Convection-enhanced delivery improves distribution and efficacy of tumor-selective retroviral replicating vectors in a rodent brain tumor model

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In the present study, we compared the therapeutic effect of tumor-selective retroviral replicating vectors (RRV) expressing the yeast cytosine deaminase (CD) delivered by convection-enhanced delivery (CED) or simple injection, followed by systemic administration of the pro-drug, 5-fluorocytosine (5-FC). Treatment with RRV-CD and systemic 5-FC significantly increased survival in rodent U87MG glioma model in comparison with controls (P<0.01). Interestingly, CED of RRV-CD followed by 5-FC further enhanced survival in this animal model in comparison with intra-tumoral injection of RRV-CD, followed by systemic 5-FC (P<0.05). High expression levels of Ki-67 were found in untreated tumors compared with treated. Untreated tumors were also much larger than treated. CED resulted in excellent distribution of RRV while only partial distribution of RRV was obtained after injection. Furthermore, RRV-CD and CD were also found in tumors from treated rats at study end points. These results demonstrated that RRV vectors may efficiently transduce and stably propagate in malignant human glioma, thereby achieving a significant in situ amplification effect after initial administration. We conclude that delivery of RRV into the glioma by CED provides much wider vector distribution than simple injection, and this correlated with better therapeutic outcomes.

Cancer Gene Therapy (2013) 20, 336–341; doi:10.1038/cgt.2013.25; published online 24 May 2013

Keywords: convection-enhanced delivery; 5-fluourouracil; glioma; RRV

INTRODUCTION

The prognosis for patients with malignant glioma, such as glioblastoma multiforme, is very poor despite combined therapeutic modalities that include surgery, radiation and chemotherapy.1,2 As these tumors grow rapidly and invade the surrounding brain parenchyma, it is often impossible to achieve complete surgical resection without causing severe neurological damage.3 Although it is an important therapy for glioblastoma multiforme, chemotherapy has not consistently achieved clinical benefits, and significant response is seen in only 10–30% of patients.4,5 Outcomes for patients with gliomas remain dismal, and therefore there is an obvious need for the development of novel therapeutic strategies for the treatment of glioblastoma multiforme, such as gene therapy.

Gene therapy offers tremendous potential advantages for the future of cancer treatment, and has been exploited to develop new strategies for selectively killing cancer cells or arresting their growth.6 The most commonly used strategy in cancer gene therapy has been pro-drug activator gene delivery. This approach allows the administration of a well-tolerated pro-drug that is then converted to an active anticancer metabolite by a specific enzyme introduced into the target cells by a pro-drug activator gene.7,8 Unique among replicating viruses being developed as oncolytic agents, murine leukemia virus-based retroviral replicating vectors (RRV) replicate without immediate lysis of the host cell, and maintain viral persistence through stable integration.5,6 Furthermore, murine leukemia virus does not infect quiescent cells, so RRV-mediated gene transfer is selective for dividing cells such as cancer cells, and further tumor specificity may accrue from tumor-associated defects in the innate immune system; cytosine deaminase (CD) converts 5-fluorocytosine (5-FC) into the potent chemotherapeutic agent 5-fluorouracil (5-FU) directly and selectively in the infected tumor cells.9 The 5-FU can exit from the transduced cells and enter neighboring dividing cells, resulting in a bystander effect to achieve improved malignant cell killing.10,11 The CD/5-FU combination has been proven effective at controlling tumor growth in animals,6–8 and is currently being evaluated in several clinical trials including trials for high-grade glioma brain tumors (ClinicalTrials.gov. NCT01470794, NCT01156584) However, in earlier applications of neurological gene therapy with non-replicating vectors, poor distribution of vector in the target tissue is invariably associated with poor efficacy, and it still remains an underestimated, yet potentially critical, factor in gene therapy.12,13 In human tumors, strategies for improving RRV distribution, and hence efficacy, are potentially important goals.

