

Chapter 3

Analysis of HSV Oncolytic Virotherapy in Organotypic Cultures

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Summary

Tumor-selective replication-competent viral vectors, such as oncolytic herpes simplex virus (HSV) type I (HSV-1), represent an attractive strategy for tumor-based therapies because these viruses can replicate and spread in situ exhibiting cytopathic effects through direct oncolytic activity. These lytic viruses offer a distinct advantage over other forms of cancer therapies in that they are self-perpetuating and can spread not only in the tumor itself, but also to distant micrometastases. Translational studies aimed at identifying novel virotherapies for human cancers are incumbent upon the appropriate experimental models. While animal models are the preferred choice for efficacy studies of HSV virotherapy, we have developed a novel complementary approach toward assessing the effectiveness of oncolytic HSV therapy in both brain and prostate cancers. This experimental model takes advantage of previously published work in which human prostate cancer biopsies and rodent brain slices can be easily maintained *ex vivo*. The advantage of these systems is that the three-dimensional structure remains intact. Thus, all of the factors that may affect viral entry and replication, such as cell–cell and cell–matrix interactions, and interstitial fluid within this three-dimensional milieu remain preserved. Moreover, with respect to the brain, this system offers the advantage of direct access to brain cells, such as microglia and astrocytes, and circumvents the problems associated with the presence of the blood–brain barrier.

Key words: Brain tumors, HSV, oncolytic virotherapy, organotypic cultures, prostate cancer.

1. Introduction

The discovery of tumor-selective replication-competent viral vectors, designated also as oncolytic viruses (OVs), represents an attractive strategy for cancer therapies. OVs can replicate in situ, spread their progeny throughout the neoplastic mass, and reach isolated migrating cancerous cells as well as distal metastases (1).

Several virus strains are being used as cancer therapeutic OV_s (1). Herpes simplex virus (HSV)-derived OV_s (HSV-OV_s) were first designated to treat brain tumors, but have been found to also be very efficient in other cancer types (2–4). The advantage of using HSV is that it can be engineered with large genetic inserts and its replication can be effectively inhibited with pharmaceuticals (1).

Although OV_s can kill tumor cells grown *in vitro* with high efficiency, they present reduced replication when applied *in vivo*, which results in a low therapeutic efficiency in clinical trials. This suggests that physiological aspects of the tumor and host decrease the OV_s's therapeutic potential, and preclinical research aimed at understanding and overcoming these physiologic aspects is currently ongoing.

Solid tumors are formed of both cancerous and noncancerous cells. The noncancerous cells include blood vessel endothelial cells, hematopoietic cells from circulating blood, and local and peripheral cells of the innate and adaptive immune system. Together, these cells form the tumor stroma, a complex and changing structure that can influence tumor formation, progression, and response to therapy. Moreover, the compact structure of solid cancers is held together by a tight extracellular protein matrix that does not allow efficient spread of drugs, including OV_s. Recent discoveries have demonstrated that disruption of the extracellular matrix and/or suppression of innate immunity from the cancer stroma can increase the efficacy of cancer virotherapy (5–18).

In vivo models are the most reliable means by which to evaluate efficacy for experimental cancer treatments. However, *in vivo* studies can present a number of limitations in understanding the physiological mechanisms that influence the outcome of a specific therapy. Indeed, studies performed *in vivo* can be time-consuming, cost-ineffective, and present difficulties in drug delivery. Moreover, they may also require complex surgeries that limit the total number of animals analyzed, thus limiting statistical analyses and parallel comparisons. Thus, to understand how physiological aspects of tumor influence the outcome of cancer virotherapy, we have developed a novel complementary approach using prostate cancer biopsies and rodent brain slices cultured *ex vivo* (19–24). The advantage of these organ cultures is that all factors that may affect viral entry and replication, such as cell–cell and cell–matrix interactions, and interstitial fluid within an intact three-dimensional milieu remain preserved. Therefore, we have been able to evaluate the role of phagocytic microglia in brain tumor virotherapy (14) and to analyze the efficacy and cancer selectivity of HSV-OV in human prostate tumors (Passer et al., unpublished).

