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Timing of Ganciclovir Administration in Glioma Gene Therapy Using HSVtk Gene-transduced Mesenchymal Stem Cells

SHINJI AMANO[1], CHUNYU GU[1,2], SHINICHIRO KOIZUMI[1], TSUTOMU TOKUYAMA[1] and HIROKI NAMBA[1]

[1]*Department of Neurosurgery, Hamamatsu University School of Medicine, Hamamatsu, Japan;*
[2]*Department of Neurosurgery, Shengjing Hospital of China Medical University, Shenyang, P.R. China*

Abstract. *Background: An established C6 glioma was successfully treated with intratumoral injection of mesenchymal stem cells transduced with HSVtk gene (MSCtk) and systemic administration of ganciclovir (GCV). The best timing of GCV administration after the MSCtk implantation was studied. Materials and Methods: GCV administration was started from 2 days before and 1, 3 and 7 days after the MSCtk administration under both in vitro and in vivo conditions. Results: The C6 cells were completely eradicated in vitro when GCV administration was started from day –2, 1, and 3. Animals with intracranial tumor survived longer when GCV was administered earlier after MSCtk administration. This may, mainly, reflect the difference in the MSCtk/C6 ratio at the time of GCV administration because this ratio drastically decreases during the delay of GCV administration. Conclusion: When using a slowly growing vector cell as MSCtk, GCV should be administered soon after MSCtk implantation.*

Tumors of glial origin (gliomas) comprise the most frequent primary neoplasia of the brain in adults. Glioblastoma, the most malignant phenotype of gliomas, is inevitably and rapidly fatal despite aggressive therapy including surgical resection of the tumor followed by radiochemotherapy, and the median survival is about one year from diagnosis (1, 2). Since highly mitotic gliomas exist in an essentially postmitotic background brain tissue and they rarely metastatize outside of the central nervous system, tumor-selective gene therapies have been extensively investigated. The herpes simplex virus thymidine kinase gene (HSVtk)/ganciclovir (GCV) system is one of the most widely used gene therapies for glioma. HSVtk converts a nontoxic GCV to GCV-monophosphate, which is then phosphorylated to the GCV-triphosphate by endogenous kinases. Integration of GCV-triphospate causes premature DNA chain termination and apoptosis of the cells. It is well known that phosphorylated GCV can pass through the gap junctions between the adjacent cells and kill neighboring dividing tumor cells. This interesting and advantageous phenomenon is called the 'bystander effect' (3).

We have demonstrated in an experimental rat model of intracranial glioma that inoculated tumors are successfully treated through the bystander effect by an intratumoral injection of bone marrow-derived mesenchymal stem cells (MSCs) transduced with HSVtk gene (MSCtk cells) followed by systemic GCV administration (4). Subsequent study further confirmed the safety of this treatment strategy towards the background normal brain tissue (5). Therefore, this treatment strategy seems to be clinically feasible. As the next step, the most effective clinical protocol needs to be developed. In our rat experiments using rat C6 glioma cells as the target tumor and MSCtk as the treatment cells, intraperitoneal administration of GCV was started from the day of or the day after the MSCtk injection (4, 5) in order to avoid changes in the MSCtk:C6 ratios due to the difference in cell proliferation; the proliferation rate of C6 cells is quite fast (doubling time of about 24 h), whereas that of MSCs is slow. Delay in GCV administration results in the drastic decrease of the MSCtk:C6 ratio, which then makes the interpretation of the results difficult.

Migratory activity of MSCs towards the glioma in the brain is another important advantage of this treatment strategy, especially under an *in vivo* situation (6-11). Since the bystander effect in HSVtk/GCV gene therapy is only

Correspondence to: Hiroki Namba, MD, Department of Neurosurgery, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu, 431-3192, Japan. Tel: +81 534352281, Fax: +81 534352282, e-mail: hnamba@hama-med.ac.jp

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generated when the transduced cells have contact with target tumor cells (3, 4, 12), an adequate period of time is required for the transduced cells to distribute throughout the tumor that may deeply infiltrate into the normal brain tissue. This would be especially true when the method is applied to human patients whose brain is much larger than that of rodents. In the present study, we aimed to seek the best timing of GCV administration after MSCtk implantation under both *in vitro* and *in vivo* conditions.

