Published in final edited form as:

Otol Neurotol. 2012 January ; 33(1): 87–92. doi:10.1097/MAO.0b013e31823dbb20.

A Cell Culture Model of Facial Palsy Resulting from Reactivation of Latent Herpes Simplex Type I

Maggie A. Kuhn, MD^{a,*}, Shruti Nayak, M.S.^{a,*}, Vladimir Camarena, M.D., Ph.D.^b, Jimmy Gardner, B.S.^a, Angus Wilson, Ph.D.^c, Ian Mohr, Ph.D.^c, Moses V. Chao, Ph.D.^b, and Pamela C. Roehm, M.D., Ph.D.^a

^aDepartment of Otolaryngology, New York University School of Medicine

^bSkirball Institute for Biomolecular Medicine, New York University School of Medicine

^cDepartment of Microbiology, New York University School of Medicine

Abstract

Hypothesis—Reactivation of herpesvirus type 1 (HSV1) in geniculate ganglion neurons (GGN) is an etiologic mechanism of Bell's palsy (BP) and delayed facial palsy (DFP) following otologic surgery.

Background—Several clinical studies, including temporal bone studies, antibody, titers, and intraoperative studies suggest that reactivation of HSV1 from latently infected GGNs may lead to both BP and DFP. However, it is difficult to study these processes in humans or live animals.

Methods—Primary cultures of GGNs were latently infected with Patton strain HSV1 expressing a green fluorescent protein-late lytic gene chimera. Four days later these cultures were treated with trichostatin A (TSA), a known chemical reactivator of HSV1 in other neurons. Cultures were monitored daily by fluorescent microscopy. Titers of media from lytic, latent, and latent/TSA treated GGN cultures were obtained using plaque assays on Vero cells. RNA was harvested from latently infected GGN cultures and examined for the presence of viral transcripts using reverse-transcription polymerase chain reaction.

Results—Latently infected GGN cultures displayed latency-associated transcript only, while lytically-infected and reactivated latent cultures produced other viral transcripts. GGN cultures displayed a reactivation rate of 65% after treatment with TSA. Media from latently infected cultures contained no infectious HSV1, while infectious virus was seen in both lytically and latently/TSA treated culture media.

Conclusions—We have shown that cultured GGNs can be latently infected with HSV1, and HSV1 in these latently infected neurons can be reactivated using TSA, yielding infectious virus. These results have implications for the etiology of both BP and DFP.

Keywords

Bell's palsy; delayed facial palsy; geniculate ganglion; herpes simplex type 1; latent herpes simplex infection; HSV1; HSV-1; viral reactivation

Address correspondence and reprint requests to: Pamela C. Roehm, M.D., Ph.D., Department of Otolaryngology, New York University School of Medicine, 530 First Avenue, Suite 7Q, New York, NY 10016, Telephone: 212.263.556 Fax: 212.263.2019, pamela.roehm@nyumc.org.

*These authors contributed equally to this work.

INTRODUCTION

Idiopathic facial nerve paralysis, or Bell's palsy (BP), is the most common cause of unilateral peripheral facial nerve weakness. It has been proposed that BP results from inflammation of the facial nerve, particularly of the labyrinthine segment, leading to neuronal pressure and ultimately dysfunction (1). Delayed facial palsy (DFP), which has been reported as a consequence in up to 1.4% of otologic cases and 35% of neurotologic cases (2–11), is also thought to result from inflammation of the facial nerve. While many potential causes of facial nerve inflammation have been studied, herpesviruses have been implicated as an underlying cause of both BP and DFP (4,8,12–16).

Herpes simplex virus type 1 (HSV1) infection is very prevalent, with 90% or more of the United States population infected by early adulthood (17). The virus can undergo lytic or latent lifecycles (Fig. 1). Non-neuronal cells can only be lytically infected, leading to production of infectious virus and eventual death of the host cell. Neurons can be either lytically or latently infected. During latent infection, no viral protein expression occurs and the latency associated transcript (LAT) is the only viral RNA produced (18). Heat, stress, ultraviolet light, or trauma can trigger HSV1 reactivation.

