Original Article
Combined effects of mesenchymal stem cells carrying cytosine deaminase gene with 5-fluorocytosine and temozolomide in orthotopic glioma model

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Abstract: Glioblastoma multiforme (GBM) is the most common and aggressive primary brain tumor, and current standard therapy provides modest improvements in progression-free and overall survival of patients. Innate tumor resistance and presence of the blood-brain barrier (BBB) require the development of multi-modal therapeutic regimens. Previously, cytosine deaminase (CD)-expressing mesenchymal stem cells (MSC/CD) were found to exhibit anticancer activity with a wide therapeutic index by converting 5-fluorocytosine (5-FC), a nontoxic prodrug into 5-fluorouracil (5-FU), a potent anticancer drug. In this study, we evaluated the efficacy of MSC/CD in a multi-modal combination regimen with temozolomide (TMZ). Cell viability test, cell cycle, and normalized isobologram analyses were performed. *In vivo* anticancer effects were tested in a mouse orthotopic glioma model. TMZ and MSC/CD with 5-FC synergistically interacted and suppressed U87 glioma cell line growth *in vitro*. Combined treatment with TMZ and 5-FU increased cell cycle arrest and DNA breakage. In an orthotopic xenograft mouse model, treatment with TMZ alone suppressed tumor growth; however, this effect was more intense with MSC/CD transplantation followed by the sequential treatment with 5-FC and TMZ. Therefore, we propose that sequential treatment with 5-FC and MSC/CD can be used in patients with GBM during the immediate postoperative period to sensitize tumors to subsequent adjuvant chemo- and radiotherapy.

Keywords: Mesenchymal stem cells, cytosine deaminase, 5-fluorocytosine, temozolomide, glioma

Introduction

Glioblastoma multiforme (GBM) is the most aggressive and common primary cancer of the adult central nervous system (CNS). GBM has a poor prognosis and high incidence of recurrence because it infiltrates the brain parenchyma, shows suboptimal resectioning potential, is resistant to therapeutic agents, and is inaccessible to most chemotherapeutic agents because of the blood-brain barrier (BBB) [1].

Temozolomide (TMZ) is a prodrug that is remarkably stable at an acidic pH, resulting in 100% oral bioavailability. Following contact with the slightly basic pH of the blood and tissues, TMZ is spontaneously hydrolyzed into its active metabolite, 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide, which sequentially methylates DNA at the N^7 and O^6 positions of guanine, and the N^3 position of adenine [2]. The guanine-thymine mismatches created by O^6-methylguanine causes DNA breaks if the lesions are not repaired by O^6-methylguanine DNA methyltransferase (MGMT), a DNA methylation repairing enzyme. TMZ crosses the BBB with tolerable systemic toxicity, to halt or slow brain cancer cell growth. The standard of care for GBM currently includes surgical resectioning followed by fractionated radiotherapy (RT) with concomitant daily TMZ administration. In a phase III trial, concurrent chemotherapy and radiotherapy increased survival and progression-free survival compared to that observed with radiotherapy only [3]. Although the survival advantage of the combined treatment lasted

for up to 5 years in the long-term follow-up study, in most patients, the tumors recurred and they died. The therapeutic efficacy of TMZ is significantly reduced by MGMT [4, 5]. Inversely, epigenetic silencing of the *MGMT* gene by promoter methylation prevents synthesis of MGMT, which sensitizes cancer cells to TMZ [6]. The finding that MGMT levels and TMZ sensitivity are correlated show the necessity of developing alternative, multi-modal therapies for patients who cannot benefit optimally from TMZ because of intrinsic resistance mainly due to MGMT.

Clinical trials have investigated other anticancer drugs that may utilize diverse pathways to induce cytotoxicity against proliferating cancer cells. One is a suicide gene therapy, which combines cytosine deaminase (CD) and 5-fluorocytosine (5-FC), an antimycotic drug. CD is a non-human enzyme that converts 5-FC to 5-fluorouracil (5-FU), an antimitotic drug commonly used in breast, skin, and gastrointestinal cancers. Unlike TMZ and other alkylating agents, 5-FU damages DNA by inhibiting thymidylate synthase and causes cellular dysfunctions by interfering with DNA and RNA synthesis [7]. Plasma membrane and the BBB are highly permeable to 5-FU, but its systemic administration causes a syndrome involving delayed myelin destruction in the CNS [8]. Therefore, delivery of a *CD* gene to brain cancer cells would be groundbreaking, and could balance therapeutic efficacy and associated toxicity with a wide therapeutic index.

Previously, we demonstrated that mesenchymal stem cells (MSCs) have a high tropism for brain tumors, and engineered MSCs to express a bacterial *CD* gene (MSC/CD) that successfully suppressed tumor growth in a rat glioma model [9]. Using the tumor-tropic MSC as a cellular vehicle for delivering the *CD* gene would enable effective conversion of 5-FC to 5-FU close to tumor sites, thereby localizing the cytotoxicity. Therefore, this stem cell-mediated suicide gene-prodrug therapy may resolve the challenges of delivering 5-FU while keeping the therapeutic index high.

This study demonstrated that the multi-modal regimen that combined TMZ and MSC/CD with 5-FC induced additional DNA damage and further suppressed glioma cell proliferation *in vitro*. Furthermore, sensitizing glioma cells with MSC/CD and 5-FC before TMZ administration enhanced *in vivo* anticancer effects, dramatically reduced tumor volumes, and increased overall survival. Therefore, based on supporting preclinical evidence, we propose that promptly suppressing infiltrative residual glioma cell growth following surgical resectioning by applying MSC/CD to surrounding brain parenchyma and systemically administering 5-FC during the immediate postoperative period, could significantly improve the prognosis of patients with brain tumors.

Materials and methods

Cell culture and preparation of cell lines

All experimental protocols using human MSCs were approved by the Institutional Review Board of the Ajou University Medical Center (Suwon, South Korea). MSCs were transduced to prepare the MSC/CD as previously described [9]. The U87 human glioma cell line was purchased from ATCC (American Type Culture Collection, Mansssas, VA, USA). Primary glioma GBL28 cells were obtained from glioblastoma patient after surgery from Seoul National University Hospital (IRB No. 1009-025-331). The U87 cells were labeled by transduction with a green fluorescence protein (GFP)-expressing lentiviral vector or LacZ-expressing retroviral vector. U87/GFP cells were enriched by using fluorescence-activated cell sorting (FACS). The U87/LacZ cells were incubated in the presence of 2 µg/mL puromycin for 2 weeks, and all cells were grown in the growth medium. A more detailed description for cell culture and preparation of cell lines can be found in the Supplementary.

In vitro suicide gene effects

MSC/CD were plated at 1×10^4 cells/well in 12-well plates for 24 hours and treated with 5-FC (Archimica, Flintshire, UK) at indicated concentrations. The medium was replaced every other day with fresh growth medium containing indicated concentrations of 5-FC. On day 7, a 3-[4, 5-dimethyl-thiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay was performed to quantify the cell viability by measuring the optical density of the reaction solution at 540 nm. The viability of the treated cells was expressed relative to that of the untreated control cells as the mean ± SEM of at least three independent experiments.

