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A heterotypic bystander effect for tumor cell killing after adeno-associated virus/phage–mediated, vascular-targeted suicide gene transfer

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Abstract

Suicide gene transfer is the most commonly used cytotoxic approach in cancer gene therapy; however, a successful suicide gene therapy depends on the generation of efficient targeted systemic gene delivery vectors. We recently reported that selective systemic delivery of suicide genes such as herpes simplex virus thymidine kinase (*HSVtk*) to tumor endothelial cells through a novel targeted adeno-associated virus/phage vector leads to suppression of tumor growth. This marked effect has been postulated to result primarily from the death of cancer cells by hypoxia following the targeted disruption of tumor blood vessels. Here, we investigated whether an additional mechanism of action is involved. We show that there is a heterotypic "bystander" effect between endothelial cells expressing the *HSVtk* suicide gene and tumor cells. Treatment of cocultures of *HSVtk*-transduced endothelial cells and non-*HSVtk*-transduced tumor cells with ganciclovir results in the death of both endothelial and tumor cells. Blocking of this effect by 18α-glycyrrhetinic acid indicates that gap junctions between endothelial and tumor cells are largely responsible for this phenomenon. Moreover, the observed bystander killing is mediated by connexins 43 and 26, which are expressed in endothelial and tumor cell types. Finally, this heterotypic bystander effect is accompanied by a suppression of tumor growth *in vivo* that is independent of primary gene transfer into host-derived tumor vascular endothelium. These findings add an alternative nonmutually exclusive and potentially synergistic cytotoxic mechanism to cancer gene therapy based on targeted adeno-associated virus/phage and further support the promising role of nonmalignant tumor stromal cells as therapeutic targets. [Mol Cancer Ther 2009;8(8):2383–91]

Introduction

Gene therapy remains a potentially viable strategy for the treatment of human cancer. It is based on the correction of pathologic gene expression patterns (e.g. by the transfer of tumor suppressor genes) or on the delivery of cytotoxic genes that directly or indirectly kill tumor cells irrespective of its gene expression. The most widely used approach for cytotoxic gene therapy involves the transfer of the herpes simplex virus type I thymidine kinase (*HSVtk*) gene (1–3) Expression of *HSVtk* results in the phosphorylation of prodrug nucleoside analogues such as ganciclovir and converts them into nucleoside analogue triphosphates. These compounds, which are incorporated into the cellular genome, inhibit DNA polymerase and cause cell death by apoptosis (4). The converted cytotoxic drug and/or toxic metabolites are able to spread from transduced cells to nontransduced cells through cellular gap junctions. This "bystander effect" may potentially overcome the requirement for all malignant cells to be transduced to achieve meaningful tumor regression (2, 5).

Although this approach has shown promise *in vitro* and *in vivo*, its wide application has been hampered by the lack of vectors that allow specific and efficient transduction of the target tissue after systemic administration. Consequently, poor efficiency of gene transfer potentially limits the number of vector-transduced tumor cells (6) and thus prevents effective systemic cancer gene therapy.

Given the estimates that up of 100 tumor cells are sustained by a single endothelial cell (7), vascular gene targeting might minimize or overcome this problem. Indeed, a small number of transduced cells that are accessible to the circulation could in theory mediate a much more pronounced effect that is relatively independent of gene transfer efficiency. The vasculature of a solid tumor is an

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Note: M. Trepel and C.A. Stoneham contributed equally to this work.

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attractive target for intervention because the angiogenic endothelium expresses several cell-surface receptors that are essentially absent or barely detectable in normal blood vessels (8, 9). Such receptors are suited for systemic gene therapy because they are readily accessible through the circulation and often mediate cellular internalization of targeting ligands (9–13).

Several technologies for design and production of vascular-targeted gene therapy vectors based on ligand-directed binding of the vector to endothelial receptors have been developed (14–19). We have reported a ligand-directed vector for systemic tissue-targeted gene delivery, termed adeno-associated virus/phage (20, 21). Adeno-associated virus/phage (AAVP) is a genomic hybrid of adeno-associated virus type 2 and of an M13 phage derivative. An established version of this vector displays the peptide ligand RGD-4C (20–22) that targets α_v integrins expressed by angiogenic blood vessels (20–27). *In vitro*, transgene expression by targeted RGD-4C adeno-associated virus/phage begins 48 to 72 hours after incubation with cells and reaches a maximum level by 1 week. Transduction efficiency varies from one cell line to another depending on the expression of the target receptor. In general, 10% to 20% of cells can be transduced in culture (20, 22, 28).

