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Therapeutic effect of genetically modified human neural stem cells encoding cytosine deaminase on experimental glioma

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ABSTRACT

The aim of this study was to determine the efficacy of neural stem cell-based suicidal gene therapy in rats bearing human glioma. F3 human neural stem cells (NSCs) were transduced to encode cytosine deaminase (CD) which converts 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU). Intratumoral or intravenouse transplantation of F3.CD human NSCs led to marked reduction in tumor burden and significantly prolonged the survival of brain tumor-bearing rats. The systemic administration of 5-FC with direct intratumoral/intravenous transplantation of F3.CD cells had remarkable therapeutic effect in rats with human glioma cells as compared with transplantation of parental F3 cells. There was 74% reduction in tumor volume in rats receiving direct transplantation of F3.CD cells into tumor site, and 67% reduction in tumor volume in rats receiving intravenous injection of F3.CD cells as compared to control animals transplanted with human glioma U373 cells alone. The combination of F3.CD and 5-FC was a highly effective in the glioma rat model. Our observations suggest that genetically engineered NSCs encoding suicide gene CD could provide clinical application of suicide gene therapy for patients with glioma.

1. Introduction

Glioma is the most common form of primary brain tumor as well as most aggressive, hence carrying the worst prognosis with less than 1 year of median survival [1]. The prognosis for the majority of patients with glioma has been dismal despite the extensive surgical excision and recent improvements in adjuvant radio- and chemo-therapy [2,3].

Thus new treatment modalities are urgently needed. Gene therapy is one of the most rapidly evolving areas of preclinical and clinical cancer research; more than 200 cancer gene therapy trials have been approved worldwide since the early 1990s. However, several issues remain to be addressed before the full potential of cancer gene therapy can be realized. Major obstacles are the low efficiency of gene transfer by currently available viral vectors and the inability of these vectors to specifically target cancer cells. Previous studies have demonstrated that neural stem cells (NSCs) infiltrate tumor mass and deliver therapeutic agent to kill the tumor cells [4–6]. Thus, NSCs with tumor-tropic property could serve as potential vectors for cell-based gene delivery to treat brain tumors.

In the present study, we used genetically modified human NSCs transduced with suicidal enzyme gene cytosine deaminase (CD) as a new tool for gene therapy of experimental glioma in rats.

2. Materials and methods

2.1. Cell culture

The human glioma cell lines U87, U251 and U373 were obtained from the ATCC (Manassas, VA) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 4 mM L-glutamine, 4500 mg/L glucose, 110 mg/L sodium pyruvate, 100 unit/mL penicillin and 100 μg/mL streptomycin. HB1.F3 (F3) is an immortalized human NSC line derived from human fetal brain at 15 weeks of gestation with the use of an ampho tropic, replication-incompetent retroviral vector containing v-*myc*[5,6]. F3 cells were grown in DMEM supplemented with 5% FBS, 4 mM L-glutamine, 4500 mg/L glucose, 110 mg/L sodium pyruvate, 100 unit/mL penicillin and 100 μg/mL streptomycin. F3 cells transfected with the cytosine deaminase gene (F3.CD cells) were cultured under the same conditions. Cells were grown at 37 °C in an atmosphere of 95% air and 5% CO_2.

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2.2. In vivo protocol of experimental tumor models

Sprague–Dawley (SD) female rats (250 g body weight, Samtaco, Osan, Korea) were anesthetized with 10% chloral hydrate (Fluka, Germany) and placed in a stereotaxic apparatus. Three microliters of Hanks' balanced salt solution (HBSS) containing 1×10^6 U373 human glioma cells were intracranially transplanted at the rate of 0.2 μL/min using a 26 gauge Hamilton micro-syringe. The target was the right caudate-putaman [antero/posterior (AP) + 0.4 mm, medial/lateral (ML) -3.1 mm, dorsal/ventral (DV) 4 mm]. After tumor cell inoculation, F3 human NSCs were transplanted at the same hemisphere. All experimental procedures were approved by the Animal Care Committee of the Ajou University School of Medicine. Most of the animal studies have done at the Ajou University while the authors were affiliated with the Ajou University.

2.3. In vitro sensitivity and cytotoxicity of F3 to 5-FC and 5-FU

The F3 and F3.CD cells at 2×10^3 cells/well were seeded in 96-well plates, and incubated in medium containing 100 μg/mL 5-fluorocytosine (5-FC; Sigma, St Louis, MO) or 10 μg/mL 5-fluorouracil (5-FU; Sigma) for 3 days. The medium was replaced with fresh medium containing 10 μl MTT solutions and incubated for 4 h. The medium was then discarded, and 100 μl of solubilization buffer [10% sodium dodecyl sulfate (Sigma) and 50% *N*, *N*-dimethyl formamide (Sigma) was then added. Cell viability was determined by measuring absorbance at 570 mm and 630 mm on a micro-plate reader.