In vitro bystander effects

U87/GFP or U87/LacZ cells were co-cultured with MSC or MSC/CD at a 1:1 ratio (each 1×10^4 cells) in 12-well plates. Twenty-four hours later, 5-FC was added at the indicated concentrations, and the medium was replaced every other day with the fresh growth medium containing the indicated concentrations of 5-FC. The U87/GFP cell images were acquired using an inverted IX71 microscope (Olympus, Tokyo, Japan) on day 7. The surviving cells were lysed in passive lysis buffer (Promega, Madison, WI, USA), and the cell lysate fluorescence was measured using a fluorometer (Molecular Devices, Sunnyvale, CA, USA). The GFP levels were expressed relative to the untreated control cell fluorescence as the means ± SEM of at least three independent experiments. The U87/LacZ cells were fixed with 4% neutral paraformaldehyde and stained with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, KOMABIOTECH, Seoul, Korea).

To determine the combined effects of 5-FC or 5-FU and TMZ (Sigma-Aldrich, St. Louis, Mo USA), 5-FC and TMZ were added at the indicated concentrations, and the medium was replaced every other day with fresh growth medium containing the indicated 5-FC and TMZ concentrations. Naïve MSCs were used as a negative control. Additionally, the combined effects of 5-FU and TMZ were further assessed in glioma cells plated at 1×10^3 cells/well in 96-well plates. Twenty-four hours later, 5-FU and TMZ were added at the indicated concentrations, and the medium was replaced every other day with fresh growth medium containing indicated TMZ and 5-FU concentrations. Cell viability was measured using an MTT assay on day 5. The absorbance at each concentration was expressed relative to that of the untreated control cells as the mean ± SEM of three independent experiments. The half-maximal inhibitory concentration (IC_{50}) values were calculated by fitting the curve using the Sigmaplot 12.5 program (Systat Software Inc., San Jose, CA, USA).

Isobologram analysis

For the isobologram analysis of the combined 5-FU and TMZ effects against glioma cells, the IC_{50} value of each drug was determined using dose response assays. The dose-dependent cytotoxicity of each drug was determined based on a fixed concentration of another agent. The synergism between 5-FU and TMZ was quantified by determining the combination index (CI), which is a quantitative evaluation of a two-drug pharmacological interaction, [10] calculated as follows: $CI = C_1/S_1 + C_2/S_2$, where, C_1 and C_2 are doses of the respective drugs in the combination required to produce the same effect that S_1 or S_2, the dose of each drug would produce. The combined effect of both drugs can be summarized as, $CI = 1$, < 1, and > 1 indicate summation (additive with no interaction), synergism, and antagonism, respectively. The CI was calculated using the CompuSyn ver 1.0 program (ComboSyn Inc, Paramus, NJ, USA) for each isobologram point.

For the isobologram analysis of combined bystander effect of MSC/CD with 5-FC and TMZ, the IC_{50} of the each test drug was first determined by evaluating its individual dose-dependent cytotoxicity, and then each value was further evaluated using a fixed concentration of another test drug. The interaction between 5-FC and TMZ was then quantified by determining the CI as described above. The IC_{50} values for all the assays were plotted, and a hypothetical additive line was drawn between two IC_{50} values from either drug alone. Above and below the additive line are the hyper- and hypo-additive regions, indicating antagonistic and synergistic interactions, respectively.

Cell cycle analysis using FACS

The U87/GFP cells (3×10^5) were plated in 100-mm dishes and treated the next day with 10 μM 5-FU or TMZ (30 or 300 μM) or a combination of both. Three days later, cells were washed with phosphate-buffered saline (PBS), fixed in 70% ice-cold ethanol at 4°C for 1 hour, washed with PBS, and stained with 20 μg/mL propidium iodide (PI, Molecular Probes, Eugene, OR, USA). After treatment with 200 μg/mL RNaseA (Sigma-Aldrich, St. Louis, MO, USA), the cellular DNA content was assessed using a BD FACS Aria system (BD Sciences, San Jose, CA, USA) and DNA histograms were analyzed using the Flowing Software 2 (Turku Centre for Biotechnology, Turku, Finland).

To analyze the phosphorylated ataxia telangiectasia mutated (p-ATM) population, cells were harvested and stained with an anti-phospho-

ATM (Ser1981) Alexa Fluor® 488 conjugate antibody against phosphorylated Ser at 1981 (Millipore, Billerica, MA, USA) using an FACS kit following the manufacturer's protocol.

In vivo anticancer activity

All animal protocols were approved by the Institutional Animal Care and Use Committee of Ajou University, Medical School. Seven-week-old female athymic Balb/C nude mice (Central Lab Animal, Seoul, Korea) were anesthetized and inoculated with U87/LacZ cells (3×10^5 cells/3 μL) in the striatum (anteroposterior, +0.05 cm; mediolateral, -0.18 cm; dorsoventral, -0.3 cm) using a stereotaxic device (Stoelting, Wood Dale, IL, USA). Six days later, MSC/CD (3×10^5 cells in 6 μL PBS) were transplanted in the same site. Then, starting the next day, mice were administered daily i.p. injections of 5-FC at 500 mg/kg for 7 days. Four days later, TMZ was administrated at 5 mg/kg for 5 days.

On day 28, animals were perfused with 4% paraformaldehyde (PFA), the brains were subsequently postfixed with 4% PFA at room temperature for 30 minutes, washed with PBS, and sliced into 1-mm thick sections using a rodent brain matrix (Stoelting). Then, the sections were incubated for 16 hours at 37°C in 1 mg/mL X-gal, 2 mM magnesium chloride ($MgCl_2$), 5 mM potassium ferrocyanide/potassium ferricyanide, and 0.02% NP40 in PBS. The images of both sides of the X-gal-positive slices were captured using a dissecting MVX10 microscope (Olympus), and the tumor area was determined as the number of pixels of X-gal-positive tumor/slice using the ImageJ (National Institutes of Health, NIH, Bethesda, MD, USA). The total tumor volume was calculated as follows: Tumor volume (mm^3) = Σ(Number of pixels of tumor area/Number of pixels per unit area) × 0.5 mm.

