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Advances in Brief

Comparative Analyses of Transgene Delivery and Expression in Tumors Inoculated with a Replication-conditional or -defective Viral Vector[1]

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Abstract

Viral vectors for cancer can be classified into those that do not replicate (replication-defective vectors) and those that selectively replicate in neoplastic cells (replication-conditional or oncolytic vectors). Both of these can deliver anticancer cDNAs for therapeutic purposes. Opposite hypotheses can be made regarding the advantages of each vector type with regard to anatomic transgene expression. For the former vector, because cDNA delivery occurs in neoplastic cells that have the ability to migrate into the tumor mass, relatively extensive anatomic and temporal expression of anticancer functions may occur. For the latter vector, active viral replication may permit anatomically and temporally extensive delivery of the foreign cDNA into the tumor mass. Herein, we performed a simple comparative analysis to test which of these hypotheses is valid. Direct inoculation of s.c. tumors with a replication-conditional or a replication-defective viral vector, each of which expressed *lacZ* cDNA, was performed. Tumors were excised and analyzed for anatomic delivery of β-galactosidase and for neoplastic viral titers. We find that *lacZ* cDNA expression is observed in approximately 40% of the tumor area 3, 7, and 14 days after injection with the replication-conditional vector, whereas approximately 10% of the tumor area expresses the transgene 3 days after injection with the replication-defective vector, with a rapid decline in expression thereafter. Titers of the replication-conditional virus remain stable within injected tumors for the 14 days of the assay (approximately 1:1,000 of the initial injection dose), whereas titers of the replication-defective vector decrease rapidly after injection (to a value of 1:100,000 of the initial injection dose). Taken in conjunction, these studies show that transgene delivery and expression in tumors last longer and are found throughout an anatomically more extensive area after injection with replication-conditional gene therapy vectors than after injection with replication-defective gene therapy vectors.

Introduction

It has been assumed that replication-conditional vectors might possess an advantage over replication-defective vectors because of their theoretical capacity to replicate within tumor cells and release progeny virions into the tumor environment, which can in turn infect and replicate in additional tumor cells (1–3). This should increase the anatomic distribution of vector, and, if a transgene is also being expressed, transgene delivery within an injected tumor mass may also be extended both temporally and anatomically. This speculative rationale justifies the use of replication-competent retroviruses (4), adenoviruses (5), and HSVs[3] (6), but formal proof of the veracity of this hypothesis is lacking in published literature.

The opposite argument and assumption can also be made. A replication-conditional vector will lyse and kill the infected tumor cell. Consistent with this argument, transgene delivery would be expected to be short-lived and may never reach an anatomically extensive area, particularly if the expected humoral and cellular response against an actively replicating virus ends up effectively limiting viral propagation in the tumor (7). Instead, when a replication-defective vector is used to deliver a cDNA into tumor cells that is not immediately toxic (such as a prodrug-activating cDNA), tumor cells can continue to proliferate and migrate throughout the tumor mass (8–11). Therefore, in this instance, a temporal and anatomic increase in cDNA expression may also be observed.

A direct comparative analysis of anatomic distribution of cDNA delivery mediated by either vector has never been reported. Herein, we compared the anatomic distribution of a foreign transgene delivered by a replication-defective and a replication-conditional HSV into human tumor xenografts. We demonstrate that expression of a transgene reaches a larger tumor area and lasts longer within these tumors after injection with the latter type of vector than with the former type of vector.

Materials and Methods

Cell Lines and Viral Mutants. Human U87DEGFR glioblastoma cells were a generous gift of Dr. H-J. Su Huang (University of California at San Diego, San Diego, CA). Cells were grown at 37°C in DMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin in an atmosphere containing 5% CO_2. Gal4 was obtained from Dr. Neal De Luca (University of Pittsburgh Medical School, Pittsburgh, PA; Ref. 12). Gal4 has mutations of both ICP4 loci due to insertions of the *Escherichia lacZ* cDNAs. hrR3 was obtained from Dr. S. Weller (University of Connecticut Medical School, CT; Ref. 13). hrR3 possesses a mutation at the ICP6 locus because of the insertion of *E. coli lacZ* cDNA. Each of these vectors is derived from the same strain of wild-type HSV-I (KOS). The above-mentioned HSV vectors were stored at −80°C before use. They were passaged on African green monkey (Vero) cells or E5 cells (14). Viral titers were obtained by plaque formation assays on Vero or E5 cell monolayers. Procedures involving viruses were performed in accordance with guidelines issued by the Harvard Office of Biological Safety.