2. Materials

2.1. Preparation of Organotypic Brain Slices

1. Five to 7 days postnatal mice or rats.
2. Vibratome with magnifier lens (Leika VT100S, Bannockburn, IL) (*see Note 1*).
3. Dissecting microscope (Nikon SMZ 1000, Melville, NY) (*see Note 1*).
4. Surgery tools (*see Note 2*): feather blades (Ted Pella, Redding, CA), knife, small spatula (4 × 150 mm), large scissors, and small dissecting scissors (Fine Science Tools Inc., Foster City, CA), the finest brushes provided by any art supply store.
5. Pasteur pipettes.
6. Super Glue (Staples).
7. Gey's balanced salt solution pH 7.2 (Gey's BSS) (Sigma-Aldrich, St. Louis, MO) enriched with 36 mM D-glucose (Fisher Scientific, Pittsburgh, PA) and with a penicillin/streptomycin (P/S) solution (Invitrogen, Carlsbad, CA) diluted 1:1,000.
8. Millicell cell culture plate insert, hydrophilic polytetrafluoroethylene (PTFE) filter 0.4 μm (Millipore, Billerica, MA).
9. Culture medium (*see Note 3*): 50 mL minimum essential medium (MEM, Cellgro, Herndon VA), 25 mL heat-inactivated fetal bovine serum (FBS; *see Note 4*) (Hyclone, Logan, UT), 25 mL Hank's balanced salt solution (Hank's BSS, Cellgro), 0.5 mL glutamine (Cellgro), 100 μL P/S solution (Invitrogen), 650 mg D-glucose (Fisher Scientific).

2.2. Tumor Establishment in Organotypic Brain Slices

1. Trypsin-EDTA (Cellgro).
2. Cell culture medium: Dulbecco's modified essential medium (DMEM, Cellgro) supplemented with 10% heat-inactivated FBS (Hyclone) and 1% P/S solution (Invitrogen).
3. Sterile phosphate buffer solution (PBS, Cellgro).
4. Cell counter.
5. 10-cm cell culture dishes.
6. Dissecting microscope (Nikon).

2.3. Prostate Organ Culture

1. Prostate organ culture complete media (POC-CM): RPMI-1640 (Cellgro) supplemented with 10% heat-inactivated FBS (Hyclone), 1 mM sodium pyruvate (Cellgro), 1 mM non-essential amino acids (Invitrogen), 1 mM P/S/glutamine (Invitrogen), Ultrafoam (collagen sponge) (Davol Inc., Cranston, RI).

2. Prostate biopsies (Massachusetts General Hospital [MGH] Pathology Department; Institutional Review Board [IRB] approved).

2.4. Oncolytic HSV Strains and Other Pharmaceuticals

1. Oncolytic HSV strain hrR3 (25): derived from HSV Kos; inactivation of the *ICP6* gene by *Escherichia coli LacZ* gene insertion. Dr. Sandra Weller at the University of Connecticut provides the virus.
2. Oncolytic HSV strain G47 (26): derived from the HSV backbone F; *ICP6* gene inactivation by *E. coli LacZ* gene insertion, deletion of both $\gamma34.5$ alleles and of the $\alpha47$ gene. MediGene Inc. (San Diego, CA) provides working preparations of this virus.
3. Dr. Robert Lee at the Ohio State University provides clodronate liposomes (14).

2.5. Cryostat Sectioning of Prostate Tissue

1. OCT freezing media (Tissue-Tek, Torrance, CA).
2. 2-Methyl butane (Sigma-Aldrich).
3. Cryomolds (Tissue-Tek).
4. Dry ice.

