INTRODUCTION

Primary brain cancer is the second most common cancer during childhood and the leading cause of cancer-related death in children aged 1–15 years. Medulloblastoma is one of the most common of these diffuse and highly disseminated brain tumors, with a mean age of onset within the first 6 years of life. Although the majority of patients with medulloblastoma can be treated with multimodality therapy, craniospinal radiation can have devastating effects on neurocognitive development and skeletal growth, and also poses a risk of initiating secondary tumors. Additional major obstacles to successful treatment of pediatric brain tumors include the blood–brain barrier, which prevents many anti-cancer agents from entering the brain, and therapeutic dose limitations due to toxicity to normal tissues. Although advances in surgical resection, refinements in radiation delivery and the addition of chemotherapy have improved survival rates for patients with medulloblastoma, it is still fatal in approximately one-third of patients. Those children who survive are often affected by delayed consequences of radiation and chemotherapy, which include permanent neurocognitive impairment, psychosocial deficits, stunted growth and endocrine deficiencies. To significantly improve clinical outcome, both in terms of survival and quality of life of these patients, novel treatment strategies must be developed that specifically target tumor cells while sparing neurons in order to maintain normal brain development and function.

Neural stem cells (NSCs), by virtue of their inherent tumor-tropic properties, offer an unprecedented advantage of therapeutic specificity over conventional cancer treatments. Because NSCs can effectively cross the blood–brain barrier and selectively target anti-cancer agents to invasive tumor cells, local drug concentrations and the therapeutic index can be increased while minimizing toxicity to normal tissues. It is especially important for pediatric patients that neurons, oligodendrocytes and endogenous NSCs are spared, in order to preserve cognitive function and normal brain development. In the studies presented here, we modified an established human NSC line (HB1.F3.CD) to deliver rabbit carboxylesterase (rCE) to enzymatically convert the prodrug camptothecin-11 (CPT-11) (Irinotecan) to the potent topoisomerase I inhibitor SN-38. HB1.F3.CD NSC tumor tropism, intratumoral distribution and therapeutic efficacy were investigated in clinically relevant experimental models. Magnetic resonance imaging was used for in vivo tracking of iron nanoparticle-labeled NSCs, and to assess the therapeutic efficacy of CE-expressing HB1.F3.CD cells.

As compared with controls, a significant decrease in tumor growth rate was seen in mice that received both NSCs and CPT-11 as their treatment regimen. Thus, this study provides proof-of-concept for NSC-mediated CE/CPT-11 treatment of medulloblastoma, and serves as a foundation for further studies toward potential clinical application.

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topoisomerase inhibitor SN-38. This NSC-mediated enzyme/prodrug therapy has demonstrated significant therapeutic efficacy in a metastatic human neuroblastoma model in mice. In the current experiments, we labeled the rCE-secreting NSCs (HB1.F3.CD.rCE) with iron nanoparticles to allow for visualization and tracking of their tumor tropism by magnetic resonance imaging (MRI). Biodistribution and therapeutic efficacy studies of NSC-mediated rCE/CPT-11 therapy were performed in immunocompetent transgenic and immunodeficient intracerebellar mouse models of medulloblastoma. High-power images of serial histopathological brain tissue sections revealed that NSCs were distributed within the tumor and adjacent to infiltrating tumor cells. MRI measurements of tumor volumes showed that mice treated with HB1.F3.CD.rCE NSCs, followed by intravenous (i.v.) administration of CPT-11, showed a significant decrease in tumor growth rate and volume when compared to mice treated with CPT-11 alone. Taken together, these studies provide proof-of-concept for NSC-mediated CE/CPT-11 treatment of medulloblastoma, and serve as a foundation for further studies toward potential clinical application.