Animal Studies. Animal studies were performed in accordance with guidelines issued by the Massachusetts General Hospital Subcommittee on Animal Care. Viral inoculation and care of animals harboring viruses were performed in approved BL2 viral vector rooms. Tumors were established in the flanks of athymic mice (NCY/sed, nu/nu; Massachusetts General Hospital breeding colony) by s.c. injection of 10^6 human U87DEGFR glioma cells in 100 μl of DMEM. Tumors were allowed to grow to a size of 200 mm^3. This measurement was determined by measuring tumor height, width, and length with external calipers and then multiplying the three values. At this time, direct intratumoral injections with viral vectors (1×10^8 pfu/100 μl/injection) were performed using a Hamilton syringe (Hamilton Co, Reno, NV).

Tumor Growth Curves. To measure the growth of tumors in the s.c. flank, direct intratumoral injections with either the replication-conditional vector (hrR3) or the replication-defective vector (Gal4) were performed every other day for 4 days (total dose = 4×10^8 pfu/animal). Control injections consisted of injections of mock-infected Vero cell lysates or of wild-type virus KOS. Tumor volumes were then measured by external caliper measurement.

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[3] The abbreviations used are: HSV, herpes simplex virus; pfu, plaque-forming unit(s).

Assays of Viral Titers in Injected Tumors. Tumors injected with either a replication-conditional vector (hrR3) or a replication-defective vector (Gal4) were excised in sterile fashion from animals at different time points. On excision, each tumor was dropped in a preweighted sterile vial and frozen immediately on in dry ice to minimize degradation of viral particles. After freezing, the tumor and vial were weighed again. In a sterile fashion, an equal amount (by weight) of frozen tumor specimens were then minced into small fragments (less than 5 mm in diameter) with a scalpel and digested with collagenase/dispase (Boehringer Mannheim) diluted in HBSS (Life Technologies, Inc.) for 60 min at 37°C in an atmosphere containing 5% CO_2. Tumor lysates were then homogenized using an ultrasonic apparatus (Sonic Dismembrator 550; Fisher Scientific) to prepare a crude viral lysate. These viral lysates were then used to infect E5 cells. Infected E5 cells were then stained with X5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside after 18 h of incubation. The number of "blue cell" forming units thus provides the number of viruses in each explanted tumor and allowed calculation of a total viral yield per gram of tumor specimen.

Analysis of *lacZ* cDNA Transfer in Tumors. To measure the anatomic distribution of infected and *lacZ* cDNA-expressing cells in injected tumors, mice treated with hrR3 or Gal4 were sacrificed 3, 7, or 14 days after the last injection of vector by *in vivo* cardiac perfusion with 4% paraformaldehyde in sodium phosphate, 0.9% sodium chloride (pH 7.4). Harvested tumors were placed in 4% paraformaldehyde in PBS for 24 h and then placed for an additional 24 h in 30% sucrose in PBS. Tumor sections were obtained by freezing in a cryotome and cutting into 20-μm sections for the entire volume of the tumor. *LacZ* cDNA expression was detected in sections by X5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining, as detailed in previously published reports (15). For computer-assisted volumetric analysis of *lacZ* distribution, tumor specimens were photographed under a microscope. The percentage of *lacZ*-positive volume in tumors was calculated from sections of each tumor sample using image analysis software (Image analyzer; Macintosh computer).

Results and Discussion

The two viral vectors are Gal4 (12) and hrR3 (13). Gal4 possesses an insertional mutation of the ICP4 gene locus, rendering the virus incapable of replication, whereas hrR3 possesses an insertional mutation of the ICP6 gene locus, rendering the virus capable of replication only in tumor and dividing cells (Fig. 1). In the ICP4 locus of Gal4, there is a *lacZ* cDNA transgene under control of the ICP4 promoter, whereas in the ICP6 locus of hrR3, *lacZ* cDNA is under control of the ICP6 promoter. Both promoters are turned on shortly after viral infection of cells. Both viral mutants are derived from the same strain of HSV (KOS), and infection of cultured cells over a period of hours revealed that the efficiency of infection is the same for both viral mutants (data not shown).