Histological analysis

After X-gal staining, 1-mm thick brain slices were embedded in paraffin blocks. The sections were obtained with 5 μm thickness and stained with H&E (Sigma-Aldrich). For immunostaining, antigens were unmasked by exposure to microwave radiation in 10 mM sodium citrate buffer (pH 6.0) and exposed to 0.3% H_2O_2 in distilled water for 30 min to block endogenous peroxidase activity. The sections were incubated first with anti-proliferating cell nuclear antigen (PCNA, mouse, 1:500; Millipore) and then with biotinylated anti-mouse antibodies (1:200; Vector Laboratories). After incubation with avidin-biotin complexes generated using a Vectastain ABC kit (Vector Laboratories), immunoreactive proteins were visualized with 3,3'-diaminobenzidine (Sigma-Aldrich) as substrate to detect horse-radish-peroxidase activity. The sections were mounted and scanned at 40 × resolution using a Scanscope CS digital slide scanner (Aperio Technologies, Vista, CA, USA).
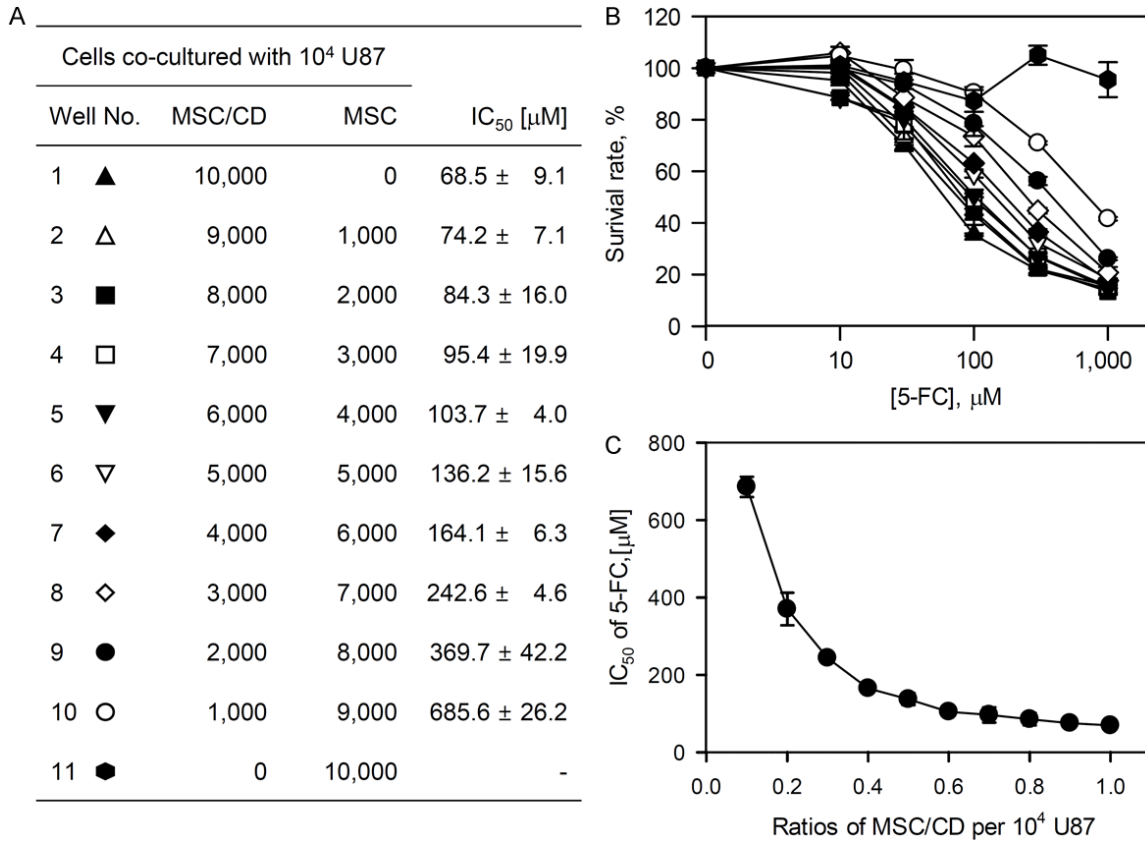
Statistical analysis

The results of at least three independent *in vitro* experiments were analyzed using the Student's *t*-test for single comparisons and Holm-Sidak method for multiple pair-wise comparisons. For the *in vivo* experiments, the results were analyzed using a one-way repeated measures analysis of variance (ANOVA) with the Holm-Sidak method. The values are expressed as the mean ± SEM ($^*P < 0.05$).

Results

Verification of MSC/CD and reporter gene-tagged glioma cell line

Transduction of the bone marrow-derived MSC with the CD-expressing retrovirus did not alter the characteristics of the MSC/CD including growth rate, differentiation ability, and surface markers as previously reported [11]. To test the effect of the suicide *CD* gene on the MSC/CD, they were cultured with 5-FC for 7 days. While the survival rate of the MSC remained consistent, the MSC/CD underwent cell death with an IC_{50} value of 36.5 μM 5-FC (Supplementary Figure 1A). The MSC growth was slightly reduced by 1 mM 5-FC, but not significantly.

Furthermore, the anticancer effect of the MSC/CD with 5-FC against U87 glioma cells was assessed. To prevent the MSC/CD from interfering in the assessment of the bystander effect against U87 glioma cells, they were tagged with a reporter gene, GFP, or LacZ. GFP or LacZ labeling did not change the cellular characteristics of anchorage-independent growth in soft agar or its sensitivity to 5-FU, an active metabolite of 5-FC [12]. Furthermore, MSC/CD suppressed the growth of both cell

A

Cells co-cultured with 10^4 U87

Well No.		MSC/CD	MSC	IC$_{50}$ [µM]
1	▲	10,000	0	68.5 ± 9.1
2	△	9,000	1,000	74.2 ± 7.1
3	■	8,000	2,000	84.3 ± 16.0
4	□	7,000	3,000	95.4 ± 19.9
5	▼	6,000	4,000	103.7 ± 4.0
6	▽	5,000	5,000	136.2 ± 15.6
7	◆	4,000	6,000	164.1 ± 6.3
8	◇	3,000	7,000	242.6 ± 4.6
9	●	2,000	8,000	369.7 ± 42.2
10	○	1,000	9,000	685.6 ± 26.2
11	⬢	0	10,000	-

Figure 1. Assessment of the effective dose of MSC/CD. (A) Indicated numbers of MSC/CD or naïve MSCs were co-cultured with 10^4 U87/GFP in the presence of 5-FC for 6 days. Survival rates of U87/GFP were measured using fluorometric analysis. (B) The fluorescence intensity was normalized to the value of U87/GFP alone without MSC/CD. (C) IC$_{50}$ values of 5-FC from (B) were presented with respect to the relative number of MSC/CD ranging 0.1~1.0. Note that IC$_{50}$ inversely decreased in proportion to an increase of the MSC/CD ratio. Data are means ± S.E. of three independent experiments.

lines treated with 5-FC with IC$_{50}$ values of 48.7 and 42.2 µM for U87/GFP and U87/LacZ, respectively (Supplementary Figure 1B and 1C).

*Determination of the effective dose of MSC/CD*

To assess the effective dose of MSC/CD for suppressing the U87 growth, we used less number of MSC/CD in co-culture with 10^4 U87 with the ratios ranging 0.1~1.0. To maintain the consistency of the culture, we kept the total number of plated cells constant by adding naïve MSCs proportionally for the ratio < 1.0 (**Figure 1B**). When 10^4 each of MSC/CD and U87 (1:1 ratio) were plated per well, the bystander effects was the highest with IC$_{50}$ of 68.5 mM (**Figure 1A**). When less number of MSC/CD was plated in the co-culture, the IC$_{50}$ value inversely increased (**Figure 1B** and **1C**). Thus, MSC/CD with 0.2 ratio required approximately 370 mM

5-FC to suppress the U87 growth by 50%, while the same dose almost completely suppressed it in the presence of 1 mM 5-FC. The result indicated that the bystander effect was dependent on both the MSC/CD dose and 5-FC concentration. All the subsequent experiments was performed with 1:1 ratio, unless otherwise specified.

