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Migratory neural stem cells for improved thymidine kinase-based gene therapy of malignant gliomas

Martin Uhl[a], Markus Weiler[a], Wolfgang Wick[a], Andreas H. Jacobs[b], Michael Weller[a], Ulrich Herrlinger[a,*]

[a] *Department of General Neurology, Hertie Institute for Clinical Brain Research, University of Tübingen, Hoppe-Seyler-Str. 3, D-72076 Tübingen, Germany*
[b] *Max-Planck Institute for Neurological Research, University of Cologne, D-50931 Cologne, Germany*

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Abstract

Gene therapy of glioma based on viral delivery of herpes simplex virus type I thymidine kinase (HSV-TK) has failed in the clinic because of low transduction efficacy. To circumvent this problem, this study evaluated highly migratory HSV-TK-transduced neural stem cells (NSC) for their ability to kill untransduced glioma cells by a gap junction-mediated bystander effect. The admixture of HSV-TK-transduced NSC to U87MG and LN-18 human malignant glioma cell lines at ratios of 1:10 or 1:1 eliminated more than 50% or 90% of glioma cells in the presence of ganciclovir (25 μM). Glioma cell cytotoxicity required cell–cell contact. Similarly, tumor cell cytotoxicity was observed in two of three primary glioblastoma cell cultures, and the presence of this bystander effect correlated with the expression of connexin 43 in the untransduced glioma target cells. In conclusion, we delineate a role for migratory HSV-transfected NSC to eliminate glioma cells purely by means of the bystander effect.

Keywords: Stem cells; Gene therapy; Thymidine kinase; Bystander effect; Glioma

With standard treatment using resection, radiotherapy, and chemotherapy, patients with glioblastoma still have a dismal prognosis. During the 1990s, prodrug activation gene therapy, e.g., using HSV-TK transduction and ganciclovir (GCV) therapy, promised to be an important step forward in the therapy for glioblastoma. Cytotoxicity in this system was not restricted to a transfected cell but also affected neighboring non-transfected cells. This bystander effect [1] is based, at least in the HSV-TK/GCV system, on the gap junction-mediated transport of phosphorylated GCV as a cytotoxic metabolite from cell to cell [2]. The formation of gap junctions depends on connexin 43 expression [2,3]. Despite impressive results in animal models of glioma [4,5], clinical trials have failed to show clinical efficacy of this therapeutic approach [6–8]. This disappointing result is not surprising since in vivo transduction efficiencies remained below the level probably required for a significant bystander effect [9,10]. The main problem of these approaches is the insufficient distribution of the gene therapy vehicles, mostly viruses, over the whole volume of the tumor. One approach to overcome this problem is the use of migratory cells that may distribute widely throughout the tumor for the delivery of toxic genes or vectors. Besides endothelial cells [11] or glioma cells [12], NSC [13] are known to extensively migrate through the brain, particularly brain tumors [14]. Recent approaches have focused on loading NSC with retroviruses [15], HSV [16], cytotoxic compounds such as cytidine deaminase (CD [14]), interleukin (IL)-4 [17], tumor necrosis

* Corresponding author. Fax: +49 7071 295260.
E-mail address: ulrich.herrlinger@uni-tuebingen.de (U. Herrlinger).

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factor-related apoptosis-inducing ligand (TRAIL) [18] or immunostimulatory molecules such as IL-12 [19]. Since, in contrast to some of the above-mentioned approaches, the HSV-TK/GCV prodrug activation system is not neurotoxic at relevant concentrations of GCV, we explored the use of NSC as carriers for HSV-TK in an in vitro model of human glioma cell bystander killing.

Materials and methods

Cell culture. U87MG and LN-18 malignant glioma cells were kindly provided by N. de Tribolet (Lausanne, Switzerland). The cells were maintained in DMEM containing 10% fetal calf serum (FCS), 2 mM glutamine and penicillin (100 IU/ml)/streptomycin (100 μg/ml). The murine neural stem cell line C17.2 was a kind gift of E.Y. Snyder (Children's Hospital, Boston, MA) and was also maintained in DMEM. Short-term cultures from human gliomas were prepared as described [20].

