Therapeutic effect of suicide gene-transferred mesenchymal stem cells in a rat model of glioma

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Abstract

We evaluated a new therapeutic strategy for malignant glioma, which combines intratumoral inoculation of mesenchymal stem cells (MSCs) expressing cytosine deaminase gene with 5-FC administration. For *in vitro* and *in vivo* experiments, MSCs were transfected with adenovirus carrying either enhanced green fluorescent protein gene (AdexCAEGFP) or cytosine deaminase gene (AdexCACD), to establish MSC expressing EGFP (MSC-EGFP) or CD (MSC-CD). Co-culture of 9L glioma cells with MSC-CD in a medium containing 5-FC resulted in a remarkable reduction in 9L cell viability. The migratory ability of MSC-EGFP towards 9L cells was demonstrated by double chamber assay. For the *in vivo* study, rats harboring 9L brain tumors were inoculated with MSC-EGFP or MSC-CD. Immunohistochemistry of rat brain tumors inoculated with MSC-EGFP showed intratumoral distribution of MSC-EGFP. Survival analysis of rats bearing 9L gliomas treated with intratumoral MSC-CD and intraperitoneal 5-FC resulted in significant prolongation of survival compared with control animals. In conclusion, molecular therapy combining suicide gene therapy and MSCs as a targeting vehicle represents a potential new therapeutic approach for malignant glioma, both with respect to the antitumor potential of this system and its neuroprotective effect on normal brain tissue.

Key words: glioma; mesenchymal stem cell; suicide gene; bystander effect.

Introduction

Although conventional therapies for malignant glioma such as surgical resection, radiotherapy and chemotherapy are available, the prognosis for patients with this disease remains extremely poor [1]. This can be traced back to the finding that malignant gliomas have the distinct ability to infiltrate the brain parenchyma and disrupt the neural extracellular matrix. Thus traces of the primary lesion frequently remain at the borders of the post-operative tumor cavity, eventually leading to tumor recurrence following initial treatment. Therefore, effective new therapeutic tools that specifically target the tumor cells, especially those cells that have escaped the main tumor mass are urgently needed [2].

Current studies suggest that stem cells are effective delivery vehicles for gene therapy against malignant glioma [3-5]. Work by Aboody *et al*. demonstrated that after intracranial injection, neural stem cells (NSCs) possess wide-ranging tropism for implanted glioma cells and show significant migratory behavior [3]. Furthermore, Ehtesham *et al.* demonstrated that intracranial injection of NSCs engineered to express interleukin-12 or tumor necrosis factor-related apoptosis-inducing ligand exhibited strong antitumor effects in experimental glioma models [5]. However, isolation of the

required amounts of autologous or allogeneic NSCs for clinical application is limited by technical barriers, ethical uncertainties and by issues with immunologic incompatibility.

Recently, bone marrow stem cells, in particular mesenchymal stem cells (MSCs) have received much attention as an alternative source of neural progenitor cells for clinical application. Mesenchymal stem cells, which exist primarily in the bone marrow, can differentiate into osteoblasts, chondrocytes, adipocytes and hepatocytes [6, 7]. Furthermore, in a rat cerebral ischemia model, MSCs have the ability to infiltrate from the initial intracranial injection site to the ischemic lesion [8, 9]. Recently, it was reported that MSCs can migrate toward malignant glioma cells and that gene-transferred MSCs have the ability improve the survival of glioma-bearing mice [10, 11]. These findings suggest that MSCs could act as surrogate NSCs and provide the vehicle for molecular therapy against glioma. Furthermore, the *in vitro* propagation of autologous MSCs for clinical use and implantation into patients with malignant glioma is not associated with the previously identified immunologic and ethical problems.

The 5-fluorocytosine (5-FC)/*Escherichia coli* cytosine deaminase (CD) system is a suicide gene therapy system that is currently used for malignant tumors [12, 13]. Expression of the CD gene within the target cell produces an enzyme that converts the prodrug, 5-FC, to the toxic metabolite, 5-fluorouracil (5-FU). The use of

5-fluorocytosine may be particularly suitable for brain tumors, because it can readily cross the blood-brain barrier (BBB). Since 5-FU is inhibitor of RNA synthesis, it is not toxic to nondividing normal neuronal cells. Moreover, gene therapy with 5-FC/CD also possesses a strong bystander effect that does not require direct cell-to-cell contact [13]. In a recent study of mice bearing melanoma, it was reported that human MSCs expressing CD were associated with tumor regression [14]. Thus, in the present study, we sought to evaluate the migratory ability of MSCs and the antitumor effects of 5-FC/CD gene therapy with MSC (5-FC/MSC-CD) as the targeting vehicle *in vitro* and *in vivo* models of glioma.

Materials and methods

Cell lines

The 9L rat glioma cells and 293 cells (transformed human embryonic kidney cells) (JCRB9068, Health Science Research Resources Bank, Osaka, Japan) were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 μg of streptomycin, and 100 U of penicillin. The 9L-DsR cells (9L cells labeled with *Discosoma* red fluorescent protein: DsRed2) were maintained in DMEM supplemented with 10% FBS and 800 μg/mL

G418. The cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

Preparation of MSCs

The MSCs were prepared from rat bone marrow as described previously [11]. Briefly, 6 week old male Fischer-344 rats (Clea Japan, Inc., Fuji Shizuoka, Japan) were euthanized with ketamine hydrochloride. The femurs and tibias were dissected free of soft tissue and the epiphyses were removed with scissors and the midshaft bone marrow tissue was flushed out into culture medium (DMEM supplemented with 10% FBS, 100 U of penicillin, 100 μg of streptomycin). A single cell suspension was obtained by drawing the marrow into syringes through needles of sequentially decreasing size (18, 20 and 22 gauge, respectively). The MSC primary cultures were seeded at a density of 5×10^7 cells/10-cm dish. To remove the non-adherent cells, the medium was replaced with fresh medium 4 days after initial culture. The MSCs were maintained at 37 °C in 5% CO_2 by exchanging the spent medium with fresh medium at 4-day intervals.

Preparation of recombinant adenovirus vectors and ex vivo gene transduction

Two recombinant replication-deficient adenovirus vectors, AdexCAEGFP (containing the gene for enhanced green fluorescent protein, EGFP) and AdexCACD (containing

the CD gene) were constructed from a serotype-5 wild-type adenovirus (Ad5) by inserting the chicken alphaglobin promoter driving the *E. coli EGFP* gene or the *E.coli CD* gene into the E1 region of the genome. The E3 region of the adenovirus was deleted. The recombinant adenovirus vectors AdexCAEGFP and AdexCACD were prepared according to previously described methodology [13]. The viral stocks were produced by infecting 293 cells with recombinant adenoviruses, purifying using CsCl density-gradient centrifugation and dialysis against phosphate-buffered saline (PBS) containing 10% glycerol. The stocks were stored at –80 °C. The viral titers were determined with an endpoint cytopathic effect assay on 293 cells and expressed as plaque forming units per milliliter (pfu). Purification of the AdexCAEGFP and AdexCACD viruses yielded concentrations of 1.0×10^{10} PFU/mL and 2.6×10^{10} PFU/mL, respectively.

Ex vivo adenoviral gene transduction of primary MSCs was performed as described previously [11]. Briefly, 1×10^{6} MSCs were plated in 10-cm dishes 1 day before adenoviral infection. The cells were infected by incubation with 3 ml of stocked viral solution containing either 1000 pfu/cells of AdexCAEGFP or 20 pfu/cells of AdexCACD at 37 °C in 5% CO_2 for 8 hours. For the first 3 hours of the infection, the cells were shaken every half hour. Eight-hours after infection, the cells were supplemented with 7 ml of

normal medium and 48 hours after infection, the infected MSCs (MSC-CD, MSC-EGFP) were used for the experiments.

CD enzyme activity assay

To show whether AdexCACD vectors produce a functional CD enzyme, we selectively measured the concentration of 5-FU converted from 5-FC in the culture medium of MSC infected with the AdexCACD vectors. A total of 1×10^5 MSC were seeded in a 6-well plate and infected with AdexCACD at a multiplicity of infection (MOI) of 20. After 24 hours, medium containing 5-FC (50–200 μg/mL) (Kyowa Yakuhin Co. Ltd, Wakayama, Japan) was added and maintained at 37 °C in 5% CO_2. After 24 hours of incubation, the supernatant was collected and its 5-FU concentration was measured by high performance liquid chromatography (HPLC) [13].