2.6. Staining of Brain and Prostate Organ Cultures

1. Glass coverslips (round glass coverslips that fit in a 24-well cell culture plate).
2. 24-Well cell culture plates.
3. Protein block (DAKO-Cytomation, Glostrup, Denmark).
4. Antibody diluent solution (DAKO-Cytomation).
5. Phosphate buffer (PBS, Sigma-Aldrich) (*see Note 5*).
6. Primary antibodies (*see Note 6*): mouse anti-rat CD68, mouse anti-rat CD163, rat anti-mouse CD68, and mouse anti-HSV-gC (AbD Serotec, Oxford, UK), mouse anti-HSV-ICP4 (US Biological, Swampscott, MA).
7. Secondary antibody (*see Note 6*): FITC-conjugated anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA).
8. Vectashield® medium containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA).
9. Acetone, ethanol (EtOH), and xylene (these do not have to be extremely pure, the cheapest brand from your local pharmacy will be good).
10. Isolectin GS-IB4 from *Griffonia simplicifolia*, Alexa Fluor® 488 conjugate (Molecular Probes, Carlsbad, CA).
11. X-gal, potassium ferricyanide, potassium ferrocyanide, and 1 M MgCl₂ solution (Sigma-Aldrich) (*see Note 7*).
12. Hematoxylin 2 (Richard Allan Scientific, Kalamazoo, MI).

2.7. Extraction and Titration of Oncolytic HSV from Brain and Prostate Organ Cultures

1. African green monkey kidney cells (Vero, American Type Culture Collection [ATCC], Manassas, VA).
2. DMEM tissue culture medium (Cellgro) supplemented with 10% heat-inactivated FBS (Hyclone) and 1% P/S solution (Invitrogen).
3. Sterile glucose-enriched PBS (Sigma-Aldrich).
4. Trypsin–EDTA (Cellgro).
5. 6-Well tissue culture plates.
6. Cell counter.
7. Human anti-HSV IgG (Gamunex, Talecris Biotherapeutics, Research Triangle Park, NC).

3. Methods**3.1. Preparation and Culture of Organotypic Brain Slices**

1. Animals are killed and plunged into a 70% EtOH solution, made with Millipore distilled water, and immediately transferred to a prechilled Petri dish maintained on ice.
2. The animals are decapitated and the skull is removed by making two horizontal cuts with small dissecting scissors; the cuts should start on each side of the head at the level just above the ears.
3. The cranial nerves are cut from the cerebrum with a small spatula, which is also used to disrupt the dura mater and to ease the brain out of the cranium.
4. The entire cerebrum is immersed in prechilled Gey's BSS in a sterile Petri dish.
5. The sagittal surface of the brain is glued with Super Glue to a chilled metal base in the chamber of a vibratome and the chamber is filled with cold Gey's BSS. Gey's BSS in the vibratome chamber should be changed after each animal.
6. Coronal sections at a thickness of 300 μm are cut from the posterior pole (remember to keep the blade and the block of tissue always moist), transferred to a separate 60-mm dish with ice-cold Gey's BSS, and incubated at 4°C for up to 3 h.
7. The slices are then transferred to the surface of the Millicell culture plate insert by means of a Pasteur pipette filled with one drop of Gey's BSS, which serves to keep the tissue moistened.
8. The Millicell culture plate insert is placed in a 24-well tissue culture dish on top of a layer of culture medium. The medium should not cover the membrane so that the explant remains well exposed to the air.

9. After the brain slice is placed on the Millicell insert, the drop of Gey's BSS is vacuum-aspirated off, and the slice is incubated at 36°C in a chamber with 5% CO₂-enriched air.
10. Brain slice cultures are fed using sterile procedures under a laminar flow hood by changing half of the growing medium every 2–3 days.
11. Three weeks later, the organotypic brain culture has matured to the point at which experimental manipulations can be performed.