Transfection. C17.2 NSC were transfected with the pHSV-TIG plasmid which contains the HSV-TK gene and the enhanced green fluorescent protein (GFP) gene driven by the cytomegalovirus (CMV) promoter and separated by an internal ribosomal entry site. For transfection, 5×10^4 C17.2 cells were seeded in 24-well plates and transfected with 0.2 μg plasmid DNA using FuGENE 6 (Roche, Mannheim, Germany) and selected in the presence of puromycin (2 μg/ml). Stable isolates positive for HSV-TK and GFP expression were identified by GFP fluorescence upon flow cytometry and were sorted for the 11.5% highest GFP-expressing cells using a FACSvantage (Becton–Dickinson, Heidelberg, Germany). Aliquots of early passages of these cells were used in all further experiments. HSV-TK expression of transduced GFP-positive C17.2 cells following FACS sorting was confirmed in cytotoxicity assays: after adherence for 24 h, 10^5 cells/well in a 24-well plate were treated with 0.01–25 μM GCV for 48 h and viable cells were counted using a Casy cell counter (Schärfe System, Reutlingen, Germany).

NSC/glioma cell coincubation assay. Cells were seeded in varying ratios of glioma cells and NSC to a total amount of 10^5/well in a 24-well plate. The cells were adhered for 24 h and then exposed to GCV at 25 μM (Cymeven, Roche, Grenzach-Wyhlen, Germany) for 48 h. Viable cells were counted using a Casy cell counter. In the vehicle group, the total number of viable glioma cells was calculated by counting the GFP-negative cells in a Becton–Dickinson FACSCalibur flow cytometer using Cell Quest software. In some experiments, NSC and glioma cells were separated using Millipore Transwell inserts with 0.4 μm pores for 24-well plates.

Immunoblot analysis. Cells grown to subconfluency were washed with cold PBS, harvested into ice-cold PBS containing phenylmethylsulfonyl fluoride (PMSF) (10 μg/ml) with a cell scraper, lysed in lysis buffer containing 50 mM Tris–HCl, pH 8, 120 mM NaCl, 0.5% NP-40, 100 μg/ml PMSF, 10 μg/ml leupeptin, and 2 μg/ml aprotinin for 15 min on ice, and centrifuged at 13,000 rpm for 15 min at 4 °C. The protein concentration was determined by the Bio-Rad Protein Assay (Bio-Rad, Munich, Germany). Soluble proteins (20 μg/lane) were separated by polyacrylamide gel electrophoresis and blotted to nitrocellulose. Equal loading was ascertained by Ponceau S staining. The membranes were pretreated for 2 h with PBS containing 5% skim milk, 0.05% Tween 20 and then incubated for 16 h at 4 °C with anti-connexin 43 antibody (Zymed, San Francisco, CA) (2 μg/ml), washed, and then incubated with horseradish peroxidase-coupled secondary antibody (1:3000) for 1 h. Visualization of protein bands was accomplished using enhanced chemoluminescence (ECL).

Results

Transduction of C17.2 NSC with HSV-TK

The HSV-TK-IRES-GFP expression cassette was transfected into early passage wild-type C17.2 cells. After cell sorting, high GFP expression was achieved in 97% of cells as demonstrated by flow cytometry (Fig. 1A). To demonstrate the activity of the transduced HSV-TK, HSV-TK/GFP-transduced C17.2 cells (TK-C17) were incubated with increasing concentrations of GCV. The EC_{50} concentration was 0.2 μM. Less than 5% of cells remained viable at 25 μM (Fig. 1B). High GFP expression and sensitivity to GCV persisted for more than 15 passages. In contrast, wild-type C17.2 cells were resistant to these concentrations of GCV.