5-FC chemosensitivity of 9L cell co-cultured with AdexCACD-infected MSCs

To evaluate the antitumor effect of the 5-FC/MSC-CD system *in vitro*, 9L-DsR cells were co-cultured with MSC-CD in medium containing 5-FC. The 9L-DsR cells (1×10^4 cells) were plated into 24-well dishes along with increasing amounts of MSC-CD to produce ratios of MSC-CD to 9L-DsR cells of 0, 10, 25, 50, 100, and 200%. As a

control, MSC-EGFP was used at the same ratio as that of MSC-CD to 9L-DsR. At 24 hours after the initial incubation, medium containing 5-FC (0 or 200 μg/mL) was added. This cell mixture was maintained in culture at 37 °C, 5% CO_2 for 72 hours, and the percentage of surviving cells was then determined with the WST-8 assay (Cell Counting Kit, Dojindo Laboratories, Kumamoto, Japan). The DsRed2-positive 9L cells were evaluated by fluorescent microscopy. To confirm the bystander effect, which does not require cell-to-cell contact, a chemosensitivity assay was performed with a double-chamber dish with 0.4-μm pores (BD FALCON, Franklin Lakes, NJ). The MSCs were infected with AdexCACD at an MOI of 0-50. The 9L-DsR cells (1×10^3) were seeded into a 24-well multiwell dish. At 48 hours after infection, the MSC-CD (1×10^4) cells were seeded into a cell-culture insert (24-well format) with 0.4-μm pores. At 72 hours after infection, the cell-culture inserts of MSC-CD were placed in the multiwell dish with 9L-DsR in medium containing 5-FU (1.0 mmol/L) or 5-FC at various concentrations ranging from 0 to 1000 μmol/L. The cells were maintained at 37 °C, 5% CO_2 for 72 hours and the percentage of surviving 9L-DsR cells was then determined with a WST-8 assay.

In vitro cell migration assay

The cell migration assay was performed in double-chamber culture dishes (BD FALCON, Franklin Lakes, NJ). The MSC-EGFP cells (5.0×10^4) were placed in the upper chamber with 8-μm pores, and the 9L cells (0, 2.5×10^4 and 2.5×10^5 cells) were placed in the lower well. The cell-culture inserts were placed at 37 °C, 5% CO_2 for 24 hours, then the number of MSC-EGFPs in the lower side in one high-power field by fluorescent microscopy were directly measured (40× magnification; Olympus; Tokyo, Japan) [11]. Each experiment was done in triplicate.

Intracerebral distribution of implanted MSCs

To evaluate the intracerebral distribution of the MSCs, rats harboring 9L-DsR brain tumors were used for the experiment. Experimental animals were housed and handled in accordance with Okayama University Animal Research Committee guidelines. To establish the brain tumor model, 6-week old male Fisher-344 rats (Clea Japan, Inc.) (n=3) were anesthetized with intraperitoneal (i.p.) nembutal (30 mg/kg) and placed in a stereotactic apparatus (Narishige, Tokyo, Japan). The 9L-DsR cells (5×10^5 cells/5 μL) were slowly injected into the basal ganglia of the right cerebral hemisphere (3 mm lateral to the midline, 1 mm posterior to the coronal suture, 4 mm deep from the dura) with a Hamilton syringe (Hamilton, Reno, Nev, USA), according to previously

published procedures [13]. Seven days after tumor inoculation, rats bearing brain tumors were re-anesthetized and received an intratumoral (i.t.) injection of MSC-EGFP (1×10^6 cells/5 μL) or PBS only (control; 5 μl) according to the same stereotactic coordinates. At 14 days after tumor inoculation, the rats were perfused with PBS and 4% paraformaldehyde while under deep anesthesia. The excised brains were postfixed with 4% paraformaldehyde overnight and then equilibrated in PBS containing 30% sucrose for 48 hours. The fixed brains were embedded in OTC (Sakura Fintek USA, Inc., Torrance, USA), snap frozen in liquid nitrogen and stored at –70 °C. The tissues were cryosectioned at 18-μm thickness and stained with hematoxylin and eosin (H&E) or with an anti-GFP monoclonal antibody (Medical & Biological Laboratories Co. Ltd, Nagoya, Japan). The sections stained with the first antibody were visualized with a Vectastain kit obtained from Vector Laboratories (Burlingame, CA, USA). The images were acquired with a fluorescent microscope (Biorevo BZ-9000, Keyence, Osaka, Japan).

The antitumor effect of the 5-FC/MSC-CD system in vivo

To assess the antitumor effect of the 5FC/MSC-CD system *in vivo*, 5.0×10^5 9L cells mixed with an equal quantity of MSC-CD cells or MSC-EGFP cells (control) were

implanted subcutaneously into the right flanks of 6–8 week old nude mice (balb/c-nu/nu, CLEA Japan, Inc, Tokyo, Japan). The mice had a median weight of 20 grams. The mice were treated i.p. with 5-FC (500 mg/kg) or PBS (control) 48 hours after tumor implantation. This treatment was repeated once a day for 2 weeks. Tumor length, height and width were measured two times per week with calipers by a researcher blinded to each animal's treatment group.

We also evaluated the antitumor effect of MSC-CD in rats harboring 9L brain tumors. To establish the brain tumor model, the 9L-DsR cells (5×10^5 cells/5 μL) were slowly injected into the right basal ganglia (3 mm lateral to the midline, 1 mm posterior to the coronal suture, 4 mm deep from the dura) of Fisher-344 rats. Five days after tumor inoculation, rats bearing brain tumors were re-anesthetized and received an intratumoral injection of MSC-CD or MSC-EGFP (1×10^6 cells/5 μL) or PBS only (control; 5 μl) according to the same stereotactic coordinates. Treatment with i.p. 5-FC (500 mg/kg) or PBS (control) was commenced 48 hours after injection of the MSC and repeated once a day for 2 weeks. The animals were monitored and sacrificed near death, and survival analysis was conducted.

Statistical analysis

The data are expressed as the mean ± standard deviation. The chemosensitivity, *in vitro* migration ability, and the size of flank tumor in each group was compared using a one-way ANOVA followed by a Scheffé's post hoc test. Kaplan – Meier curves were compared using the log-rank test. All statistical tests were two-sided. A *P*-value of less than 0.05 was considered significant. All statistical analyses were performed with the use of SPSS statistical software (version 14.0; SPSS, Inc., Chicago, IL).

Results

CD enzyme activity

The conversion of 5-FC to 5-FU was measured by HPLC after 24 hours of incubation. The concentration of 5-FU increased in a dose-dependent fashion in the supernatant of the medium containing MSCs infected with the AdexCACD vector, with 1.84, 2.92, 5.42μg/ml of 5-FU concentration when the medium containing 50, 100, 200μg/ml of 5-FC, respectively. Data are expressed as 5-FU concentration in the supernatant relative to the 5-FC added in the culture medium (Figure 1).

The antitumor and bystander effects of the 5-FC/MSC-CD system in vitro

To evaluate the antitumor effect of the 5-FC/MSC-CD system *in vitro*, the 9L-DsR cells

were co-cultured with MSC-CD or MSC-EGFP in medium with or without 5-FC. The number of DsRed-positive cells was determined. As shown in Figure 2, the proliferation of 9L-DsR cells was significantly inhibited by co-culture with only 10% MSC-CD in the medium containing 5-FC relative to the control medium. Next, to confirm that the bystander effect did not require cell-to-cell contact, a chemosensitivity assay was performed using double-chamber dish. The MSCs were infected with AdexCACD at an MOI of 0–50 (Figure 3). A marked, dose-dependent suppression of 9L-DsR cell growth in the medium with 5-FC was observed, regardless of the MOI (10, 20, 50). There was no statistical difference in growth suppression between either MOI at any concentrations of 5-FC.