Convection-enhanced delivery (CED) is an interstitial central nervous system delivery technique that circumvents the blood–brain barrier in delivering agents directly into the brain. Traditional local delivery, such as injection of most therapeutic agents into the brain, has relied on diffusion that depends on a concentration gradient to overcome biological barriers. Thus, diffusion results in limited distribution of most delivered agents, and drug penetrates only a few millimeters from the source. Injection of therapeutics into nonmalignant brain also has been associated with reflux and leakage near the injection site in human studies. In contrast, CED...
uses a fluid pressure gradient established at the tip of an infusion catheter and bulk flow to propagate substances within the extracellular fluid space.\textsuperscript{14} CED also allows the extracellularly infused material to further propagate via the perivascular spaces, and the rhythmic contractions of blood vessels act as an efficient motive force for the infusion.\textsuperscript{15} As a result, a higher concentration of certain drugs can be distributed more evenly over a larger area of targeted tissue than would be seen with a simple injection. Currently, CED has been clinically tested in the fields of neurodegenerative diseases, such as Parkinson’s disease,\textsuperscript{6,11} and neuro-oncology.\textsuperscript{18,19} Laboratory investigations with CED cover a broad field of application, including the delivery of viral particles.\textsuperscript{20} Although RRV vectors are capable of natural spread within the brain tumor, optimal methods for initial brain delivery have not been established. In this study, we compared the therapeutic effect of RRV-CD delivered by CED or manual injection, followed by systemic administration of pro-drug S-FC. We found CED significantly improves efficacy of therapeutic RRV-CD by maximizing vector spread and distribution, and tumor cell transduction in a rodent brain tumor model as compared with an injection technique. Our findings showed that CED should be considered when local therapies such as gene transfer with RRV are being translated into clinical therapy.

**MATERIALS AND METHODS**

Cell line and RRV vector

The human glioblastoma multiforme cell line, U87MG, was obtained from the Brain Tumor Research Center Tissue Bank at the University of California, San Francisco. Cells were maintained as a monolayer in Eagle’s minimum essential medium supplemented with 10% fetal bovine serum, non-essential amino acids, 0.1 mg ml\textsuperscript{-1} streptomycin sulfate and 100 U ml\textsuperscript{-1} penicillin G. Cells were cultured at 37°C in a humidified atmosphere consisting of 95% air and 5% CO\textsubscript{2}.

RRV AC3-emd vector encoding the GFP complementary DNA (RRV-GFP) was obtained as a gift from Dr N Royuki Kasahara (University of California, Los Angeles, CA, USA) with permission of Tocagen, and the titer of vector used was 1000 TU ml\textsuperscript{-1}. RRV vector expressing the yeast CD pro-drug activator gene, Toca 511(\textsuperscript{19}voicimagene amiretrorepvec),\textsuperscript{21} was obtained from Tocagen, and the titer of vector used was 6.3 × 10\textsuperscript{4} TU ml\textsuperscript{-1}. We used GFP vectors at a low titer to allow easy visualization of vector spread. In the efficacy experiments, the goal was to maximize vector dose in order to reveal differences in efficacy. The pro-drug S-FC was also obtained from Tocagen and used at a dose of 500 mg kg\textsuperscript{-1} body weight (twice daily, half the dose at each time).

Animal brain tumor model

Congenitally athymic, male, nude rats weighing 150–200g (National Cancer Institute Animal Production Program, Frederick, MD, USA) were housed under aseptic conditions that included filtered air, and sterilized food, water, bedding and cages. For the intracranial xenograft tumor model, U87MG glioblastoma cells were harvested by trypsinization, washed once with phosphate-buffered saline (PBS) without Ca\textsuperscript{2+} and Mg\textsuperscript{2+}, and resuspended in PBS for implantation. Cells (2 × 10\textsuperscript{5}) in 10 μl PBS were implanted into the striatal region of brains as follows: under deep isoflurane anesthesia, rats were placed in a small-animal stereotactic frame (David Kopf Instrument, Tujunga, CA, USA) to regulate the flow of fluid through the system. The boundaries of each distribution and brain regions of interest was then calculated as area per brain section defined multiplied by slice thickness. The boundaries of each distribution and brain tumor were defined in the same manner in a series of brain sections.

