Endothelial Cell Vehicles for Delivery of Cytotoxic Genes as a Gene Therapy Approach for Carcinoma of the Ovary

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Abstract

Human umbilical vein endothelial cells (HUVECs) were evaluated for utility as a vector to achieve a bystander effect and killing of ovarian carcinoma cell lines. After demonstrating that HUVECs could be transduced with the reporter gene LacZ encoded by an adenoviral vector, appropriate cell killing of the AdCMVHSV-TK-transduced HUVECs was exhibited after treatment with 20 μM ganciclovir. Mixing experiments were then performed to determine whether the transduced HUVECs would demonstrate a bystander effect with the ovarian cancer cell lines. When 50% AdCMVHSV-TK-transduced HUVECs were mixed with untransduced ovarian cancer cells, >70% of all cells were killed. Finally, s.c. and i.p. injections of herpes simplex-thymidine kinase-expressing HUVECs and SKOV3ip1 tumor cells were performed to evaluate the effects of HUVECs in vivo models. These studies showed a decrease in tumor growth s.c. as well as a statistically significant survival prolongation (P < 0.05) in the i.p. model. These findings suggest that endothelial cells may be used as a vehicle for the delivery of cytotoxicity (bystander effect) in molecular chemotherapy.

Introduction

The cancer gene therapy approach of molecular chemotherapy is designed to achieve toxin gene expression selectively in tumor cells using DNA-based methods. To this end, a variety of enzyme/prodrug combinations have been developed, including HSV-TK/GCV, cytosine deaminase/5-fluorouracil, and purine nucleoside phosphorylase/6-methylpurine-2′-deoxyxribonucleoside (1–3). For delivery of these toxin genes to tumor cells, developed vector approaches have included retroviral vectors, retroviral producer cells, adenoviral vectors, and liposome/DNA complexes. Some of these experimental approaches have been translated into human clinical gene therapy trials, including protocols for primary tumors of the central nervous system, ovary, mesothelium, and liver (4). In addition, approaches to metastatic disease of the peritoneum have been implemented (5).

In the various human clinical gene therapy approaches using toxin genes, the disease stage considered most suitable has been locoregional, whereby tumor is contained within an anatomical compartment. This situation potentially allows vector concentrations favoring optimal tumor cell transduction. In addition, vector containment within the compartment theoretically limits vector dissemination, thereby preventing the potentially deleterious consequences of non-tumor cell transduction. Based on these considerations, recombinant adenoviral vectors have shown the greatest vector utility for toxin gene delivery, demonstrating significant antitumor effects in animal models of glioma, mesothelioma, carcinoma of the ovary, and disseminated breast carcinoma based on high levels of in situ tumor cell transduction (6–9).

Despite the rapid clinical translation of these molecular chemotherapy strategies, significant limitations to this approach have become apparent. In this regard, human clinical studies have demonstrated relatively low efficiencies of tumor cell transduction at used magnitudes of adenoviral vector concentrations (10). In addition, host inflammatory and immunological responses to the vector have resulted in limited vector efficiency and treatment-related toxicity (11, 12). In the latter regard, it has also been demonstrated that vectors may disseminate beyond the tumor compartment (13). In selected instances, the resultant non-tumor cell transduction could be shown to be the basis of serious toxicity (14, 15). It is thus clear from these studies that even in the cancer compartment context, current vectors may not be the optimal approach to use for application of molecular chemotherapy.

Recognizing these defined limitations of current generation vector approaches, we have investigated the application of non-transformed endothelial cell as vehicles for toxin gene therapy. In this approach, normal human endothelial cells expressing toxin genes function as the vector for achieving delivery to tumor cells in situ. In this report, we show that such cellular vehicles can achieve an antitumor effect in a murine model of carcinoma of the ovary. This method of exploiting cells as gene delivery/cytotoxicity vehicles may offer appreciable advantages...
over currently used viral and nonviral vectors for molecular chemotherapy approaches.

