J. Neurovirol. (2012) 18:456–461
DOI 10.1007/s13365-012-0123-0

Human trigeminal ganglionic explants as a model to study alphaherpesvirus reactivation

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Received: 26 June 2012 / Revised: 9 July 2012 / Accepted: 11 July 2012 / Published online: 1 August 2012

Abstract Varicella zoster virus (VZV) latency is characterized by limited virus gene expression and the absence of virus DNA replication. Investigations of VZV latency and reactivation have been hindered by the lack of an in vitro model of virus latency. Since VZV is an exclusively human pathogen, we used naturally infected human trigeminal ganglia (TG) obtained at autopsy to study virus latency. Herein, we report optimization of medium to maintain TG integrity as determined by histology and immunohistochemistry. Using the optimized culture medium, we also found that both herpes simplex virus-1 (HSV-1) and VZV DNA replicated in TG explants after 5 days in culture. The increase in HSV-1 DNA was fourfold greater than the increase in VZV DNA. Overall, we present a model for alphaherpesvirus latency in human neurons in which the key molecular events leading to virus reactivation can be studied.

Keywords VZV · HSV-1 · DNA · Trigeminal ganglion · Latency

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Introduction

Varicella zoster virus (VZV) is a ubiquitous human neurotropic alphaherpesvirus. Over 95 % of the world's population has been exposed to the virus which is typically acquired in childhood (Virgin et al. 2009). Primary VZV infection causes varicella (chickenpox) during which time the virus establishes latency in neurons of multiple cranial, sensory, and autonomic ganglia along the entire neuraxis (Gilden et al. 2003). Latency is characterized by the presence of the VZV genome in an episomal state where most of the ~70 virus genes are transcriptionally silent (Cohrs et al. 1992; Clarke et al. 1995). VZV can remain in this repressed state indefinitely, but the virus will reactivate in 10–20 % of individuals during their lifetime (Ragozzino et al. 1982). VZV reactivation can cause zoster (shingles) and multiple other serious neurologic disorders (postherpetic neuralgia, vasculopathy, myelopathy, and retinal necrosis) (LaGuardia and Gilden 2001). Importantly, VZV reactivates in the absence of rash producing chronic radicular pain (zoster sine herpete) as well as the disorders indicated above (Gilden et al. 1992). In severity and duration of disease as well as reduced quality of life, VZV reactivation is more important than primary infection, yet the molecular events underlying virus reactivation remain unknown.

VZV is an exclusively human virus. Most of our knowledge concerning VZV latency is derived from studies of human ganglia removed at autopsy. VZV latency is restricted to neurons where the "endless" virus genome is associated with histones and virus gene transcription is limited to a small set genes (Cohrs et al. 1992, 2003; Clarke et al. 1995; Kennedy et al. 1998, 1999, 2000; Grinfeld and Kennedy 2004; Gary et al. 2006; Nagel et al. 2011). Our understanding of VZV latency and reactivation is hindered by the lack of an in vitro model. While infection of mice (Wroblewska

et al. 1982), rats (Debrus et al. 1995), and guinea pigs (Myers et al. 1980) results in seroconversion, virus does not become latent. Thus, experimental infection of animals does not fulfill all the rigorous criteria to be regarded as true models of VZV latency. The best model to study VZV latency in which reactivation can be induced experimentally is simian varicella virus (SVV) infection of non-human primates (White et al. 2001; Mahalingam et al. 2010). SVV, the primate equivalent of human VZV, becomes latent in multiple ganglia when SVV-seronegative monkeys are exposed to monkeys with varicella (Mahalingam et al. 2002). The SVV model has provided important information concerning virus pathogenesis and latency in an immunocompetent host; however, animal cost and availability have limited its general use.

In vitro models of VZV latency by necessity involve terminally differentiated human neurons. VZV does not infect human embryonic stem cells (Dukhovny et al. 2012), but under strictly defined conditions, the stem cells will differentiate to yield a highly enriched culture of neurons. VZV will infect differentiated neurons, but does not lead to productive (lytic) infection that occurs in all other human cells infected with VZV in vitro (Pugazhenthi et al. 2011). Further characterization of VZV in differentiated neuron will shed light on the latent state of virus; however, care must be taken since the presence of even small numbers of non-neuronal cells leads to productive VZV infection (Markus et al. 2011).