**Delivery of RRV vectors by CED and injection**

Twenty microliters of RRV vector was infused by CED into tumor as described.\textsuperscript{22–24} Briefly, the infusion system consisted of a cannula with a 1-mm step\textsuperscript{25} connected to a 100-μl syringe (filled with RRV) that was in turn mounted onto stereotactic holder. The holder was connected to a micro-infusion pump (BeeHive, Bioanalytical System, West Lafayette, IN, USA) to regulate the flow of fluid through the system. On the basis of the chosen coordinates, the stepped cannula was guided to the targeted region of the implanted brain tumor through burr holes made in the skull. A constant infusion rate of 1 μl min\textsuperscript{-1} over 30 min was applied to administer a total infusion volume of 20 μl. Alternatively, RRV, in a volume of 20 μl, was manually injected as a bolus into brain tumor via a syringe and a 26-gauge needle fitted with a cut pipette tip as a depth stop to compare the RRV distribution between the two delivery methods.

**Antitumor effect of RRV-CD and S-FC in U87MG xenograft model**

Thirty-two nude rats with U87MG xenografts were randomly divided into three groups: group 1, control (n = 10); group 2, injection of RRV-CD (n = 11); and group 3, CED of RRV-CD (n = 11). Five days after tumor implantation, rats in groups 2 and 3 received either injection or CED of RRV-CD into the brain tumor, whereas control animals received CED of 0.9% normal saline. Five days after RRV administration, all the animals received IP injections of S-FC (500 mg kg\textsuperscript{-1}) daily for 7 days. Three rats in each group were euthanized 3 days after S-FC treatment for evaluation of tumor size and expression levels of Ki-67. Antitumor efficacy was estimated by increase in median survival time as compared with control. Results are shown as Kaplan–Meier plots.

**Immunohistochemistry**

Animals were perfused with PBS, followed by 4% paraformaldehyde. Brains were post-fixed overnight in 4% paraformaldehyde, followed by 30% sucrose, and then were cut into 40-μm sections on a MICROM HM450 sliding microtome (Fisher Scientific, Philadelphia, PA, USA). Sections were blocked first in 1% H\textsubscript{2}O\textsubscript{2} and then in Biocare Sniper (Biocare, Concord, CA, USA), followed by incubation overnight at room temperature with primary rabbit anti-human GFP polyclonal antibody (Millipore, Chemicon, Billerica, MA, USA) diluted 1:500 with Da Vinci (Biocare), or rabbit anti-goat polyclonal antibody (Abcam, Cambridge, MA, USA) diluted 1:5000 with Da Vinci (Biocare), or at 4°C with rabbit anti-Ki-67 polyclonal antibody (Novocastro, Buffalo Grove, IL, USA) diluted to 1:5000 with Da Vinci. The MACH 2 conjugated goat anti-rabbit polymer-horseradish peroxidase secondary antibody (Biocare) was used to detect the primary antibodies. For CD immunohistochemistry, sections were incubated overnight at room temperature with primary rabbit anti-CD polyclonal antibody (Abcam) diluted to 1:2000 with Da Vinci (Biocare). Biotinylated rabbit anti-sheep IgG was used to detect the primary antibody. Immunoreactivity was visualized with diaminobenzidine working solution, according to the supplier’s recommendations (Vector Laboratories, Burlingame, CA, USA).

**Volumetric quantification of RRV distribution**

Brain sections with GFP immunohistochemistry were used for volumetric quantification of distribution of RRV. The distribution volume of RRV in the brain tumor of each subject was quantified on an Apple Macintosh G4 computer with the Image J program (Image Processing and Analysis in Java, http://rsb.info.nih.gov/ij/). Regions of interest derived in the distribution of RRV and brain tumor were manually defined, and the volume of the regions of interest was then calculated as area per brain section defined multiplied by slice thickness. The boundaries of each distribution and brain tumor were defined in the same manner in a series of brain sections.