Migratory ability of MSCs in vitro

The migratory nature of MSC towards glioma cells was evaluated by double-chamber assay *in vitro.* Although the MSC-EGFP did not migrate without 9L cells in the lower chamber, they were stimulated to migrate by the addition of 9L cells into the lower chamber. Of the MSC-EGFP (5.0×10^4 cells) placed in the upper chamber, 9.0% (4.5×10^3 cells) moved to the lower side in the presence of 2.5×10^4 9L cells. In contrast, 38% (1.9×10^4 cells) moved to the lower side in the presence of 2.5×10^5 9L cells. The

migration of MSCs increased in a dose-dependent manner with increasing numbers of 9L cells ($P < 0.005$) (Figure 4A and 4B).

Intracerebral distribution and tumor tropism of implanted MSC

After the confirmation of the *in vitro* migratory ability of MSCs, we investigated whether implanted MSCs could migrate toward an intracranial glioma *in vivo*. The 9L-DsR glioma cells were inoculated into the right basal ganglia, and the MSC-EGFP were injected directly into the glioma 7 days later. The rats (n=3) were sacrificed 14 days after tumor inoculation and the brain sections were prepared. The EGFP-labeled MSCs infiltrated into the established tumor in a relatively uniform manner (Figure 5A). Moreover, the GFP-labeled MSCs were densely localized at the border between the tumor and normal parenchyma. By fluorescent microscopy, the GFP-labeled MSCs, most of which retained their spindle-like shape, infiltrated into the DsRed-labeled 9L tumor (Figure 5B). Additionally, the MSCs were observed around the glioma cells, which spread out from the main tumor (Figure 5C). By fluorescent microscopy, no GFP-positive MSC was observed in distal brain parenchyma where no DsRed-labeled 9L tumor cell invaded. Similar distribution of MSC was observed in all animals. Thus, the MSCs demonstrated significant migratory capability and glioma tropism in the

established *in vivo* tumor model.

In vivo antitumor effect of the 5-FC/MSC-CD system

The antitumor efficacy of 5-FC/MSC-CD was tested on subcutaneous tumors in athymic mice. The 9L cells mixed with MSC-CD or MSC-EGFP (control) were implanted subcutaneously into the right flanks of athymic mice. The mice harboring subcutaneous tumors (150–250 mm^3) were treated i.p. with 5-FC (5-FC/MSC-CD), while the control animals were treated i.p. with 5-FC (5-FC/MSC-EGFP) or PBS (PBS/MSC-EGFP). The animals were closely monitored for tumor growth. Figures 6A and B shows the tumor growth of individual mice from the groups treated with PBS/MSC-EGFP (top panel), 5-FC/MSC-EGFP (middle panel) and 5-FC/MSC-CD (bottom panel). All mice treated with i.p. PBS rapidly developed subcutaneous tumors. There was a significant difference in tumor size in mice treated with 5-FC/MSC-CD compared with those treated with PBS/MSC-EGFP or 5-FC/MSC-EGFP.

Survival of the rats bearing the 9L gliomas treated with 5-FC/MSC-CD were also analyzed (Figure 7). Control rats treated with PBS/PBS and PBS/MSC-EGFP had both median survivals of 28 days after tumor cell implantation. The rats treated with PBS/MSC-CD and 5FC/MSC-EGFP had median survivals of 31 and 31.5 days after

tumor cell implantation, respectively. Compared with the animals treated with 5-FC/MSC-EGFP, those treated with 5-FC/MSC-CD had a significantly prolonged survival (31 days versus 44 days, log-rank $P < 0.001$). In the control groups, there was little difference in the survival of the animals treated with PBS/PBS compared with other control groups. No systemic toxicity due to 5-FC was observed when the rats were treated with i.p. injections of 5-FC at 500 mg/kg/day for 14 days without intracerebral injection of 9L rat glioma cells (data not shown).

Discussion

Glioma tropism of MSC

In this study, we demonstrated that MSCs have the ability to migrate towards glioma cells *in vitro* and *in vivo*. While our study was subjected by using rat MSCs and rat glioma cell line, the migratory activity of MSCs was demonstrated in human cell lines similarly. Park et al. reported that human umbilical cord blood-derived MSCs (hUCB-MSCs) were able to migrate toward the human glioma cell lines (U-87MG, U-251MG, three primary glioma cell lines)[15]. These results suggested that soluble factors released from 9L glioma cells were responsible for the tropism of the MSCs for the glioma cells. This is in agreement with previous reports, which suggest that soluble

factors, or chemoattractants, may include various chemokines, cytokines and growth factors. For example, platelet-derived growth factor (PDGF-BB), epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF-A) have all been shown to enhance tumor tropism of MSCs [10, 16]. Birnbaum *et al*. reported that the tumor tropism of MSCs is dependent on interleukin-8 (IL-8), transforming growth factor-β1 (TGF-β1) and neurotropin-3 (NT-3) [17]. In comparison, Xu *et al*. reported that monocyte chemoattractant protein-1 (MCP-1) and stromal-cells derived factor-1 alpha (SDF-1α) play a role in migration of MSCs toward gliomas [18]. Furthermore, Park et al. reported that overexpression of the SDF-1α receptor, CXCR4, on hUCB-MSCs enhanced the migratory capacity of MSCs toward gliomas [15]. In addition, it has been suggested that MSC migration requires extracellular matrix (ECM) as the toehold. To this end, Schichor *et al.* reported that laminin and tenascin were involved in MSC migration [16]. In fact, it has been reported that laminin, tenascin, fibronectin, vitronectin and different types of collagen are upregulated within the glioma stroma and at the advancing edge of the tumor within the brain parenchyma [19, 20].

Advantages of MSCs as a glioma-targeting vehicle

Despite the genetic heterogeneity of malignant gliomas, common aberrations in the signaling elements involved in their angiogenesis and invasion pathways are known to exist [21]. Although anti-VEGF therapy (e.g., bevacizumab) seems to be effective in normalizing abnormal tumor vasculature, leading to an enhanced response to radiation and chemotherapy, tumors eventually become resistant to the therapy and adopt a highly infiltrative and invasive phenotype. Therefore, it is important to develop a therapeutic option with the ability to track the tumor cells and coupled with one that has multiple mechanisms through which the antitumor activity can occur. There are several advantages of MSCs as a targeting vehicle for malignant glioma. As shown by our experiments, MSCs show active *in situ* targeting capabilities toward invading glioma cells, whereas conventional antitumor agents including chemotherapeutic drugs and viral vectors have only passive distributive effects after administration. Findings by Nakamura *et al* showed the intracranial distribution of MSCs resembled capsule-like structures around the tumor mass [11]. This unique intracranial distribution of MSCs might act as a barrier preventing the spread of glioma cells into the normal parenchyma. Recently, findings suggest that MSCs secrete large amounts of angiogenic factor angiopoietin-1 (Ang1) [22]. Coupled to this is the knowledge that Ang1 can inhibit tumor-vascular leakage and also tumor

growth *in vivo*. Therefore, Ang1 released from MSCs could represent an additional antitumor effect of MSC. Moreover, other neurotrophic factors are released from MSCs, including nerve growth factor (NGF) [23], which can induce the differentiation and the growth-inhibition of C6 glioma cells *in vitro*. This may act as a potential mechanism underlying the antitumor effect exerted by MSCs [24]. In the cerebral infarction model, it has been reported that implanted MSCs mediate neural protection through the inhibition of neuronal apoptosis and this protective effect is thought to be because neurotrophic factors such as NGF, are released from the MSCs [23]. Therefore, the implantation of MSCs for the treatment of gliomas might be beneficial both with respect to its antitumor potential and its protective effect on normal brain tissues.

Application of genetically engineered-MSCs for malignant glioma

Our results add to the list of applications for MSCs that have been presented to date. Previous studies have shown that the genetic manipulation of MSCs, either by overexpressing targeting molecules or by introducing exogenous genes for the expression/secretion of a desired therapeutic factor, have the ability to improve the migratory efficiency to specific tumor cells and enhance the antitumor effect of MSCs. Nakamura *et al*. reported antitumor effect of genetically engineered MSC expressing

human IL-2[11]. Their findings showed prolonged survival in rats bearing 9L brain tumors when treated with MSCs expressing IL-2 compared to those treated with unmodified MSCs. Therefore in this study, IL-2 gene modification of MSCs conferred additional therapeutic benefits. Chen *et al* [25]. transduced MSCs with an adenovirus engineered to secrete interleukin-12. Human MSCs, engineered to express interferon-β (IFN-β), have been used for targeted delivery of IFN-β, a potent antiproliferative and proapoptotic agent, in both metastatic [26] and gliomas [10] models. Studies have also shown the antiproliferative, antitumor and immunomodulatory effects of IFN-α [27], a multifunctional regulatory cytokine. Mohr *et al*. reported the ability of an adenoviral vector expressing TRAIL to transduce MSCs and the subsequent therapeutic efficacy of these MSCs in a lung cancer model [28]. The potential of mesenchymal progenitor cells to assist the delivery of an oncolytic virus which targets virus-mediated lysis of tumor cells has been evaluated and may be a novel approach for human glioma therapy [29-32].