Evaluation of interaction of MSC/CD with 5-FC and TMZ

To evaluate the combinatorial interaction of MSC/CD with 5-FC and TMZ, MSC/CD and U87/GFP were co-cultured with 5-FC and TMZ. The fluorescence U87/GFP images demonstrated gradual 5-FC and TMZ concentration-dependent decreases (**Figure 2A**). The fluorometric cell lysate analysis indicated that the IC$_{50}$ values of 5-FC and TMZ alone were 58.3 and 313.4 µM, respectively (**Figure 2C** and **2D**).
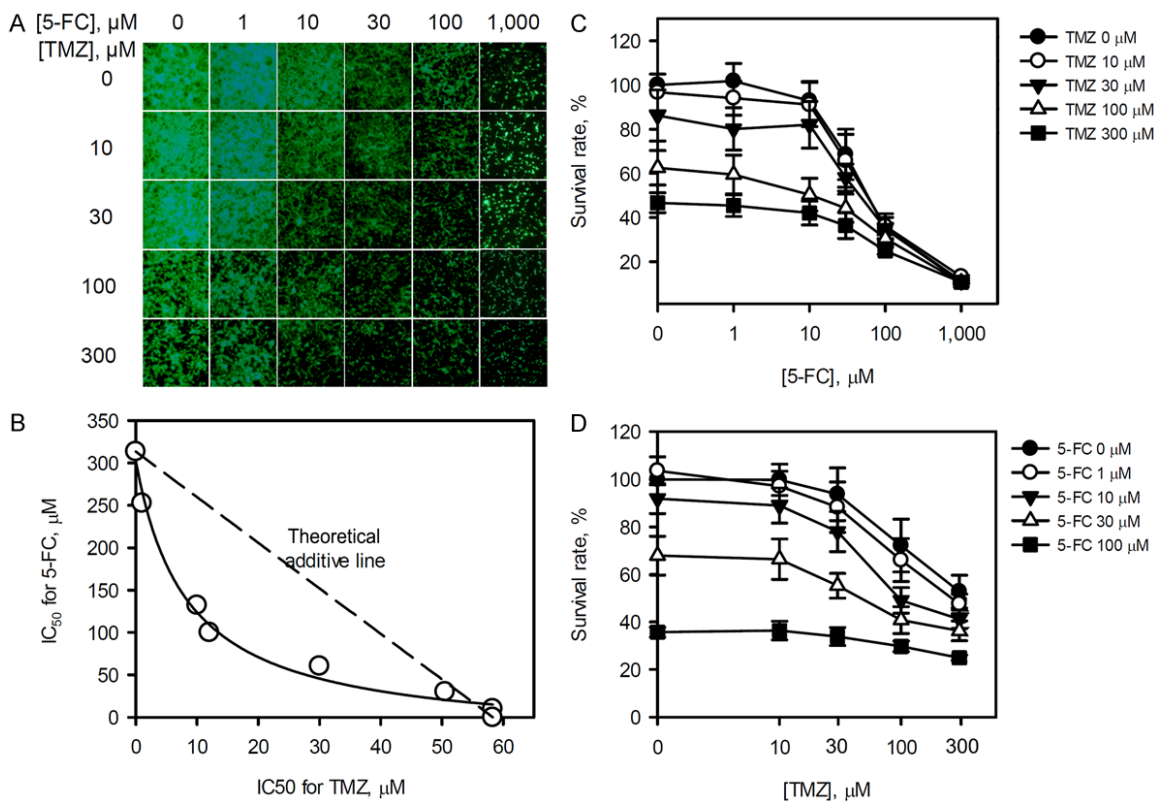
Figure 2. Synergistic effects of combined treatment with MSC/CD with 5-FC and TMZ against U87/GFP. (A) Fluorescence images of U87/GFP co-cultured with MSC/CD and indicated 5-FC and TMZ concentrations. (B) IC_{50} of 5-FC and TMZ obtained from (C and D) were plotted in isobologram using Chou-Talalay method, indicating synergism. Dashed line indicated a theoretical additive line. (C and D) Fluorescent intensity decreased with increasing 5-FC or TMZ concentrations. Data are means ± S.E. of three independent experiments.

Importantly, combined treatment lowered the drug's individual IC_{50} values interactively. To evaluate the combinatorial interactions, the IC_{50} values shown in **Figure 2C** and **2D** were plotted using the Chou-Talalay method (**Figure 2B**) [10] and the combination indices were < 1, which corresponding to hypo-additive points and indicated 5-FC and TMZ acted synergistically.

Mechanism of synergism between MSC/CD with 5-FC and TMZ

To investigate the mechanism of the synergistic effects of MSC/CD with 5-FC and TMZ, we used 5-FU, because unlike other small chemical compounds, the MSC/CD interfered with the cell cycle phase distribution. The IC_{50} value of 5-FU alone was 6.0 μM for U87. The MTT assay used various TMZ concentration combined with different fixed 5-FU concentrations (**Figure 3A**). Using the dose-response viability data, a normalized isobologram was constructed, and IC_{50}

values from **Figure 3A** were plotted using the Chou-Talalay method (**Figure 3B**). The CIs of both glioma cells were < 1, which corresponded to hypo-additive points and verified that 5-FU and TMZ interacted synergistically, similar to MSC/CD with 5-FC and TMZ. We also carried out similar experiments with GBL28, primary glioma cells obtained from glioblastoma patient. The IC_{50} values of 5-FU and TMZ alone were 7.8 and 523 μM, respectively. The results indicated that the combined treatment could be applicable to other glioma cells.

The unsynchronized U87/GFP cells were exposed to 10 μM 5-FU or TMZ (30 or 300 μM) or a combination of both for 3 days and PI-stained, followed by FACS analysis (**Figure 4A**). Exposure to 30 μM TMZ increased the G_2/M phase arrest from 37.3 to 74.9% at the expense of the G_1 population while combination 30 μM TMZ and 10 μM 5-FU further increased the S phase arrest from 8.1 to 15.3%. However, exposure to 300 μM TMZ did not further increase the G_2/M
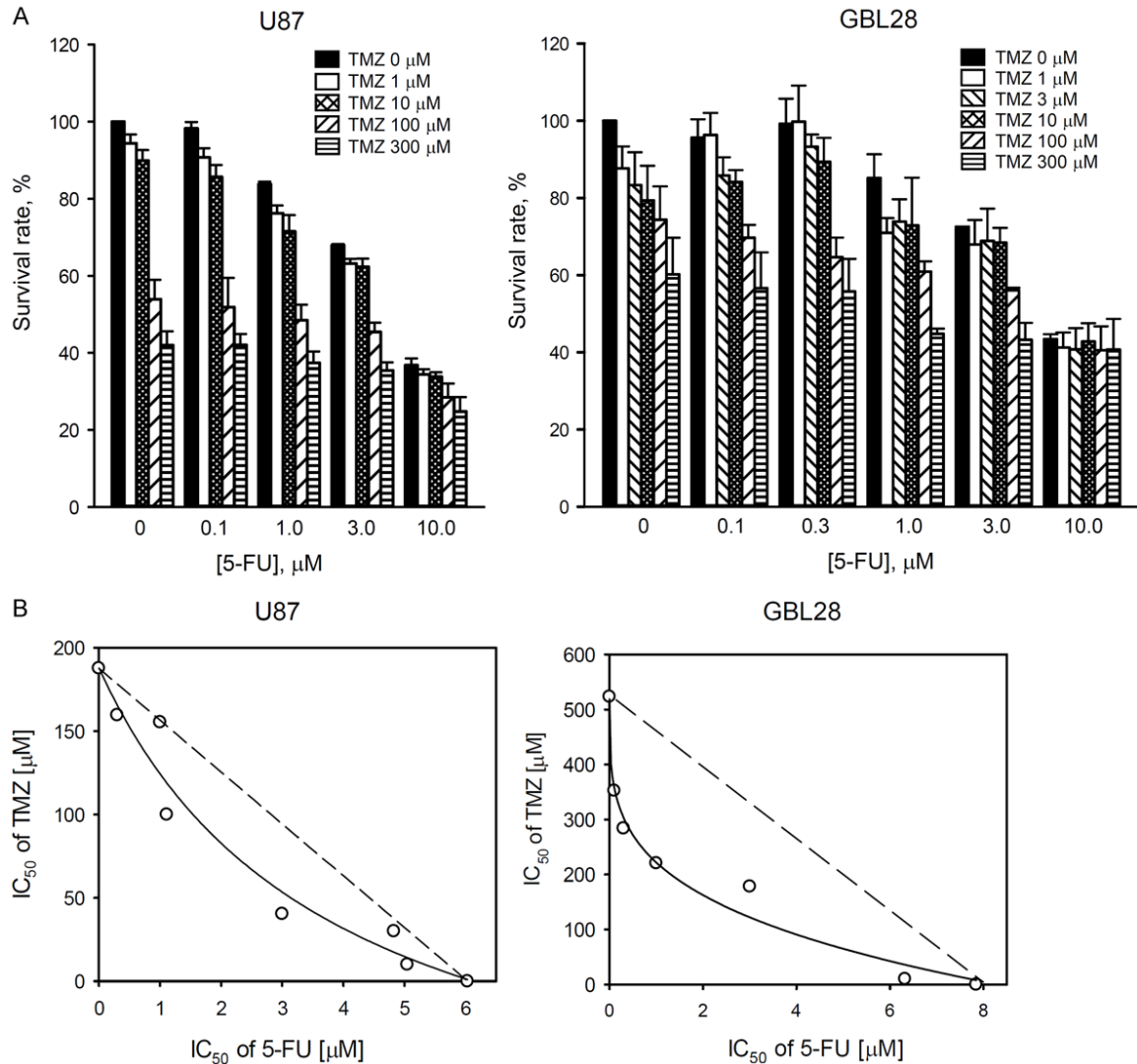
Figure 3. Cytotoxicity of 5-FU and TMZ against U87 and GBL28 cells. (A) Cell viability was measured using MTT assay with increasing 5-FU or TMZ concentrations. Data are means ± S.E. of three independent experiments. (B) IC_{50} values of 5-FU and TMZ obtained from (A) were plotted in isobologram using Chou-Talalay method, indicating synergism. Dashed line indicated a theoretical additive line.