Materials and Methods

Cell Lines. The human ovarian carcinoma cell line derivative SKOV3ip1 was obtained from Janet Price (University of Texas M. D. Anderson Cancer Center, Houston, TX). The human ovarian carcinoma cell lines PA-1 and OVCAR-3 were obtained from the American Type Culture Collection (Rockville, MD). All cell lines were maintained in DMEM:F12 (50:50 mixture; Cellgro, Herndon, VA) supplemented with l-glutamine (200 mg/ml), penicillin (100 mg/ml), streptomycin (25 mg/ml), and 10% heat-inactivated FBS (HyClone Laboratories, Logan, UT) at 37°C in a humidified 5% CO2 atmosphere.

Primary Cells. HUVECs were obtained from the laboratory of F. M. Booyse (University of Alabama, Birmingham, AL) from freshly obtained umbilical cords using collagenase digestion as described previously (16) and grown on 1% gelatin-coated flasks with Media 199 (Cellgro) supplemented with l-glutamine (200 mg/ml), penicillin (100 mg/ml), streptomycin (25 mg/ml), heparin sodium (10 units/ml; Elkins-Sinn, Inc., Cherry Hill, NJ), endothelial mitogen (0.1 mg/ml; Biomedical Technologies, Stoughton, MA), and 10% FBS (HyClone Laboratories). The media were changed every 3 days while the cells were maintained at 37°C in a humidified 5% CO2 atmosphere. Cells were used in the experiments for no more than seven passages.

Recombinant Adenoviruses. A replication-incompetent recombinant adeno viral vector encoding HSV-TK (AdCMVHSV-TK) was prepared using standard homologous recombination techniques and has been described previously (17). The recombinant adeno viral vector encoding the Escherichia coli a-galactosidase gene under the control of the cytomegalovirus promoter/enhancer (AdCMVLacZ) was used as a control (provided by Robert Gerard, The Center for Transgene Technology and Gene Therapy, Leuven, Belgium).

In Vitro Recombinant Adenoviral Transduction. Recombinant adenoviral vectors were used to transduce cells as described previously (18). In brief, the cells were plated 24-48 h before infection, with all experiments performed after the cells reached a confluence of 90-95%. The transduction was performed with the virus diluted in appropriate cell-specific medium containing 2% FBS. Infection was done in a small volume for 1 h at 37°C, after which complete medium was added to each flask. The cells were transduced with 100 PFU/cell either with AdCMVHSV-TK or AdCMVLacZ.

Toxin Gene-killing Experiment. In vitro cell-mixing experiments were performed to assess for a bystander effect as described in our prior work (9). Briefly, a population of either HUVECs or SKOV3ip1 cells was transduced with AdCMVHSV-TK and then proportionately mixed with a non-HSV-TK-expressing cell line (SKOV3ip1, PA-1, OVCAR-3, and HUVECs). The ratios of TK-expressing cells to untransduced cells were as follows: 0:100; 10:90; 50:50; 90:10; and 100:0. Cell mixtures were plated in triplicate in 96-well plates at a total density of 20,000 cells/well. Twenty-four h later, half of the samples were treated with GCV (20 μM). Cell viability was then ascertained 6 days later by a colorimetric cell proliferation assay measuring the conversion of a tetrazolium salt to formazan by viable cells as described by the manufacturer (MTS assay, Cell Titer 96 Aqueous Non-radioactive Cell Proliferation Assay; Promega, Madison, WI). The plates were incubated for 4 h at 37°C after the addition of 20 μl of assay solution. Subsequently, the absorbance was measured at 490 nm (Molecular Devices, Menlo Park, CA). To quantify the cell numbers, a standard curve was created by plating cells in triplicate wells at the following concentrations: 0; 2,000; 5,000; 10,000; 20,000; and 50,000 cells/well. Total numbers of cells were calculated based on the standard curve using SOFTmax computer software (Molecular Devices).