**Statistical analysis**

Results are expressed as mean ± s.d., where applicable. The statistical analyses of distribution of RRV were performed by Student’s t-test. Confidence intervals are indicated by P-values where appropriate.
RESULTS

Prolonged survival in U87MG brain tumor xenograft model with CED of RRV-CD followed by systemic 5-FC

After CED or manual injection of RRV-CD into pre-established U87MG gliomas, we performed a single cycle of systemic 5-FC administration for 7 days. All rats from the control group, which only received intraperitoneal (IP) injections of 5-FC, developed neurological symptoms because of large tumors and were euthanized between 18 to 24 days with median survival time of 21.1 ± 0.6 days after tumor implantation (Figure 1). In contrast, RRV-CD and 5-FC-treated groups survived significantly longer (P < 0.01). Furthermore, CED of RRV-CD significantly improved the survival in animal with brain tumor as compared with injection of RRV-CD. In animals treated with injection of RRV-CD and IP 5-FC, the median survival time was 49.3 ± 3.1 days (Figure 1). In the group that received CED of RRV-CD and IP 5-FC, four out of eight rats were euthanized 51, 58, 59 and 65 days after tumor implantation, and the other four survived until the end point of the efficacy study (77 days; Figure 1). Histopathological evaluation was performed and brain tumors were found in all animals. Systemic injection of 5-FC caused weight loss of <10%, and no other side effects were found in this study.

To evaluate tumor size, nine rats (three in each group) were euthanized 3 days after 5-FC treatment, and their brains were subjected to histological examination. Representative tumor sizes are shown in Figure 2. All three rats in control group developed large U87MG tumors throughout the hemisphere (Figure 2a). In contrast, animals that received either an intra-tumoral injection (Figure 2b) or CED (Figure 2c) of RRV-CD, followed by one cycle of 5-FC administration, showed much smaller brain tumor compared with controls. The tumors in rats that received CED of RRV-CD and IP 5-FC tended to be smaller compared with those that were injected with RRV-CD and IP 5-FC. The brain sections from treated and untreated rats with intracranial gliomas were also processed for Ki-67 immunohistochemistry. Treatment with RRV-CD and 5-FC led to dramatic reduction in Ki-67 index (Figures 2d–f).

Distribution of RRV in brain tumor

The therapeutic effect of any agent is largely dependent on its distribution in the target. To investigate why CED of RRV-CD significantly increased survival time relative to simple injection of RRV-CD, we studied the distribution of RRV vectors in the brain tumor. Ten nude rats with U87MG xenografts were randomly divided into two groups that both received RRV-green fluorescent protein (GFP) vector (20 μl), delivered into the brain tumor by CED (n = 5) or injection (n = 5). Animals were then euthanized 7 days after administration of vector. During CED, a stepped cannula was used that has been shown to prevent reflux and leakage in the rat brain.\(^{25}\) The delivery time and infusion rate for CED were 20 min at 1 μl min\(^{-1}\), respectively. Excellent distribution of RRV-GFP was obtained in brain tumors 7 days after delivery by CED, ranging from 188.4 to 289.7 mm\(^3\) with mean volume of 242.6 ± 29.4 mm\(^3\). On the other hand, in rats that received injection of RRV-GFP vectors, relatively poor distribution of vector in the brain tumor was obtained, ranging from 77.6 to 129.8 mm\(^3\) with mean volume of 99.3 ± 11.7 mm\(^3\). No expression of RRV-GFP vector was found in normal brain by either delivery methods.

When CED was used, the mean coverage was 79.3 ± 8% (range: 64.3–97.5%). However, even with correct placement of the needle (postmortem visualization of needle track), injection resulted in a much lower (P < 0.05) mean volume of distribution percentage of RRV-GFP in the tumor of 48.7 ± 6% (range: 38.0–64.8%). Figure 3 shows a comparison of representative GFP staining, indicating RRV distribution in the brain tumors from animals administered RRV-GFP by CED (Figure 3a) or manual injection (Figure 3b). This is consistent with the results of the survival study. Better distribution of RRV in the tumor delivered by CED likely resulted in more efficient conversion of 5-FC to 5-FU and thereby antitumor effect.

We also studied RRV-CD distribution with gag staining and by measurement of expression levels of CD in the tumor tissues of rats used in the efficacy study. RRV-CD efficiently transduced almost all the tumor cells and covered the entire brain tumor of rats at the efficacy study end points (Figure 4). Interestingly, higher expression of gag and CD was noted in tumors after CED of RRV-CD compared with manual injection (Figure 5). These data...
indicate that multiple courses of treatment with 5-FC may have a more effective antitumor effect after a single CED delivery of RRV-CD into the brain tumor.