Suicide-gene transferred-MSC for malignant glioma

In our therapeutic system, the 5-FC/CD suicide gene therapy was found to exert its antitumor effect mainly via a bystander effect. The CD gene-transferred MSCs navigated to the glioma cells among the normal brain parenchyma, and the conversion

of 5-FC into 5-FU by the CD gene in the vicinity of the migrated glioma cells can impair these single tumor cells. The strong bystander effect of the 5FC/MSC-CD system means that tumor regression is possible using nontoxic levels of 5-FC, even if only a small percentage of MSCs expresses CD. Amano *et al*. reported that GCV/HSVtk gene therapy combined with MSCs was effective in the treatment of rat glioma [33]. In that system, however, the bystander effect of GCV/HSVtk gene therapy required direct cell-to-cell contact and was depended upon the formation of gap junctions between contact cells and upon the expression of connexin-43 in the cells [34]. In contrast, the bystander effect of 5-FC/CD gene therapy does not require direct cell-to-cell contact [10, 13]. Therefore, invasive glioma cells may become extensively disordered if MSCs reach the vicinity of tumor cell.

Future perspectives

Although treatment with the 5-FC/MSC-CD system was shown to be effective for the highly invasive malignant glioma, hurdles must be overcome before this therapeutic system could be implemented. First, the experiments conducted in this study were performed in a small animal model. Nevertheless, the capacity of MSCs to migrate

through the brain parenchyma suggests that these cells can survive within the CNS for a prolonged period of time, a prerequisite condition for use of human MSCs within the much larger human brain. Primate study is desirable before clinical application. Second, the available volume of autologous MSCs is limited. Although our studies have focused on bone marrow–derived MSCs, recent work suggests that other cells in the bone marrow, such as the marrow-derived neural-competent cell (MDNCC), may also be a useful delivery vehicle for brain tumors [35]. Third, preclinical data and the results of early patient trials with cell-based gene therapy suggest that the generation of secondary malignancies is a potential risk [36, 37]. In our system, tumorigenic transformation of MSCs may be avoided because the MSCs themselves, in theory, are killed by the 5-FU.

Conclusions

In this study we demonstrated the tumor homing capabilities and antitumor effects of the 5-FC/MSC-CD system *in vitro* and *in vivo*. This system demonstrated a potent bystander effect with the ability to kill tumor cells even when the MSCs and tumor cells were not in direct contact leading to the invading glioma cells becoming extensively disordered. This system may represent a promising new therapeutic approach for highly invasive malignant glioma.

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Conflict of interest

None of the authors have any conflicts of interest to declare.

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Figure legends

Figure 1. The conversion of 5-FC to 5-FU mediated by cytosine deaminase (CD) *in vitro*

The MSCs were infected with AdexCACD at an MOI of 20 and treated with 5-FC. The concentration of 5-FU in the supernatant increased in a dose-dependent manner, with 1.84, 2.92, 5.42μg/ml of 5-FU concentration when the medium containing 50, 100, 200μg/ml of 5-FC, respectively.

Figure 2. The antitumor and bystander effects of the 5-FC/MSC-CD system *in vitro*

The 9L-DsR cells were co-cultured with MSC-CD or MSC-EGFP in medium with or without 5-FC, and the number of DsRed-positive cells was then determined. The proliferation of 9L-DsR co-cultured with MSC-CD in media containing 5-FC was significantly inhibited (MSC-CD/5FC(+)) (mean ± SD, n = 4).

Figure 3. Validating the bystander effects of the 5-FC/MSC-CD system *in vitro*

To confirm that the bystander effect does not require cell-to-cell contact, a chemosensitivity assay was performed in a double-chamber dish. Proliferation of 9L-DsR cells in the lower chamber was inhibited significantly when the MCS-CD cells

were cultured in the upper chamber with medium containing 5-FC (mean ± SD, n = 4).

Figure 4. Migratory ability of MSCs *in vitro*

A: MSC-EGFPs in the upper chamber were stimulated to migrate to the lower chamber by the addition of 9L cells into the lower chamber. The number of migrated MSCs increased in a dose-dependent manner with increasing numbers of 9L cells (*P = 0.0012, **P = 0.0005) (mean ± SD, n = 3). **B:** A fluorescent microscopic view of the migrated MSC-EGFPs in the lower chamber. Magnification, ×40.

Figure 5. Intracranial distribution and tumor tropism of implanted MSCs

The MSC-EGFP cells were injected at the center of the 9L-DsR brain tumor. The rats were sacrificed and their brains were excised 7 days after the MSC injection.

A: Macroscopic photograph of immunostaining with anti-GFP monoclonal antibody. The GFP-positive MSCs (brown) infiltrated into the established tumor in a relatively uniform manner. The GFP-labeled MSCs were densely localized at the border between the tumor and normal parenchyma. Scale bar = 1mm.

B, C: Immunofluorescence microscopy showing the border zone between the

DsRed-labeled 9L tumor (red) and normal parenchyma (B) and the invading tumor cells in the normal brain parenchyma (C). The GFP-labeled MSCs (green), most of which retained their spindle-like shape, accumulated in the tumor border zone (B), although some tracked, or navigated, to the tumor cells adjacent to the main mass (C). Scale bar = 100 μm.

Figure 6. *In vivo* antitumor effects of the 5-FC/MSC-CD on mice subcutaneous tumors

A: 9L cells mixed with MSC-CD or MSC-EGFP (control) were implanted subcutaneously into the right flanks of nude mice. The mice were treated with i.p. 5-FC or PBS. There was a significant difference in tumor size in the mice treated with 5-FC/MSC-CD compared with those treated with 5-FC/MSC-EGFP and PBS/MSC-EGFP ($P < 0.005$) (mean ± SD, n = 4).

B: Representative figure showing the size of subcutaneous tumors in the anesthetized mice. At 35 days after tumor inoculation, significant growth suppression was observed in mice treated with 5-FC/MSC-CD (bottom panel) compared with those treated with PBS/MSC-EGFP (top panel) and 5-FC/MSC-EGFP (middle panel).

Figure 7. Survival analysis of rats bearing the 9L brain tumors

Rats bearing 9L brain tumors were divided into five treatment groups; PBS/PBS, PBS/MSC-EGFP, PBS/MSC-CD, 5-FC/MSC-EGFP and 5-FC/MSC-CD. Compared with animals treated with PBS/PBS, PBS/MSC-EGFP, PBS/MSC-CD and 5-FC/MSC-EGFP, those treated with 5-FC/MSC-CD had a significantly prolonged survival (44 days, log-rank $P < 0.001$).