In Vivo s.c. Experiments. To study the effects on tumor growth, nude mice were separated into two groups; each group contained four animals injected in each flank with tumor cells (four injections/mouse). The groups varied according to the injection they received and GCV treatment: (a) HUVEC-TK (transduced with AdCMVHSV-TK) mixed with SKOV3ip1 cells; and (b) HUVEC-LacZ (transduced with AdCMVLacZ) mixed with SKOV3ip1 cells. Each animal received a total of 2.5 × 10⁶ tumor cells s.c. HUVECs were infected in vitro with their respective adenoviral vector (AdCMVHSV-TK or AdCMVLacZ) at a MOI of 100. Sixteen h postinfection, the transduced HUVECs were mixed with SKOV3ip1 cells at a ratio of 50:50 (2.5 × 10⁶ transduced HUVECs:2.5 × 10⁶ SKOV3ip1 cells) and injected s.c. into the flanks of nude mice. Twenty-four h after injection of the cellular mixtures, the animals were treated with daily i.p. injections of GCV (50 mg/kg) for 14 days. Tumor weight was measured at termination of GCV treatment.

In Vivo Survival Experiments. Female CB17/SCID mice ages 6–8 weeks were obtained from the National Cancer Institute Animal Program (Frederick, MD). To study the effects on survival, the mice were separated into five groups; each group contained seven animals. The groups varied according to the cell mixture they received: (a) HUVEC-TK (transduced with AdCMVHSV-TK) mixed with SKOV3ip1 cells; (b) HUVEC-LacZ (transduced with AdCMVLacZ) mixed with SKOV3ip1 cells; (c) SKOV3ip1-TK (transduced with AdCMVHSV-TK) mixed with SKOV3ip1 cells; and (d) SKOV3ip1 cells only. The animals received a total of 2 × 10⁵ tumor cells i.p. The transduced cells were infected in vitro with their respective adenoviral vector (AdCMVHSV-TK or AdCMVLacZ) at a MOI of 100. Twelve h postinfection, the transduced cells were mixed with untransduced SKOV3ip1 cells at a ratio of 50:50 (2 × 10⁵ transduced cells:2 × 10⁵ untransduced SKOV3ip1 cells) and injected i.p. in SCID mice. Twenty-four h after injection of the cellular mixtures, the treated animals received daily i.p. injections of GCV (50 mg/kg) for 14 days. Survival differences between control and experimental groups were then compared, and the statistical significance was analyzed using the log-rank test. All animal protocols were approved by the University of Alabama at Birmingham and are in compliance with the standards established by the NIH.

Results

The practical realization of an endothelial cell vehicle approach for gene therapy is based on endothelial cells achiev-
demonstrate that such a vector strategy could achieve high levels of expression of the HSV-TK toxin gene. For this analysis, HUVECs were transduced with AdCMVHSV-TK at a MOI of 100 PFU/cell with a recombinant adenoviral vector encoding the E. coli LacZ gene. Forty-eight h postinfection, the transduced HUVECs were fixed and stained with 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-Gal). The photomicrograph was taken at ×100 magnification. B. toxin gene killing demonstrated in AdCMVHSV-TK-transduced HUVECs at 100 PFU/cell. HUVECs (4 x 10^5) were plated in a gelatin-coated 6-well plate. Twenty-four h later, half of the samples were treated with 20 μM GCV (+GCV). Six days later, the samples were stained with crystal violet. The photomicrographs were taken at ×20 magnification.

Fundamental to the efficiency of the molecular chemotherapy strategy is the bystander effect, whereby toxin gene-expressing cells exert a noxious effect on surrounding untransduced tumor cells. This bystander phenotype is an intrinsic property of target cells that dictates the potential efficacy of this approach in a given context. A crucial determinant of the utility of our approach was the degree to which toxin gene-expressing endothelial cells could establish a bystander effect with the intended target tumor cells.