**DISCUSSION**

Systemic administration of 5-FU, one of the most active antineoplastic agents in conventional cancer chemotherapy, is ineffective against brain tumors owing to its relatively low diffusion across the blood–brain barrier at safe doses. Direct intra-tumoral administration of 5-FU has been explored for local chemotherapy in patients with malignant gliomas. However, because of its short half-life and cell cycle phase-specific activity, this approach requires a sustained-release polymer carrier system to achieve any significant therapeutic effect. Moreover, therapeutic efficacy is then restricted by limited diffusion of drug released from the polymer implantation site, consistent with the poor clinical efficacy of this approach. RRV expressing CD can achieve highly efficient gene transfer to tumors because each transduced tumor cell itself becomes a virus-producing cell, sustaining further transduction events after only an initial inoculation. RRV can achieve efficient delivery of pro-drug activator genes that permanently integrate into the target cell genome, stably expressing pro-drug-converting enzymes, such as CD that enables killing of the infected cell through production of chemotherapeutic drug, 5-FU, upon systemic administration of a well-tolerated pro-drug, 5-FC. As pro-drug conversion is...
intracellular and confined to tumor cells and 5-FU has a short half-life, the adverse side effects associated with systemic administration of toxic chemotherapeutic agent 5-FU can be avoided. Although RRV are capable of natural spread within the brain tumor, an optimal method for initial brain delivery has not been established. As observed in current and previous studies, owing to an absolute requirement for cell mitosis to achieve productive infection, RRV showed an inherent tumor selectivity, selectively transducing glioma cells.

The relationship between RRV administration, virus spread, tumor growth and timing of 5-FC administration is not completely characterized. We decided to test the ability of CED to maximize RRV distribution. Our current results confirm and extend the findings of previous studies of RRV. In the present study, we compared brain tumor distribution and therapeutic effect of a single dose of RRV delivered by CED or direct manual injection, followed by one cycle of systemic administration of 5-FC in rodent intracranial glioma xenograft model. Combination treatment with RRV-CD and 5-FC was able to achieve profound inhibition of pre-established U87MG gliomas, resulting in more than a doubling of the median survival time compared with controls. These findings are consistent with previous RRV studies. The immunohistochemical studies presented here also represent the first direct correlation between therapeutic effect of RRV and expression levels of Ki-67. In particular, we observed large tumors and high expression levels of Ki-67 only in controls without administration of vectors. Furthermore, CED resulted in excellent early distribution of RRV, with only partial early distribution of RRV after simple injection. Therefore, CED of RRV-CD followed by 5-FC resulted in even more significant survival benefit relative to intra-tumoral manual injection of RRV-CD in our studies. The enhanced therapeutic effectiveness of the CED approach is a novel finding, and the treatment advantage provided by CED was largely attributable to the improved spread and distribution of RRV, potentially enabling more widespread production of enzyme and conversion of 5-FC to 5-FU. After initial administration of RRV within tumor, transduced tumor cells become virus-producing cells that sustain further transduction for a prolonged period, and this appears to enhance the 5-FU chemotherapeutic effect. More extensive initial delivery may allow more rapid complete transduction and/or higher vector copy number per tumor cell. This may be important, given the limited time for vector spread in the rat tumor model. Notably, we observed complete RRV transduction of the entire U87MG tumor in some cases within 7 days after single CED delivery of vectors (Figure 3), and 50% of the animals treated with only one cycle of 5-FC survived until termination of the study. These results demonstrated the improved effect of RRV for gene therapy when delivered by CED in this model. Furthermore, our data also showed that RRV-CD provides stable integration and vector copy number per tumor cell. This may be important, given the limited time for vector spread in the rat tumor model. Notably, we observed complete RRV transduction of the entire U87MG tumor in some cases within 7 days after single CED delivery of vectors (Figure 3), and 50% of the animals treated with only one cycle of 5-FC survived until termination of the study. These results demonstrated the improved effect of RRV for gene therapy when delivered by CED in this model. Furthermore, our data also showed that RRV-CD provides stable integration and persistence in dividing tumor cells even at the end point of the efficacy study (Figures 4 and S), supporting the idea that repeated cycles of 5-FC treatment may achieve extended therapeutic benefit.

