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 additional 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
Cells targeted with anti-VEGF-R2 antibodies showed different results. The results demonstrated that anti-VEGF-R2 antibodies could inhibit the proliferation of tumor cells and induce apoptosis in vitro. These findings suggest that targeting VEGF-R2 may be a promising strategy for the treatment of HCC.

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).

Methods

The experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee. Tumor xenografts were generated by subcutaneous injection of tumor cells into nude mice. Tumor growth was measured using a caliper and the tumor volume was calculated using the formula: volume = length x width x height / 2. The efficacy of the treatment was assessed by monitoring tumor growth and comparing it to the control group.

Results

The results showed that the combination of anti-VEGF-R2 antibodies and a targeted gene therapy approach resulted in significant inhibition of tumor growth compared to the control group. The targeted gene therapy approach involved the use of a viral vector that targeted the VEGF-R2 receptor in tumor cells. The results demonstrated that this approach could be effective in the treatment of HCC.

Conclusion

In conclusion, the current study demonstrated that targeting VEGF-R2 with anti-VEGF-R2 antibodies in combination with a targeted gene therapy approach could be effective in the treatment of HCC.

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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 (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.

Results

EF43-FGF4 Tumor Cells Barely Express αν 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, αν 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 αν integrins on their surface (Fig. 1A). Kaposi’s sarcoma cells (KS1767), which served as a positive control (20, 23, 24), showed strong expression of αν integrins. The corresponding negative control, in which species-matched immunoglobulin G isotype control antibodies were used, lacked αν 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).

αν 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 mm³) received 5 × 10¹⁰ 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 Ps were equal to 0.0079 (significant at significance level α = 0.05).
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 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,
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

![Figure 4](image-url)
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 extent 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 transfer 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...
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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