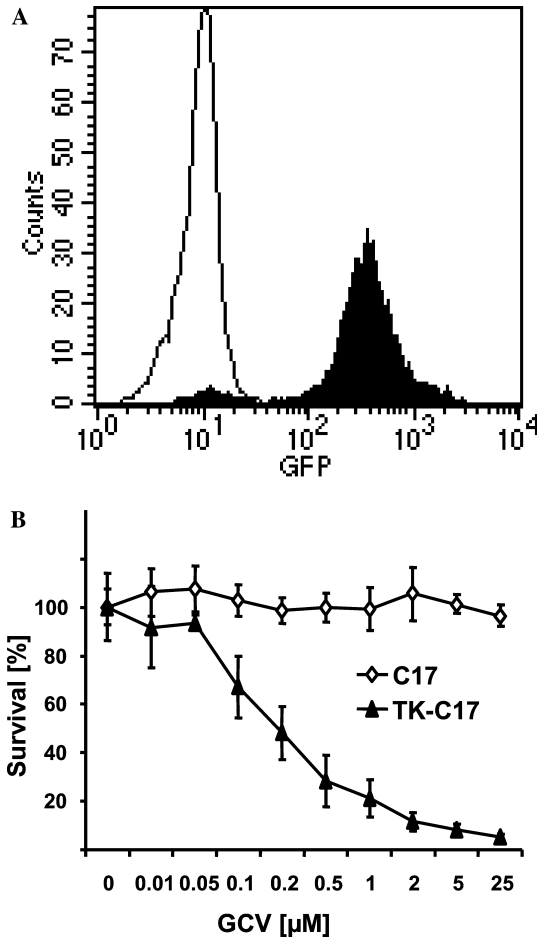

Fig. 1. Expression and activity of HSV-TK in C17.2 NSCs. (A) Flow cytometric analysis of GFP expression in wild-type and HSV-TK-transduced cells. (B) Viability of control cells or HSV-TK-transduced C17.2 NSC after 48 h treatment with GCV [mean ± standard error of mean (SEM); $n = 3$].

In vitro cytotoxicity mediated by HSV-TK-transduced NSC in glioma cell lines and primary glioblastoma cultures

To analyze the bystander effect of GCV-treated TK-C17 cells on U87MG and LN-18 glioma cells, the glioma cells were mixed with TK-C17 cells at different ratios from 1:20 to 1:1 and treated with GCV (25 μM) for 48 h. GCV killed all TK-C17 cells and reduced the number of viable glioma cells in a cell:cell ratio-dependent manner. The EC_{50} ratios were approximately 1 TK-C17 cell per 10 untransduced U87MG and 2 TK-C17 cells per 10 untransduced LN-18 cells (Fig. 2). At ratios exceeding 1:1, fewer than 10% of U87MG cells survived. The bystander effect was strictly depending on cell–cell contact. When TK-C17 and glioma cells were separated by a membrane allowing the penetration of diffusible agents, but not cells, the cytotoxic bystander effect on glioma cells was abolished in both cell lines (Fig. 2). No cytotoxic effect was seen when the glioma cells were coincubated with untransfected C17.2 cells in the absence or presence of GCV (data not shown). Similar coincubation experiments were also performed with early passages of three primary glioblastoma cultures. In two of these cultures, Tu-113 and Tu-159, a pattern similar to U87MG cells with a significant bystander effect at a ratio of 1:10 was seen (Fig. 3). In contrast, Tu-132 was refractory.

Correlation of the bystander effect with connexin 43 expression in glioma cells

Connexin 43 is a major constituent of gap junctions that allow the transfer of phosphorylated GCV from cell to cell. TK-C17 cells expressed connexin 43 at low levels; U87MG glioma cells and LN-18 glioma cells expressed connnexin 43 slightly above the detection limit. Among the primary cultures, the bystander effect appeared to depend on the level of connexin 43 expression. The