RESULTS

NSC migration and tumor cell killing assays in vitro

To investigate the directed migration of HB1.F3.CD NSCs to medulloblastoma cells in vitro, we performed Boyden chamber cell migration assays. Conditioned media derived from transgenic mouse medulloblastoma (cell lines 144-4, 108-2, 157-7) and human medulloblastoma (Daoy, UW228) cells were used as chemoattractants for NSCs. HB1.F3.CD cells demonstrated robust migration to tumor-conditioned media from all murine and human medulloblastoma cells, with the greatest migration to human Daoy and UW228 (primary human medulloblastoma cells), when compared with positive (10% fetal bovine serum (FBS)) and negative (2% bovine serum albumin (BSA)) controls (Figure 1a). HB1.F3.CD cells were labeled with iron nanoparticles (Ferumoxide and protamine sulfate (FE-Pro)), as previously described. Neither labeling of cells with iron nor adenovirally transducing them with the rCE gene significantly affected their in vitro migration (Supplementary Figure S1).

To determine CPT-11 and SN-38-mediated growth inhibition and cell killing of medulloblastoma cells, we generated mouse medulloblastoma cell cultures from dissociation of transgenic tumors (144-4) or used established human medulloblastoma cell lines (Daoy and UW228). Cells were incubated for 72 h in culture media containing various concentrations of CPT-11 (0 - 100 μM) or SN-38 (0 - 100 nM) alone, or CPT-11 (0 - 100 μM) in the presence of NSC-secreted rCE in the culture media. After incubation, the numbers of viable cells were determined by WST-1 (water soluble tetrazolium salts) or Guava ViaCount assay, and drug toxicity was calculated compared with zero drug controls. SN-38 was ~1000-fold more potent than CPT-11; medulloblastoma cells had an IC50 for CPT-11 in the concentration range of 0.18 – 17.5 μM, while that for SN-38 was 0.2 – 2.2 nM (Figures 1b and c). The IC50 values for Daoy.CD cells were 41 μM and 25.9 nM for CPT-11 and SN-38, respectively (data not shown). Conditioned media from rCE-secreting HB1.F3.CD cells (rCE enzyme activity at 341 units per min) were used in combination with CPT-11 to sensitize human UW228 cells to the CPT-11 prodrug (Figure 1c). The IC50 for CPT-11 for UW228 cells was 17.5 μM and that for SN-38 was 2.2 nM. However, in the presence of rCE, the IC50 for CPT-11 was decreased by ~300-fold to 53 nM. The rCE expressed by the NSCs sensitized the target tumor cells to CPT-11 by producing SN-38 as confirmed by liquid chromatography – mass spectrometry analysis (Supplementary Figure S2).

In vivo migration of iron-labeled HB1.F3.CD.rCE NSCs to medulloblastoma

To determine the tumor tropism and intratumoral distribution of HB1.F3.CD.rCE NSCs in vivo, two mouse models of medulloblastoma were used: (1) an immunocompetent transgenic model that mimics the tumor histopathology of human patients (Figure 2a) (Shackelford et al., manuscript in preparation) and (2) an intracerebellar human medulloblastoma (Daoy) xenograft model in nude/nude mice (Figure 2b). In the transgenic model, medulloblastomas were induced in the developing cerebellum of...
mice by activating the Sonic Hedgehog (Shh) and Mycn pathways in granule neuron progenitor cells of the external granular layer that were targeted using an avian retrovirus, and tumor growth was monitored by firefly luciferase luminescence and MRI. Advantages of the transgenic medulloblastoma model include (1) endogenous emergence of tumors by activation of signaling pathways involved in human disease in the presumed cell-of-origin and (2) non-invasive real-time monitoring of tumor development by bioluminescence imaging or MRI. Among the advantages of the orthotopic human medulloblastoma model is the ability to evaluate biodistribution of NSCs in a human tumor xenograft model in vivo.

FE- Pro-labeled HB1.F3.CD.rCE NSCs were administered by intracerebellar (i.c.) injection (1 × 10^5 cells per 2 μl phosphate-buffered saline (PBS)) into mice with detectable transgenic medulloblastoma tumors and human Daoy xenografts (Figure 3). NSCs injected i.c. ipsilateral to transgenic and Daoy tumors migrated and distributed into the tumor parenchyma and invasive tumor areas (Figures 3c, d, h and i). The NSC migration was monitored by MRI on days 1 and 4 after injection (Figures 3a and f).

