Potent Bystander Effect in Suicide Gene Therapy Using Neural Stem Cells Transduced with Herpes Simplex Virus Thymidine Kinase Gene

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Key Words
Herpes simplex virus thymidine kinase  ·  Ganciclovir  ·  Bystander effect  ·  Neural stem cell  ·  Suicide gene therapy

Abstract
Objective: The herpes simplex virus thymidine kinase (HSVtk)/ganciclovir suicide gene therapy system has been considered as one of the most promising therapeutic strategies for malignant gliomas. We have been using HSVtk gene-transduced neural stem cells (NSCtk) that possess an ability to migrate toward a tumor mass for the treatment of experimental brain tumors. In the present study, we evaluated the potency of anti-tumor effect mediated by the bystander effect between NSCtk and C6 glioma cells in the HSVtk/ganciclovir suicide gene therapy system. Methods: NSCtk and C6 glioma cells were mixed at various ratios (NSCtk:C6 cell ratios of 1:1 to 1:64) and the bystander effect was evaluated both under in vitro and in vivo conditions. Results: In vitro co-culture experiment showed a complete tumor growth inhibition at the NSCtk:C6 ratios as low as 1:16. In vivo co-implantation study in the rat brain showed no visible tumors at the NSCtk:C6 ratios as low as 1:16 and all those rats survived more than 100 days. Conclusion: The results clearly demonstrated an extremely potent bystander effect between NSCtk and C6 cells, and the minimum number of NSCtk cells needed for the treatment of tumors was roughly estimated.

Introduction
The infiltrative and invasive nature of glioma, as well as the proximity to critical intracranial structures, presents a major hurdle to effective treatment by conventional means of surgery and radiotherapy [1]. In fact, despite the continuous refinement of the techniques of diagnosis, surgical intervention, radiotherapy and/or chemotherapy, the prognosis for patients with malignant gliomas remains poor [2]. Fortunately, the encouraging advances in molecular biology have been contributing to the development of gene therapies. To date, a number of target genes have been used or are expected to be applied. Among them, the most feasible and well-established approach is the herpes simplex virus thymidine kinase/ganciclovir (HSVtk/GCV) system, one of the suicide gene therapies. This strategy is also called the enzyme/prodrug system because the suicide gene encodes an enzyme that modifies a nontoxic prodrug into a toxic molecule in the
cells. The HSVtk converts the nontoxic nucleoside analogue GCV into GCV monophosphate, which is then further phosphorylated to GCV triphosphate by the cellular kinases. Incorporation of GCV triphosphate into elongating DNA during cell proliferation results in premature chain termination and eventually selectively kills dividing cells [3]. One of the most attractive elements in the HSVtk/GCV system is the so-called ‘bystander effect’, where brain tumor cells that were not transduced with the HSVtk gene were also eliminated along with HSVtk gene-transduced tumor cells [4, 5]. Though its mechanism is still not clear, it is considered that phosphorylated GCV is capable of passing through cellular gap junctions to confer cytotoxic effects on nontransduced neighboring cells. The biosafety and clinical safety of the strategy have already been tested in clinical trials by direct injection of the HSVtk retroviral vector-producing cells into the tumor or the brain walls around the excised tumor, although only limited therapeutic benefit was achieved [6, 7]. Since the extracellular virus moves by passive diffusion or fluid convection, it is not possible for the virus to spread far from the site of injection. On the other hand, glioma cells are known to migrate a long way along the white matter beyond the therapeutic radius.

In order to increase the ability of targeting the infiltrating tumor cells, we tried to treat an experimental brain tumor through the bystander effect by injecting HSVtk gene-transduced tumor cells (TK tumor cells) in the vicinity of the tumor followed by GCV administration [8, 9]. Tumor growth was drastically suppressed through the potent bystander effect achieved between the pre-existing tumor cells and the injected TK tumor cells. Though, theoretically, all the injected TK tumor cells were to be completely killed by GCV, ethical problems still exist with the use of viable tumor cells even in fatal gliomas. Therefore, we tested the use of neural stem cells (NSCs) transduced with HSVtk gene (NSCtk) instead of TK tumor cells, because NSCs exhibit an innate tumor-homing capability in experimental brain tumor models. NSCs implanted at distant sites from the tumor, even in the opposite side of the brain, migrate through normal brain tissue targeting the tumor cells [10–13]. In our previous study, we showed a potent bystander effect between NSCtk and C6 cells, and complete tumor eradication was obtained by the ‘NSCtk therapy’ when the same number of NSCtk cells as the tumor cells were intracranially implanted [14]. The bystander effect observed between NSCtk and C6 cells was much more potent than that observed between tumor cells [8, 9], and therefore, this strategy seemed appropriate for clinical use not only from the aspect of clinical safety, but also for its anti-tumor potency. In the present study, we precisely evaluated the potency of anti-tumor effect mediated by the bystander effect between NSCtk and C6 cells by a stepwise decrease in the ratios of the NSCtk cells against C6 cells both under in vitro and in vivo conditions in order to provide basic experimental data for clinical application of the ‘NSCtk therapy’.