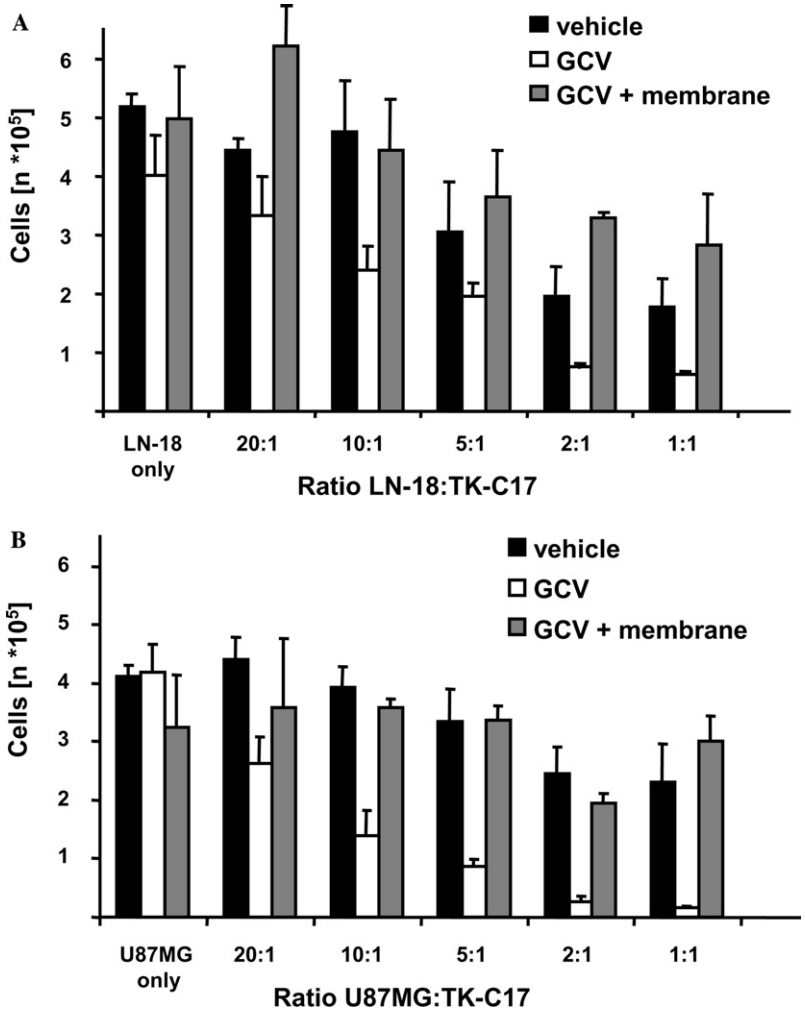

Fig. 2. TK-C17/GCV-induced killing of glioma cells. Different ratios of TK-C17 and LN-18 (A) or U87MG (B) cells were incubated for 48 h in the absence or presence of GCV (25 μM) with or without a diffusion-permeable membrane. The number of surviving glioma cells was determined by flow cytometry (mean ± SEM; $n = 3$).