phase arrest even with 5-FU treatment, which increased the G_1 and S phase cells. U87/GFP cells in the G_2/M phases increased to 74.9% and 63% by exposure to 30 and 300 µM TMZ, respectively. Corresponding decreases in relative G_1 phase cell numbers were observed while, contrastingly, 5-FU treatment alone increased the relative number of G_1 and S phase cells from 60.0 to 71.2%. Co-treatment with 5-FU and TMZ increased S phase cells from 8.1 to 15.3 and 13% with 30 and 300 µM TMZ, respectively, while increasing G_2/M phase cells to ca. 60%. The data suggest that these anticancer drugs modulate the U87 glio-

ma cell cycle similarly *in vitro*, as reported previously [13] with other cells.

The DNA damage induced by these drugs was assessed indirectly by monitoring phosphorylation of p-ATM kinase at Ser198 (**Figure 4B**). The p-ATM-positive cell population was minimal (3.2%) under the culture conditions, but increased to 10.6 and 11.8% with 5-FU- and TMZ treatment, respectively. Co-treatment with 5-FU and TMZ further increased the p-ATM-positive cells to 27.3 and 38.0% at 30 and 300 µM TMZ, respectively. The results indicated that 5-FU and TMZ synergistically induced DNA damage and cell cycle arrest.
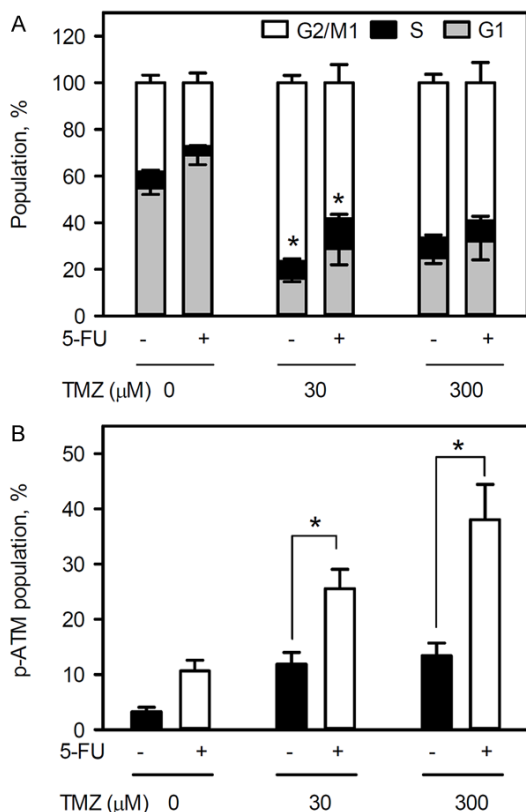
Figure 4. Cell cycle arrest and DNA damages induced by 5-FU and TMZ. A. Cell cycle arrest was analyzed using PI staining and FACS. 5-FU increased G_1 and S phase cell populations, whereas TMZ induced G_2/M arrest. Combined 30 μM 5-FU and 30 μM TMZ increased S-phase cell population significantly compared to 5-FU alone. B. p-ATM population significantly increased with 30 and 300 μM 5-FU and TMZ, respectively. Data are means ± S.E. of three independent experiments (*P < 0.05; one-way analysis of variance (ANOVA) using Holm-Sidak method).

Sensitization of glioma cells to TMZ by pretreatment of MSC/CD with 5-FC in vivo

We evaluated the efficacy of combination MSC/CD and 5-FC treatment in sensitizing glioma cells to TMZ treatment in an orthotopic xenograft mouse brain tumor model (**Figure 5A**). U87/LacZ was transplanted into the mice, and tumors were allowed to develop for 6 days. The anticancer drugs were cytotoxic to both cancer and normal cells when administered systemically. We found that TMZ caused cell death of MSC/CD with an IC_{50} value of 550 μM (Supplementary Figure 2). To avert TMZ's cytotoxicity on the MSC/CD and retain the synergy, we sequentially treated the mouse glioma model with MSC/CD followed by 5-FC first and then TMZ.

On day 28, the brain tumors were X-gal-stained for histological analysis (**Figure 5B**), and tumor masses were quantified using the ImageJ software (**Figure 5C**). The control tumor volume was 60.4 mm³, and it decreased significantly with MSC/CD treatment with 5-FC or TMZ (16.7 and 9.9 mm³, respectively). Importantly, the tumor volume decreased more dramatically with the combination treatments (2.6 mm³) than it did with either treatment alone.

The Kaplan-Meier graph indicated that the median survival of the control group alone was 40 days while that of the MSC/CD with 5-FC- or TMZ-treated groups showed a slight increase of 10 days (**Figure 5D**). The group treated with combination MSC/CD with 5-FC and TMZ exhibited a more significant improvement in median survival of up to 64 days, and 38% of the mice survived until the observation ended while the other mice died.

*Histopathological analysis of in vivo tumor mass*

H&E staining revealed typical anaplastic gliomas with increased cellularity and mitotic activity in the tumor mass (**Figure 6A**). In the PBS control group, the nucleus-to-cytoplasm ratio was so high that hypercellular tumor nodules were sharply demarcated. In the TMZ or MSC/CD with 5FC treatment group, the size of tumors was reduced compared to that of the control group. Importantly, combination treatment induced the smallest tumor size as well as the lowest cellularity with scar-like necrotic margins with pseudopalisading cells. Regardless of treatment (MSC/CD with 5-FC, TMZ, or both), the remaining tumor cells were spindle-shaped or plump with a large eosinophilic cytoplasmic mass with nuclear pleomorphism. Immunostaining with anti-PCNA indicated that the proportion of PCNA-positive cells was high in the control group but slightly reduced in the MSC/CD with 5-FC or TMZ group (**Figure 6B**). As expected, combination treatments robustly reduced the number of PCNA-positive cells and the cellularity in tumor mass.