Materials and Methods

Isolation of NSCs and Establishment of the NSCtk Cells

All of 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. As we described previously, fetal cerebral hemisphere tissue was obtained from 14-day Sprague-Dawley rat embryos and mechanically dissociated in NSC growth medium using the Neural Stem Cell Expansion Kit/Neurosphere system (R&D Systems, Inc., Minneapolis, Minn., USA) [14]. The cells were plated at a concentration of $4 \times 10^5$ per milliliter in an ultra-low attachment plate and incubated at 37°C under 5% CO$_2$. The medium was supplemented with 20 ng/ml human epidermal growth factor and human fibroblast growth factor basic each day. Thereafter, bulk cultures were generated by passaging the cells repeatedly.

The PA317 cells (HSVtk retrovirus-producing mouse fibroblast cell line, provided by Genetic Therapy Inc., Gaithersburg, Md., USA) were cultured in NSC growth medium with 20 ng/ml of human fibroblast growth factor basic to prepare the HSVtk supernatant. After the supernatant of HSVtk retrovirus and 8 µg/ml polybrene (Aldrich Chemical Company Inc., Milwaukee, Wisc., USA) were added, the NSCs were incubated for 3 h. Then the cells were washed and cultured for an additional 2 days. The drug-resistant neurospheres were collected after drug selection with 150 µg/ml G418 (Sigma-Aldrich Japan K.K., Tokyo, Japan) for 1 week. The clone, having a high GCV sensitivity and the same in vitro proliferative activity as wild-type NSCs, was selected (NSCtk cells). All the NSCtk cells were used as a single cell suspension for the following experiments by mechanically dissociating them with a fire-polished Pasteur pipette.

In vitro Bystander Effect between NSCtk and C6 Cells

The C6 rat glioma cell line was purchased from ATCC (Manas- sas, Va., USA). To determine the lowest necessary number of NSCtk cells which could provide effective anti-tumor effect in our strategy, we co-cultured NSCtk cells with $5 \times 10^5$ C6 cells at NSCtk: C6 cell ratios of 1:1, 1:4, 1:16, 1:32 and 1:64 in DMEM medium (including 10% fetal bovine serum) containing 1 µg/ml GCV in a 96-well tissue culture plate. C6 cells alone ($5 \times 10^3$, with or without GCV) or mixed with the same number of NSCtk cells (NSCtk:C6 cell ratio of 1:1, without GCV) were also cultured in the DMEM medium. The conditional medium was changed every 2 days and the number of living cells was determined by tetrozolium-based colorimetric assay (MTT assay) on day 10 [15]. Sensitivity to GCV was expressed as the percent absorbance of the C6 cells alone, cultured without GCV.
In vivo Bystander Effect between NSCtk and C6 Cells in Sprague-Dawley Rats

Seventy-two male Sprague-Dawley rats (280–320 g, 9 weeks old; Nippon SLC, Hamamatsu, Japan) were anesthetized with 0.4 ml/100 g equithesin and placed in a stereotaxic apparatus (Narishige Scientific Instrument Lab., Tokyo, Japan). NSCtk cells mixed with $1 \times 10^6$ C6 cells at ratios of 1:1, 1:4, 1:16 and 1:32 (n = 12 for every group) in 10$\mu$l DMEM medium were infused with a 50-µl microsyringe to the point of 5 mm ventral from the dura through the burr hole (coordinates with respect to bregma: 2 mm posterior, 3 mm right). The animals were administered intraperitoneally with 15 mg/kg GCV twice daily from day 0 for 10 days.

Another 12 rats, implanted similarly with $1 \times 10^5$ NSCtk cells mixed with $1 \times 10^5$ C6 cells in 10$\mu$l DMEM medium, were given physiological saline (0.9% NaCl solution) intraperitoneally for 10 days. The animals were kept under the same laboratory conditions. No steroids or antibiotics were used. Half of the animals (n = 6) for each group were sacrificed and histologically examined on day 14, the other half (n = 6) served for the survival study group. When the rats developed symptoms such as severe paresis and/or ataxia, or when their body weight decreased to less than 80%, they were sacrificed. The total volume of the tumor (in cubic millimeters) was calculated by summing up the cross-sectional areas. Survival was analyzed by a log-rank test based on the Kaplan-Meier survival analysis using Statview 5.0 software.

Results

In vitro Bystander Effect between NSCtk and C6 Cells

No proliferation inhibitions of C6 cells were detected when they were cultured with GCV (fig. 1, black column). When C6 cells were co-cultured with the same number of NSCtk cells in the medium without GCV (fig. 1, gray column), we did not observe proliferation inhibition either, as compared with the condition of C6 only without GCV, which, therefore, was used as the control for other experimental conditions. The anti-tumor effect mediated by the bystander effect was quite evident in the co-cultured NSCtk/C6 cells at ratios from 1:1 to 1:16 when cultured in the medium containing 1 µg/ml GCV, where we could hardly detect any viable cells on day 10. Even at the NSCtk:C6 cell ratios of 1:32 and 1:64, over 80 and 20% growth inhibition could be achieved (fig. 1, white columns).