Targeted RGD-4C adeno-associated virus/phage was also used to systemically deliver the *HSVtk* gene to α_v integrin-positive cells in either isogenic EF43-*FGF4* mouse mammary tumors (20) or nude rats bearing human sarcoma xenografts (21). EF43-*FGF4* tumor cells themselves have a barely detectable expression level of α_v integrin receptors that does not allow their transduction by RGD-4C adeno-associated virus/phage; nevertheless, systemic administration of targeted RGD-4C adeno-associated virus/phage-*HSVtk* to mice bearing established EF43-*FGF4* tumors resulted in marked suppression of tumor growth after ganciclovir treatment (20). Such antitumor effect was accompanied by extensive tumor vascular disruption caused by apoptosis of the blood vessels (20). It is not currently known, however, whether subsequent inhibition of tumor growth by RGD-4C adeno-associated virus/phage-*HSVtk* plus ganciclovir was simply a consequence of the lack of blood supply or whether tumor cell killing was also mediated by a heterotypic bystander effect between *HSVtk*-transduced vascular endothelium and tumor cells.

Herein, we have evaluated the hypothesis that a heterotypic bystander effect exists in this targeted system. We show that EF43-*FGF4* tumor cells, which are not transduced by *HSVtk*, can also be eliminated *in vitro* and *in vivo* by a vascular cell-mediated bystander effect through gap junction intercellular communication between endothelial and tumor cells.

Materials and Methods

Reagents and Cells

SVEC4-10–transformed murine small vessel endothelial cells and KS1767 Kaposi's sarcoma cells were from American Type Culture Collection. MDA-MB435 breast carcinoma cells were a gift from Jane Price (The University of Texas M. D. Anderson Cancer Center) and 9L rat glioblastoma cells were a gift from Dr. James Basilion (Case Western Reserve University). The EF43-*FGF4* cells were derived from the EF43 BALB/c mouse mammary cell line by infection of the latter cells with a retroviral vector carrying the *FGF4* oncogene, as described (20, 29). KS1767 cells were maintained in Minimal Essential Medium (Irvine Scientific). All other cell lines were cultured in DMEM (Gibco). All media were supplemented with 10% FBS (Gibco), L-glutamine, and penicillin G plus streptomycin.

Plasmids and Transfections

HSVtk was expressed in endothelial SVEC4-10 cells by transfection of a adeno-associated virus (pAAV)-*HSVtk* plasmid containing the cDNA encoding the *HSVtk* mutant SR39 (30). To generate the pAAV-*HSVtk* plasmid, we removed green fluorescent protein (*GFP*) from the pAAV-enhanced green fluorescent protein (*eGFP*) plasmid (Stratagene) by digestion with *Bam*HI-*Not*I and replaced this DNA with a *Bam*HI-*Not*I fragment containing the *HSVtk*-SR39 (referred to in this work as *HSVtk*). DNA sequencing and analysis of restriction enzyme digests served to verify the correct orientation of the insert in the constructs. The plasmids were transfected into SVEC4-10 cells with the FuGENE 6 transfection reagent (Roche).

Immunostaining of Connexins

For detection of connexins 26 and 43 by immunofluorescence, cells were grown for 2 to 4 d to a subconfluent monolayer. Cells were rinsed with PBS and fixed with 100% ethanol for 20 min at room temperature. Subsequently, the cells were saturated for 45 min with PBS containing 2% bovine serum albumin and were incubated for 1 h at 37°C with 10 to 20 μg/mL rabbit polyclonal antibodies against either connexins 26 or 43 (Zymed Laboratories) diluted in PBS containing 2% bovine serum albumin. After extensive washing with PBS, cells were incubated for 1 h with a 1:40 dilution of a FITC-conjugated porcine anti-rabbit immunoglobulin G (Dako). The cells on coverslips were washed thrice with PBS, mounted on glass slides, and viewed under an Olympus fluorescence microscope.

Determination of the Bystander Effect *In vitro*

SVEC4-10 cells transiently expressing *HSVtk* were mixed in a 1:9 ratio with nontransduced tumor cells, as indicated, and were grown to a subconfluent layer. The cocultures were treated with 20 μmol/L ganciclovir. In subsequent experimental settings, the long-term inhibitor of gap junctional intercellular communication, 18α-glycyrrhetinic acid (Sigma), was added at 70 μmol/L to the medium during the treatment with ganciclovir. Media containing ganciclovir, 18α-glycyrrhetinic acid, or both was renewed every 2 d, and the viable cells were counted after 5 d by the trypan blue–exclusion methodology.

Production, Purification, and Titration of Adeno-Associated Virus/Phage Vectors

Targeted RGD-4C adeno-associated virus/phage particles as well as nontargeted controls were amplified, isolated, and purified from the culture supernatant of host bacteria (*Escherichia coli* MC1061), as we previously described (20,