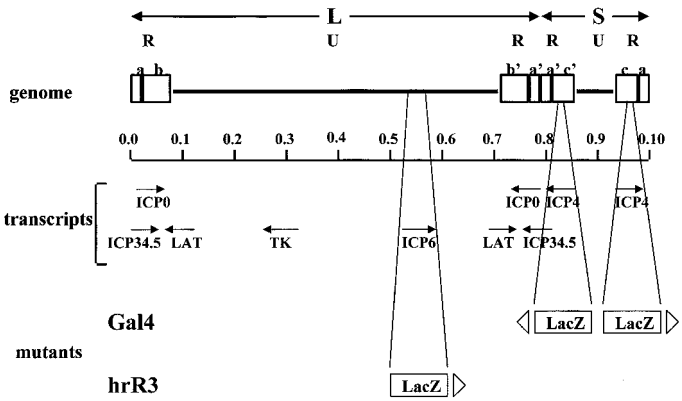

Fig. 1. A schematic of the Gal4 and hrR3 viral vectors. The *top line* represents a schematic of the HSV1 genome labeled with its long (*L*) and short (*S*) fragments and its repeat (*R*) and unique (*U*) regions, designated as *a*, *b*, *c*, and *a′*, *b′*, and *c′*. Map units are located below the HSV genome. Examples of the location of HSV1 transcripts (ICP34.5, Icp0, LAT, TK, ICP6, and ICP4) are provided beneath this. Finally, a map of the genetic mutants Gal4 and hrR3 is at the very bottom. In Gal4, the two copies of ICP4 are disrupted by the insertion of two copies of a *lacZ* cDNA, whereas in hrR3, ICP6 is disrupted by insertion of one copy of *lacZ*.

We first sought to address the thesis of this report: s.c. human tumor xenografts were directly inoculated with the same dose and schedule of Gal4 or hrR3. Tumors were then explanted at different time points and stained for expression of the delivered *lacZ* cDNA. Fig. 2*A* provides a representative photomicrograph of these tumors over the 14-day period of the assay. Computer-assisted analysis of several sections was then performed (Fig. 2*B*). It is evident that *lacZ* cDNA expression within tumors injected with hrR3 remained relatively stable for the first 7 days (at 40%) and then began to decline to a mean of 25% by the second week, as tumors were regressing. Instead, *lacZ* expression within tumors inoculated with Gal4 was approximately 10% on day 3 and declined to 1% or less within 1 week. These results thus show that cDNA delivery mediated by the replication-conditional vector viral vector was more prolonged and anatomically more extensive than that mediated by the replication-defective vector.

To ensure that the above result was due to the fact that one vector replicated but the other did not, viral titers within tumors were also measured. This revealed a continuous presence of hrR3 within tumors, at least for the 14 days of the assay, whereas tumor titers of Gal4 dropped precipitously during the first week (Fig. 3). Because 4×10^8 pfu of each vector were injected initially, there was an initial drop of 3 log units of virus recovered from tumors injected with hrR3, whereas there was a drop of 5 log units of virus recovered from tumors injected with Gal4. However, titers of hrR3 then remained stable within injected tumors due to continued viral replication, whereas this was not observed for titers of Gal4 due to its lack of replication. These results thus showed that viral vector replication in tumors correlated with both temporal and anatomic increases in the delivery of *lacZ* cDNA.

In spite of these results, it is still possible that the replicating vector would be more toxic to mice than the replication-defective vector or that the replicating vector was not that oncolytic. We thus determined the clinical toxicity and efficacy of these vectors in a parallel group of athymic mice harboring human tumor xenografts. There were no signs of neurological impairment, skin lesions, or weight loss in either group of treated animals (Table 1). Furthermore, the growth curves of tumors treated with hrR3 stabilized or regressed in terms of growth, whereas tumors treated with Gal4 continued to grow (Fig. 4), indicating that hrR3 was oncolytic. As a control to ensure that toxicity would be clinically apparent in these animals, tumors were also injected with the wild-type parental strain KOS. In these animals, clinical evidence for a systemic viral infection was observed with cutaneous herpetic plaques, weight loss, and neurologic morbidity (Table 1). Interestingly, these tumors exhibited regression that was even more complete than that of tumors injected with hrR3 (Fig. 4). These studies thus showed that in this animal model, toxicity was absent for the replication-conditional vector at the same doses required to achieve an anticancer effect.

The evidence presented in this report argues that, at least in this animal model, the replication-conditional vector, in spite of its oncolytic effects, was able to deliver and express its *lacZ* transgene in both a temporally and anatomically more extensive region of tumor than the replication-defective vector. Tumor masses injected with a replicating *lacZ*-expressing HSV vector displayed *lacZ* expression in at least 40% of the tumor mass for a period of 1 week, whereas those injected with a replication-defective HSV vector expressed the same transgene in less than 10% of the tumor for a few days.