3.2. Tumor Establishment, Drug Treatment, and Virus Infection in Organotypic Brain Slices

1. 10 mL of warmed sterile PBS are added to the bottom part of a 10-cm culture dish.
2. Trypsinized brain tumor cells are resuspended in 10% FBS supplemented with the P/S solution at a final density of 100,000 cells/mL (*see Note 8*).
3. A drop of cell suspension is gently placed (~20 µL or ~2,000 cells) on the upper lid of a 10-cm cell culture dish. About 25 drops can be placed in one dish.
4. The bottom part of the dish, containing sterile PBS, is carefully covered with the upper lid where the cell drops are placed, and the hanging drops are allowed to grow for 3–4 days to allow the cells to aggregate in spheres (27).
5. Under a dissecting microscope, one sphere is removed (pipette setting: 1.2 µL) and placed onto the brain slice surface without damaging the slice.
6. The sphere will adhere to the brain slice through interaction with surrounding astrocytes and small tumor structures will be formed. Depending on the tumor model, this process may take only 8 h or as long as 1 week; this has to be established empirically with different cancer cells.
7. Phagocytic microglial cells can be depleted with the use of clodronate liposomes. One milliliter of liposome solution (0.2 mg/mL in feeding medium) is placed below the insert holding the brain slice and on top of the brain slice. The slice is incubated with the liposome solution for 24 h, after which it is rinsed twice and fresh medium is put under the slice for further growth. Treatment with liposomes is performed before tumor establishment to avoid the disruption of the tumor. The tumor growth is not altered by this treatment.
8. OV infection of the tumor is performed by placing a sterile plastic ring (cut from the top edge of a 200-µL pipette tip) on top of the tumor and by filling it with 40–50 µL of OV suspension in sterile PBS (*see Note 9*). The brain slice is then incubated for 3 h at 37°C, after which the virus is rinsed out with several changes of feeding medium.

3.3. Virus Infection and Spread in Prostate Organ Cultures

All procedures described below should be performed in a laminar flow hood. To maintain the structural integrity of the tissue, prostate biopsies must be manipulated immediately after receiving.

1. Human prostate cancer tissues are obtained from the MGH Pathology Department (IRB-approved) after radical prostatectomies.
2. Prostate tissues are placed into a 10-cm Petri dish containing RPMI media supplemented with a P/S/glutamine solution and cut into 2–4 mm³ fragments.
3. Tissue fragments are placed in a 48-well plate containing 250 µL/well of POC-CM and inoculated with 5×10^4 – 5×10^6 plaque-forming units (pfu) of G47Δ for 1–2 h at 37°C in a humidified incubator with 5% CO₂-enriched air.
4. During inoculation, the collagen sponge is cut into a ~10 × 10-mm square under sterile conditions and placed into 2 mL of POC-CM. Tumor fragments are then transferred to the semi-submersed sponge and incubated at 37°C in a 5% CO₂-humidified incubator for up to 2 weeks.
5. At various time points after infection, tissues are removed and placed in OCT in a cryomold. Tissues are then allowed to slowly freeze in a 2-methyl butane bath prechilled in dry ice, and can either be stored at –80°C or immediately cut with a cryostat in 5 µM sections.

3.4. Staining of Brain and Prostate Organ Cultures (13, 14)

All staining procedures are performed on tissues that were dried overnight, fixed in ice-cold acetone, and stored at –20°C or –80°C (*see Note 10*).

3.4.1. Immunohistochemistry Staining of HSV and Microglia (see Notes 11 and 12)

The incubation times required for the brain slices are in bold; the other incubation times refer to the prostate slices. If only one time is indicated, it refers to both the prostate and brain slices.

1. The brain and prostate slices are rehydrated in 300 µL PBS for 30 min at room temperature (RT).
2. Endogenous protein background is blocked with 300 µL serum-free protein block for 10 min or **2 h** at RT.
3. Sections are then incubated with the primary antibody appropriately diluted in 300 µL of antibody diluent solution overnight or for **48 h** at 4°C.
4. After three washes in 500 µL PBS at RT for 30 min each, the slices are incubated with the secondary antibody for 45 min at RT or **24 h** at 4°C.
5. After three washes in 500 µL PBS at RT for 30 min each, the slices are mounted with Vectashield® medium containing DAPI for nuclear counterstaining.