The hypointense MRI signal around the tumor likely reflects NSCs that are in the process of migration from the NSC injection site to tumors, but may not have reached their tumor target yet or have accumulated in the vicinity of tumor.

The brain tissue was harvested at day 6 post-NSC administration and histological sections were processed for Prussian blue staining to visualize iron-labeled NSCs. NSCs were detected as single cells or groups of NSCs within the tumor, as well as at the tumor periphery (Figures 3b–d, g–i). Figures 3c and h show Prussian blue-labeled NSCs deep in the tumor or close to the tumor edge (within 150 μm of the tumor edge). The Prussian blue-labeled NSCs in Figure 3i (lower right arrow) indicate the NSC injection site. NSCs were not detected in normal-appearing regions of the cerebellum or forebrain. These data suggest tumor-directed distribution of NSCs.

Both human and mouse tumors stained for the proliferation marker Ki-67 displayed areas of high proliferative activity as compared with islands of more differentiated tumor cells, a histopathological hallmark of medulloblastoma in human patients (Figures 3e and j). When NSCs were injected i.v. (1 × 10^6 cells per...
100 μl PBS), we observed homing of these cells to the tumor in a similar patchy distribution, with extravasated NSCs often found along blood vessels or in perivascular spaces (Supplementary Figure S3).

NSC-mediated rCE/CPT-11 therapeutic efficacy in medulloblastoma-bearing mice

We then evaluated the in vivo efficacy of NSC-mediated enzyme/prodrug (rCE/CPT-11) therapy in the transgenic murine medulloblastoma model. Transgenic mice that received Shh and Mycn gene-carrying viruses on postnatal day (PND) 3 were MR imaged every 2 weeks for tumor detection. Approximately 1.5 months after virus injection (PND 50), mice that developed tumors detectable by MRI were selected for NSC and CPT-11 treatment (mice that developed hydrocephalus were excluded from the study). A total of 14 mice were divided into four groups. Two treatment groups of four mice each received an i.c. injection of HB1.F3.CD.rCE cells (1 x 10^5 cells), followed by CPT-11 4 days later. The two i.v. CPT-11 treatment regimens used reflect clinical dosing schedules: (T1) a single high-dose injection of CPT-11 (38 mg kg^{-1}) or (T2) multiple low-dose injections of CPT-11 (20 mg kg^{-1} per day x 4 days). One week later, each treatment group received a second round of NSCs plus CPT-11. Each experimental group was paired with a CPT-11 only control group of three mice each that were treated with the same CPT-11 regimens but without NSCs (C1 and C2, respectively).

Evaluation of tumor growth and treatment efficacy was performed by MRI beginning at 1 month (PND 30) and continuing thereafter (PND 50, 58 and 71). Tumor growth was inhibited in mice that received either therapeutic regimen T1 or T2 when compared with control groups C1 and C2. In addition, MRI revealed a reduction in tumor size and edema in the brains in comparison with control groups C1 and C2. In addition, MRI mice that received either therapeutic regimen T1 or T2 when thereafter (PND 50, 58 and 71). Tumor growth was inhibited in mice that received either therapeutic regimen T1 or T2 when compared with control groups C1 and C2. In addition, MRI revealed a reduction in tumor size and edema in the brains of these mice.

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Statistical analysis of the kinetics of tumor growth was performed using linear mixed effects models. Comparisons of tumor growth rates between the treatment groups were done for pre-specified contrasts using t-tests. The average tumor growth rate between days 50 and 71 was 4.17 mm^3 per day in the C1 control group, but was only 0.73 mm^3 per day in the T1 treatment group (difference = -3.44, s.e. = 1.56, P = 0.04). The difference in tumor growth rates between the C2 control group (2.26 mm^3 per day) and the T2 treatment group (-0.46 mm^3 per day) was not significant (difference = -2.72, s.e. = 1.86, P = 0.16) and the difference in tumor growth rates between the T2 and T1 treatment groups was not significant (difference = -1.19, s.e. = 1.56, P = 0.45). The between mouse standard deviation for the tumor growth rates was 2.03 mm^3 per day and the within mouse standard deviation was 2.88 mm^3. Figure 5b depicts the mean tumor growth rates and actual data points before and after treatment for each of the four groups. Figure 5c shows the tumor growth rates after treatment, as calculated using simple linear regression for each mouse and graphed by study group.