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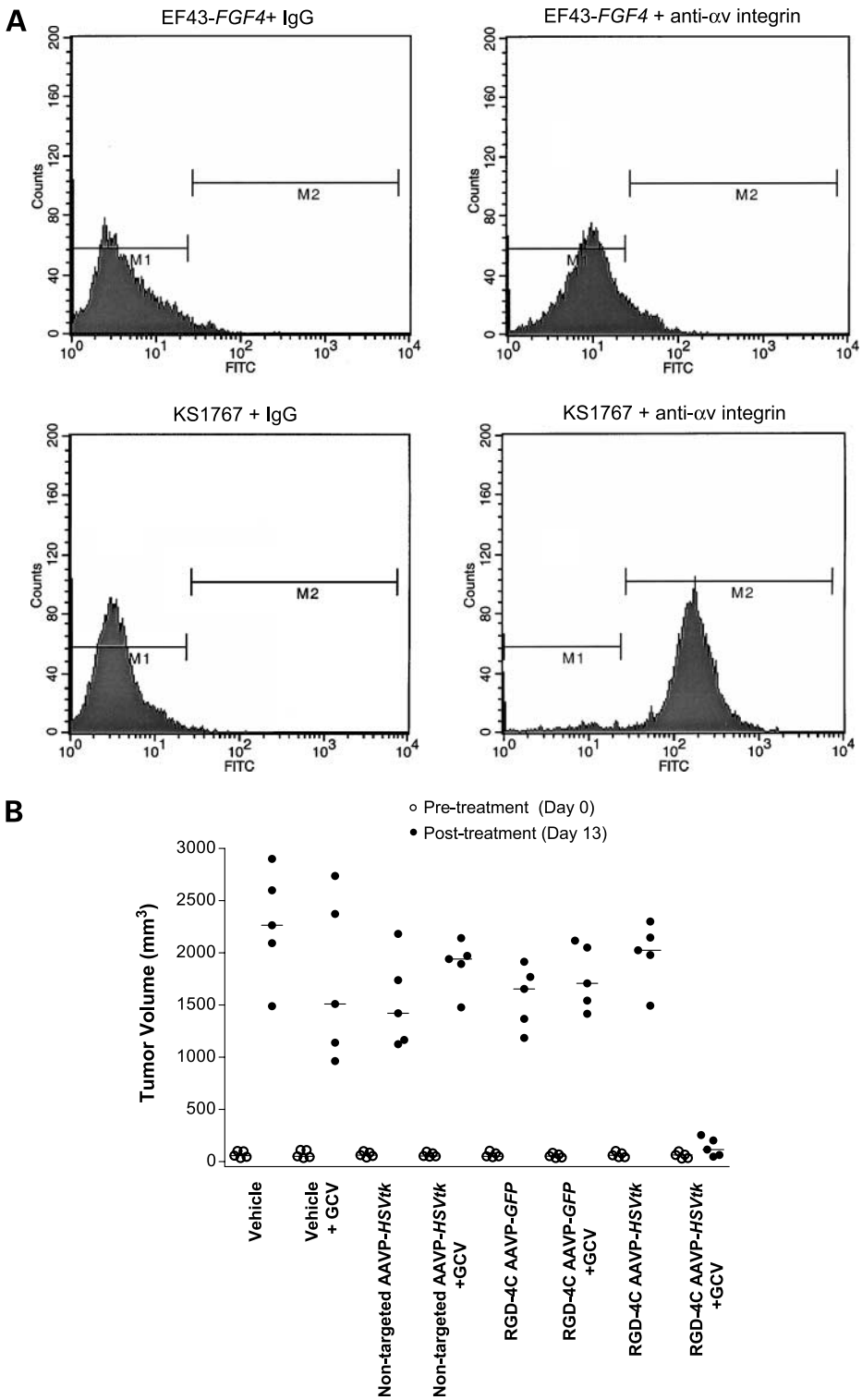

Figure 1. EF43-*FGF4* tumor growth is inhibited by targeted RGD-4C adeno-associated virus/phage–*HSVtk* independently of expression of the target receptor on tumor cells. **A,** EF43-*FGF4* tumor cells barely express α_v integrins. EF43-*FGF4* cells grown *in vitro* were detached and subjected to fluorescence-activated cell sorting analysis with an anti-α_v integrin antibody (*right*) or a species-matched immunoglobulin G control antibody (*left*). Kaposi's sarcoma (KS1767) cells served as a positive control. **B,** systemic administration of α_v integrin-targeted RGD-4C adeno-associated virus/phage–*HSVtk* vector mediates marked suppression of EF43-*FGF4* tumors after ganciclovir treatment. EF43-*FGF4* tumors were established in BALB/c immunocompetent mice (100 mm^3). A single dose of RGD-4C adeno-associated virus/phage–*HSVtk* vector (5×10^{10} transducing units) was administered i.v. Treatment with ganciclovir (80 mg/kg/d) i.p. started 2 d later. A comprehensive panel of negative experimental control groups, including vehicle alone, vehicle plus ganciclovir, nontargeted adeno-associated virus/phage–*HSVtk*, nontargeted adeno-associated virus/phage–*HSVtk* plus ganciclovir, targeted RGD-4C adeno-associated virus/phage–*HSVtk*, targeted RGD-4C adeno-associated virus/phage–*GFP*, and targeted RGD-4C adeno-associated virus/phage–*GFP* plus ganciclovir (mock transduction) is shown. Data are presented as the volumes of five individual tumors and their medians before and at day 13 after treatment.