Replication-conditional vectors, such as hrR3, exhibit relatively good tumor selectivity. However, because the virus infects and replicates better in human cells than in murine cells, it is generally impossible to study selectivity in human xenograft models in mice. In

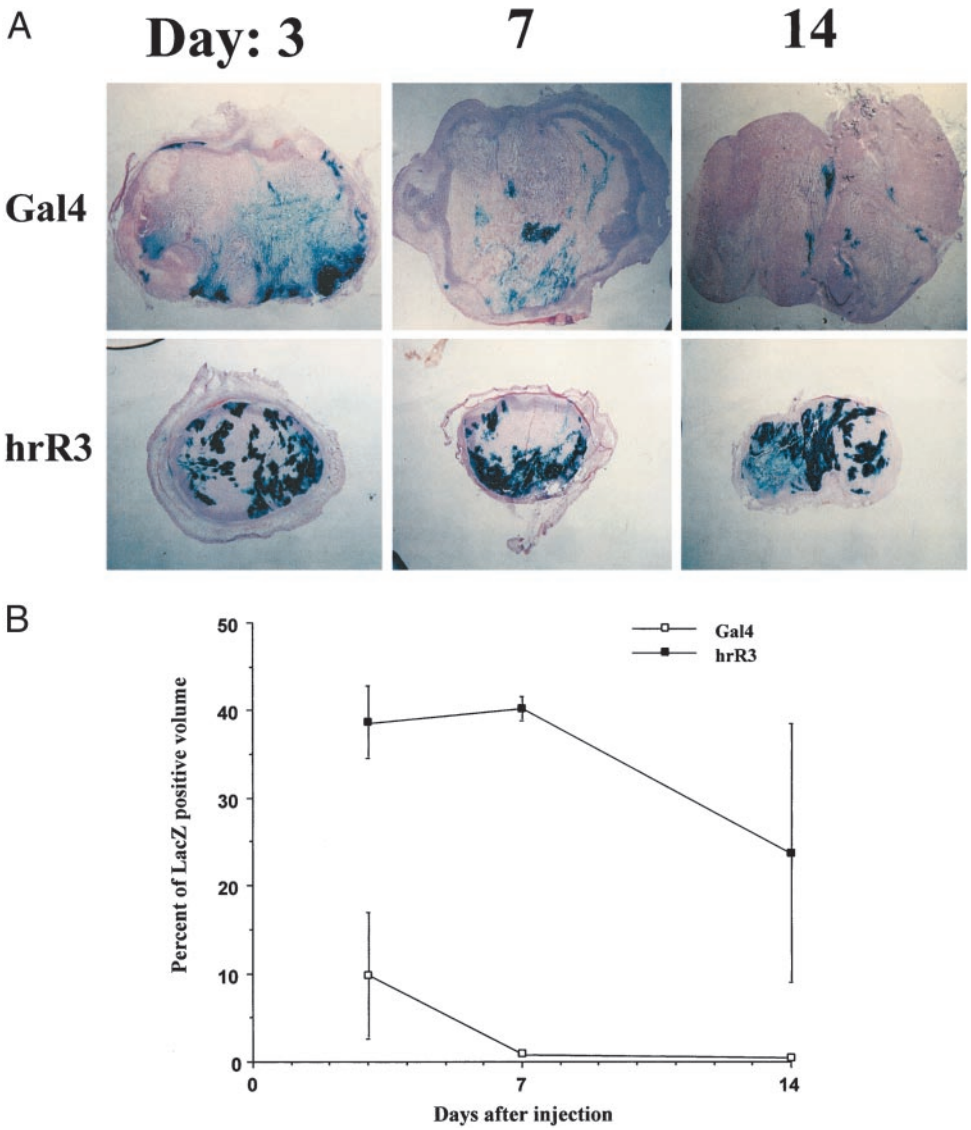

Fig. 2. *LacZ* cDNA transfer and expression in injected tumors. In *A*, tumors injected with Gal4 or hrR3 and then explanted at different time points were stained for *lacZ* cDNA expression. In *B*, quantitative analysis of *lacZ* cDNA expression within tumors was accomplished by computer-based calculation of areas of *lacZ* cDNA expression and unstained areas. Values represent the average percentage of *lacZ* cDNA staining ± SE.