We thus investigated if the HSV-TK-expressing HUVECs could functionally accomplish a bystander effect with the different human ovarian carcinoma cell lines in vitro. A mixing experiment was performed in which HSV-TK-expressing HUVECs were mixed at various ratios with nonexpressing HUVECs or human ovarian cancer cells. GCV killing was measured using an MTS assay. Initial studies validated the bystander assay, whereby SKOV3ip1 ovarian cancer cells demonstrated a bystander phenotype such that a mixture of 50% AdCMVHSV-TK-infected SKOV3ip1 cells achieved a net tumor cell kill of more than 90% (Fig. 2A). When the same experiment was performed with HUVECs to determine whether these cells could independently demonstrate a bystander phenotype, as few as 10% of AdCMVHSV-TK-infected HUVECs could achieve killing of greater than 50% of the total cell population of HUVECs (Fig. 2B). This established that HUVECs possess the physiological capacity to accomplish a bystander effect using an HSV-TK/GCV enzyme/prodrug approach. Next, the ability of AdCMVHSV-TK-transduced HUVECs to achieve a bystander effect with tumor targets was determined. This experiment established a scenario with the highest level of analogy to our planned cellular vehicle approach. In Fig. 2, C–E, it could be demonstrated that a cell mixture of 50% HSV-TK-expressing HUVECs and 50% untransduced ovarian cancer cells resulted in an overall cell kill of >70%. It thus seems that HSV-TK-expressing HUVECs could function to achieve a functional bystander effect with the human
Fig. 2 Bystander effect exhibited *in vitro* by AdCMVHSV-TK-transduced HUVECs when mixed at varying percentages with uninfected HUVECs (B) and the human ovarian carcinoma cell lines SKOV3ipl (C), PA-1 (D), and OVCAR-3 (E). AdCMVHSV-TK-transduced SKOV3ipl cells mixed with untransduced SKOV3ipl cells served as a control for the bystander assay (A). Twenty-four h after mixing, half of the cells were treated with GCV. The percentage of viable cells was measured using an MTS assay.

We next investigated if the HSV-TK HUVECs could achieve a bystander effect and mediate killing of tumor cells *in vivo*. In this experiment, nude mice were inoculated s.c. with a mixture of 50:50 HSV-TK-expressing HUVECs and SKOV3ipl- or LacZ-expressing HUVECs and SKOV3ipl ovarian cancer cells, respectively. Half of the animals were treated with GCV i.p. (50 mg/kg). Tumor weight was measured after 14 days of GCV treatment and plotted in Fig. 3. The animals that had received HSV-TK-expressing HUVECs and SKOV3ipl cells and were treated with GCV showed no growth of s.c. nodules, whereas all of the control groups developed tumor nodules. These results thus demonstrated that the HSV-TK-expressing HUVECs can achieve a functional bystander effect with human ovarian cancer cells and mediate killing of these cells *in vivo*.

Having established that HUVECs are an effective vehicle for the delivery of cytotoxicity to tumor cells *in vitro* and *in vivo*, we next evaluated the therapeutic utility of this cellular vehicle approach in an *in vivo* model of human carcinoma of the ovary. For this analysis, SCID mice were xenografted with SKOV3ipl cells and HUVECs expressing either the HSV-TK or LacZ gene at a ratio of 50:50, as described previously. The control groups consisted of those animals receiving SKOV3ipl cells only, SKOV3ipl cells transduced with AdCMVHSV-TK, and those animals that did not receive any tumor cells. All experimental groups then received daily i.p. injections of GCV (50 mg/kg) for 14 consecutive days. The results of this analysis are shown in Fig. 4. Using the log-rank test, a significant difference in survival was observed with regard to the animals receiving a combination of SKOV3ipl and HUVECs expressing HSV-TK when compared to those animals receiving only SKOV3ipl (*P* < 0.05). There was also a trend toward a survival advantage when the SKOV3ipl + HSV-TK-transduced HUVEC group was compared to the SKOV3ipl + LacZ-transduced HUVECs (*P* = 0.058). A larger sample size may have yielded enough statistical power to attain a significant difference. In addition, one animal within the SKOV3ipl + HSV-TK-transduced HUVEC group died on day 25. This animal was noted from the beginning of the experiment to be smaller than other cohorts and experienced thinning fur and other symptoms consistent with GCV-related toxicity at the time of death. If this animal was excluded from the analysis, the difference between the groups receiving SKOV3ipl + HSV-TK-transduced HUVECs and SKOV3ipl + LacZ-transduced HUVECs would have been statistically significant (*P* = 0.018). Importantly, there was no significant difference between the group receiving SKOV3ipl + HUVECs expressing HSV-TK when compared to the group receiving SKOV3ipl and AdCMVHSV-TK-transduced SKOV3ipl (*P* = 0.18). In addition, selected necropsy cases revealed decreased tumor volume between the group treated with HUVECs expressing HSV-TK and the control groups (data not shown). Thus, HUVECs, as a cellular vehicle,
are effective in prolonging survival in this model of human ovarian carcinoma.