21, 28, 31). Next, vector particles in suspension were sterile filtered through 0.45-μm filters, then titrated by infection of host bacteria for colony counting on Luria-Bertani agar plates under a double antibiotic selection and expressed as bacterial transducing units.

EF43-*FGF4* Tumor Model and Systemic RGD-4C Adeno-Associated Virus/Phage Therapy

Tumor-bearing mice were established and tumor volumes were calculated as described (20, 21, 29). Mice were anesthetized by gas (2% isoflurane and 98% oxygen)

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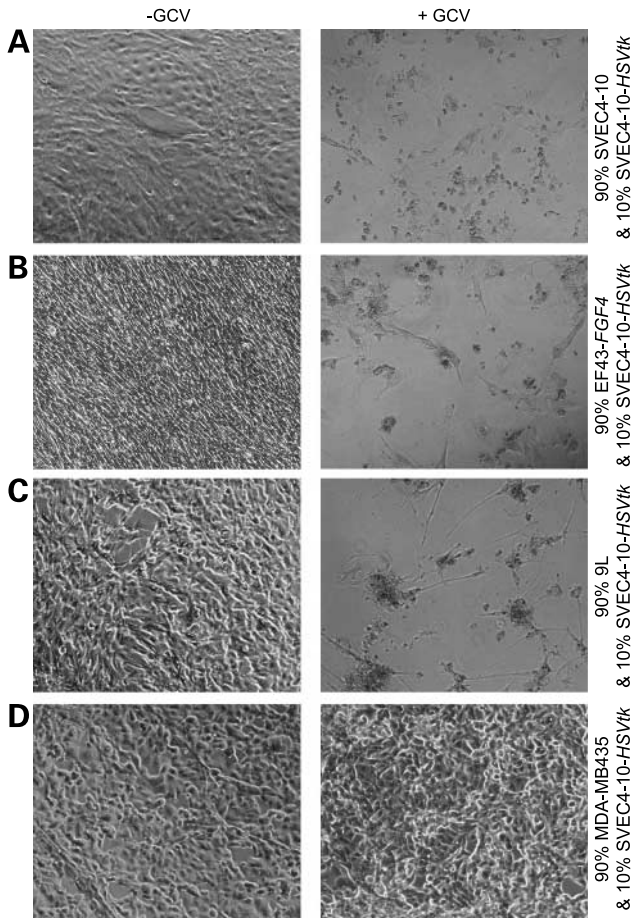

Figure 2. *In vitro* bystander effect between *HSVtk*-transduced SVEC4-10 endothelial cells and nontransduced tumor cells. *HSVtk*-transduced endothelial SVEC4-10 cells were mixed at a 1:9 ratio with nontransduced SVEC4-10 cells (**A**), EF43-*FGF4* breast cancer cells (**B**), 9L malignant glioma cells (**C**), or MDA-MB435 breast cancer cells (**D**) in 24-well plates. After 24 h, the cocultures were treated with 20 μmol/L ganciclovir (*right*) or vehicle (*left*) for 5 d. Phase-contrast microscopy 5 d after initiation of drug treatment.

inhalation. Tumor cells were released by exposure to trypsin, counted, centrifuged, and resuspended in serum-free medium. A total of 5×10^4 cells from the EF43-*FGF4* mouse mammary tumor were implanted s.c. into 6-wk-old female BALB/c immunocompetent mice. When tumors reached a volume of ~100 mm^3, tumor-bearing mice received a single i.v. dose of RGD-4C adeno-associated virus/phage–*HSVtk* (5×10^{10} transducing units) or controls. Treatment with ganciclovir (80 mg/kg/d, i.p.) was initiated later in cohorts of size-matched, tumor-bearing mice as indicated. Tumor growth was monitored daily and measured by caliper twice weekly. Each experimental cohort contained at least 15 tumor-bearing mice divided into 3 groups of 5 mice each. Simple hypothesis test (Mann-Whitney test statistic) was applied to assess whether differences among groups were significant. Statistical significance level was set to $\alpha = 0.05$.

Determination of the Bystander Effect *In vivo*

The Institutional Animal Care and Use Committee approved all experimentation described here. Mice were anesthetized by 2% isoflurane and 98% oxygen inhalation. Cultured cells were detached with a solution of trypsin-EDTA, counted, centrifuged, and resuspended in serum-free medium. The EF43-*FGF4* tumor cells (5×10^4) were mixed with *HSVtk*-transduced SVEC4-10 (5×10^4) or parental SVEC4-10 cells (5×10^4), then the resulting cell mixtures (1×10^5 cells at a 1:1 ratio) were injected s.c. into the back of 6-wk-old female athymic *nu/nu* (nude) mice. Treatment of mice with ganciclovir started 6 d after cell implantation into mice and was administered daily by i.p. injection of 80 mg/kg. Tumor growth was monitored thrice a week by caliper measurement of two diameters and expressed as mean tumoral volume ± SD. In each assay, the number of mice per group (*n*) was 10.