2.4. In vitro 5-FU cytotoxicity to human glioma cells

U373 human glioma cells at 2×10^3 cells/well in 96-well plates were seeded with fresh culture medium. After 1 day, the medium was replaced with fresh culture medium. The cells were then incubated with media containing 0.2, 0.5, 1, 1.3, 2, 5, 10 or 15 μg/mL 5-FU for 3 days. The medium was discarded and fresh medium was added along with 10 μL MTT solution for 4 h. Then, the medium was discarded and 100 μL of solubilization buffer was added. The cell viability was determined by measuring absorbance at 570 mm and 630 mm on micro-plate reader.

2.5. In vitro bystander effect between F3.CD and U373

To determine the lowest necessary number of F3.CD cells which could provide effective anti-tumor effect in our study and to confirm the "bystander effect" of 5-FU released from F3.CD cells, we co-cultured F3.CD:U373 cell ratios of 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, and 9:1 in culture medium (DMEM containing 10% FBS) containing 100 μg/mL 5-FC in 96-well tissue culture plates. Also, we co-cultured F3.CD cells with equal number of U251 or U373 in DMEM medium in 96-well culture plates with 100 μg/mL to 2000 μg/mL 5-FC to evaluate the lowest concentration of 5-FC which could provide effective anti-tumor effect. The number of living cells was determined by MTT assay on day 3.

2.6. In vitro and in vivo therapeutic efficacy of F3.CD NSCs

Therapeutic efficacy of F3 and F3.CD human NSCs was analyzed by co-culture with U373 after 5-FC treatment. One group contained U373 and F3, and the other group U373 and F3.CD cells. Each cell line (3×10^3) was plated in 96-well plates and analyzed as follows: On day 1, the media of both groups were changed, and 5-FC (100 μg/mL) and 5-FU (10 μg/mL) were added to the mixed cell cultures. On day 3, cell viability was estimated as described above, and expressed as mean ± SE in percentage of the control viability (100%). All experiments were conducted in quadruplicate. To evaluate the therapeutic efficacy of F3.CD NSCs in vivo, we first transplanted U373 cells alone into the right caudate-putamen, and then F3.CD NSCs were injected into the same site in ipsilateral hemisphere 5 days later. Rats were injected daily with 500 mg/kg 5-FC (10 mg/mL in sterilized saline) into the peritoneum for 7 days.

2.7. In vivo therapeutic efficacy according to transplantation of F3.CD cells

Five days after U373 glioma cell transplantation, F3.CD cells were injected into the same site in ipsilateral hemisphere or intravenously, followed by daily injection of 5-FC (500 mg/kg) for 7 days. Five days after U373 transplantation, the rats were divided into 4 groups (total $n = 29$), U373 transplantation alone as control ($n = 6$), U373 + intratumoral injection of F3.CD ($n = 9$), U373 + intravenous injection of F3.CD ($n = 6$), and U373 + combined direct and intravenous injection of F3.CD ($n = 8$). The tumor volume was determined by one center area of the tumor mass on 13 days post-transplantation of the U373 cells.

2.8. Measurement of tumor size by magnetic resonance imaging (MRI)

After rats were anesthetized with 10% chloral hydrate, coronal T1-weighted images (TR 500 ms, TE 11 ms, 3 mm thickness, gapless) were obtained with a 1.5 tesla MR device (Signa Exite, General Electric, Milwauke, WI). MRI studies were started on 7 days after tumor inoculation and continued at 7 days intervals on 14 days and 21 days. The cross sectional area of the tumor mass was measured on each axial slice by drawing ROI (region of interest). These were multiplied by gap (3 mm) between consecutive axial slices and total tumor volume was calculated as the sum of all slice volumes.

2.9. In vitro assay for conversion of 5-FC to 5-FU by HPLC

F3.CD cells were plated in a culture dish and incubated in the presence of 10, 50, 100, 120 and 200 μg/mL 5-FC for 1 or 3 days. An aliquot (50 μg) of the medium was extracted with 500 μl of a mixture of ethyl acetate: isopropanol: acetic acid (84:15:1). HPLC was performed with Variable Wavelength Detector (HP 1100 Series) at 254 nm using a LUNA 5u C18 (2) column (250×4.6 mm 5u micron). (Column oven temperature was 25 °C). The HPLC system consisted of a HP 1100 Series, Quaternary HPLC pump, HP 1100 Series, Autosampler and HPLC injector. Injection volume of standard 5-FC and 5-FU was 2 μL and concentration was 0.5 mg/mL. 5-FC and 5-FU were eluted isocratically at a flowrate of 1 mL/min with an isocratic mobile phase consisting of methanol and 0.025 M KH2PO4 (5:95 and pH 2.5). The retention times for 5-FC and 5-FU were 3.4 and 5.2 mins, respectively.