Representative phase-contrast photomicrographs of the bystander anti-tumor effect between NSCtk and C6 cells at the ratio of 1:1 co-cultured in the medium with and without 1 µg/ml GCV on day 1 through day 10 are shown (fig. 2, bottom two rows). The C6 cells showed no obvious change on day 1 even after GCV exposure. However, the proliferation of C6 cells began to be held down from day 2 in the existence of GCV, and dramatic cell death could be observed from day 4. The C6 cells, together with NSCtk cells, degenerated and gradually detached from the bottom of the plate, and finally, no viable cells could be seen in the plate on day 10 (fig. 2, bottom row). But the NSCtk cells did not exert any inhibiting effect on the surrounding C6 cells when they were not exposed to GCV (fig. 2, third row). C6 cells alone were also cultured with or without GCV to examine the direct effect of GCV on C6 cells (fig. 2, top two rows).

In vivo Bystander Effect between NSCtk and C6 Cells in Sprague-Dawley Rats

On histological examination on day 14, no visible brain tumors were found in the groups implanted with an NSCtk/C6 mixture (1:1, 1:4 and 1:16) and treated with GCV (fig. 3c–e), while all the rats in the group implanted with C6 alone (fig. 3a) and in the group implanted with NSCtk/C6 cells (1:1) but not treated with GCV (fig. 3b) developed a large tumor. All 6 rats in the group implanted with an NSCtk/C6 mixture at the ratio of 1:32 and treated with GCV showed a small tumor along the needle path (fig. 3f). The measured tumor volume is shown in figure 3g.

On survival study, all the rats in the groups implanted with an NSCtk/C6 mixture (1:1, 1:4 and 1:16) and treat-
ed with GCV survived more than 100 days. All the rats in the group implanted with C6 alone and in the group implanted with NSCtk/C6 cells (1:1) but not treated with GCV died 2–3 weeks after tumor implantation due to tumor growth, and there was no statistical significance between them (p = 0.89, log-rank test). The mean survival time of the 6 rats in the group implanted with an NSCtk:C6 mixture at the ratio of 1:32 and treated with GCV was 28 days and was significantly longer than in the previous two control groups (p < 0.01, log-rank test) (fig. 3h).

**Discussion**

In the present study, we have demonstrated the feasibility of the ‘NSCtk therapy’ for the treatment of experimental gliomas and observed an extremely potent bystander effect between NSCtk and C6 glioma cells. We have shown in the co-implanted study that an NSCtk:C6 ratio of at least 1:16 is required for complete eradication of the tumor. This information would be of importance when preparing a clinical protocol of this strategy.

There have been several intracranial co-implantation studies using neural stem/progenitor cells and tumor cells similar to the present study. Barresi et al. [16] implanted
8 × 10^4 immortalized neural progenitor cells expressing cytosine deaminase and 2 × 10^4 C6 glioma cells in Sprague-Dawley rats and administered 5-fluorocytosine. Though four times more effector cells were used, the cytosine deaminase/5-fluorocytosine system, which was another suicide gene therapy and also generated the bystander effect, only produced 50% reduction in tumor size 10 days after implantation. Shah et al. [12] implanted with an NSCtk/C6 mixture at ratios of 1:1 to 1:32 and treated with GCV showed potent growth inhibition of tumor (white column, p < 0.01 compared with C6 alone treated with GCV). h Kaplan-Meier survival curve. The rats in the group of C6 alone treated with GCV and in the group of NSCtk/C6 (1:1) treated with physiological saline (PS) died 2–3 weeks after tumor implantation, while all the rats in the groups of NSCtk/C6 (1:1, 1:4, 1:16) treated with GCV survived more than 100 days. The mean survival time in the group of NSCtk/C6 (1:32) treated with GCV prolonged to 28 days (p < 0.01 compared with any group of C6 alone treated with GCV or NSCtk/C6, 1:1, treated with physiological saline).
of the present study strongly indicate that the ‘NSCtk therapy’ is extremely potent as compared with other strategies using neural stem/progenitor cells.

In the present study, we obtained NSCs from the embryos of the Sprague-Dawley rat, from which C6 glioma was also derived. A strong bystander effect was achieved by GCV treatment in the co-implantation experiments of NSCtk and C6 cells derived from a syngeneic animal. This bystander effect was much stronger than that observed between the HSVtk gene-transduced and HSVtk gene-nontransduced C6 rat glioma cells and even more potent than that observed between the HSVtk gene-transduced and HSVtk gene-nontransduced 9L rat glioma cells that had a higher expression of connexin43 than C6 cells [8, 17]. The reasons for this extremely potent bystander effect by NSCtk cells in the present study are not fully understood, and further studies are needed to elucidate the mechanism. A higher in vivo resistance of differentiated NSCs to GCV might partly explain why the bystander effect observed between NSCtk and C6 cells is much more potent than that observed between tumor cells. Whatever the mechanism, this surprisingly stronger bystander effect, as well as the higher biological safety, suggests a broad prospect of the ‘NSCtk therapy’ in clinical applications.

References