Discussion

We aimed to develop an anticancer therapy that mechanistically functions via pathways distinct from that of TMZ while limiting its cytotoxicity to targeted cancer cells. In the present study, we showed that MSC/CD combined with
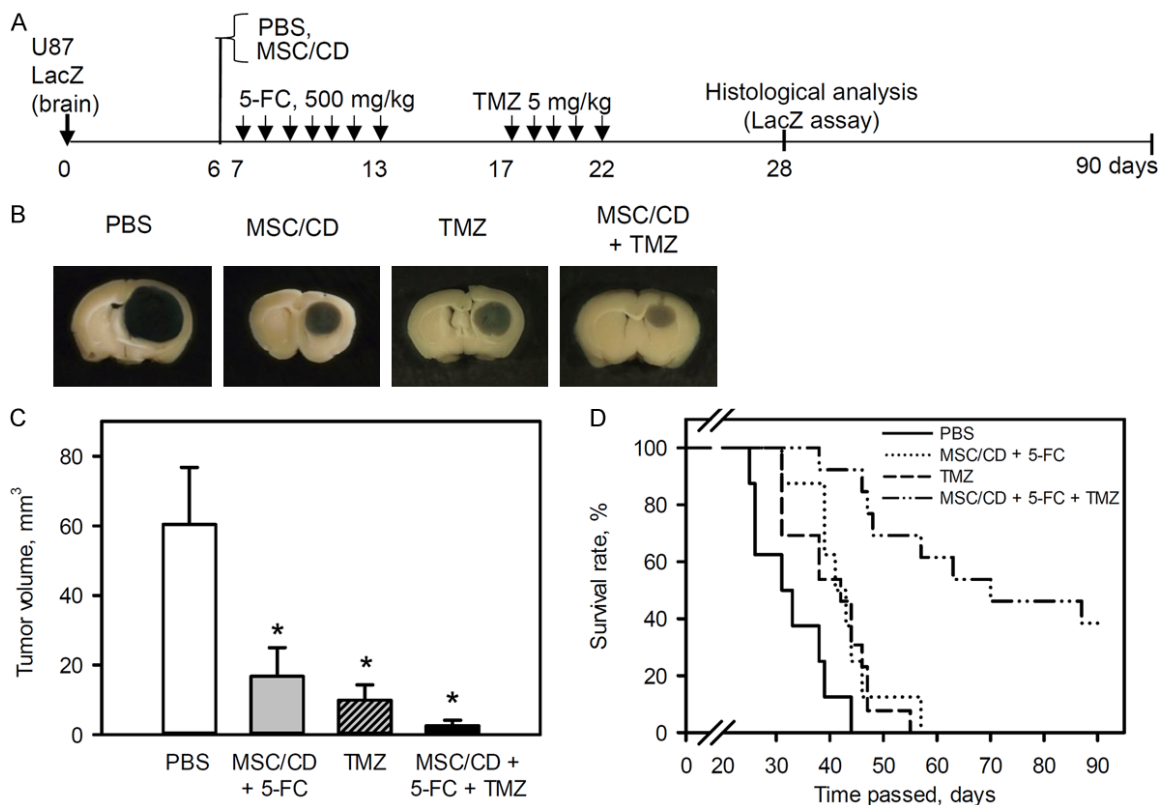
Figure 5. Synergistic effect of serial treatment of MSC/CD with 5-FC and TMZ on orthotopic xenograft model. A. Schematic presentation of experiments. U87/LacZ (3×10^5 cells) were inoculated into the brain. MSC/CD (3×10^5 cells) was transplanted 6 days later. 5-FC (500 mg·kg^{-1}·day^{-1}, i.p.) was administered for 7 days between day 7 and 13. Then, TMZ (5 mg/kg, i.p.) was administered for 5 days between day 17 and 22. On day 28, brains were sliced and X-gal stained. B. Representative brain slices of each group. C. X-gal-positive tumor volume of each group as means ± S.E. of at least four animals/group. ($^*P < 0.05$; one-way analysis of variance (ANOVA) using Holm-Sidak method). D. Overall survival of at least eight animals/group represented in Kaplan-Meier graph. Note that sequential treatment with MSC/CD with 5-FC and then TMZ significantly increased the overall survival.

5-FC sensitized the glioma cells to TMZ and, thereby, effectively suppressed the glioma with a safe therapeutic index in an orthotopic brain tumor model. TMZ is a prodrug, which is remarkably stable at acidic pH, allowing a 100% oral bioavailability. These characteristics, its rapid absorption, excellent biodistribution, and high BBB permeability have made TMZ a standard drug option for GBM [14, 15]. Numerous GBMs have intrinsic or acquired resistance to TMZ, primarily determined by the MGMT level and activity [16]. MGMT promoter methylation and gene silencing are associated with favorable outcomes following TMZ treatment [6]. To restore the sensitivity and increase the therapeutic effectiveness of TMZ, O[6]-benzylguanine (O[6]-BG), an irreversible inhibitor of MGMT was developed [17, 18]. By depleting MGMT, O[6]-BG restores the TMZ sensitivity of patients with TMZ-resistant anaplastic glioma, but not of those with TMZ-resistant GBM. Moreover, systemic administration of O[6]-BG combined with TMZ was reported to be cytotoxic to the hematopoietic system [19]. Therefore, it is imperative to develop adjuvant therapies that do not share the same pathways with TMZ with safe therapeutic indices.