Results

EF43-*FGF4* Tumor Cells Barely Express α_v Integrin

To understand the lack of transduction of EF43-*FGF4* breast cancer cells by the targeted RGD-4C adeno-associated virus/phage vector, we assessed the expression of the receptors of RGD-4C ligand, α_v integrins, in EF43-*FGF4* cells. We carried out fluorescence-activated cell sorting analysis *in vitro*. The data revealed that EF43-*FGF4*–derived tumor cells barely express the α_v integrins on their surface (Fig. 1A). Kaposi's sarcoma cells (KS1767), which served as a positive control (20, 23, 24), showed strong expression of α_v integrins. The corresponding negative control, in which species-matched immunoglobulin G isotype control antibodies were used, lacked α_v integrin expression. These data are consistent with our findings of nontransduction of EF43-*FGF4* cells by the RGD-4C adeno-associated virus/phage vector (data not shown).

α_v Integrin-Targeted RGD-4C Adeno-Associated Virus/Phage-*HSVtk* Vector Mediates a Marked Growth Suppression of EF43-*FGF4* Tumors After Ganciclovir Treatment

For assessment of the therapeutic efficacy of the RGD-4C adeno-associated virus/phage–*HSVtk* vector on the growth of EF43-*FGF4* tumors *in vivo*, mice with established EF43-*FGF4* tumors (~100 mm^3) received 5×10^{10} transducing units of targeted RGD-4C adeno-associated virus/phage–*HSVtk* i.v. Treatment with ganciclovir (80 mg/kg/d, i.p.) was initiated 2 days later. There was marked growth suppression of established tumors in the presence of targeted RGD-4C–displaying vector but not with nontargeted control vectors (Fig. 1B); moreover, tumor growth was not affected in several negative experimental control groups (Fig. 1B). For statistical analyses, we compared the median tumor growth of the group treated with RGD-4C adeno-associated virus/phage–*HSVtk* against all other seven control groups (Fig. 1B). For all comparisons, we applied the nonparametric Mann-Whitney test statistic. All *P*s were equal to 0.0079 (significant at significance level $\alpha = 0.05$).

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These results establish that targeted suicide gene therapy to the tumor tissue after systemic administration of the RGD-4C adeno-associated virus/phage vector results in efficient antitumor therapy despite the lack of direct tumor cell transduction.

Heterotypic Bystander Killing of EF43-*FGF4* Tumor Cells Can Be Induced by *HSVtk*-transduced Endothelial Cells

First, we determined that the EF43-*FGF4* cells can be killed by a bystander effect *in vitro*. EF43-*FGF4* cells transiently expressing *HSVtk* (100%) were mixed in a 1:9 ratio with parental nontransduced (i.e., *HSVtk* negative) EF43-*FGF4* cells and grown in coculture to subconfluent monolayers. Addition of 20 μmol/L ganciclovir resulted in cell death in >98% of the coculture, as confirmed by trypan blue exclusion (data not shown). This suggests a potent bystander effect among EF43-*FGF4* cells. Next, we investigated a potential heterotypic bystander effect between the EF43-*FGF4* tumor cells and *HSVtk*-transduced SVEC4-10 mouse endothelial cells from an SV40-transformed murine small blood vessels. We chose these cells after verifying that they display a strong bystander effect in homoculture after transduction with *HSVtk* and subsequent ganciclovir treatment (Fig. 2A). SVEC4-10 cells transiently expressing *HSVtk* were grown in a mixed heteroculture with nontransduced (i.e., *HSVtk* negative) EF43-*FGF4* cells (1:9 ratio). After 5 days of ganciclovir treatment, >90% of the cells in the heteroculture were killed indicating a strong bystander effect between *HSVtk*-transduced endothelial cells and the nontransduced tumor cells (Fig. 2B). To show that this result was not a unique phenomenon between endothelial cells and a particular tumor cell line, we reproduced the effect with malignant glioma 9L cells (Fig. 2C). The cell line MDA-MB435 served as a negative control because it does not display bystander killing in homoculture (data not shown). Consistently, no bystander killing was observed between MDA-MB435 cells and *HSVtk*-transduced endothelial cells (Fig. 2D).

The Bystander Effect between Endothelial Cells and Tumor Cells Is Mediated by Gap Junctional Intercellular Communication

Next, we attempted to identify the mechanism of the observed heterotypic bystander effect between *HSVtk*-expressing endothelial cells and tumor cells. Gap junctional intercellular communication plays a central role in mediating bystander effects (32). Therefore, we investigated gap junctional intercellular communication function in our heterotypic coculture system and analyzed the effect of a selective gap junctional intercellular communication inhibitor, 18α-glycyrrhetinic acid, on the heterotypic bystander killing that was observed *in vitro*. In heterotypic cocultures of 1:9 *HSVtk*-transduced SVEC4-10 endothelial cells and nontransduced EF43-*FGF4* cells, or 9L cells, respectively, the addition of 70 μmol/L 18α-glycyrrhetinic acid substantially inhibited bystander killing upon ganciclovir treatment (Fig. 3). For statistical analyses, we compared in each coculture the mean of cell survival between control and ganciclovir treatment, as well as between ganciclovir and 18α-glycyrrhetinic acid