Therapeutic efficacy was also evaluated by histopathological analysis (hematoxylin and eosin staining) and 3D reconstruction of medulloblastoma tumors (mice were euthanized at PND 71) (Supplementary Figure S4). These results corroborated the data obtained by MRI and showed that mice treated with i.c. injections of NSCs and CPT-11 had an overall smaller tumor volume when compared with mice treated with i.v. injection of CPT-11 alone on the same schedule of administration (Supplementary Figure S4).

Taken together, these results provide the first evidence for a therapeutic effect of NSC-mediated rCE/CPT-11 enzyme prodrug therapy for orthotopic medulloblastoma in preclinical animal models.

DISCUSSION

Advances in the treatment of childhood medulloblastoma have dramatically improved the survival rates of standard-risk patients. However, overall therapeutic improvement has come with the
cost of significant adverse effects among survivors, notably cognitive dysfunction.18 Patients with high-risk medulloblastoma, metastatic disease or post-operative residual tumor, when treated with currently available treatments have relatively low 5-year event-free survival (25–40%).

The efficacy of CPT-11 was previously investigated in a phase II trial for children with high-risk malignant brain tumors, as well as in preclinical mouse models of medulloblastoma.19,20 Limitations of current therapies include the low achievable CPT-11 concentration at the tumor site, and consequent high rate of tumor progression after initial treatment. Furthermore, CPT-11 therapy in children with brain tumors causes systemic toxicities such as myelosuppression and diarrhea.19,21 Our study aims at development of an NSC-mediated enzyme/prodrug therapy to treat medulloblastoma, which may help to overcome limitations of currently available therapies. Our previous in vitro studies demonstrated that SN-38 is ~1000-fold more effective at killing melanoma cells than is CPT-11 alone.

When injected in vivo, NSCs can migrate long distances and target tumor areas, making them ideal for delivery of therapeutic genes, including prodrug-activating enzymes and antibodies.23,24 NSC-mediated enzyme/prodrug therapy provides a strategy to circumvent the blood–brain barrier and overcome systemic toxicities by localized production of the active drug (SN-38) at the tumor site and providing a heretofore unachievable therapeutic specificity for malignant pediatric brain tumors.6,7,23,26 Because NSCs home to hypoxic regions of brain tumors where cancer stem cells reside, they may aid in the eradication of this population of cancer cells as well.27 It will also be important to investigate whether our NSC-mediated rCE/CPT-11 therapy can target the various subtypes of medulloblastoma and eliminate tumor-initiating cells.28,29

To our knowledge this is the first report to investigate NSC-mediated therapy in a transgenic mouse model of medulloblastoma. We also used an orthotopic human Daoy medulloblastoma xenograft model to evaluate NSC homing, biodistribution in the tumor, as well MRI-based in vivo monitoring of iron nanoparticle (Feridex)-labeled NSC migration. For the in vivo therapeutic study, we have chosen a new retrovirus-based Shh pathway-induced transgenic mouse model that closely replicates the conditions of spontaneous human medulloblastoma tumors (Shackleford et al., manuscript in preparation). Treatment of mice bearing murine medulloblastoma with NSC.rCE + CPT-11 resulted in reduction of the tumor growth rate and volume in these mice as compared with mice treated with CPT-11 alone.