2.10. Statistical analysis

All values were calculated as mean ± SD or expressed as percentage of control ± SD. Significant differences between viability of the tumor cell lines and viability to 5-FU at 0, 1, 3, 5 or 7 days points were performed by one-way ANOVA. Significant differences between assessment of cell viability and tumor volume were determined using the SPSS (version 11.5, SPSS, Chicaco, IL) followed by paired *t*-test. A *p* values less than 0.05 were considered significant.

3. Results

3.1. F3.CD human neural stem cells

Expression of CD transcript in F3.CD was confirmed by reverse transcription-PCR (RT-PCR). The CD transcript was found to be expressed only in F3.CD cells, but not in the parental F3 cells.

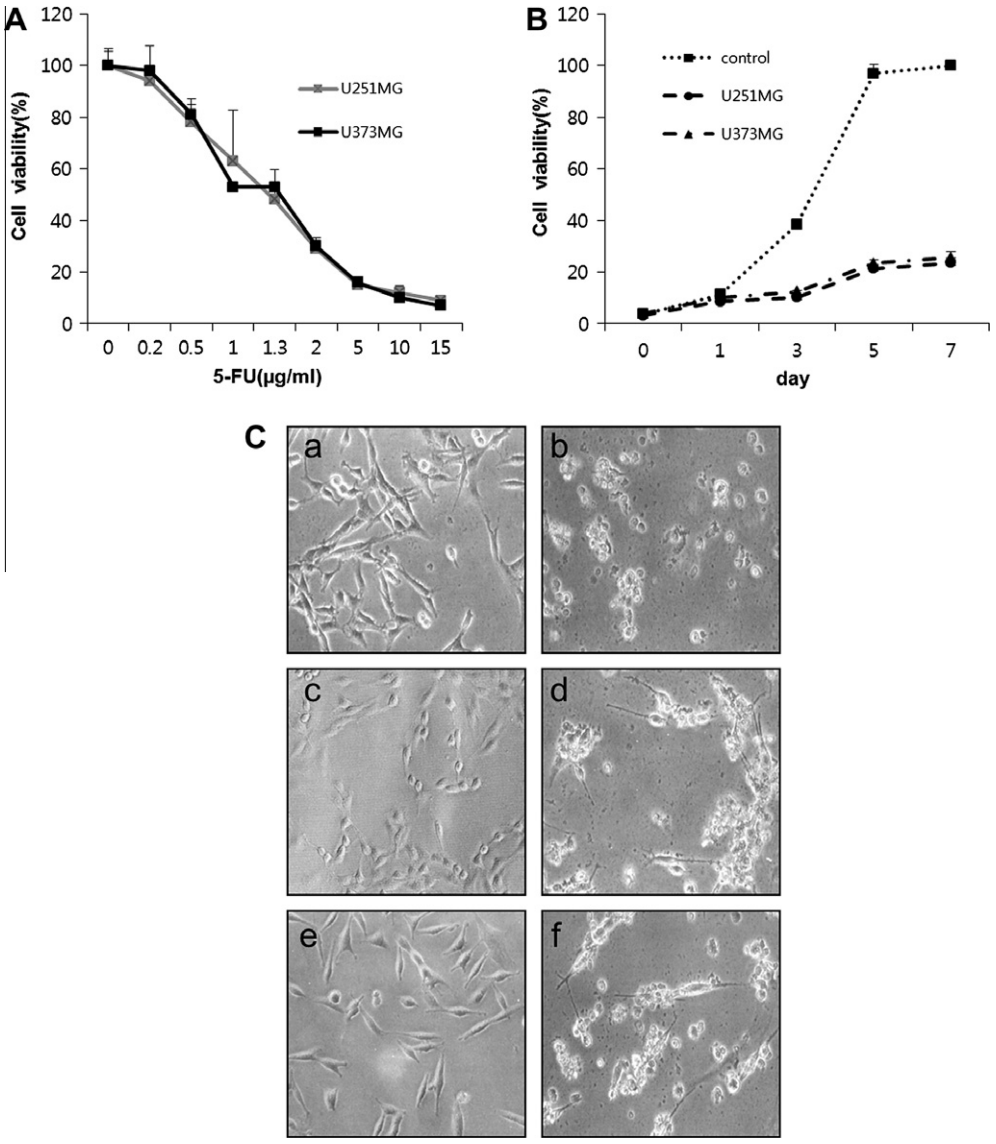

Fig. 1. Graph demonstrating cytotoxicity of varying concentrations of 5-FU on human glioma cells infected at elapsed time and the human glioma cell lines (U251 and U373) in 96-well plates were assessed for their cell viability by MTT assay. (A) Viability of the tumor cell lines were assessed after incubation with 0.2, 0.5, 1, 1.3, 2, 5, 10 or 15 μg/mL concentrations of 5-FU. (All data are $^{*}p < 0.05$ (0.5 μg/mL; $p = 0.001$,1 μg/mL; $p = 0.000$, 1.3 μg/mL, $p = 0.000$; 2 μg/mL; $p = 0.000$, 5 μg/mL; $p = 0.000$, 10 μg/mL; $p = 0.000$, 15 μg/mL; $p = 0.000$) except at 0.2 μg/mL ($p = 0.522$) inU251andU373). (B) The survival was assessed at 0, 1, 3, 5 or 7 days points. (5-FU; 1 μg/mL) (control means average of 2 human glioma cell lines without 5-FU treatment) (each group $n = 6$, (All data are $^{*}p < 0.05$ (1 day; $p = 0.001$, 3 days; $p = 0.000$, 5 days; $p = 0.000$, 7 days; $p = 0.000$) except at 0 day ($p = 0.313$) in U251 and U373. (C)The human glioma cells [U373 (b), U251 (d) and U87 (f)] showed a marked reduction in tumor cell population after treatment with 5-FU (10 μg/mL) as compared with untreated controls (a, c, e).