Figures

Figure 1

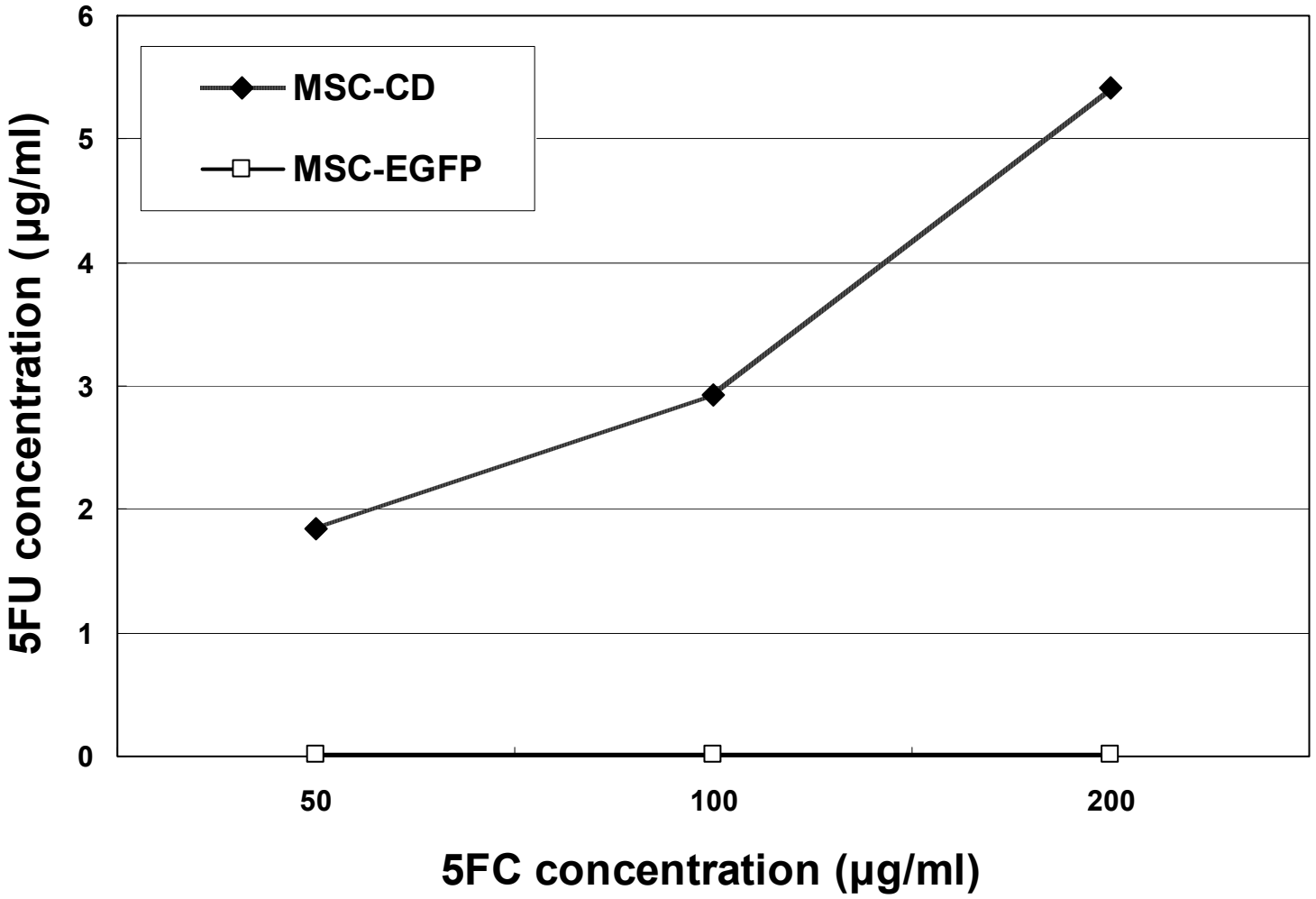

MSC-CD
MSC-EGFP
5FU concentration (μg/ml)
0
1
2
3
4
5
6
50
100
200
5FC concentration (μg/ml)

Figure 2

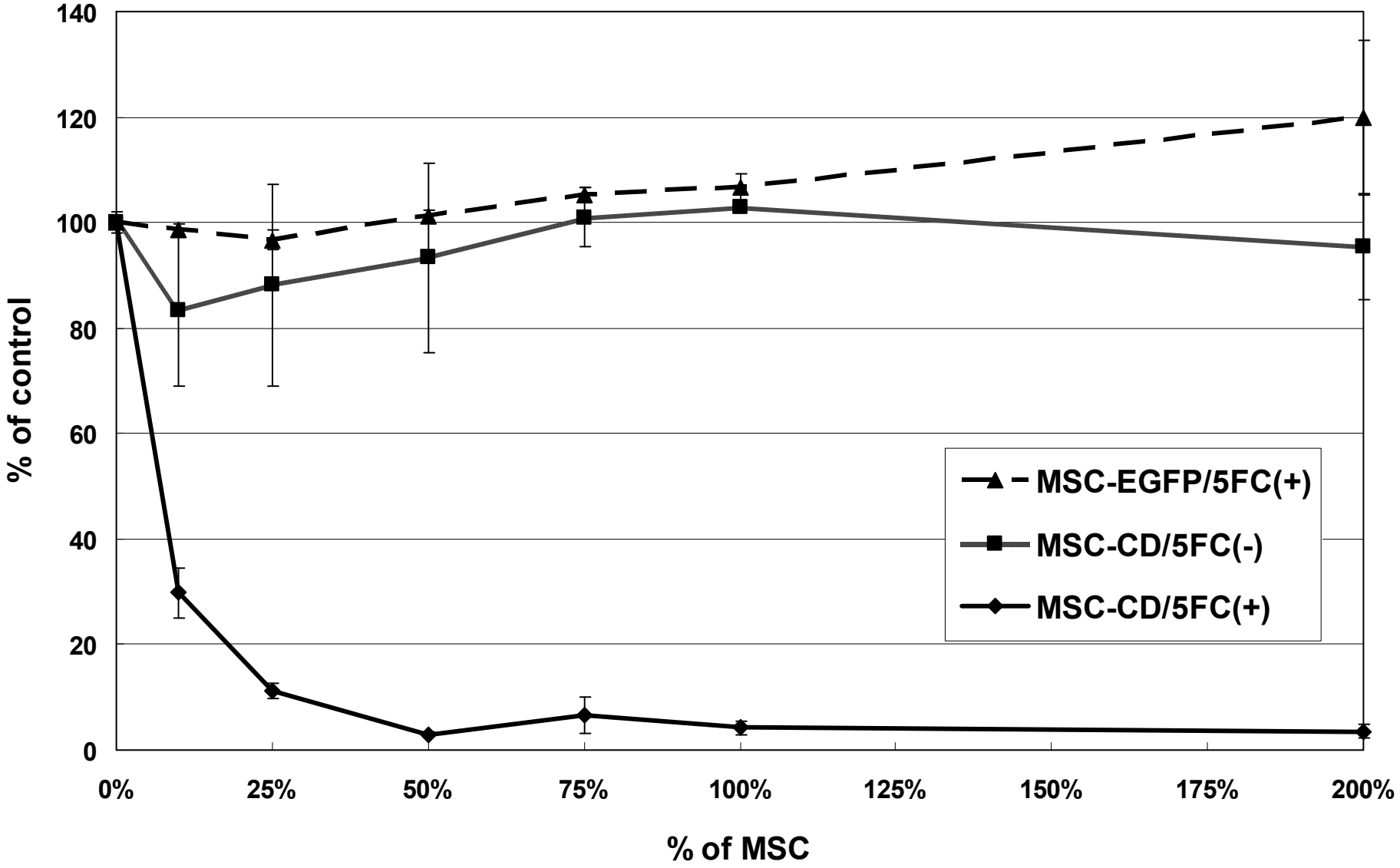

140
120
100
80
60
40
20
0
% of control
0%
25%
50%
75%
100%
125%
150%
175%
200%
% of MSC
MSC-EGFP/5FC(+)
MSC-CD/5FC(-)
MSC-CD/5FC(+)