The ability to monitor the in vivo migration and fate of therapeutic NSCs is an important aspect of stem cell-mediated gene therapy. Several studies have investigated the labeling of stem cells with reporter genes or various contrast agents to enable non-invasive cell tracking, as well as quantification of the fate of the administered stem cells in vivo.20 Although labeling of stem cells with fluorescent and bioluminescent reporter genes for visualization can be used in animal models, it cannot be used in humans. For this reason, we loaded NSCs with iron oxide nanoparticles before administration in order to track their migration and tumor distribution over time by MRI.31 MRI is already the primary method of assessing therapeutic responses in brain tumor clinical trials.

In preclinical studies, our group has demonstrated the effective use of MRI to track iron-labeled NSCs, as well as the retention of NSC properties after iron labeling, including viability, tumor tropism, transgene expression and lack of tumorigenicity and acute toxicity.13 Other work has shown the utility of preclinical MRI for tracking iron-labeled leukocytes and mesenchymal stem cells.32 The safe use of iron oxide MRI contrast agents has been demonstrated in clinical research studies for central nervous system tumor visualization, and for diagnostic MRI purposes following i.v. administration.33 Based on these reports, we believe the superparamagnetic iron oxide labeling technique may be safely and effectively used in the clinical setting to track NSCs introduced into the brain after tumor resection.

NSC-mediated gene therapies have been previously validated in preclinical models of subdural medulloblastoma, neuroblastoma, melanoma brain metastases and glioma.6,11,22,34 The current results provide preclinical proof-of-concept in support of NSC-mediated CE/CPT-11 enzyme/prodrug therapy for medulloblastoma, warranting...
further studies toward translation of this treatment strategy to clinical application. Of translational significance, the same NSC line used in these studies are currently being used in clinical phase I trials in adult patients with recurrent high-grade glioma (clinical trial ID # NCT01172964; http://clinicaltrials.gov/ct2/show/NCT01172964). This trial is the first-in-human to use an immortalized, clonal, expandable NSC line, and the first to use NSCs to deliver a therapeutic agent for cancer treatment. We envision further modification of this GMP NSC Master Cell Bank to secrete CE, followed by injection directly into the cerebellum of medulloblas-toma patients at the time of surgical resection or biopsy. Patients would then receive i.v. administration of CPT-11 to generate SN-38 locally at the tumor sites, to effect a significant anti-cancer response while minimizing toxicities to normal tissues. Although these NSCs are human leukocyte antigen class II negative, patients would be placed on dexamethasone during each course of NSC and prodrug administration to minimize any potential immune response. Larger, longer-term preclinical medulloblastoma studies are planned to optimize the CE/CPT-11 therapeutic regimen.

MATERIALS AND METHODS

Cell lines

Human Daoy medulloblastoma cells (ATCC, Manassas, VA, USA; HTB-186) and UW228 human medulloblastoma cells (gift from Dr Rolando F Del Maestro, McGill University) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS. Murine medulloblastoma cells were obtained at necropsy of tumor-bearing transgenic mice at Children’s Hospital Los Angeles. To generate cell lines, cells were dissociated from murine tumors, and were cultured initially in Neurobasal medium with B27 (Invitrogen) and 20% FBS and later in DMEM supplemented with 10% FBS. Human medulloblastoma Daoy xenografts were established in nude/nude mice (1 x 10^5 cells per 2 μl). HB1.F3.CD NSCs were cultured (6% CO2, 37 °C) as an adherent monolayer in DMEM supplemented with 10% FBS. These cell lines were further genetically modified using an adenovirus carrying the rCE gene (obtained from Dr Philip Potter at St Jude Children’s Research Hospital). Adenoviral transduction was performed with a secretable version of the rCE enzyme (Ad.zrCE), as previously described.12,13

FE-Pro labeling of NSCs

HB1.F3.CD cells were labeled with FE-Pro as previously described.16,27 Briefly, FE-Pro (Feridx IV, Berlex Laboratories, Wayne, NJ, USA) with a total iron content of 11.2 mg ml^-1 was combined with Pro (1 mg ml^-1) stock solution (American Pharmaceutical Partners, Schaumburg, IL, USA), added to HB1.F3.CD cells in serum-free DMEM (FE, 100 μg ml^-1; Pro, 3 μg ml^-1 final concentration) and incubated for 2 h (6% CO2, 37 °C). After 2 h, an equal volume of DMEM plus 10% FBS containing Ad.zrCE (multiplicity of infection = 20) was added to the cells and were further incubated for 24 h. After 24 h, cells were washed in PBS containing 10 U ml^-1 of heparin to remove the excess FE-Pro and were used for i.v. injections into tumor-bearing mice.