**Discussion**

We report here the development of a cellular vehicle for achievement of a molecular chemotherapy approach for carcinoma of the ovary. This strategy of cell-based cytotoxicity delivery was shown to accomplish an antitumor effect comparable to viral vector-mediated toxin gene delivery. Most importantly, this approach may offer distinct advantages from the standpoint of safety and vector toxicity that may allow an enhanced therapeutic index with toxin gene therapy approaches.

Our studies have established key concepts with respect to the endothelial cellular vehicle strategy. High-efficiency transient expression of the HSV-TK gene can be achieved within the endothelial cells with adenoviral vector-mediated gene transfer. In this regard, means of toxin gene induction could effectively sensitize these cells to the prodrug GCV with quantitative cell killing. Fundamental to the efficiency of the molecular chemotherapy approach is the bystander effect, whereby toxin gene-expressing cells exert a noxious effect on surrounding untransduced tumor cells. The importance of this effect is that it potentially overcomes the requirement to achieve near-universal quantitative tumor cell transduction for cancer gene therapy efficiency. Thus, in the molecular chemotherapy approach, the establishment of a bystander effect is an important achievement contributing to the overall efficiency of this gene therapy strategy. To this end, studies have examined the biological basis of the bystander effect. It seems that the presence of intercellular communications is an important determinant of the bystander phenotype (20). In addition, apoptotic bodies generated during toxin gene delivery killing may also function as a vehicle for disbursement of toxin gene metabolites to untransduced tumor cells (21). Functional studies to establish bystander cell killing are based on mixing experiments, whereby toxin gene-expressing and nonexpressing cells are combined at various ratios to determine the magnitude of bystander-induced cell killing that is out of proportion to the percentage of toxin gene-expressing cells. We have established that HUVECs can manifest a bystander phenotype in conjunction with human ovarian tumor cells in vitro and in vivo. This achievement suggests that delivered HSV-TK-expressing HUVECs can couple functionally with tumor targets; therefore, the resulting bystander effect may allow an amplified tumor cell killing effect.

There have been reports using cellular vehicles in distinct gene therapy contexts. In this regard, passive immunotherapy approaches using tumor-infiltrating lymphocytes have been modified by genetic methods to enhance tumor cell killing and/or tumor cell localization. In these schema, the antitumor lymphocytes are exploited in a manner in which their basic antitumor physiology is enhanced by genetic methods. Alternatively, the use of endothelial cell vehicles has been developed by Ojeifo et al. (22). In their schema, endothelial cells achieve tumor cell localization by incorporation into areas of tumor localization by incorporation into areas of tumor
angiogenesis. Such targeting has been used in various model systems to localize antitumor gene products to tumor sites with significant antineoplastic effects.

In our system, endothelial cells are not exploited in any manner relating to their basic physiology. In this regard, the HUVECs function as a simple vehicle for transport of toxin genes mediating cytotoxic effect to the site of tumor cells. Key to their functioning is their ability to achieve a bystander effect with tumor targets. This capacity sets them apart from other mobile cells that might have been used, such as lymphocytes or monocytes, because these cells cannot achieve a substantial bystander capacity. This ability of the HUVECs to establish a bystander effect is responsible for the tumor burden reduction and significant survival prolongation in a human ovarian carcinoma mouse model. We have previously used an orthotopic human tumor xenograft model of carcinoma of the ovary to determine the efficacy of antitumor gene therapy approaches (9). This model exploits the biological behavior of the ovarian carcinoma cell line SKOV3ip1 in this context, whereby a valid murine model of human carcinoma of the ovary is achieved. In addition, our s.c. nodule results are consistent with the i.p. studies and likewise provide evidence for the efficacy of this novel approach. It remains to be determined if the issues of safety and toxicity related to this system will recommend its use for molecular chemotherapy approaches in preference to viral vectors. However, this basic efficiency profile suggests these vehicles warrant further development for molecular chemotherapy applications.

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References

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