This study demonstrated that 5-FU produced by combination treatment with MSC/CD and 5-FC exhibited synergism with TMZ (**Figure 2**). Such combination treatment can be applicable to other GBM cells since the synergy between 5-FU, an active metabolite of 5-FC and TMZ was also evident in primary gliomblastoma GBL28 cells (**Figure 3**). TMZ induces G_2/M phase arrest by alkylating the guanine residues and consequently induces DNA breakage [20]. In comparison, 5-FU inhibits thymidylate synthase, an important enzyme in the *de novo* syn-
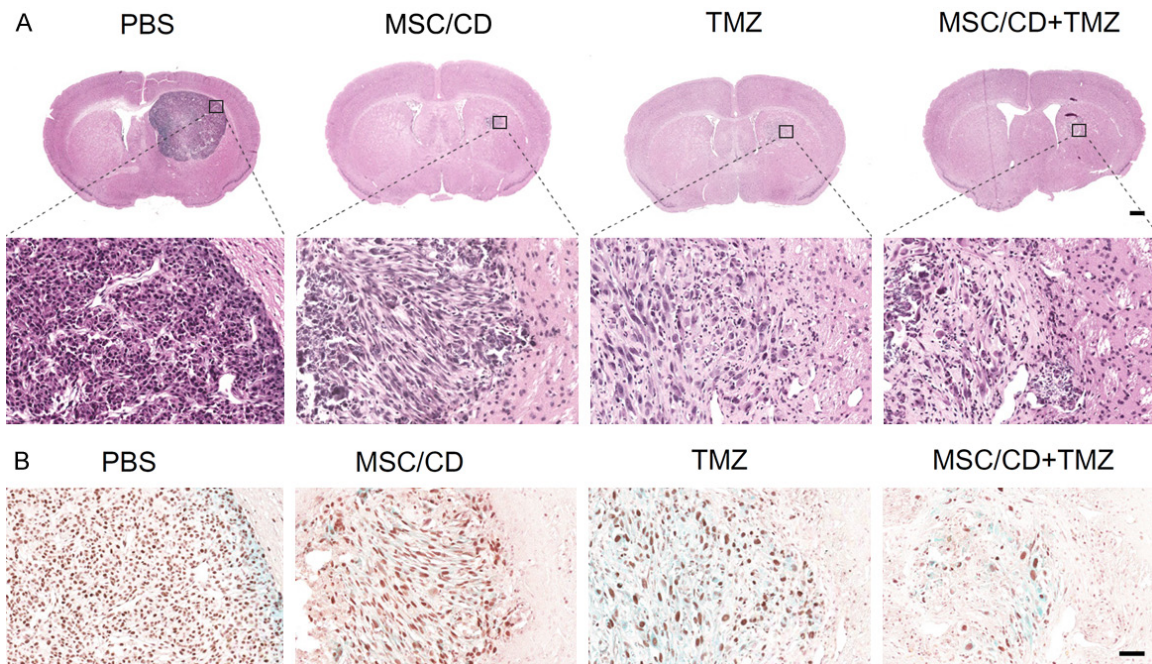
Figure 6. Histopathological analysis of *in vivo* tumor mass. A. H&E staining indicated high cellularity with demarcating margin in the PBS control group. The tumor size was reduced after treatment with MSC/CD with 5-FC or TMZ. Note that combined treatment induced the smallest tumor size. Tumor cells remained after the treatment were spindle shaped with large masses of eosinophilic cytoplasm and pleomorphic nuclei. B. Immunostaining with anti-PCNA antibody revealed the PCNA-positive proliferating cells in the tumor mass. Combined treatment of MSC/CD with 5-FC and TMZ was the most effective to reduce the number of PCNA-positive cells and the cellularity in tumor mass. Note that traces of X-gal-staining were detectable. Scale bars: A=500 µm; B=50 µm.

thesis of DNA. Additionally, 5-FU metabolites integrate into RNA and DNA. DNA synthesis inhibition by 5-FU reduces outflows from the early S-phase and consequently induces cell accumulation in the G_1/S phase [13]. This study showed that treatment with 30 mM TMZ alone induced G_2/M arrest by damaging DNA as evidenced by increased p-ATM-positive cell numbers (**Figure 4**). Treatment with TMZ alone (300 mM) did not further increase the G_2/M accumulation or p-ATM-positive cell population. Furthermore, 5-FU alone increased the relative G_1/S cell number and DNA breakage. Importantly, DNA breakage dramatically increased when glioma cells were co-treated with 5-FU and TMZ. These results provide evidence supporting the relevance of our combination MSC/CD with 5-FC and TMZ approach to inducing DNA breakage efficiently via multimodal pathways, thereby inducing synergistic therapeutic effects *in vivo*.

Despite the importance and usefulness of 5-FU in cancer care, it is not indicated for treating patients with GBM because its systemic admin-

istration causes a syndrome involving delayed CNS myelin destruction [8]. The selective delivery of this drug to tumors may reduce the CNS and hematopoietic system side effects while still providing its anticancer effects. Viral- and stem cell-mediated gene therapies are attracting attention because of their targeting efficiency in GBM. Therefore, oncolytic polioviruses targeting CD155 receptors overexpressed in glioma cells were designed [21]. Conditionally replicative adenoviruses exhibit synergism with TMZ and exert cytopathic effect against tumor cells [22]. The therapeutic effect of those oncolytic adenoviruses was enhanced further when the viruses were loaded into neural stem cells [23]. The adeno-associated virus encoding soluble tumor necrosis factor (TNF)-related apoptosis-inducing ligand (sTRAIL) suppressed glioma growth in a subcutaneous mouse model [24]. Furthermore, MSC engineered to express sTRAIL also exerted anticancer activity against glioma cells following co-administration with TMZ [24, 25]. Treatment with a replication-competent retrovirus carrying a *CD* gene followed by 5-FC administration suppressed tumor

growth [26] and immortalized neural stem cells were used to deliver a *CD* gene close to a glioma site [27].

The bystander effect of a single treatment with MSC/CD and 5-FC was comparable to the impressive effects of 5 mg/kg TMZ treatment in our model (**Figure 5D**). The timing and transplantation routes for therapeutic viruses or stem cells are critical to their optimal therapeutic efficacy when they are combined with the standard of care because TMZ induces dysfunctions of proliferating cells and, thus, delays the viral dissemination [27, 28]. We also found that TMZ exerted cytotoxicity against MSC/CD with an IC_{50} of 550 μM (Supplementary Figure 2). Therefore, we chose to administer the treatments sequentially, by first transplanting MSC/CD (day 6), followed by TMZ administration (day 17). Histopathological analysis indicated that the combination treatment regimen effectively suppressed U87 glioma cell proliferation (**Figure 6**) and consequently suppressed the tumor growth, leading to an increase in overall survival of the animals (**Figure 5**). Treatment of MSC/CD with 5-FC and TMZ altered tumor cell morphology to spindle-shapes with a large eosinophilic cytoplasmic mass and nuclear pleomorphism. Such morphological changes might be attributed, if not all, to the cell cycle arrest at S and G_2/M induced by 5-FU and TMZ (**Figure 4**). As shown in the *in vitro* assays (**Figure 2**), the treatment efficacy can be explained by the generation of high 5-FU amounts by MSC/CD from the 5-FC in nearby tumor sites, which sufficiently suppressed tumor growth and sensitize tumor cells to TMZ.

Our results can be clinically extrapolated where a single transplantation of MSC/CD following 5-FC administration during the recovery period after tumor mass removal, could synergistically enhance TMZ's efficacy. Furthermore, this effect can be achieved without interfering with the standard treatment procedure, consisting of a 4-week fractionated radiotherapy session and oral administration of TMZ. Glucocorticoids have been commonly used to treat brain tumor patients to reduce tumor-associated vasogenic edema and minimize intracranial pressure post-operatively and during radiotherapy phases [29]. Glucocorticoid-induced effects are derived from their immunomodulatory actions that reduce pro-inflammatory cytokine and che-

mokine expressions [30, 31]. However, immune system suppression following the prolonged use of glucocorticoids can increase the vulnerability to opportunistic infections.

Considerable data currently support the anti-inflammatory effect of MSC on immune cells mediated by diverse cytokine and chemokine secretion [32]. The immunomodulatory functions of MSC promote wound healing and tissue repair [33]. We also previously reported that MSC secreted transforming growth factor (TGF)-β, which suppressed immune responses in an acute stroke model [34]. The transduction of MSC with a *CD* gene did not change their original characteristics, therefore, the MSC/CD may retain innate immunomodulatory functions of MSC cells. Further studies are necessary to determine the immunosuppressive functions of MSC/CD that may facilitate patient recovery following brain resectioning or preclude the necessary administration of glucocorticoids, or both.

This study demonstrated that combination MSC/CD and 5-FC exerted a localized bystander effect on nearby tumor cells and enhanced the therapeutic effects of TMZ in an orthotopic glioma model. Early treatment with MSC/CD and 5-FC may be effective in two ways. First, this combination suppresses residual tumor cell growth, which would otherwise robustly regrow during the interim period before conventional drug therapy, and the treatment sensitizes residual tumors to subsequent TMZ treatment. Therefore, we propose combination MSC/CD and 5-FC as a candidate therapy, which could be incorporated in current regimens with flexibility. Furthermore, the regimen may be used in the immediate postoperative recovery period for patients who have undergone a cytoreductive surgery, particularly with suboptimal resectioning.