**3.4.2. Staining of Oncolytic
HSV β -Galactosidase
Activity**

Because both hrR3 and G47 contain the *E. coli LacZ* gene, X-gal staining on tissue sections can be performed to evaluate viral spread.

1. Rehydrated tissues are covered with a freshly prepared X-gal solution containing: 0.5 mL of X-gal stock in DMSO, 0.05 mL of 1 M MgCl₂, 1 mL of 125 mM potassium ferricyanide, 1 mL of 125 mM potassium ferrocyanide, and 1X PBS and 22.45 mLs of 1X PBS (total volume 25 mL).
2. Tissues are then incubated for 2–4 h (until neat blue HSV colonies appear) at 37°C in the dark, and excessive X-gal solution is rinsed off by submerging the tissues in clean PBS two to three times.
3. The tissues are counterstained through a 2 min incubation in hematoxylin.
4. Excess of hematoxylin is rinsed off with large volumes of deionized water. The tissues are submerged in fresh clean water for 5 s; this is repeated several times until the rinsing water remains colorless.
5. The stained tissues are dehydrated in increasing concentrations of ethanol (2 min at 75% EtOH, 2 min at 95% EtOH, and 2 min at 100% EtOH) and mounted in xylene.

**3.4.3. Live Microglia
Immunofluorescence**

Isolectin binds specifically to microglia cells and fluorescent conjugated forms of this agent can be used for live microglia staining.

1. The slices are submerged in an isolectin solution (5 μ L/mL) in culture medium.
2. Next, the slices are washed three times with 500 μ L PBS for 30 min each and can be analyzed under an inverted fluorescence microscope.

**3.5. Virus Extraction
from Brain and
Prostate Organ Cul-
tures Slices (12, 14)**

1. The tissues are resuspended in an appropriate volume of DMEM supplemented with a P/S solution diluted 1:100.
2. The tissues are then manually homogenized by pipetting them up and down.
3. The homogenized tissues are frozen in a dry ice–EtOH bath and thawed at 37°C three consecutive times.
4. After the final thaw, the freeze-thawed material is sonicated for 10 s in an ice bath to ensure release of the virus.
5. The cellular debris from the tissue lysates is pelleted through centrifugation at 14,000*g* for 10 min at 4°C.
6. Virus-containing supernatants are collected in new tubes and stored at –80°C.
7. Virus titers can then be determined as described next.

3.6. Virus Titration

1. Vero cells are plated at 400,000 cells/well in complete DMEM in 6-well plates and allowed to grow overnight.
2. The following day, serial dilutions (1:10) of the OV stock are made in glucose-enriched PBS.
3. OV inoculum (0.7 mL) is added to duplicate wells and incubated for 1 ½ h at 37°C. During this incubation, the plates must be rocked every 15 min to avoid drying of the cells.
4. OV inoculum is removed and 2 mL of DMEM media containing 1% FCS, 1% P/S solution, and human anti-HSV IgG is added to each well.
5. The virus is allowed to grow for 4 days. The presence of anti-viral IgG in the culture medium will not allow the spread of OV particles to distant cells. Therefore, only the cells adjacent to the initially infected cell will be infected by the virus, thus allowing the formation of distinct virus plaques that will correspond to the initial pfu of the OV stock.
6. After staining of virus β -galactosidase activity as described in **Subheading 3.4.2**, the OV plaques can be easily counted (*see Note 13*).