Furthermore, only F3.CD cells were sensitive to 10 μg/mL concentration of 5-FC treatment ($p < 0.05$), indicating enzymatic conversion of pro-drug 5-FC to toxic 5-FU (Data not shown).

3.2. In vitro and in vivo 5-FU cytotoxicity study

5-FU was highly cytotoxic for two human glioma cell lines tested (Fig. 1A). The size of the tumor decreased remarkably after daily injection of 5-FU for 7 days in comparison to the control group with saline injection (Fig. 3C).Tumor masses of 5-FC treated group were much smaller than those of untreated or saline injected groups (Fig. 3D).

3.3. Analysis on conversion of 5-FC to 5-FU by CD activity

We identified 5-FC and 5-FU in the extracts of conditioned media (Fig. 2A) by HPLC system. When treated with various concentrations of 5-FC for 1 day, F3.CD cells did not produce any detectable amount of 5-FU (Data not shown). However, after 3 days we could detect approximately 50% of 5-FU from each concentration of 5-FC, and the amount of 5-FU increased in proportion to the concentration of 5-FC applied (Fig. 2A).

3.4. In vitro bystander effect between U373 glioma cells and F3.CD NSCs

After 5-FC treatment, the cell viability of U373 glioma cells was markedly reduced with the presence of F3.CD cells, as compared with controls containing only U373 cells (Fig. 2B). The bystander effect increased gradually in proportion to the increasing ratio of F3.CD cells over tumor cells. The CD gene with 5-FC treatment markedly inhibited glioma cell growth in 5-FC concentration-dependently (from 100 μg/mL to 2000 μg/mL, Fig. 2C). Consequently, the cell viability of glioma cells with F3.CD cells was decreased by