Figure 3

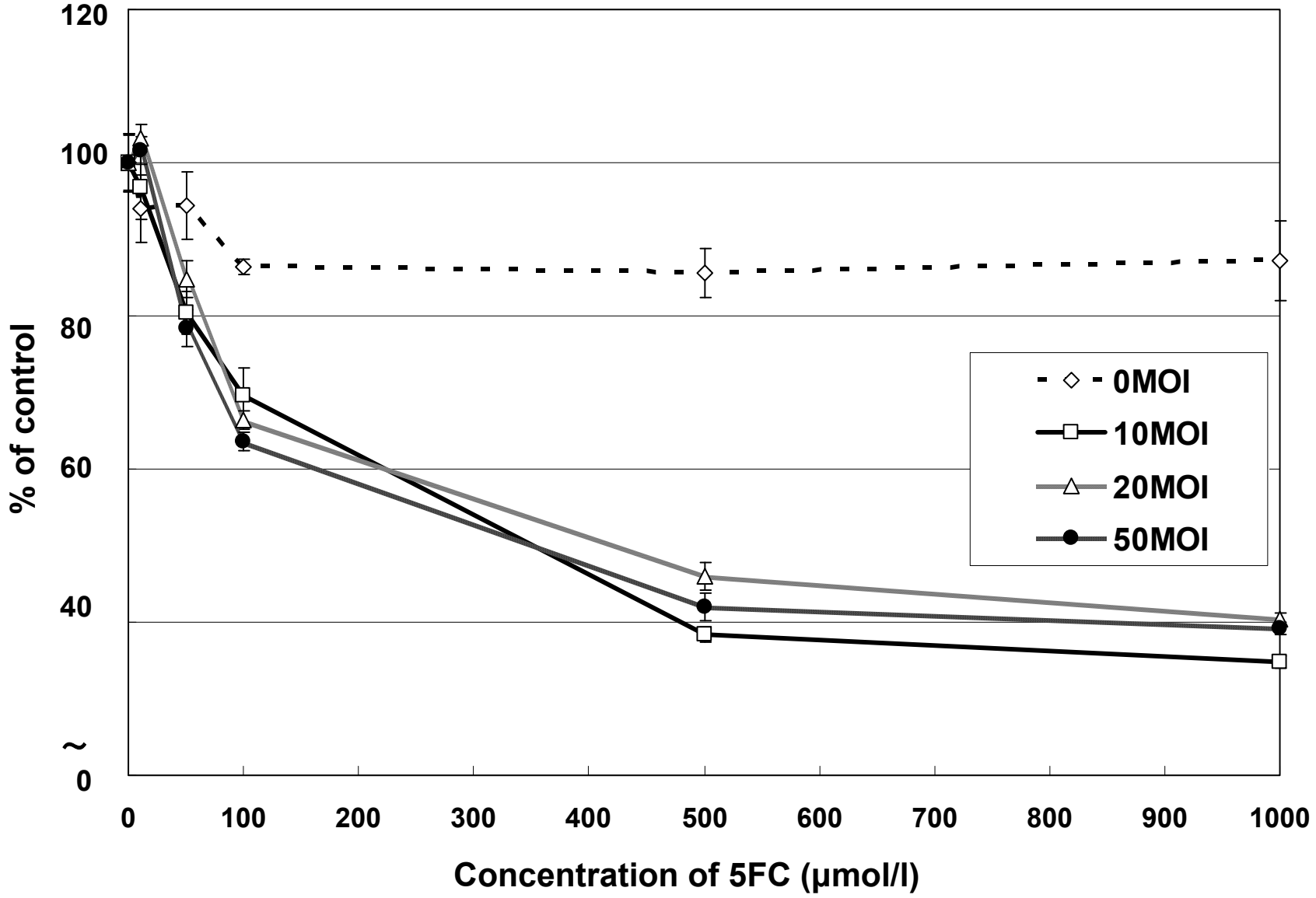

120
100
80
60
40
~
0
% of control
0
100
200
300
400
500
600
700
800
900
1000
Concentration of 5FC (μmol/l)
0MOI
10MOI
20MOI
50MOI

Figure 4A

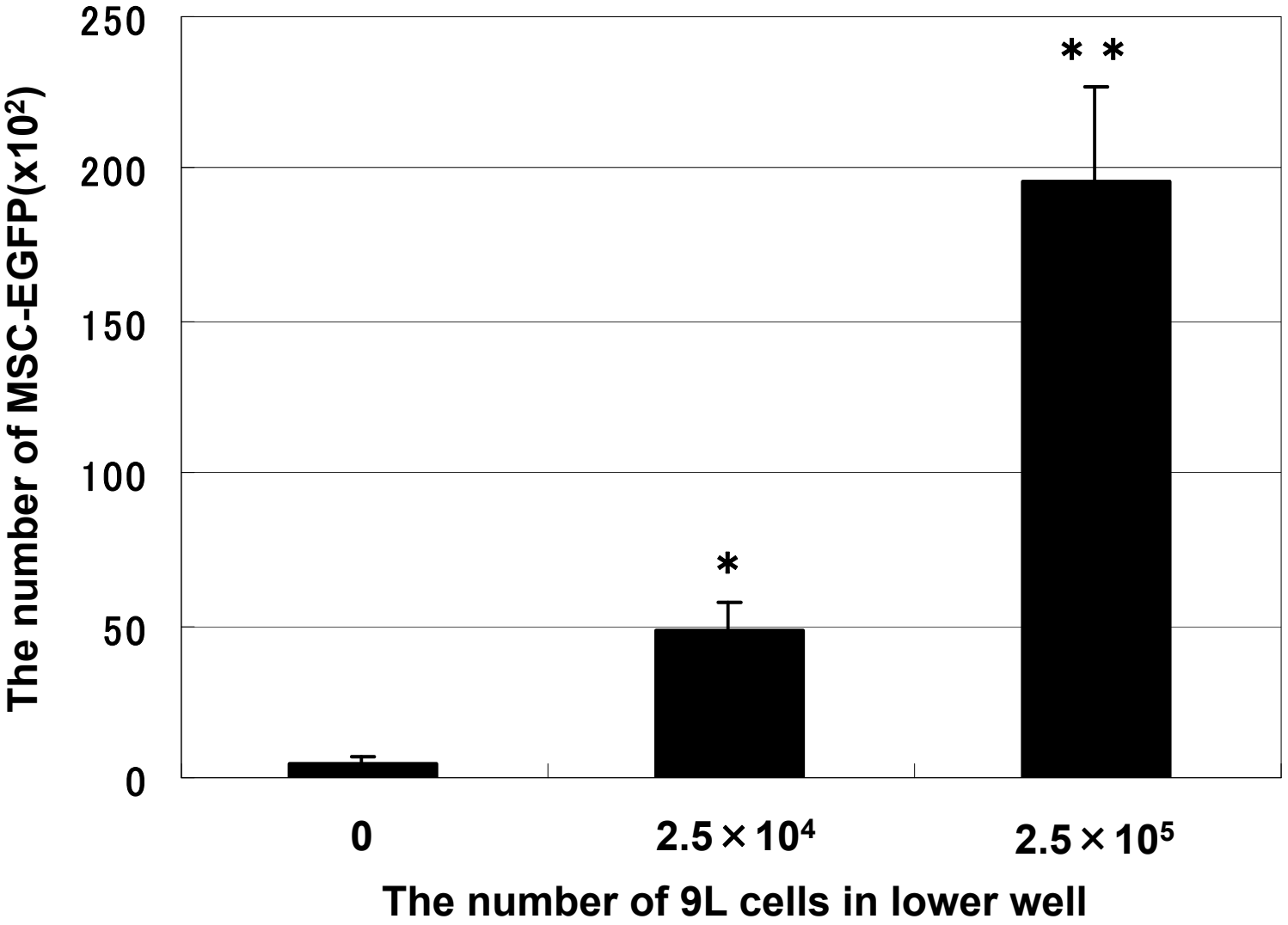

250
200
150
100
50
0
The number of MSC-EGFP(x10²)
* *
*
0
2.5×10⁴
2.5×10⁵
The number of 9L cells in lower well