Materials and Methods

Isolation of MSCs from Sprague-Dawley rat. All the following experiments were performed according to the Rules of Animal Experimentation and the Guide for the Care and Use of Laboratory animals of the Hamamatsu University School of Medicine. Sprague-Dawley rats (9 weeks old) were obtained from Nippon SLC, Hamamatsu, Japan. Rata were sacrificed with ether. The pelt of the rat was wet thoroughly with 70% isopropyl alcohol and femurs and tibias were removed by severing them from the animal at the hip and ankle, respectively. The interior of the marrow tissue was flushed out using 21-gauge needle attached to a 5-ml syringe. A single cell suspension was obtained by gently aspirating the tissue several times using the same needle and syringe in 5 ml Murine MSC Growth Medium (MMSCGM; StemCell Technologies Inc., British Columbia, Canada) and washed once with 10 ml fresh MMSCGM and incubated at 37˚C under 5% CO_2. The non-adherent cells were removed by replacing the medium 24 h after the initial culture. The residual attached cells were maintained at 37˚C in 5% CO_2 by exchanging the medium with fresh medium at 5-day intervals. These cells are designated as MSCs in the present study.

Establishment of MSCs with HSVtk gene. The HSVtk retrovirus-producing cells (PA317; mouse fibroblst cell line with HSVtk gene) were kindly provided by Genetic Therapy Inc. (Gaithersburg, MD, USA). After culturing the PA317 cells in Dulbecco's Modified Eagle Medium (DMEM) for 48 h, the supernatant was collected.

After removing non-adherent cells 24 h after the initial culture (MSC fraction), 1 ml of the supernatant of HSVtk retrovirus was added into the 25-cm^2 tissue culture flask with 1 ml MMSCGM. The MSCs were incubated in the presence of 8 μg/ml Polybrene (Aldrich Chemical Company Inc., Milwaukee, WI, USA) for 6 h. After three washings, the cells were maintained with fresh MMSCGM. Drug selection with 100 μg/ml G418 (Sigma-Aldrich Japan K.K., Tokyo, Japan) was performed for 4 weeks. The drug-resistant cells were collected and cultured in MMSCGM (containing 100 μg/ml G418) to prepare sufficient numbers of cells. The drug-resistant cells, HSVtk gene-transduced MSCs, were used for further experiments (MSCtk cells).

Timing of GCV administration in in vitro bystander effect. The C6 rat glioma cell line was purchased from ATCC (Manassas, VA, USA). The MSCtk (5×10^3) and C6 cells (5×10^3) were co-cultured in MMSCGM in a 24-well tissue culture plate (day 0). GCV administration (1 μg/ml) to the medium was started from day –2, 1, 3, 7 and continued for 7 days (days –2 to 4, days 1 to 7, days 3 to 9, or days 7 to 13). The cells were cultured in the medium without GCV thereafter. The conditional medium was changed every 2 to 3 days and the number of living cells was determined by counting under a phase-contrast microscope on days 7, 14 and 21. Living cell numbers were counted and averaged in 3 fields for 3 wells.

Duration of GCV administration in in vitro bystander effect. To estimate the minimum duration of GCV administration needed for complete tumor eradication, the MSCtk (5×10^3) and C6 cells (5×10^3) were co-cultured in MMSCGM in a 24-well tissue culture plate as in the previous experiment and GCV administration (1 μg/ml) to the medium was started from day 2 for different durations (1 to 6 days). Living cell numbers were counted and averaged in 3 fields for 3 wells.

Timing of GCV administration in an intracranial tumor model. Studies were performed on 30 adult male Sprague-Dawley rats weighing 300±20 g. Following initial induction with 4% isoflurane, rats were anesthetized intraperitoneally with chloral hydrate (400 mg/kg) and placed in a stereotaxic apparatus (Narishige Scientific Instrument Lab., Tokyo, Japan). After incising the scalp, a burr hole was made at 1 mm rostral and 3 mm right from the bregma. A mixture of MSCtk and C6 cells (1×10^5 each in 10 μl DMEM) was co-injected *via* the burr hole by 23-gauge needle to a point 5 mm ventral from the dura using a micro-injector (1 μl/min). The needle was kept in place for an additional 5 min before removal. The rats were intraperitoneally administered 15 mg/kg GCV twice daily for 7 days and GCV administration was started from day –2, 1, 3 and 7 (days –2 to 4, days 1 to 7, days 3 to 9, or days 7 to 13, n=6 for each group). Control rats were not injected with GCV (n=6). Survival was analyzed by a log rank test based on Kaplan-Meier survival analysis using SigmaStat 3.0 software.