4. Notes

1. The vibratome and dissecting microscope are placed under the laminar hoods during preparation and sectioning.
2. All tools for preparing the organ cultures must be sterile. The instruments are sterilized with a high-temperature heater with glass beads after dissecting each animal, and cooled with ice-cold sterile phosphate buffer (PBS). All surfaces of the slicing apparatus that come in contact with the tissue, as well as the blades and the fine brushes, are sterilized with 70% alcohol.
3. To avoid excessive autofluorescence during the imaging experiments, all media reagents must be without phenol red. The medium is sterilized through a 0.2- μ m filter before use (SCGVU02RE, Millipore).
4. The serum (FBS) is stored at -20°C. Before using, it is thawed at room temperature, and incubated in a water bath at 56°C for 30 min for complement inactivation.
5. Dissolve 1 packet of the buffer powder in 1 L of deionized water for a 1X ready-to-use solution, and store at room temperature.
6. The optimal dilution of primary and secondary antibodies will have to be determined empirically for each model. In all cases, we have used a 1:100 dilution for the primary antibody and a 1:200 dilution for the secondary antibody.

7. Prepare X-gal stock solutions by dissolving 50 mg/mL X-gal in DMSO and store in the dark at -20°C ; prepare 10 mL of 125 mM potassium ferricyanide (0.41 g in 10 mL 1X PBS) and 10 mL of 125 mM potassium ferrocyanide (0.53 g in 10 mL 1X PBS) and store at room temperature.
8. Different tumor cells require different culture treatments. In our experiments, we have used mainly U87 human glioblastoma and D74 rat glioma cells transduced with the HSV receptor HveC (D74-HveC (12)). Both of these cell lines are detached from the culture dish through trypsinization. Briefly, cells are washed with prewarmed sterile PBS, incubated with trypsin-EDTA at 37°C until all cells are detached from the culture dish, and resuspended in complete growth medium. The serum of the medium inactivates completely the trypsin enzymatic activity.
9. The quantity of virus to be used depends on the OV strain, tumor model, and experiment. We have used 10 plaque-forming units (pfu) of hrR3 in the D74-HveC rat glioma model.
10. The tissues are dried overnight and submerged in a bath with ice-cold acetone for 10 min. Then, the tissues are air-dried for 30 min at RT before storage. It is important to use large volumes of acetone to avoid evaporation of the acetone during the fixation procedure. To avoid melting of the 24-well cell culture plate, the organotypic brain slices are fixed on a glass cover slip. Then, they are placed and stored in 24-well cell culture plates. Because of the thickness of the organotypic brain slices, staining of these tissues requires prolonged incubation time to allow the reagents to penetrate the whole tissue in a homogeneous way. Indeed, even though these slices are too thick for high-resolution IHC, they are too thin to be cut into thinner, 2- to 6- μm slices. The same problems were not present with the prostate organ cultures, which were easily cut into 5- μm slices (*see Subheading 3.3*) before staining.
11. Staining of the tumor implanted on the ex vivo brain slices is very difficult. Indeed, because of its fragile structure, the tumor tends to easily detach from the brain slice. To avoid this problem, the staining and washing passages must be reduced to a minimum. Thus bright light staining with DAB detection is not recommended, and fluorescent staining is preferred.
12. Microscopy imaging of 300- μm -thick tissues is very challenging. Good cellular resolution can be achieved only at high magnifications (40×10 or higher). A broad view of the tumor tissue and surrounding brain performed at low magnification has very poor resolution. The best way to image the tumor is on unstained slices under bright light microscopy (14). The tumor is thick enough to give good contrast

and under these conditions the tissue is not disrupted. DAPI staining of the tumor tissue at low magnification gives a dark-black contrast because of autofluorescence caused by the thickness of the tumor (14).

13. Before staining the cells for β -galactosidase activity, the cells must be fixed to the cell culture dish. This is performed by incubating the cells for 5 min at 4°C within the following freshly prepared fixing solution: 2.7 mL of 37% formaldehyde plus 0.4 mL 25% glutaraldehyde in 50 mL of 1X PBS. There is no need to rinse the cells before fixation or before adding the LacZ staining solution.

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