Figure 4B

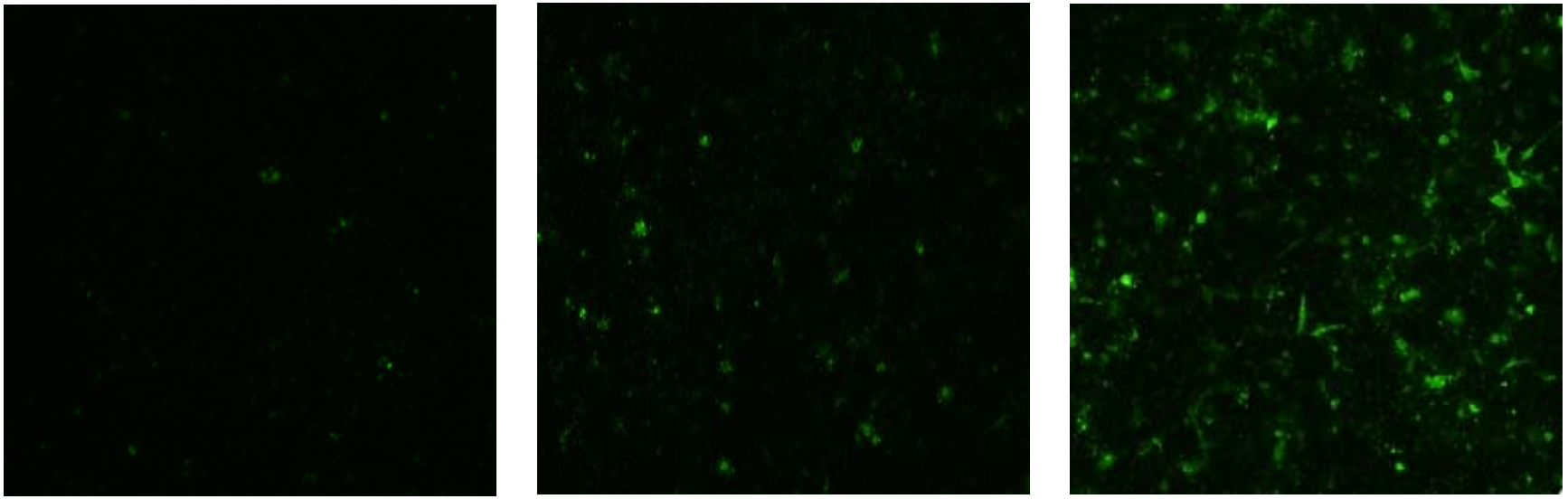


Figure 5

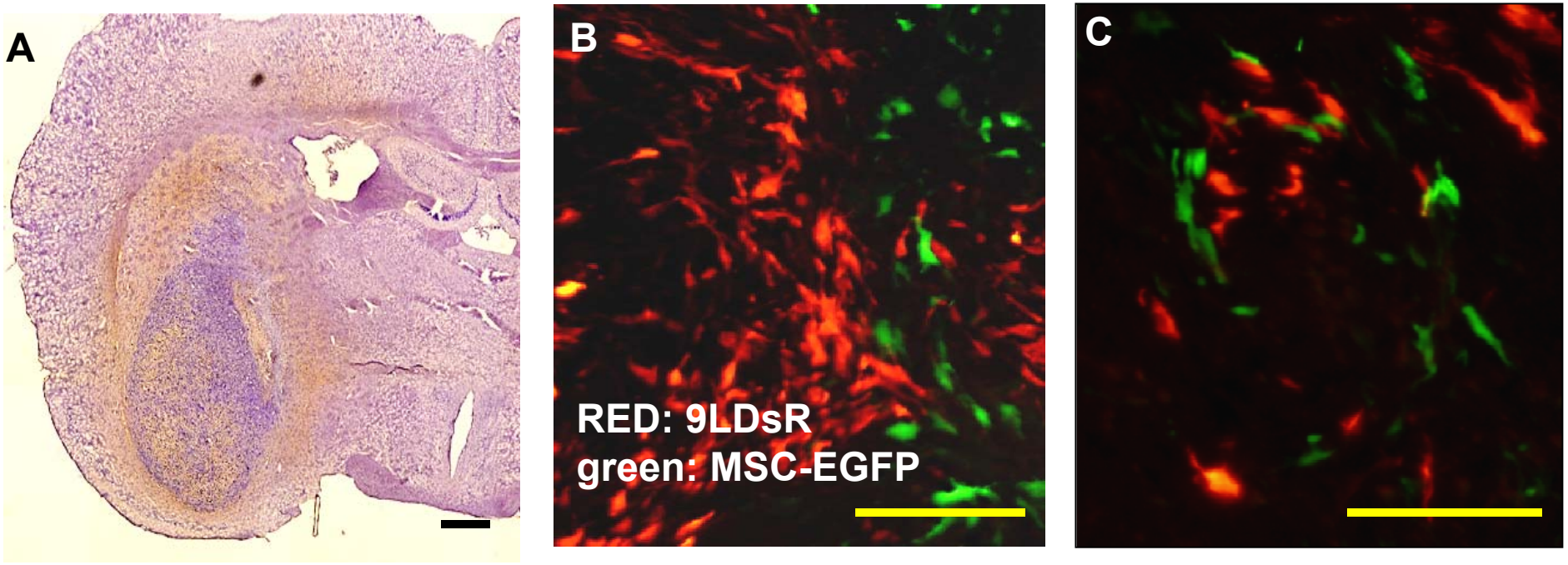


Figure 6A

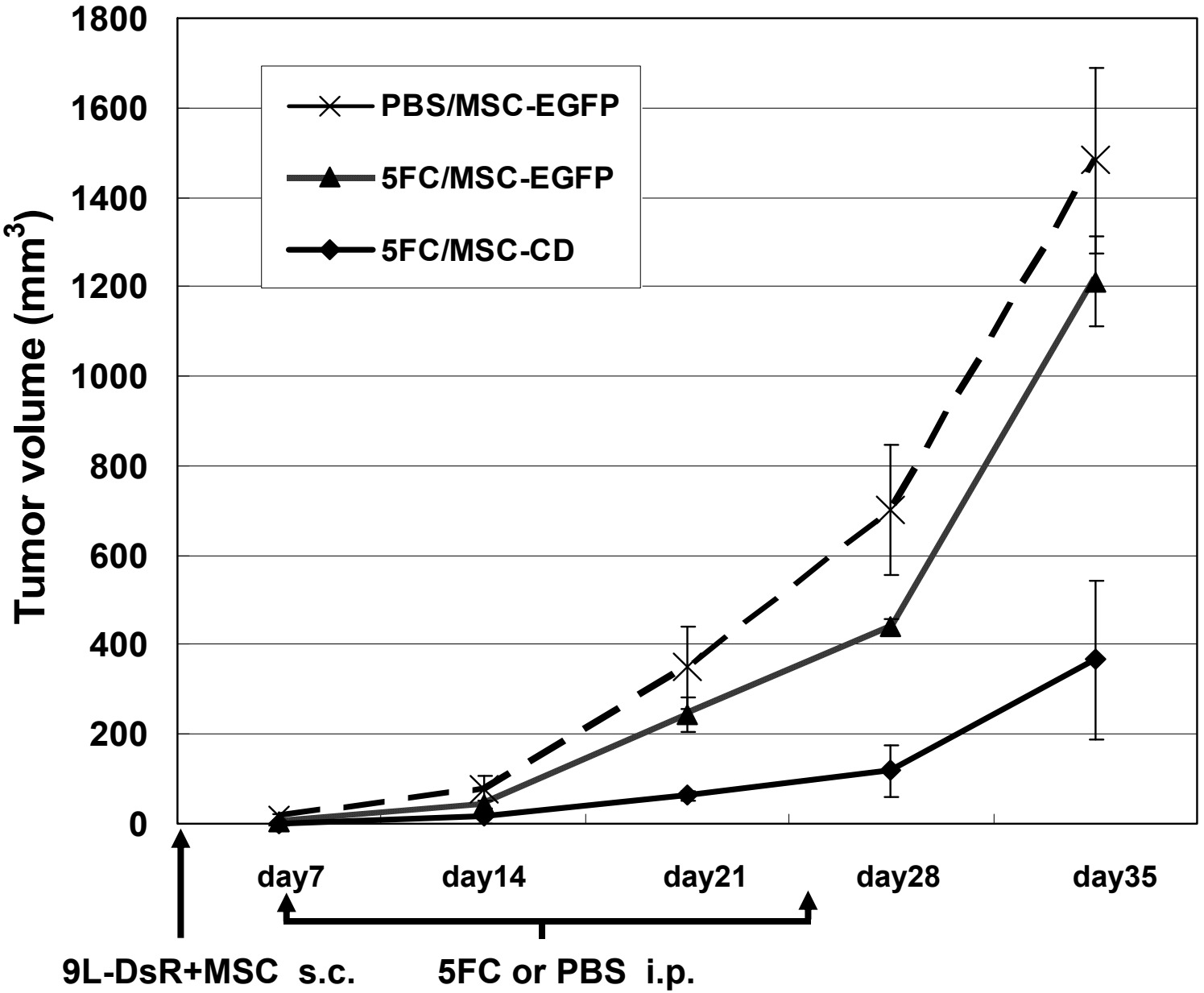

1800
1600
1400
1200
1000
800
600
400
200
0
Tumor volume (mm3)
PBS/MSC-EGFP
5FC/MSC-EGFP
5FC/MSC-CD
day7
day14
day21
day28
day35
9L-DsR+MSC s.c.
5FC or PBS i.p.