Results

In vitro bystander effect and the timing of GCV administration. C6 cell proliferation was completely inhibited in the co-culture of C6 and MSCtk cells when GCV administration was started from day –2, 1, or 3 and continued for 7 days, even after GCV administration was ceased, but not when GCV administration was started from day 7 (Figure 1). Although the cell number of the latter group (GCV administration from day 7 to 13) was significantly smaller than that of the control group up to day 21 (Figure 1, $p<0.01$), the tumor proliferation ratio was not suppressed after day 14. One of the main reasons why the tumor proliferation ratio was not suppressed when GCV was administered from day 7 is probably because the MSCtk:C6 ratio at the time of GCV administration of this group was too small.

Duration of GCV administration needed for complete tumor eradication. Because complete tumor eradication was observed when GCV administration was started from day 1 or day 3 in the previous co-culture study, we then changed the duration of GCV administration. GCV administration was started from day 2 and continued for different durations (for 1 to 6 days). When GCV administration was continued only for 1 or 2 days, tumor regrowth occurred thereafter. On the other hand, no such regrowth was observed when GCV

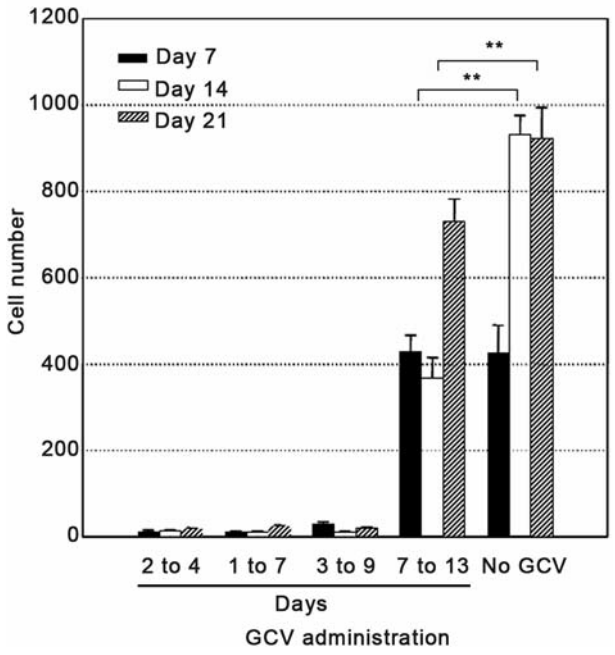

Figure 1. *In vitro bystander effect and the timing of GCV administration The MSCtk and C6 cells were co-cultured and GCV administration (1 μg/ml) was started from day –2, 1, 3, 7 and continued for 7 days (days –2 to 4, days 1 to 7, days 3 to 9, or days 7 to 13). C6 cell proliferation was not observed when GCV administration was started from day –2, 1, and 3 even after GCV administration. When GCV administration was started from day 7, significant tumor growth inhibition was still observed (**p<0.01) but complete tumor cell eradication was not achieved.*

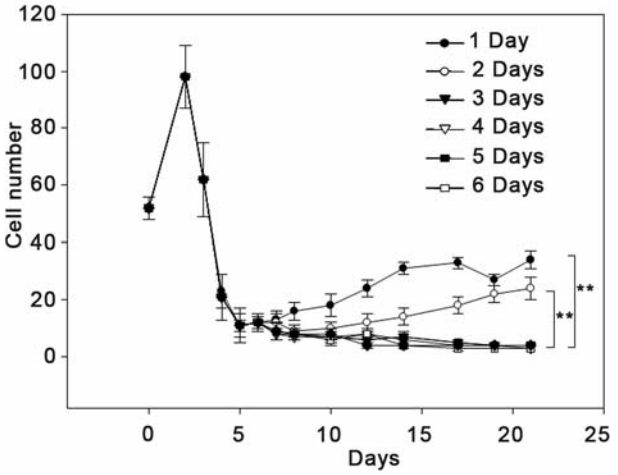

Figure 2. *Duration of GCV administration needed for complete tumor cell eradication. The MSCtk and C6 cells were co-cultured and GCV administration (1 μg/ml) was started from day 2 for different durations (1 to 6 days). When GCV administration was continued for only 1 or 2 days, tumor regrowth occurred, while no regrowth was observed when administration continued for more than 3 days (**p<0.01 on day 21).*