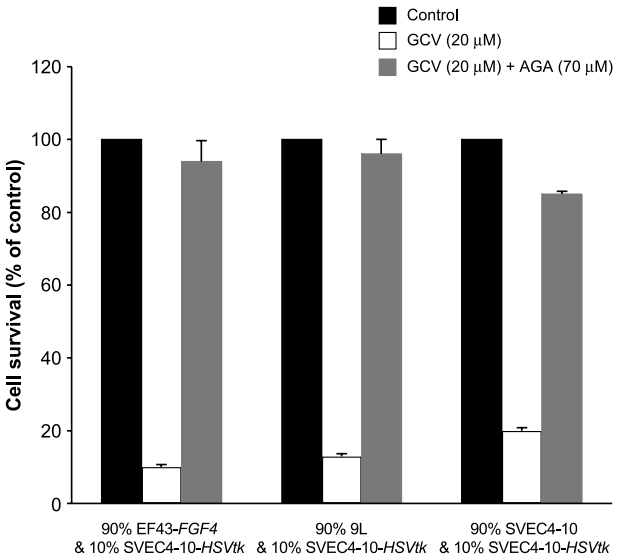

Figure 3. The heterotypic bystander effect is mediated by gap junctional intercellular communication. Heterotypic cocultures in a 1:9 ratio of *HSVtk*-expressing SVEC4-10 cells and *HSVtk*-negative EF43-*FGF4* or 9L tumor cells were treated for 5 d with either 20 μmol/L ganciclovir alone or a combination of 20 μmol/L ganciclovir plus 70 μmol/L 18α-glycyrrhetinic acid. Homotypic cocultures in a 1:9 ratio of *HSVtk*-expressing SVEC4-10 cells and *HSVtk*-negative SVEC4-10 were treated similarly. After treatment, cell survival was assessed by trypan blue exclusion and compared with that of the untreated control coculture (set to 100%). Data are means ± SD ($n = 3$).

treatments (Fig. 3). We applied the t test statistical and for all pairwise comparisons $P < 2.2 \times 10^{-16}$ (significant at significance level $\alpha = 0.05$).

This result indicates that the heterotypic bystander effect is related to gap junctional intercellular communication. It therefore became relevant to analyze the expression of connexins 43 and 26 in the cell types involved in the bystander effect. Connexins are proteins composing the channels of the gap junctional intercellular communication, through which toxic phosphorylated ganciclovir and/or other toxic intracellular metabolites are exchanged between one cell and another (33, 34). Immunofluorescence staining showed strong expression of connexins 43 and 26 in all cell types that exhibited bystander effect (Fig. 4). Consistently with our hypothesis, MDA-MB435 cells, which are not susceptible to the bystander effect, did not express these connexins (Fig. 4). We conclude that gap junctions mediate the heterotypic bystander effect between tumor and endothelial cells and that such cell junctions contain the connexins 43 and 26.

The Heterotypic Bystander Effect Can Be Elicited *In vivo*

Finally, we determined whether the bystander killing that was induced in EF43-*FGF4* tumor cells by the *HSVtk*-transduced SVEC4-10 endothelial cells *in vitro* could also be observed *in vivo*. Therefore, *HSVtk*-expressing SVEC4-10 or untransduced SVEC4-10 cells, respectively,

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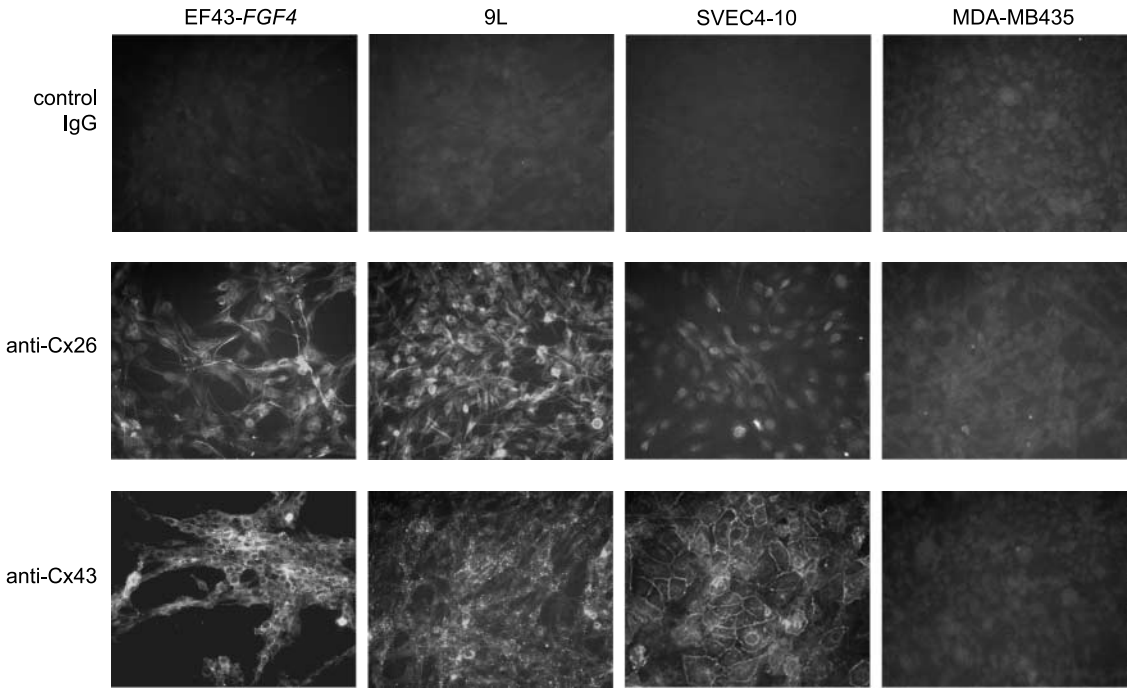