An alternative approach to study VZV latency in vitro takes advantage of the fact that most human trigeminal ganglia harbor latent virus (Azarkh et al. 2010). Early attempts to reactivate latent VZV from human trigeminal ganglia did not yield infectious virus (Plotkin et al. 1977), which may reflect the experimental conditions employed. Herein, we revisited this early report and tested various culture media with the goal of maintaining human trigeminal ganglia viability for sufficient time to initiate replication of latent virus DNA.

Methods

Human trigeminal ganglia

All human cadaver analysis was reviewed by the University of Colorado School of Medicine Internal Review Board and collected after obtaining informed consent from next of kin. Subject 1 was a 42-year-old man and subject 2 was a 51-year-old woman, and the cause of death for both subjects was drug overdose. The time between death and autopsy was 13 h (subject 1) and 18 h (subject 2). At autopsy, there was no cutaneous sign of herpesvirus reactivation, and neither subject was immunosuppressed before death. After the brain and dura were removed, the trigeminal ganglia were exposed. Trigeminal ganglia and connective tissue were removed and transferred to the laboratory at 4 °C in ~10 ml medium containing 5 % fetal bovine serum and antibiotics. Connective tissue was removed from the ganglia and washed in phosphate-buffered saline (PBS). The left and right trigeminal ganglia from subject 1 were cut into five equal-sized pieces; one trigeminal ganglia (TG) piece was fixed overnight in 2 % formaldehyde/PBS, and the remaining eight pieces (four from each TG) were divided between four wells of a six-well tissue culture plate. At 5 days, the TG pieces were fixed in 2 % formaldehyde/PBS. The left and right trigeminal ganglia from subject 2 were each cut into three equal-sized pieces, placed into optimized culture medium (see below), harvested at various times postcultivation, quick frozen in liquid nitrogen, and stored at −80 °C.

Culture medium and conditions

The following culture components were obtained: Dulbecco's minimal essential medium (DMEM, Sigma-Aldrich, St. Louis, MO), neurobasal medium (Invitrogen, Carlsbad, CA), 10 mg/ml gentamicin (Sigma-Aldrich), 200 mM glutamine (Invitrogen), 100 μg/ml nerve growth factor 2.5S (NGF; Invitrogen), 10 μg recombinant human bFGF (Invitrogen), and B27 supplement (Invitrogen). All pieces from each pair of TG were incubated for 1 or 5 days at 37 °C in 5 % CO_2 in one of the four freshly prepared culture media (Table 1).

Histology

The formaldehyde-fixed tissue was dehydrated in graded ethanols, imbedded in paraffin, and 50-μm sections (Lica RM2135 microtome, Buffalo Grove, IL) were placed on superfrost glass slides (Cole-Parmer, Vernon Hills, IL). Hematoxylin and eosin (H&E) staining of xylene-deparaffinized sections was as follows: sections were hydrated in graded ethanol, stained 8 min in Harris hematoxylin, differentiated in 1 % acid alcohol for 30 s, fixed in 0.2 % aqueous ammonium for 30 s, counterstained with eosin–phloxine for 1 min, dehydrated through graded ethanols, cleared by washing twice with xylene, mounted, and sealed with clear nail polish.

Immunofluorescence staining of xylene-deparaffinized sections was as follows: sections were hydrated in graded ethanols, epitopes unmasked in 0.01 M citrate buffer, pH 6 for 13 min at 95 °C, blocked in 20 % normal donkey serum at 22 °C for 30 min, incubated with a 1:1,000 dilution of α-NFH (Santa Cruz Biotech, Santa Cruz, CA) at 22 °C for 1 h, counterstained in 0.3 % Sudan black solution for 5 min, and incubated in a 1:1,000 dilution of Alexafluor 488-conjugated

Table 1 Complete culture medium

Medium 1		Medium 2		Medium 3		Medium 4	
DMEM	80 %	DMEM	80 %	Neurobasal	78 %	Neurobasal	98 %
FBS	20 %	FBS	20 %	FBS	20 %	B27	2 %
Gentamicin	20 μg/ml	Gentamicin	20 μg/ml	B27 supplement	2 %	Glutamine	0.4 mM
		NGF	50 μg/ml	Glutamine	1 mM	Glutamax	0.6 mM
		bFGF rec hu	5 μg/ml	Gentamicin	20 μg/ml	Gentamicin	20 μg/ml
				NGF	50 μg/ml		
				bFGF	5 μg/ml		