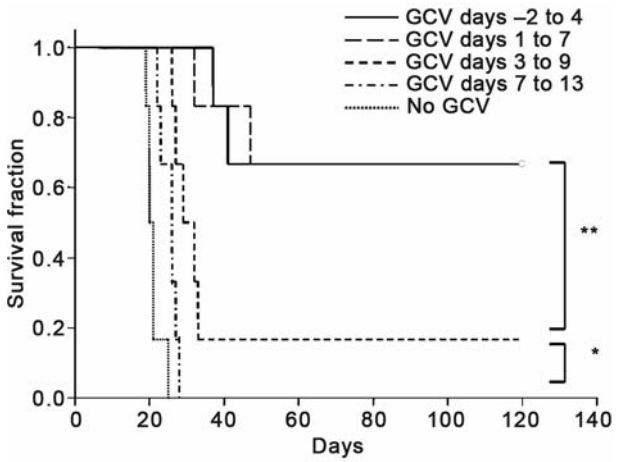

Figure 3. *Animal survival and the timing of GCV administration. A mixture of MSCtk and C6 cells was co-injected into rat brain. Intraperitoneal GCV administration (15 mg/kg twice daily) was started from day –2, 1, 3 and 7 and continued for 7 days (days –2 to 4, days 1 to 7, days 3 to 9, or days 7 to 13). All the rats administered GCV survived significantly longer than the control rats (*p<0.05, not marked). The survival time of the rats administered GCV from day –2 and day 1 was significantly longer than that of rats receiving GCV from day 3 (**p<0.01). The survival of the rats administered GCV from day 3 was still significantly longer than that of rats receiving GCV from day 7 (*p<0.05).*

administration was continued for more than 3 days (Figure 2). The cell numbers of the groups with 1-day and 2-day GCV administration were significantly larger than those of the other groups on day 21 ($p<0.01$).

Animal survival and the timing of GCV administration. The rats implanted with C6 and MSCtk but not administered GCV died of tumor in about three weeks (Figure 3), which was similar to the survival of the rats implanted with C6 only (data not shown). When the rats were administered GCV (15 mg/kg GCV twice daily) either from day –2, 1, 3 and 7 for 7 days, all the rats survived significantly longer than the rats not receiving GCV (Figure 3, $p<0.05$). The survival time of the rats administered with GCV from day –2 and day 1 was significantly longer than that with GCV from day 3 ($p<0.01$). There was no difference between the groups administered with GCV from day –2 and day 1. The survival of the rats administered GCV from day 3 was still significantly longer than that of rats receiving GCV from day 7 ($p<0.05$). Four out of six rats in the groups treated with GCV from day –2 and 1 and one out of six in the group with GCV from day 3 survived more than 120 days and no tumor was histologically shown on day 120.

Discussion

The HSVtk/GCV suicide gene therapy was first introduced in 1992 (3) and then clinically tested using the retroviral vector system (13, 14). In this system, the HSVtk gene is

transduced selectively into the tumor cells because the neural tissues rarely divide. Moreover, even if the gene is not transduced into all the brain tumor cells, there is an additional bystander killing effect on the HSVtk-negative tumor cells. The complete eradication of the tumors was observed in the rat brain tumor model, but clinical studies only demonstrated the safety but not the efficacy of the HSVtk/GCV suicide gene therapy using the retroviral vector system (13, 14). It was thought that retrovirus-producing fibroblast and/or the retrovirus itself was unable to permeate into the glioma and cover the whole tumor including the tumor microsatellites that were the ultimate cause of recurrence. In order to circumvent this shortcoming of retroviral HSVtk/GCV gene therapy, systems using neural stem cells and MSCs as vectors have been developed because these cells are known to have potent tumor-homing activities (4, 15-19). We already demonstrated that tumor implanted in one of the rat cerebral hemispheres could be successfully treated by the injection of HSVtk-transduced neural stem cells in the contralateral cerebral hemisphere followed by systemic GCV administration (20).