Figure 4. Connexin expression in tumor cells and endothelial cells involved in bystander killing. EF43-*FGF4* cells, 9L glioma cells, and SVEC4-10 endothelial cells as indicated were grown to saturation, after which they were fixed. Immunofluorescence with primary rabbit polyclonal anti–connexin 26, anti–connexin 43, or control antibodies was done. The MDA-MB435 cell line, which is not susceptible to bystander killing, served as a negative control.

were mixed at a 1:1 ratio with EF43-*FGF4* or SVEC4-10 cells and then implanted into nude mice. Tumors were formed only from cell mixtures containing EF43-*FGF4* cells, whereas the SVEC4-10 cells alone were nontumorigenic. Systemic treatment with ganciclovir started at day 6 after implantation, and the drug was administered daily until day 20. Mice injected with the control cell mixture of parental (*HSVtk* negative) SVEC4-10 and EF43-*FGF4* cells developed large tumors with rapid invasive growth (Fig. 5), comparable with that of homotypic parental EF43-*FGF4* tumors. In contrast, almost total inhibition of tumor growth was achieved in mice implanted with SVEC4-10–*HSVtk*–positive and EF43-*FGF4* cell mixtures (Fig. 5). These results establish that the heterotypic bystander effect between endothelial and tumor cells can also be elicited *in vivo*.

Discussion

Tumor vascular endothelium has certain properties that render it an attractive target for cancer gene therapy, such as accessibility to circulating vectors, expression of endothelial surface receptors distinct from those of normal quiescent vasculature, and a potential amplifying effect caused by hypoxia. Ligand-directed adeno-associated virus/phage can mediate targeted *HSVtk* suicide gene transfer to tumor vascular endothelium in several experimental models (20, 21). However, the precise cytotoxic mechanism of such profound antitumor effects after vascular-targeted suicide gene transfer has not as yet been entirely understood. Here, we show that vascular targeted *HSVtk* suicide gene delivery results in efficient cell killing mediated by a heterotypic bystander effect between endothelial and parenchymal tumor cells *in vitro* and *in vivo*.

The bystander effect has initially been described as a phenomenon in homotypic cultures, in which neighboring (bystander) *HSVtk*-nonexpressing tumor cells were also killed by ganciclovir (1, 2, 35, 36). Later, similar bystander effects were reported in other suicide gene systems (37), and it was observed after ionizing radiation as well (38). These observations suggest that not only activated cytotoxic drugs but also other toxic metabolites can be transferred from treated to adjacent untreated cells. Although well documented *in vitro*, the bystander effect after suicide gene transfer has been less studied in animal models (1, 35, 39) and only in experimental systems in which the parenchymal tumor cells were the primary target of the gene transfer. Clearly, there is room to improve the knowledge about this particular cell killing mechanism in an *in vivo* setting and perhaps to develop new translational applications.

We show that gap junctions mediate the heterotypic bystander effect in our system because it can be blocked by

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18α-glycyrrhetinic acid, a potent inhibitor of gap junctional intercellular communication. The intercellular junctions formed by endothelial cells and tumor parenchymal cells contain connexins 43 and 26, overexpression of which in gap junctions have been shown to potentiate the bystander effect (33, 34, 40). Consequently, gene transfer–mediated forced expression of these connexins in cells with low levels of gap junctions can result in potent induction of a bystander effect in cells lacking expression of the suicide gene (41). Therefore, the expression of these connexins selectively in the cell types displaying bystander effects in this study suggests that these proteins are involved in the heterotypic bystander killing described here. It is generally assumed that the level of the bystander effect is determined by the characteristics of the non-*HSVtk*-transduced cell population (40, 41). Consistent with this assumption, we did not observe bystander killing in cocultures of connexin-expressing, *HSVtk*-transduced endothelial cells and non-connexin-expressing, non-transduced MDA-MB435 cells.