Acknowledgements

Disclosure of conflict of interest

None.

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Supplementary methods

Cell culture and preparation of cell lines

Bone marrow-derived MSC were transduced with a CD-expressing retrovirus for 8 hours in the presence of 4 µg/mL polybrene (Sigma-Aldrich, St Louis, MO, USA) and 10 ng/mL basic fibroblast growth factor (Dong-A Pharmaceutical Co., Youngin, Korea). The transduced cells were enriched for 2 weeks in the presence of 2 µg/mL puromycin (Sigma-Aldrich) and maintained by subculturing every 5 to 7 days in the growth medium, which was composed of Dulbecco's modified Eagle's medium (Welgene, Daegu, Korea) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA), 100 units/mL penicillin, and 100 µg/mL streptomycin (Gibco, Grand Island, NY, USA). The differentiation potential and the surface antigenicity of MSC/CD were assessed using previously described methods [1].

The U87 human glioma cell line was purchased from ATCC (American Type Culture Collection, Mansssas, VA, USA). Primary glioma cells GBL28 were obtained from glioblastoma patient after surgery from Seoul National University Hospital (IRB No. 1009-025-331). Both cell lines were cultured and expanded in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin [2].

The U87 cells were labeled by transduction with a green fluorescence protein (GFP)-expressing lentiviral vector, Ll3.7-GFP, which was produced by transfecting pll3.7-GFP with Δ8.9 and VSV-G into 293T cells. Then, the culture medium supernatant was syringe-filtered through a 0.45-µm filter 2 days after transfection, the U87 cells were subsequently transduced with the supernatant, and the U87/GFP cells were enriched by using fluorescence-activated cell sorting (FACS). The U87 cells were also transduced with an MSCV-LacZ, LacZ-expressing retroviral vector, as previously described [3]. The U87/LacZ cells were incubated in the presence of 2 µg/mL puromycin for 2 weeks, and all cells were grown in the growth medium.

Supplementary References

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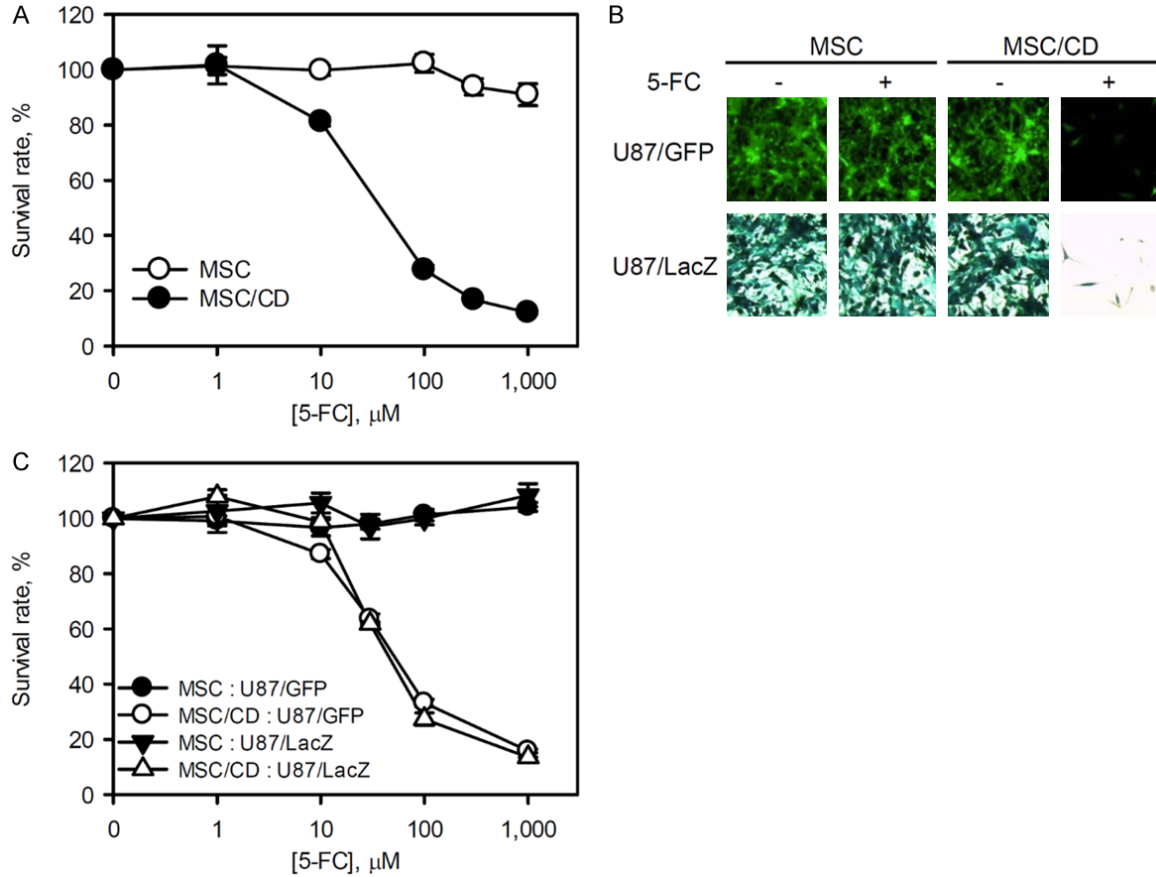
Supplementary Figure 1. Cytotoxicity of MSC/CD in the presence of 5-FC. A. Effects of MSC/CD suicide gene incubated with 5-FC for 6 days measured using MTT assay. B. U87/GFP or U87/LacZ cell images were co-cultured with MSC or MSC/CD with 5-FC. C. Bystander effects of MSC/CD with 5-FC against U87/GFP and U87/LacZ were measured using fluorometric analysis and β-gal assay, respectively. Data are means ± S.E of three independent experiments.
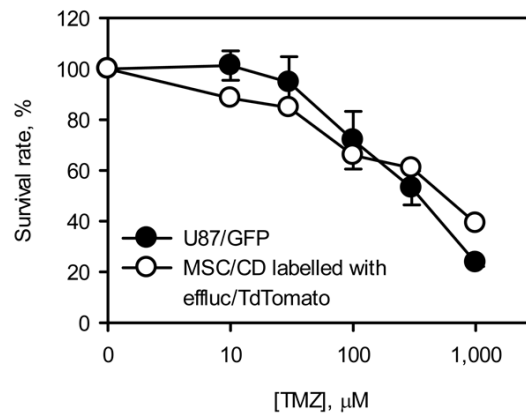
Supplementary Figure 2. Cytotoxic effects of temozolomide (TMZ) in cytosine deaminase CD gene-expressing mesenchymal stem cells (MSC/CD) and U87 cells expressing green fluorescence protein (U87/GFP). TMZ was added to the co-culture of MSC/CD labelled with effluc/TdTomato and U87/GFP. TMZ suppressed the survival MSC/CD and U87/GFP with IC_{50} values of 550 and 313 μM, respectively. Data are presented as means of duplicate experiments for MSC/CD labelled with effluc/TDTomato and means ± standard error of the mean (SE). from three independent experiments for U87/GFP. IC_{50}, half-maximal inhibitory concentration.