In our previous studies using MSCtk and HSVtk-transduced neural stem cells, systemic GCV administration was started immediately after the implantation of transduced cells into the intracranial tumors (4, 5, 16, 20, 21). In the present study, we aimed to seek the optimum timing of GCV administration after the intratumoral injection of MSCtk cells, because there would be an adequate duration for the treatment cells to distribute throughout the tumor. However, we found that this experiment was quite complicated because the MSCtk:C6 ratio in the brain also drastically changed during the delay of GCV administration due to the difference in the proliferation of MSCtk and C6 cells: C6 cells proliferate very rapidly, whereas MSCtk cells proliferate very slowly. Assuming the doubling time of C6 cells to be about 24 h, the MSCtk:C6 ratio on day 3 and 7 is estimated to be 1:8 and 1:128, respectively. Our previous co-culture study at various MSCtk:C6 ratios demonstrated that complete tumor eradication was obtained up to the MSCtk:C6 ratio of 1:32 (4). The results of the present *in vitro* studies agree well with this previous finding because complete tumor cell eradication was obtained with GCV administration no later than 3 days after co-culture of MSCtk and C6 cells (estimated MSCtk:C6 of about 1:8) (Figure 1). Similarly, the *in vivo* study also demonstrated that the earlier GCV administration resulted in better survival of the rats implanted with MSCtk and C6 cells at the ratio of 1:1 (Figure 3). From the present experimental setting, it is concluded that early GCV administration is necessary for better tumoricidal effect and better survival of the tumor-bearing animals. Because most of the established tumor cell lines usually have rapid proliferation rates and this would be a limitation of the rat experiments using tumor cell lines.

Another finding implicated in the present *in vitro* study is that GCV administration needs to be continued at least for 72 h (Figure 2). When the duration of the GCV administration was less than 48 h, the C6 tumor cells, whose proliferation had been suppressed by GCV, started to regrow. On the other hand, no tumor regrowth was observed when GCV administration was continued more than 72 h. Since the duration of GCV administration used as an antiviral drug in the clinical setting is 5 to 7 days, a similar duration of GCV administration is recommended for this treatment strategy.

An important factor for the success of MSCtk therapy in the clinical setting is how the MSCtk cells distribute to the cells not only in the tumor bulk but also tumor satellites infiltrating the surrounding normal brain tissues. Because human brains are much larger than those of rodents, the time needed for MSCtk cells to migrate and distribute to the whole tumor should be taken into account. In our previous *in vitro* co-culture study of MSCtk and C6 cells in the presence of GCV, C6 cells died rapidly, whereas the MSCtk cells survived longer than 48 h and continued exerting bystander effects towards the proliferating tumor cells (4). Therefore, as long as HSVtk-expressing cells with low proliferation, such as MSCtk cells, are used, it is not likely that MSCtk cells are rapidly eradicated by GCV before the bystander effect has been fully exerted.

However, it would be ideal if we could monitor the distribution and amount of exogenously administered transduced cells (MSCtk cells in this case) before GCV administration. There have been studies using radiotracers evaluating exogenously administered HSVtk gene expression with positron-emission tomography (22-26). If the presence of MSCtk cells in the human brain can be continuously traced using superparamagnetic iron oxide nanoparticle-labeled cells by widely available magnetic resonance imaging, the optimum timing for GCV administration would be individually determined (10, 27-29). Advances in these techniques could improve the treatment protocol and efficacy of HSVtk/GCV gene therapy in the future.

In summary, the present study suggests that when treating a rapidly growing tumor with HSVtk/GCV gene therapy using a slowly growing vector cell with low proliferative activity, early GCV administration continued for more than 72 h is needed for effective tumoricidal effect. Alhough the results of the present study are not conclusive and universally applicable, there are some implications when this strategy is clinically applied to human gliomas with lower proliferative activity than experimental tumor cell lines. For success of the cell-based HSVtk/GCV gene therapies, many factors including the proliferative activity and volume of the target tumor and *in vivo* migratory activity and survival period of the

therapeutic MSCtk cells should be taken into account. The optimal timing of GCV administration could be extrapolated from this information. Further studies, especially on the fate of MSCtk cells implanted in brain tumor by *in vivo* monitoring of HSVtk-expressing cells, are needed for preparing better clinical protocols.

Conflict of Interest

None declared.

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