Figure 6B

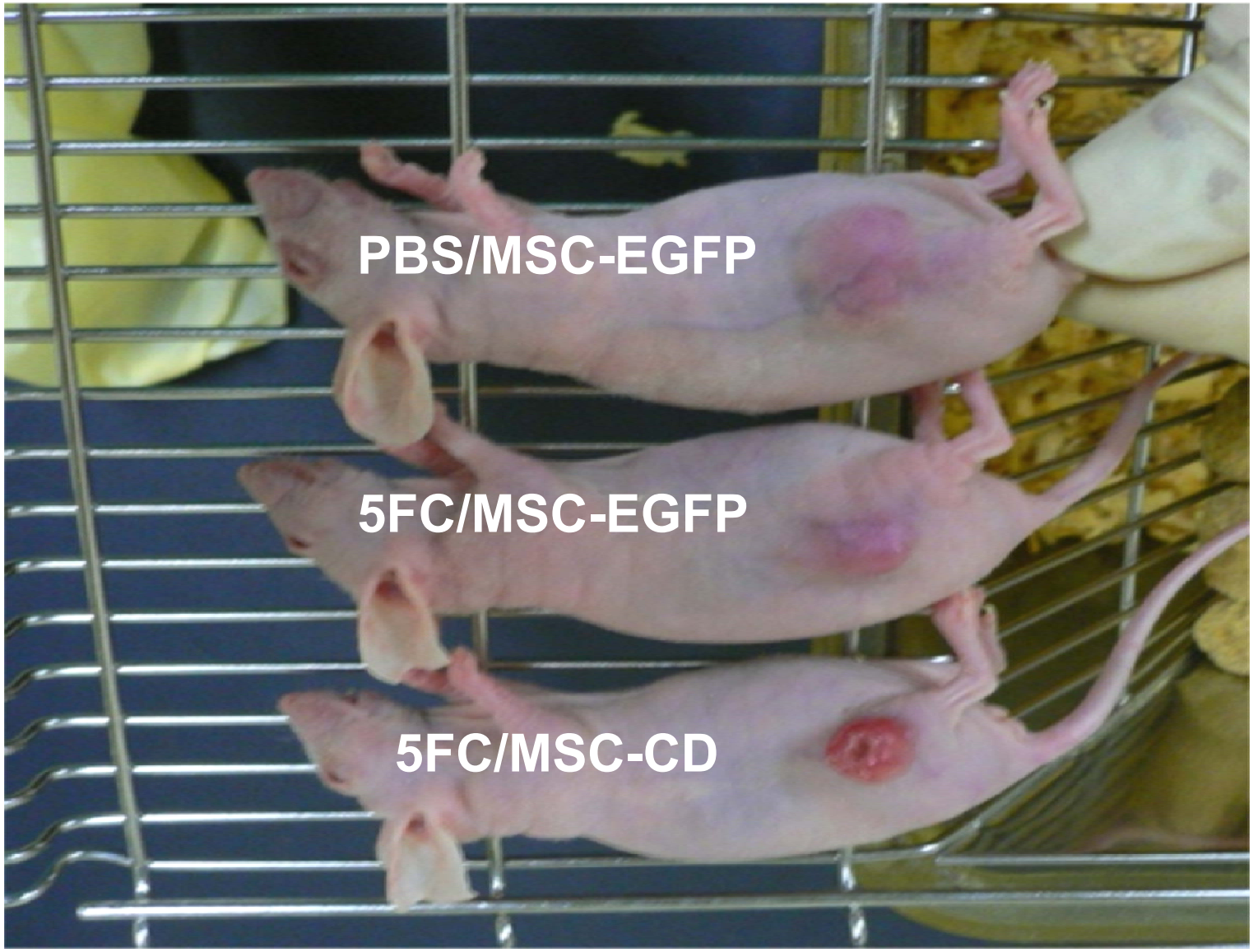
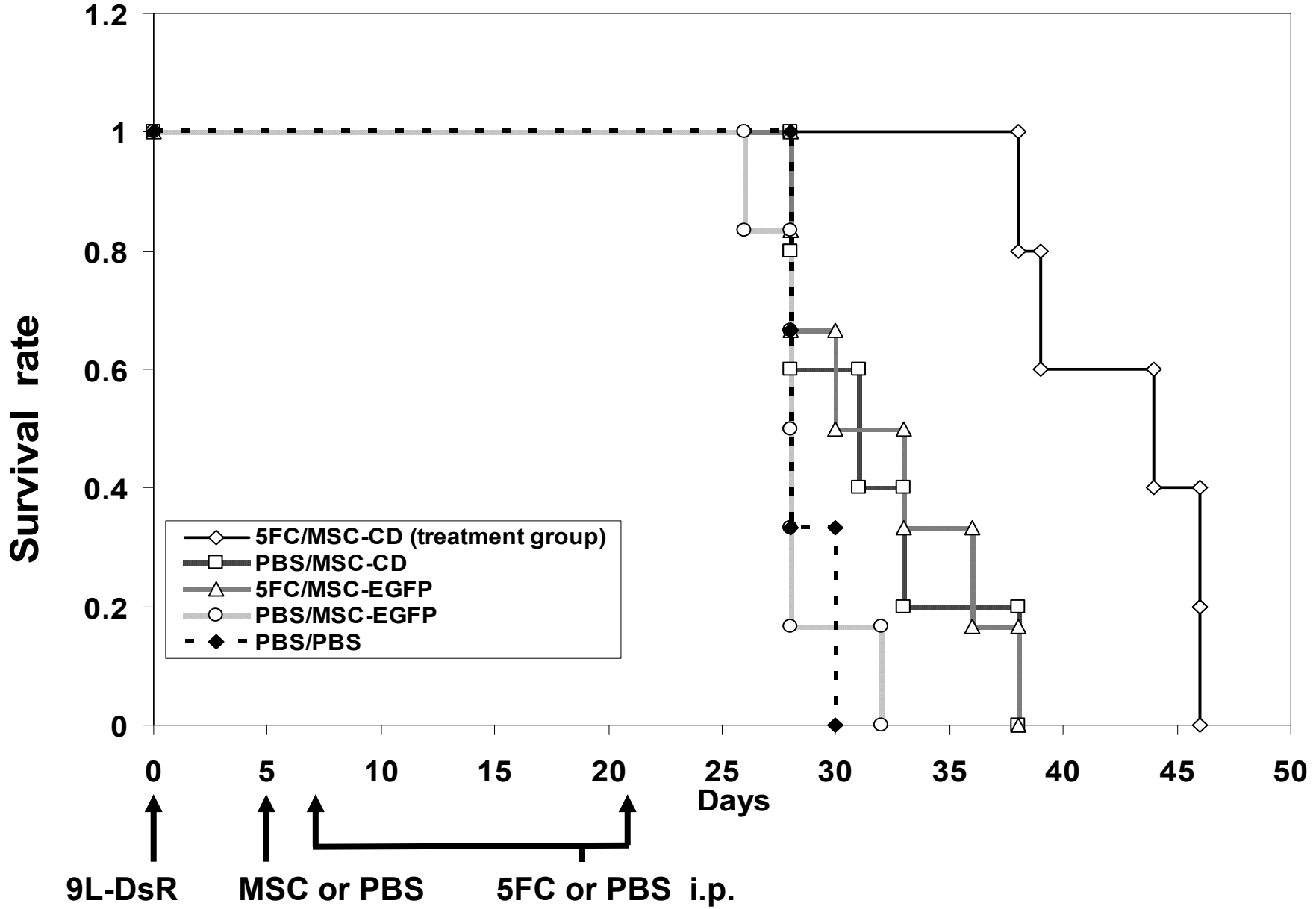


Figure 7


1.2
1
0.8
0.6
0.4
0.2
0
Survival rate
0
5
10
15
20
25
30
35
40
45
50
Days
5FC/MSC-CD (treatment group)
PBS/MSC-CD
5FC/MSC-EGFP
PBS/MSC-EGFP
PBS/PBS
9L-DsR
MSC or PBS
5FC or PBS i.p.