CE enzyme activity assay

Media were derived from HB1.F3.CD.rCE cells (96h culture) and CE enzyme activity was measured by conversion of α-nitrophenyl acetate substrate to α-nitrophenol, as determined by spectrophotometry.25

NSC migration assay

In vitro cell migration assays were conducted using 24-well cell culture plates with polycarbonate inserts (Millipore, Billerica, MA, USA) with pore diameters of 8 μm. Briefly, conditioned media derived from medulloblastoma cells were prepared by addition of serum-free medium to cultured cells (~75% confluence), followed by incubation (37 °C, 6% CO2, 48 h). Conditioned media were collected and added to the lower chamber of 24-well plates (600 μl). Inserts were placed into wells, and a suspension of HB1.F3.CD cells was added in the upper chamber (1 x 10^5 cells per 400 μl) in DMEM supplemented with 2% BSA. After incubation (4 h, 37 °C), cells that did not migrate were removed from the upper surface of the filter, whereas migrated cells were detached from the lower surface of the insert by trypsinization. Detached cells were centrifuged at 1500 r.p.m. for 5 min and counted using the Guava ViaCount assay (Guava Technologies, Hayward, CA, USA). Migration assay controls were as follows: negative control, NSCs resuspended in 2% BSA were added to the upper chamber, with 2% BSA in the bottom chamber; positive control, 10% FBS was added to the lower chamber as a chemoattractant.

Cytotoxicity assays

Cells derived from medulloblastoma were used for cytotoxicity assays. Human and murine medulloblastoma cells were placed into 96-well plates (3000 cells per well, in triplicate) with final CPT-11 concentrations of 0, 0.1, 1, 10 or 100 μg ml^-1, or SN-38 concentrations of 0, 0.1, 1, 10 or 100 ng ml^-1. After incubation for 72 h, viable and dead cells were counted using the Guava ViaCount assay; only viable cells were included in data analysis.

Liquid chromatography - mass spectrometry assay for CPT-11 and SN-38

Liquid chromatography - mass spectrometry analysis was performed using a Waters Acquity UPLC system (Milford, MA, USA) interfaced with a Waters Quattro Premier XE Mass Spectrometer (Milford, MA, USA). High-performance liquid chromatography separation was achieved using a Synergi Hydro-RP 4 μm, 150 x 2.0 mm analytical column (Phenomenex, Torrance, CA, USA) proceeded by a Phenomenex C18 guard column. The column temperature was maintained at 30 °C, and the flow rate was 0.4 ml per minute. The mobile phase consisted of A (20 mM ammonium acetate buffer, pH 3.5) and B (acetonitrile). The following gradient program was used: 20% B (0 - 3 min), 68% B (6 min), 68% B (6 min), 20% B (6.5 min), 20% B (8 min). The total run time was 8 min. The auto-injector temperature was maintained at 5 °C. The electrospray ionization source of the mass spectrometer was operated in positive ion mode with a cone gas flow of 801 per hour and a desolvation gas flow of 7001 per hour. The capillary voltage was set to 0.6 kV, and the cone and collision cell voltages were optimized to 60 V and 36 eV for CPT-11, 48 V and 26 eV for SN-38 and 45 V and 23 eV for CPT (internal standard), respectively. The source temperature was 125 °C and the desolvation temperature was 450 °C. A solvent delay program was used from 0 to 4.7 min and from 6.1 to 8 min to minimize the mobile phase flow to the source. Masslynx version 4.1 software (Waters Corporation, Milford, MA, USA) was used for data acquisition and processing. Positive electrospray ionization of CPT-11, SN-38 and CPT produced abundant protonated molecular ions (MH^- + 1) at m/z 587.31, 393.21 and 349.15, respectively. Fragmentation of these compounds was induced under collision-induced dissociation conditions and acidic mobile phase. The precursor → product ion combinations at m/z 587.31 → 124.14 for CPT-11, 393.21 → 349.20 for SN-38 and 349.15 → 305.11 for CPT were used in multiple reaction monitoring mode for quantitation. Under optimized assay conditions, the retention times for CPT-11, SN-38 and CPT were 5.25, 5.43 and 5.62 min, respectively.