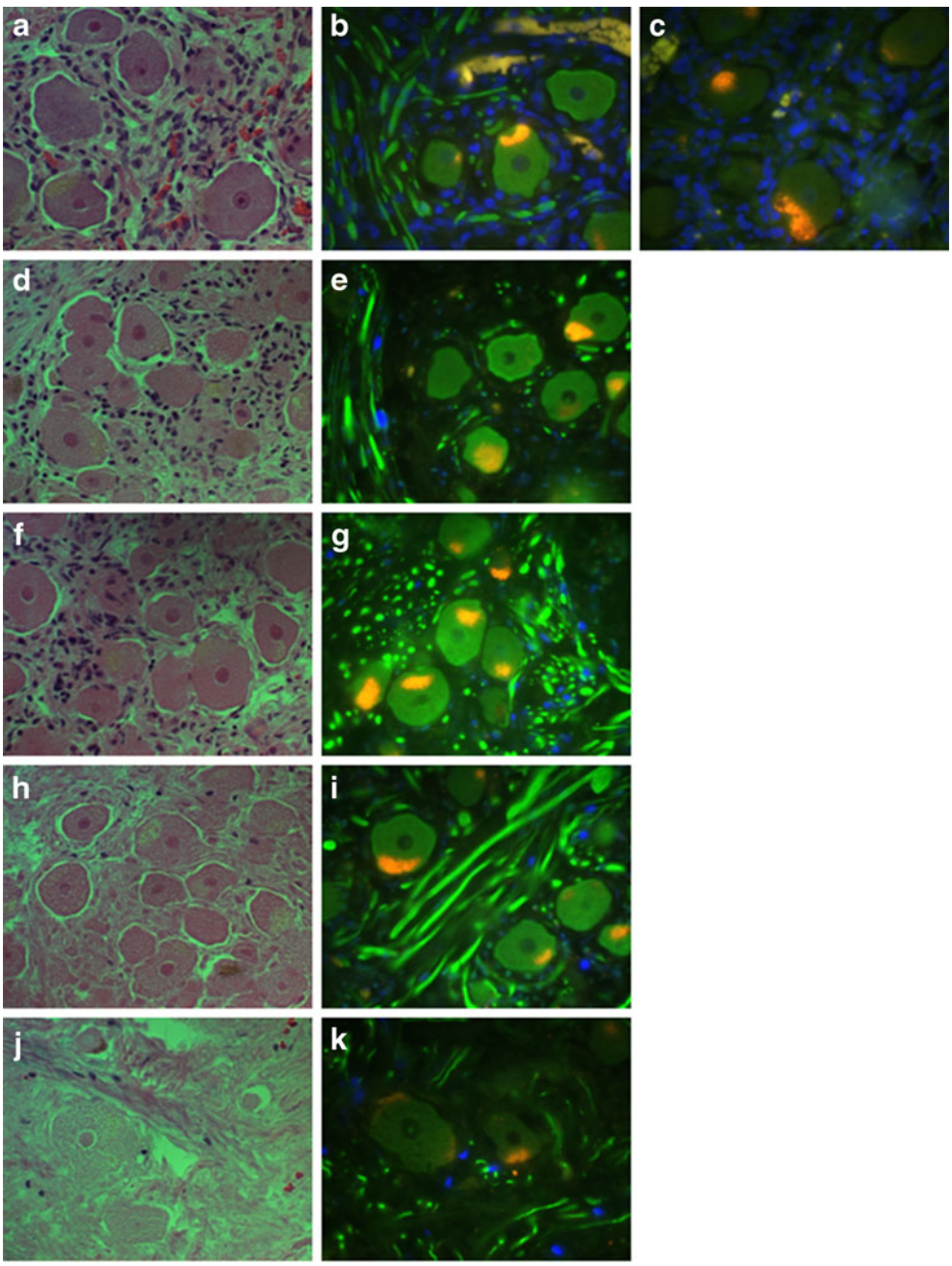

Fig. 1 Integrity of human trigeminal ganglia. Human trigeminal ganglia were cultured for 0 days (**a**–**c**) or 5 days (**d**–**k**) in culture medium 1 (**d**, **e**), 2 (**f**, **g**), 3 (**h**, **i**), and 4 (**j**, **k**). Sections were stained with hematoxylin/eosin (**a**, **d**, **f**, **h**, and **j**) or immunostained with antibodies directed against the heavy subunit of human neurofilament (**b**, **e**, **g**, **i**, and **k**). To detect background autofluorescence, normal IgG was substituted for neurofilament antibody (**c**)