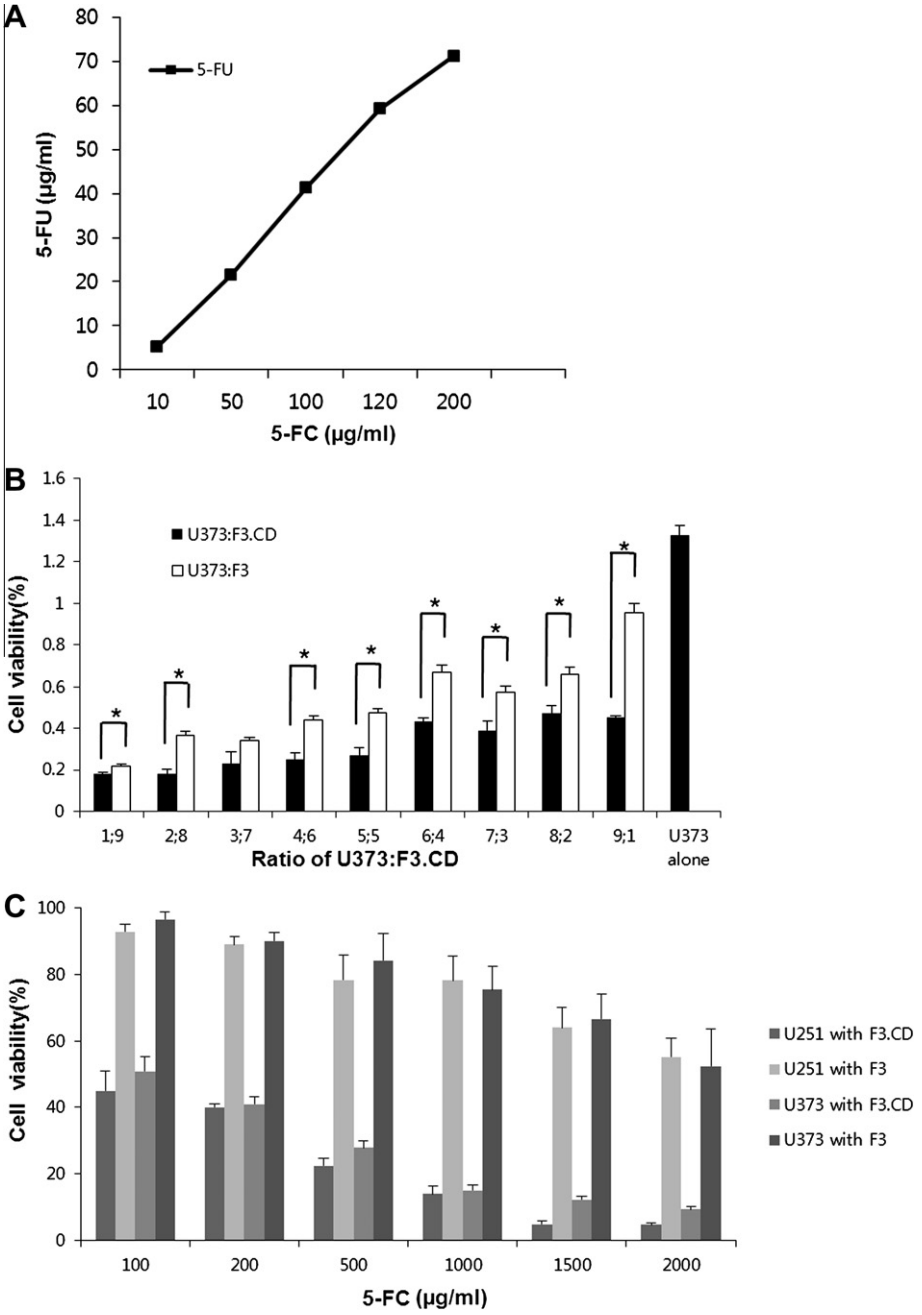

Fig. 2. High performance liquid chromatography (HPLC) analysis and in vitro bystander effects in co-culture of F3.CD and U373 cells. (A) F3.CD human neural stem cells were incubated with different concentrations of 5-FC, and the medium was analyzed by HPLC. CD enzyme converted non-toxic 5-FC to the highly potent chemotherapeutic agent 5-FU. This graph shows almost equal amounts of 5-FC and 5-FU after 4 h of incubation. (B) Bystandere ffects of F3.CD were investigated at various ratios. No proliferation inhibition was observed in the plates of U373 with 5-FC alone. (MTT assay, triplicate, mean ± standard error, error bars smaller than icons do not appear. All data are $^{*}p < 0.05$ except 3:7 ratio). (C) Co-cultured glioma cell lines (U251, U373) and F3 human neural stem cells with or without CD gene were treated with various concentrations of 5-FC (columns = mean, bars = SD, all data are $^{*}p < 0.05$).

approximately 90% (Fig. 3A, B). To confirm the "bystander effect" by F3.CD cells, co-culture system was used. When co-cultured with F3.CD cells, the growth of U373 glioma cells was significantly inhibited by 100 mg/mL 5-FC, where as no growth inhibition of U373 cells was observed without F3.CD cells alone. These results indicate that F3.CD cells converted sufficient amount of 5-FC to 5-FU to effectively to kill U373 human glioma cells in vitro.

3.5. In vivo therapeutic efficacy of HB1.F3.CD

There was 74% reduction in tumor volume in rats receiving direct transplantation of F3.CD cells into tumor site, and 67% reduction in tumor volume in rats receiving intravenous (IV) injection of F3.CD cells as compared to control animals transplanted with human glioma U373 cells alone (Fig. 4D). Injection of F3 cells only did not reduce the tumor size (Fig. 3B). The direct intratumoral transplantation of F3.CD cells was more effective than IV injection of the cells; however, there was no statistically significant difference in reduction of tumor size between two groups (Fig. 4D).

3.6. MRI study of F3.CD cells on in vivo tumor growth

Tumor volumes on 7 and 14 days after intracranial inoculation of F3.CD cells were monitored by MRI. We delivered F3.CD or