Animal models

To initiate an orthotopic xenograft model of human medulloblastoma, 4-week-old nude/nude mice received a stereotactic injection of Daoy medulloblastoma cells into the cerebellum (1.0 mm lateral and 1.0 mm posterior from lambda, and 3.0 mm deep from the surface of the skull). NSCs were injected ipsilaterally to the tumor into the cerebellum of mice (1.5 mm lateral, 1.5 mm posterior from lambda and 3.0 mm deep from the surface of the skull). Migration of the iron-labeled NSCs was monitored by MRI at 1, 4 and 6 days after NSC injection. We used a transgenic mouse model of medulloblastoma that expresses the Tva avian retrovirus receptor in the cerebellar granule neuron precursor cells (Shackelford et al., unpublished data). Medulloblastomas were produced by i.c. injections at PND 1 - 3 of non-tumorigenic chicken DF-1 cells that produced two avian RCAS (Replication-Competent Avian Sarcoma-Leukosis Virus) retroviral vectors encoding Shh-N1, a pre-processed Shh, and a stabilized Mycn mutant (TSBA, S62A). The transgenic...
mice were crosses of the C57BL/6 and DBA/2 strains. Transgenic experiments and tumor cell line isolations were performed with Children’s Hospital Los Angeles (IACUC Protocol 190-09). For both models, mice were monitored by MRI every 2 weeks for tumor development. Medulloblastoma-bearing mice received ir-labeled therapeutic NSCs i.v. into the retro-orbital sinus (10^6 cells per 100 μl PBS) or i.c. (10^5 cells per 2 μl PBS). NSCs were injected into the cerebellum of transgenic mice bearing tumors laterally to Bregma 2.0 mm, 6.7 mm posterior and 2.5 mm deep from the surface of the skull in order to target region of the multiple medulloblastoma foci (2.0 mm lateral, 6.7 mm posterior from bregma and 2.5 mm deep from the surface of the skull). All animals were MRI imaged at days 1, 4 and 6 after NSC injection to track the distribution of NSCs in the brain. Brain tissue was harvested at day 6 post-NSC administration, and histologic sections were processed for Prussian blue staining to visualize ir-labeled HB1.F3.CD.rCE NSCs. NSC-mediated therapy was evaluated in the transgenic mouse model using the following regimens: (1) HB1.F3.CD.rCE i.c. + CPT-11 at 20 mg kg^-1 per day i.v. for 4 days and (2) HB1.F3.CD.rCE i.c. + CPT-11 at 38 mg kg^-1 per day i.v. one dose. The administration of CPT-11 in both regimens was started 4 days after NSC injections. Control mice received CPT-11 only at the same doses as above, and did not receive NSC injections. All therapeutic regimens were repeated once 2 weeks after the start of the first round of treatments (the second NSC injection was into the opposite hemisphere of the cerebellum). Tumor size was monitored by MRI. Mice were euthanized 2 weeks after the start of the second cycle of therapy (total duration of therapy was 30 days). Tissues (brain, liver, spleen, kidneys, bone marrow, lungs, pancreas) were collected and used for immunohistochemistry and Prussian blue staining of ir-labeled NSCs.