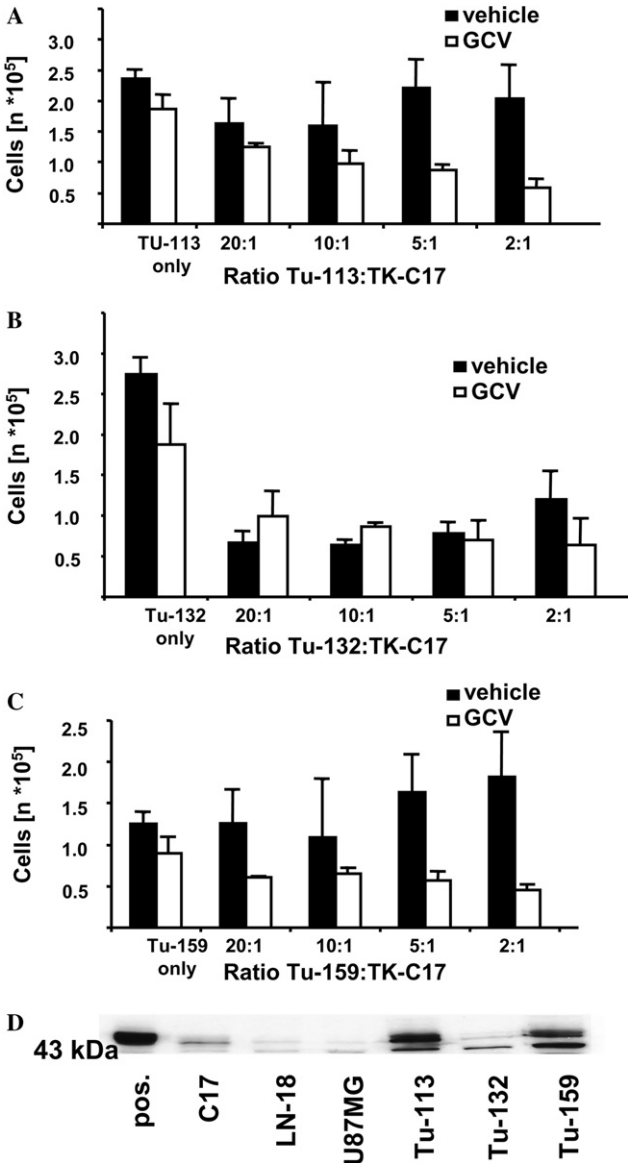

Fig. 3. TK-C17/GCV-induced cytotoxicity in three primary glioblastoma cultures: correlation with connexin 43 expression. (A–C) TK-C17 and Tu-113 (A), Tu-132 (B) or Tu-159 (C) cells were incubated at different ratios for 48 h with GCV (25 μM) and the number of viable glioma cells was determined. (D) Connexin 43 expression in TK-C17 NSC, established glioma cell lines (U87MG, LN-18), and glioma primary cultures (Tu-113, Tu-132, and Tu-159) was assessed by immunoblot, using extracts from rat heart as a positive control.

two cell cultures that showed an extensive bystander effect, Tu-113 and Tu-159, expressed connexin 43 at high levels whereas the primary cell culture that expressed only low levels of connexin 43, Tu-132, showed no bystander effect (Fig. 3D).

Discussion

The present report provides a proof of principle that HSV-TK-transduced non-neoplastic NSC can be used to confer a probably gap junction-dependent cytotoxic bystander effect to malignant glioma cells. Since these NSC are highly migratory, this offers the possibility that HSV-TK-transduced NSC can be used to distribute phosphorylated cytotoxic GCV throughout a tumor. The classical bystander effect of HSV-TK-mediated gene therapy for glioma has so far relied on the transduction of glioma cells by viral or non-viral means as a prerequisite and consists of the transfer of cytotoxic phosphorylated GCV from a transduced glioma cell to a neighboring non-transduced glioma cell [1,2,4]. Our data demonstrate that gap junction-mediated transfer of cytotoxic GCV can not only be achieved between glioma cells, but also between non-neoplastic NSC and glioma cells. The bystander effect that could be generated with HSV-TK-transduced NSC (Figs. 2 and 3) was as prominent as in paradigms of a glioma cell-mediated bystander effect [4,21]: with the admixture of 10% of transduced cells, a substantial cytotoxic effect on non-transduced cells was induced. Here, this was not only shown in established cell lines but also in the clinically more relevant primary cultures (Fig. 3).

The data provided here support the notion that the extent of the bystander effect depends on the formation of gap junctions by the target cells as demonstrated by connexin 43 immunoblotting (Fig. 3B). This corresponds to published data on tumor cell-tumor cell interactions [22,23] and on the importance of target cell connexin 43 expression as a major prerequisite of the bystander effect [3]. The amount of connexin 43 expression needed for a substantial bystander effect appears to be much lower in established glioma cell lines than in primary glioblastoma cultures. One can speculate that glioma cells of established cell lines with a high proliferation rate are highly susceptible to phosphorylated GCV so that even low transfer rates of phosphorylated GCV are sufficient for a substantial cytotoxic effect whereas glioma cells in primary cultures with low proliferative activity may only show a cytotoxic effect with high transfer rates of phosphorylated GCV, thus requiring high connexin 43 expression.

With the HSV-TK-transfected cells, a new and safe NSC-based system to confer cytotoxicity to glioma cells has been established. Previous experiments [14,24] had used NSC transfected with CD which may be a less safe approach for prodrug activation therapy in the brain. In contrast to HSV-TK, the metabolites produced by CD do not only affect DNA replication but also RNA processing. Also, the bystander effect induced by CD does not only depend on gap junctions but also on free diffusion of metabolites. All these mechanisms contribute to the neurotoxicity that has been observed with CD/5-FC-based gene therapy of brain tumors [25]. Also, the use of non-neoplastic NSC for the delivery of phosphorylated GCV is safer than the previously described use of neoplastic glioma cells as carriers [12,22].

In conclusion, the potent bystander effect induced by HSV-TK-transduced NSC may provide a relatively safe approach to confer cytotoxicity to glioma cells. Due to the migratory capacity of NSC, these cells may provide an excellent tool to distribute cytotoxicity throughout a glioma and deserve further evaluation.

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