goat anti-rabbit IgG (Invitrogen) at 22 °C for 30 min. DNA was stained with DAPI contained in the mounting medium (Vectashield; Vector, Burlington, CA), and coverslips were sealed with clear nail polish.

Sections were viewed under incandescent or UV light with a Nikon fluorescent microscope and images digitally captured with an Axiovision CCD camera. Image documentation was performed using Photoshop without altering relative channel intensities or picture aspects.

DNA extraction

Total DNA was extracted from each TG sample as described by Cohrs et al. (2000). Briefly, at 0, 1, and 5 days in culture, TG pieces were snap frozen, powdered under liquid nitrogen, and thawed in ATL dissociation buffer containing 10 mg/ml proteinase K (DNeasy, Invitrogen). DNA was collected on affinity columns, washed, eluted in 200 µl nuclease-free water, and quantified by optical adsorption at 260 nm (Nanodrop, Thermo Scientific, Wilmington, DE).

PCR

VZV and HSV-1 DNA were quantified by TaqMan-based real-time PCR using the following primers/probes synthesized by IDT (Coralville, IA): HSV-1, forward (TGG TAT TGC CCA ACA CTT TCC), reverse (GCG CCA GGC ACA CAC AT), probe (FAM/CGT GTC GCG TGT GGT/3BHQ); VZV, forward (CGA ACA CGT TCC CCA TCA A), reverse (CCC GGC TTT GTT AGT TTT GG), probe (FAM/TCC AGG TTT TAG TTG ATA CCA/3BHQ); GAPdH, forward (CAC ATG GCC TCC AAG GAG TAA), reverse (TGA GGG TCT CTG TCT TCC TCT), probe (FAM/TCC AGG TTT TAG TTG ATA CCA/3BHQ). Quantitative PCR was performed in a 96-well format in 20-µl reactions using a 7500 ABI with conditions as described (Cohrs et al. 2000).

Results

Optimization of human trigeminal ganglion culture medium

Morphology of trigeminal ganglion (subject 1) was determined by H&E staining and neurofilament immunostaining. At autopsy, the trigeminal ganglion showed numerous large polygon-shaped neurons surrounded by satellite cells whose small nuclei were clearly identified by intense basophilic stain (Fig. 1a). Anti-NFH immunostaining identified neurofilament in neuronal cell bodies and axons (Fig. 1b). Control immunostaining in which normal rabbit IgG was substituted for anti-NHF antibody showed only autofluorescent granules of lipofuscin (Fig. 1c).

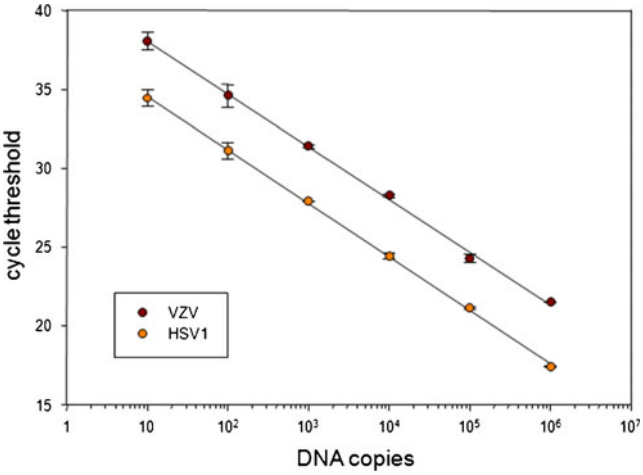

Fig. 2 Efficiency of PCR amplification. Dilutions of VZV or HSV-1 DNA were PCR-amplified with primers specific for each virus