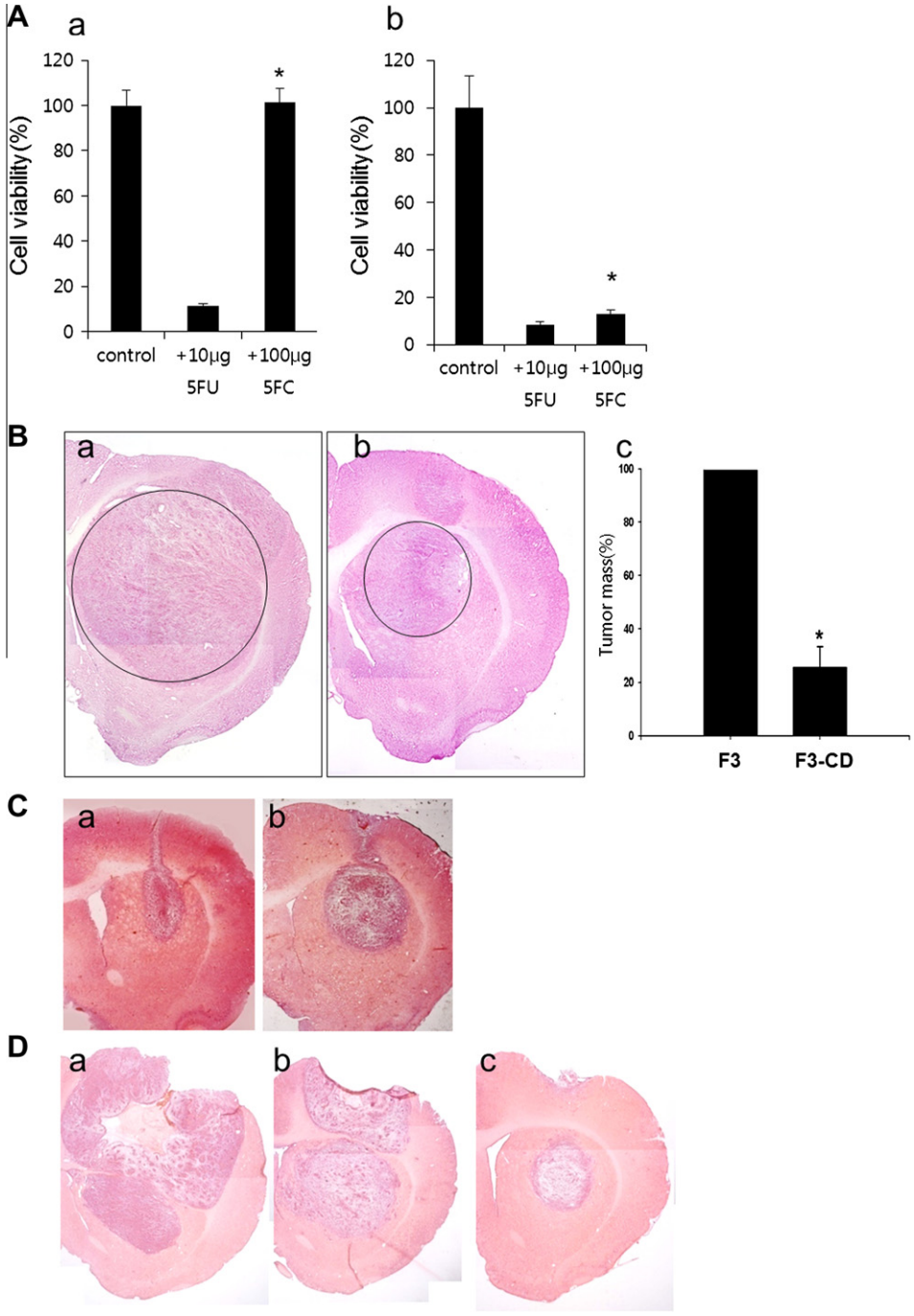

Fig. 3. Bystander effects of F3.CD human neural stem cells in vitro and in vivo. (A) F3 and F3.CD human neural stem cells were assessed for cell death due to conversion of prodrug 5-FC to highly cytotoxic5-FU. The cell lines were treated with 100 μg/mL 5-FC and 10 μg/mL 5-FU concentrations. (a) Cell number decreased in control F3 cells only by 5-FU treatment. (b) Cell number decreased in F3 cells encoded with CD gene following treatment with both 5-FC and 5-FU (each group $n = 6$). (B) After U373 (1×10^6 cells) transplantation, human neural stem cells (F3 and F3.CD) were transplanted at the same hemisphere on day 6 and 5-FC (500 mg/kg) was injected daily for 7 days. (a) This model represents daily injection of 5-FC for 7 days after F3 transplantation ($n = 6$). (b) This model was injected with 5-FC daily for 7 days after F3.CD cells transplantation ($n = 9$). (c)The tumor mass with F3.CD cells transplantation was reduced to 30% compared to that with F3 cells transplantation (columns = mean, bars = SD, $^*p < 0.05$). The tumor model rats were injected daily with 10 μg of 5-FU for 7 days and sacrificed (C). (C,a): 5-FU injection, (C,b): saline injection. U373 tumor inoculation control model (D,a), treated with saline injection (D,b) and treated with 5-FC (D,c). Each group was injected daily for 7 days.

control F3 cells into intracranial tumors in rats 7 days after inoculation with U373 human glioma cells. As shown in Fig. 4E and G, progressive growth of U373 and U251 glioma cells was observed in the brain of control untreated rats, reaching a lethal volume 21 days after tumor inoculation. In contrast, significantly smaller tumor volumes were observed in the brain of rats treated with F3.CD with 5-FC prodrug administration (Fig. 4F and H) ($P < 0.01$ compared to untreated controls 21 days after tumor inoculation). Intratumoral inoculation of F3.CD significantly prolonged the survival of human glioma bearing rats: The mean survival time of glioma-bearing rats injected with F3 was significantly less than that of rats injected with F3.CD.