The heterotypic bystander effect between endothelial and epithelial tumor cells can also be induced *in vivo*. Thus, such phenomenon likely accounts for the extend of tumor cell killing observed in various tumor cell models after endothelial cell–directed suicide gene transfer by targeted adeno-associated virus/phage vector and potentially other vascular-targeted gene therapy vectors. Often, a regular tumor graft model in which the endothelium is destroyed by vascular-targeted suicide gene therapy followed by secondary tumor eradication does not allow a rigorous dissection of underlying cytotoxic mechanisms. The model we have used in this study potentially circumvents this limitation. Co-administration of tumor cells and endothelial cells results in the formation of chimeric tumors, in which a predetermined fraction of the cells is endothelial, but it does not contribute to the circulation that is host dependent. Notably, killing of the graft endothelial cell population by suicide gene transfer and subsequent treatment with ganciclovir can most likely be explained by a heterotypic bystander effect rather than indirect tumor killing due to destruction of the vasculature and subsequent hypoxia. Such heterotypic bystander effects have been considered but not proven in previous models (36), and our study supplies for the first time systematic evidence to show that this hypothesis is valid. Nevertheless, additional mechanisms mediating tumor cell killing after suicide gene transfer in vascular endothelial cells *in vivo* cannot be excluded. Such additional conceivable mechanisms include bystander cell phagocytosis of apoptotic factors released into the extracellular space by dying cells (35, 42) or host immune responses following the *HSVtk* plus ganciclovir treatment (39, 43–45). As such, one must speculate that many of these putative mechanisms are nonmutually exclusive, may be context dependent, and may also occur between tumor cells and other nonvascular tumor stromal cells.

In summary, our demonstration of a heterotypic bystander effect *in vivo* may have implications for cancer

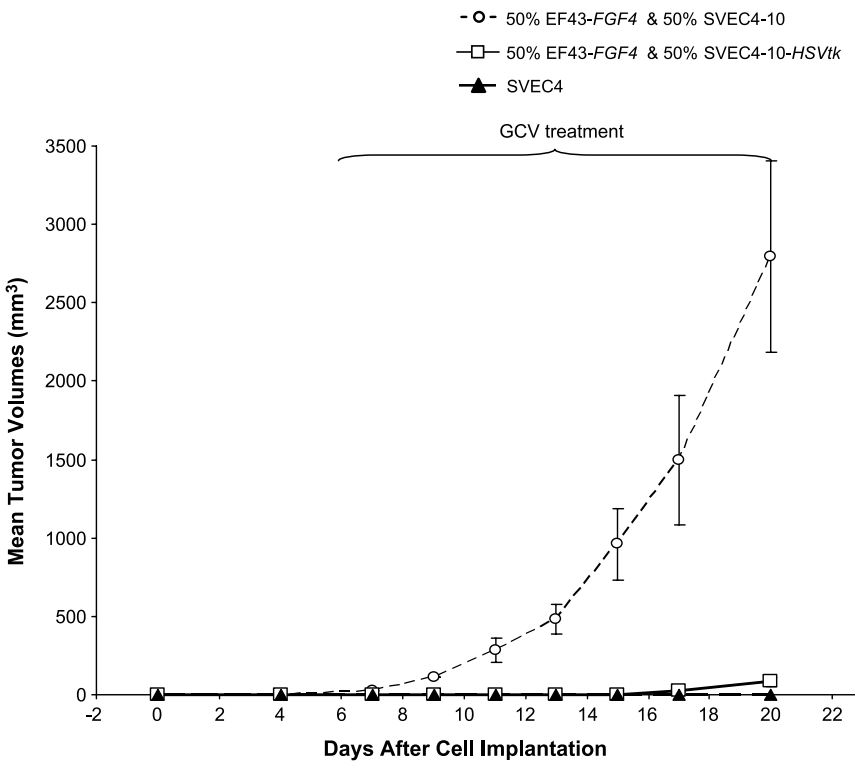

Figure 5. The heterotypic bystander effect between SVEC4-10–*HSVtk* cells and EF43-*FGF4* tumor cells can be induced *in vivo*. Mixtures of 10^5 cells containing 50% each of *HSVtk*-expressing SVEC4-10 endothelial cells and EF43-*FGF4* or parental SVEC4-10 cells, respectively, were injected s.c. into 6-wk-old female athymic nude mice at day 0. Starting at day 6, all mice received ganciclovir (80 mg/kg/d). Note the selective ablation of tumors containing *HSVtk*-transduced endothelial cells.

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gene therapy. Systemic, effectively targeted suicide gene delivery to nonparenchymal cells within a tumor may yield significant tumor responses in preclinical systems, even if the targeted cell population constitutes only the genetically nonmalignant fraction of the tumor. Together, these data add another potential cytotoxic mechanism to suicide gene therapy based on targeted adeno-associated virus/phage and support the promising role of nonmalignant tumor vascular and/or stromal cells as candidate therapeutic targets.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Molecular Cancer Therapeutics

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