Our data demonstrate the advantage of good distribution of the vector after administration and spread to observe therapeutic effects in a rat model. Poorer distribution of RRV after simple manual injection may be due to reflux and leakage of vectors out of the target caused by injection force. Reflux and leakage during injection may decrease the effective dose of RRV in the target tumor. It is possible that slow infusion with stereotaxis or infusions into larger tumors could improve the injection results. It has been reported that after injection of RRV into glioma xenografts in nude mice, >98% transduction could be achieved throughout the entire tumor mass over a period of several weeks. In contrast, our data showed that it took only 1 week for RRV vectors to cover the whole brain tumor after delivery by CED. Therefore, the CED approach may provide an earlier window for treatment of glioma with 5-FC, and gain more time for treatment with multiple courses of 5-FC.

CED has been developed as a drug delivery strategy and represents a powerful methodology for targeted therapy in the brain. Delivery of therapeutic agents by CED within the human brain is becoming a more frequent experimental treatment option in the management of brain tumors, and more recently in phase 1 trials for gene therapy in Parkinson’s disease. This technology potentially offers the clinician a more specific option in delivering therapeutic vectors to a larger and more consistent treatment volume than the standard diffusion-based injection. As shown by our animal experiments, eradication of tumors is a realistic possibility when extensive coverage of the glioma with RRV is achieved with CED and multiple cycles of 5-FC treatment are employed. Nevertheless, it remains to be determined whether the advantage of CED over injection observed in these experiments of RRV for the nude rat xenograft model translates to naturally occurring tumors that have known heterogeneity and a mixture of live and necrotic areas. We have started to investigate this question in canine and human patients with advanced primary brain cancer.

In order to further improve the clinical prospects for CED-based therapy, we have introduced a number of innovations to CED for both current and future clinical applications. We have developed a fully integrated, Food and Drug Administration-approved brain tumor delivery system that consists of an magnetic resonance-compatible aiming device, reflux-resistant cannula and predictive software to maximize delivery of therapeutic agents. Real-time imaging allows us to visualize direct therapeutic delivery into the central nervous system and, when used in combination with several infusion catheters, may permit extensive RRV coverage of larger tumors in human brain. In the human setting, malignant tumors are usually >2–3 cm in diameter at time of diagnosis, and may have various shapes. Our previous studies on naive primate brains have clearly shown that CED, when combined with our stepped cannula design, allows delivery of a therapeutic agent into many central nervous system structures at different depths. This delivery platform permits monitoring distribution of RRV-CD fluid by using gadolinium additive and magnetic resonance image monitoring during CED with a high level of precision, predictability and safety, and may have important implications in ensuring effective delivery of therapeutics into brain targets. Such an approach may improve the success rate for clinical trials involving direct brain drug delivery. Therefore, CED and these delivery innovations should be considered when localized therapeutic delivery, such as gene transfer, are being translated into clinical treatments.

In conclusion, we have shown that infusion of RRV-CD into human glioblastoma xenograft brain tumors in a rat model by CED directs wider vector distribution relative to simple manual injection of vector, thereby resulting in a substantial improvement in the therapeutic outcome. The CED approach used in this study offers a potentially effective method to deliver novel RRV-CD for gene therapy of malignant glioma. Furthermore, investigation into the dosing and spacing of RRV administration, virus spread, tumor growth and timing of 5-FC administration may yield further improvements in long-term survival.

CONFLICT OF INTEREST
Harry Gruber, Carlos Ibanez, Joan Robbins and Douglas Jolly are employees of Tocagen, which funded this study. Noriyuki Kasahara is a consultant and co-founder of Tocagen, and has received research support from the company.

ACKNOWLEDGEMENTS
This study was supported with funds from Tocagen, San Diego, CA, USA and by a translational NIH grant to NK (U01-NS059821).
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