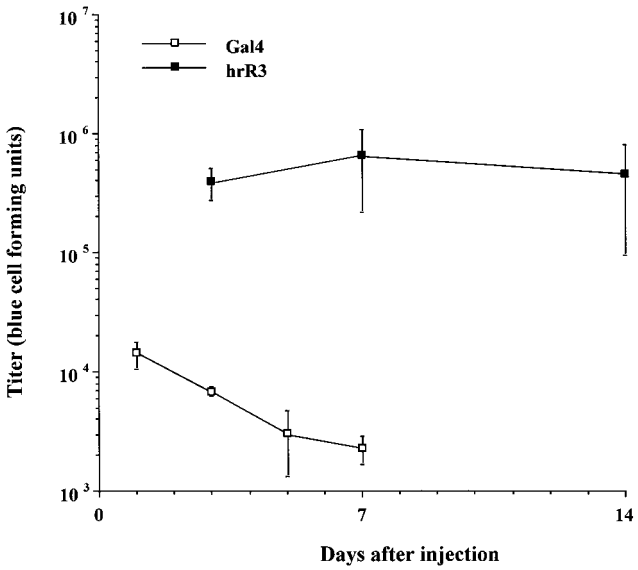

Fig. 3. Titers of virus in tumor explants. Tumors were harvested from each animal at different time periods and weighed, and viral vectors were released by mincing tumor tissue using freeze-thawing and ultrasonication. Shown are the mean ± SE at each time point. Values in the *Y* axis are in logarithmic units.

this respect, the results of Table 1 prove interesting due to the inclusion of the wild-type HSV KOS control. In fact, because KOS demonstrated clinical replication and infection not only of human tumor but also of mouse cells (epidermis and nervous), an argument can be made for the relative selectivity of hrR3, which was observed to infect and replicate only in tumor cells. Furthermore, the KOS-treated tumors showed evidence for significant tumor regression, even more so than hrR3-treated tumors. This might suggest that further refinements in the genetic make-up of oncolytic viruses could render their tumor-selective replication more akin to that of a wild-type virus, although the lack of replication and infection of normal tissue should be maintained. Such refinements could incorporate the use of tumor-

Table 1 *Side effects of viral therapy*

	Gal4[a]	HrR3	KOS
Skin lesions[b]	0/8[c]	0/8	8/8
Weight loss	0/8	0/8	5/8
Neurological deficits[d]	0/8	0/8	4/8

[a] Gal4 represents the replication-defective HSV injected into tumor; hrR3 represents the replication-conditional virus; KOS represents wild-type virus.
[b] Necrotic skin sores.
[c] Number of affected animals/total number of treated animals.
[d] Manifested as hind limb paresis.

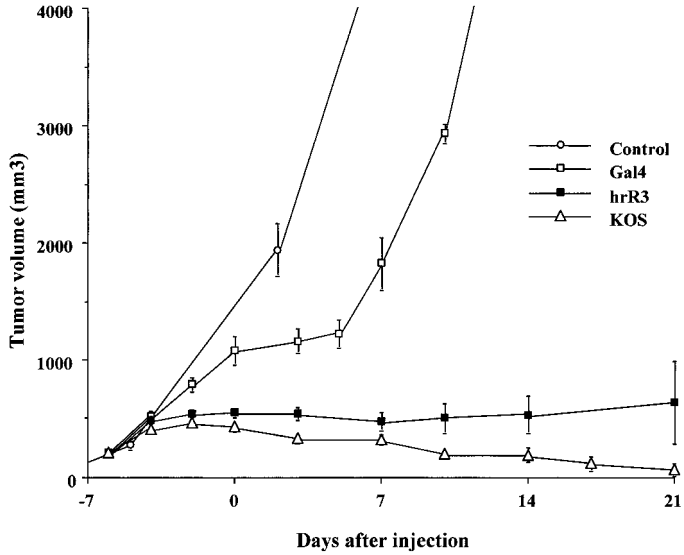

Fig. 4. Tumor growth curves after treatment with each viral vector. Tumors were inoculated when they reached a volume of 200 mm^3 with either control saline, Gal4, hrR3, or KOS by injections on day −6, −4, −2 and 0. Tumor growth was then monitored twice weekly. Values represent the mean ± SE.

selective promoters to drive expression of viral genes needed for replication (16).

Oncolytic viruses are currently being used in clinical trials in humans affected with a variety of cancers (17–19). Combining these viruses with available therapies such as chemotherapy (18) and radiotherapy (20, 21) or arming them with additional anticancer cDNAs, such as prodrug-activating genes (22), greatly enhances viral oncolytic effects, producing complete regressions in experimental tumor models. Therefore, the use of oncolytic viruses should be viewed as an addition to the therapeutic armamentarium available to the oncologist in the fight against tumors.

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