Culture medium had a pronounced effect on morphology and neurofilament expression in trigeminal explants cultured for 5 days. The tissue morphology of TG explants cultured in medium 2 was normal (Fig. 1f, g). In contrast, multiple abnormalities were seen with medium 4. The total number of both neurons and satellite cells in H&E-stained sections was decreased. Some neurons appeared shrunken, while other neurons were missing, thus exposing large holes in the tissue; the cytoplasm of neurons was stained in a patchy eosinophilic pattern, and the perinuclear zone of many neurons was not stained (Fig. 1j, k). Anti-NFH stain of the sections from TG explants cultured in medium 4 revealed a marked decrease in neurofilament heavy peptide, which was detected only in small sections of axons. Media 1

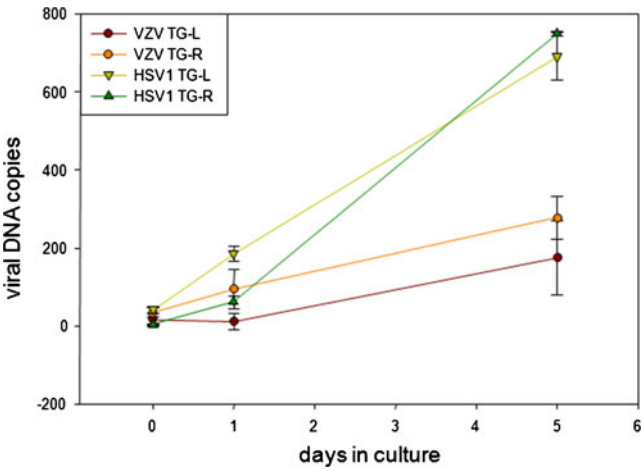

Fig. 3 Quantification of VZV and HSV-1 DNA in human trigeminal ganglion explants. Human trigeminal ganglia from subject 2 were divided into three portions and incubated for various times as indicated in "Methods" section. Total DNA was extracted and the abundance of VZV and HSV-1 DNA quantified by real-time PCR. *TG-L* left trigeminal ganglion, *TG-R* right trigeminal ganglion

(Fig. 1d, e) and 3 (Fig. 1h, i) had an intermediate effect on explant integrity.

Replication of alphaherpesvirus DNA in human trigeminal ganglia explants

Trigeminal ganglia (subject 2) were cultured for 0, 1, and 5 days in medium 2 after which HSV-1 and VZV DNA were quantified by real-time PCR. Both HSV-1 and VZV primer/probes detected as little as ten copies of virus DNA with equal efficiencies (Fig. 2). Low but detectable amounts of both viral DNAs were detected in all samples at day 0 and increased during the 5-day incubation period (Fig. 3). In all instances, the HSV-1 DNA copy number exceeded the VZV DNA copy number.

Discussion

With respect to the media tested, fetal bovine serum is the single most important factor in maintaining trigeminal ganglion explants in vitro. Explant integrity was enhanced by supplementing the medium with growth factors NGF and bFGF. In contrast, cell integrity was markedly reduced where cells were maintained in a serum-free medium even when supplemented with B-27 or when neurobasal medium was substituted for DMEM. Overall, DMEM supplemented with 20 % FBS and growth factors were optimal for maintaining trigeminal ganglion explants in culture for at least 5 days.

Both the HSV-1 and VZV DNA copy numbers increased during the 5-day study period, indicating that virus DNA replicated, a prerequisite to reactivation. Interestingly, the increase in HSV-1 DNA was about fourfold greater than the increase in VZV DNA. The finding that HSV-1 DNA replication was more pronounced than VZV DNA replication may explain why HSV-1 but not VZV can be isolated by explanting human ganglia in tissue culture (Baringer and Swoveland 1973). Since most human ganglia contain both latent HSV-1 and VZV, future studies will test various inducers of herpesvirus reactivation for their ability to initiate alphaherpesvirus DNA replication in latently infected human ganglia. An interesting extension of our model will be its application to other areas of herpesvirology, such as viral egress from reactivated neurons followed by entry into neighboring cells, and possible isolation of virus mutations which reactivated with increased frequency.

Acknowledgments This work was supported in part by Public Health Service grants NS007321 and AG032958 from the National Institutes of Health.

Conflict of interest The authors report no conflicts of interest.

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