3D reconstruction of histological sections

Automatic Cellular Imaging System (ACIS from ChromaVision Medical Systems, Inc., San Juan Capistrano, CA, USA) was used to generate high-resolution images of histological sections (10 μm thick). Three-dimensional reconstruction was performed using Reconstruct (version 1.1.0.1) software (Dr Kristen M Harris et al., SynapseWeb, Laboratory of Synapse Structure and Function, Austin, TX, USA; http://synapses.clm.utexas.edu/tools/reconstruct/reconstruct.stm). For each tumor, 10 serial brain sections separated by 200 μm were imported into Reconstruct and aligned manually. To produce a 3D image, structures of interest were segmented based on color (Prussian blue-label for NSCs) and cell density (tumor areas), and volumes containing 3D image, structures of interest were segmented based on color (Prussian blue-label for NSCs) and cell density (tumor areas), and volumes containing.

Magnetic resonance imaging

Anesthesia was induced and maintained with Isoflurane throughout the imaging procedure. Mice were inserted in the prone position into a small animal MRI scanner (PharmaScan 300, Bruker BioSpin Division, Billerica, MA, USA) 7T magnet using the 19-mm inner diameter transmit receive coil. ParaVision 4.0 scanner software (BRIUKER BioSpin MRI GmbH, Ettlingen, Germany) was set to use RARE (RAPid Acquisition with Relaxation Enhancement) spin echo sequence for fast T2-weighted imaging (echo time = 50, repetition time = 3000, RARE Factor 8) with a 256 × 256 in-plane matrix and 2.56-mm field of view. After scanning, if needed, mice were gently warmed on a thermo-statically controlled heating pad until awake enough to be returned to their home cage. MRI images were reconstructed at native resolution. For each mouse, we acquired 22 axial images with 0.4 mm thick slices and 0.02 mm gap between slices. This produced 0.1 × 0.1 mm^2 per pixel in-plane resolution with an effective slice thickness of 0.02 mm. eFilm Workstation 1.8.1 (eFilm Medical Inc, Toronto, Canada) was used to transfer reconstructed images from DICOM files into TIFF files for further analysis. MetaMorph 6.3 ( Molecular Devices, Sunnyvale, CA, USA) was used to open the TIFF files, outline the tumors, count the number of pixels inside each tumor slice and catalog the data. The number of pixels per slice was converted into tumor volume per slice (tumor volume = pixel count × 0.1 × 0.1 × 0.042 mm^3). Tumor volume per slice measurements were summed to produce the final tumor volume per animal.

Statistical analysis

The tumor volume analysis included 14 mice. There were two treatment groups (four mice per group) and two control groups (three mice per group). Measurements were made on each mouse at day 0, day 8 and day 21 after the start of treatment. Two mice died before day 8 (one in control group C2, one in treatment group T2) and these data were not included in the analysis. Three additional mice died before day 21. Day 21 measurements were imputed for these mice using linear regression analysis of the individual mouse’s data. This avoids the bias that mice with generally larger tumors died, leaving only the mice with small tumors by day 21.

The analysis was performed for experimental groups as described above using linear mixed effects models implementing the R package nlm.le. The starting model included treatment, days, centered baseline (baseline-mean of the baseline values) and the associated two-way interactions. Tumor growth rate (days) was defined to be random, that is varied with each mouse. Non-significant fixed effects were removed based on F-values from t-tests on REML (restricted maximum likelihood) estimates. The final fixed effects portion of the model included center baseline, interaction between days and treatment and associated main effects. The question of interest was: Did the tumor volume growth rates differ between specific treatment and control groups? Comparisons were defined using contrasts and tested with t-tests based on REML estimates. We repeated the analysis, calculating the tumor growth rates for each mouse using simple linear regression, and the results of the predefined contrasts were similar to those of the mixed effects models analysis. The level of significance was set at P<0.05.

CONFLICT OF INTEREST

KSA, AIA are founders, CSO and CEO (respectively) of TheraBiologics, Inc., a clinical stage biopharmaceutical company supporting development of NSC-mediated cancer treatments. The remaining authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Gene Therapy website (http://www.nature.com/gt)