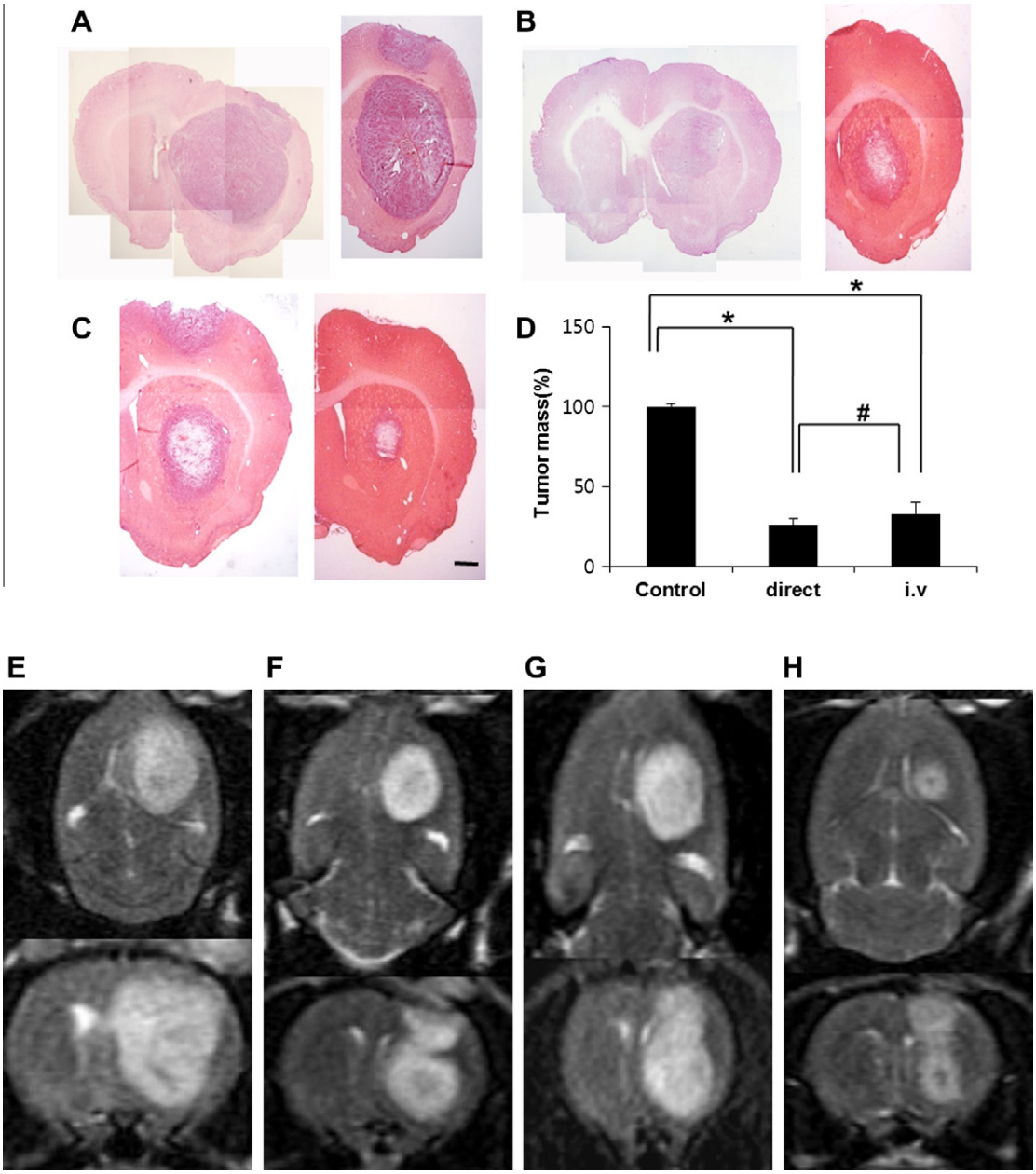

Fig. 4. Therapeutic effect according to the treatment modality and effect of F3.CD human neural stem cells on tumor growth evaluated by MRI. The large areas of tumor are seen in control groups transplanted with human glioma U373 cells alone (A, $n = 6$). The tumor models were direct (B, $n = 9$) and intravenous injection(C, $n = 6$) with 5-FC on day 6 after U373 transplantation, followed by daily injection of 5-FC. Barindicates 2 mm. The histogram shows that the systemic treatment with 5-FC reduced the size of tumor with direct and intravenous injection of F3.CD or both, compared to control group (D). Tumor volume reduction of 74% indirect injection of F3.CD cells, 67% in intravenous(iv) injection of F3.CD and 63% in combined direct and iv injection of F3.CD cells (columns = mean, bars = SD, $^*p < 0.05$). Representative MRI (T1-weightened axial and coronal images) data are shown. The rats with U373MG tumors ($n = 3$) (E) and U251MG ($n = 3$) (G) as controls were not treated for 21 days after tumor inoculation. U373MG tumors ($n = 5$) (F) and U251MG ($n = 5$) (H) were treated with F3.CD implantation for 7 days after tumor inoculation, followed by daily intraperitoneal injection of 5-FC for 14 days.

4. Discussion

In the present study, intratumoral transplantation of F3.CD human NSCs led to marked reduction in tumor burden and significantly prolonged the survival of brain tumor-bearing rats. The systemic administration of 5-FC with direct intratumoral transplantation of F3.CD cells had remarkable therapeutic effect in rats with human glioma cells as compared with transplantation of parental F3 cells. The direct transplantation method appears to be more effective than intravenous injection. The direct transplantation method produced reduction of 74% of the entire tumor volume whereas intravenous injection reduced to 67% of tumor volume.

Results of the present study indicate that the suicide gene therapy employing genetically modified human NSCs as an effectively targeting vehicle is promising as a new therapeutic approach for malignant glioma. Because of their high rate of cell proliferation and diffuse infiltrating properties into surrounding brain parenchyma, gliomas are known to be fatal. Indeed, radical surgical resection is practically impossible because of the disseminated infiltration and growth beyond the tumor boundaries, invisible even on modern neuro-radiological imaging [7]. Therefore, selective

targeting to treat the infiltrating tumor cells may be the goal for a new therapeutic approach.

