic gene, these stem cells could provide a practical antitumor modulatory effect for this inoperable tumor.

There may be some limitation to our study. Firstly, the NSCs and BM-MSCs were established cell lines, whereas the AT-MSCs and UC-MSCs were primary cells. This discrepancy may interfere with the study as a confounding factor. However, there was no clear difference in the tumor-tropic migration of the cell lines and the primary cells. Secondly, we could only show cellular differentiation in a qualitative way. A laser-captured microdissection technique might be useful in effectively quantifying cellular differentiation. Finally, no long-term outcome, such as the survival rate of the animals after stem cell–based gene therapy, was examined. However, our short-term results provide some clues: 3 of 10 rats in the PBS-treated group
and one rat in the HB1.F3-treated group died during the test period.

In conclusion, this study shows that not only NSCs but also MSCs from various sources can be used as useful vehicles for gene therapy directed against brainstem glioma, based on their tumor-tropic capacities. Furthermore, genetically modified NSCs display therapeutic efficacy against brainstem glioma.

These results suggest a potential role for stem cell–based gene therapy for brainstem glioma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Targeting Rat Brainstem Glioma Using Human Neural Stem Cells and Human Mesenchymal Stem Cells


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