Recently the use of NSCs encoded with suicide gene has received wide attention for cell-based gene therapy for glioma because of their being an ideal vehicle for gene transfer and strong tropism for glioma [8–10]. Successful preclinical studies utilizing human NSCs encoding CD suicide gene in animal models of brain tumors including glioma and medulloblastoma have previously been reported by us [11–18]. We have demonstrated, in animal models, the safety, feasibility, and efficacy of NSCs to track invasive tumor cells and distant micro-tumor foci and to deliver therapeutic gene products to tumor cells, thereby providing an effective anti-tumor response overcoming obstacles facing current gene therapy strategies. In November 2010, the FDA approved the City of Hope Medical Center in metropolitan Los Angeles to proceed with the first human NSC clinical trial to treat glioma patients. This trial uses F3.CD human NSCs to deliver an anticancer therapeutic agent. Stably immortalized human NSCs that have been retrovirally transduced to express a therapeutic transgene (F3.CD NSCs) have been transplanted in surgically resected bed of glioma lesion in patients with recurrent high grade glioma. Malignant glioma is among the most devastating and difficult cancers to treat due to their highly invasive nature, disseminating extensively away from the main tumor mass and causing disease spread and recurrence. Because these aggressive tumor cells infiltrate normal brain, they are not readily treatable by surgery, irradiation or chemotherapy. NSCs, modified to express the CD suicide gene, hold great promise for glioma therapy due to their inherent tumor-tropic properties.

Previous studies have demonstrated that NSCs administrated intracranially possesses extensive tropism for experimental brain tumors and significant migratory behavior [11–19]. NSCs distribute throughout the primary tumor bed and migrate together with widely outgrowing tumor microsatellites after intratumoral implantation. Moreover, when NSCs are implanted intracranially at sites distant from the tumor, they migrate through the normal parenchyma and localize in the tumor sites, known as the ‘chasing down’ phenomenon [19]. We also found that F3 human NSCs were found migrating far from the injection site, but not to the cerebellum: F3 cells were observed mostly in the area of the tumor, as well as in the corpus callosum, hippocampus, and auditory cortex [17].

Suicide gene therapy strategy has several advantages such as avoiding all of the toxic side effects generated by systemic 5-FU administration, and it allows regression of distant unmodified tumor via a “distant bystander effect” induced by immune system stimulation during 5-FU-mediated regression of the suicide gene-modified tumor [10,20,21]. In the present study, 1 μg/mL 5-FU decreased the cell viability by approximately 50% (Fig. 1A).

Cytosine deaminase, a fungal or bacterial enzyme, catalyzes the deamination of nontoxic 5-FC, resulting into highly cytotoxic drug 5-FU [19,22–25]. HPLC analysis of the conditioned medium of F3.CD cells with 5-FC detected no 5-FU after 1 day. However, after 3 days, approximately 50% of 5-FC was converted to 5-FU indicating that 5-CD gene was activated and deaminated 5-FC into 5-FU after 3 days.

Acknowledgments

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