Despite clinical evidence that HSV1 reactivation is a potential cause of BP and DFP, the precise etiology and mechanism remain unknown. *In vivo* systems to study the relationship of HSV1 and facial palsy have been developed but such models have limited application to the pathophysiologic process underlying BP and DFP in adult humans (15,19–21).

We have developed a cell culture system to study HSV1 infection in geniculate ganglion neurons (GGNs). Using this system, both lytic and latent HSV1 infections can be established in these neurons. Reactivation of HSV1 in latently infected GGNs leads to production of infectious virus particles. These studies will further our understanding of factors which lead to HSV1 reactivation within GGNs and potentially aid in our prevention and treatment of facial palsy.

METHODS

GGN purification and cell culture

GGNs were harvested from postnatal day 5 Sprague-Dawley rat pups, trypsinized (0.0125%, Sigma), triturated, and filtered through a 70 micron cell culture filter (BD Falcon). Cells were plated on 96-well culture plates (BD Falcon) coated with poly-L-ornithine (0.1mg/cc; Sigma) and laminin (20µg/cc; Sigma). Cell culture media was Neurobasal media (Gibco) with B27 supplements (Invitrogen), L-glutamine (Gibco), 0.37% glucose, 5% fetal bovine serum (Gibco), 20µM Z-VAD-FMK (Z-VAD; Calbiochem), and ofloxacin (0.0003%; Daiichi Pharmaceutical Corp). Cultures were initially treated with 20µM 5-fluoro-2'-deoxyuridine (5-FU; Sigma) and 10µM aphidicolin (Calbiochem) for 2 days.

HSV1 strain

HSV1 used in these experiments was Patton strain with a US11-GFP chimera (HSV1:GFP) (22). HSV1 stocks were grown from Vero cell cultures infected at low multiplicity of infection (MOI). Cell-free lysates were harvested by multiple freeze-thaw cycles. Viral titers were obtained by plaque assay of serial dilutions on cultured Vero cells.

Induction of primary lytic HSV-1 infection

On day *in vitro* (DIV) 4, GGN cultures were infected with HSV1:GFP at an estimated MOI of 0.004 for 1.5 hours. The virus was removed and replaced with fresh cell culture media

(NBM/B27/FBS/ZVAD/floxin). GFP-positive neurons were detected at 18–19 hours post-infection by fluorescent microscopy. Two wells of each experiment were lytically infected in the subsequently described experiments as a positive infection control to determine the activity of the HSV1:GFP stock.

Induction of latent HSV-1 infection and reactivation

A time course of induction of latent HSV1 infection in GGNS is shown in Figure 2. GGNS were treated at DIV 2 with 100 μ M acyclovir (ACV; Calbiochem). At DIV 4, these neurons were infected with HSV1. ACV was removed at DIV 8 and fresh media was added. Wells that demonstrated GFP-positive cells before ACV removal were not used to determine rates of viral reactivation.

On DIV 8, cells were treated with 100 μ M trichostatin A (TSA; Sigma). In six wells of the 96-well plate, cells were plated in fresh cell culture media and carrier and observed for GFP positivity. These served as a baseline viral reactivation control.

RT-PCR for viral transcripts

Total RNA was isolated from cultured GGNS from the lytically-infected, latently-infected, and uninfected cells using Qiagen RNAeasy micro isolation kits per the manufacturer's directions (Qiagen Corp). cDNA was produced from each RNA sampled, and reverse-transcription polymerase chain reaction (RT-PCR) was performed using Qiagen One-Step Micro RT-PCR kits per the manufacturer's directions. The primers conformed to the LAT transcript (which is produced in both latent and lytic infections), ICP27 (a late lytic HSV1 gene), and GAPDH (a housekeeping gene) (23). GAPDH served as an RNA control. An H₂O control was performed with each primer set to evaluate for cross-contamination. RT-PCR reactions were run in separate wells on 1.25% agarose:TAE-ethidium bromide gels.

Viral quantification from infected cultures

Media from infected GGN cultures was collected at specific time points to determine viral titers. On day 4 following the HSV1 infection (DIV8), media was collected from lytically infected wells. On day 7 following the HSV1 infection (DIV 13) 3 days after the removal of ACV, media was collected from latently infected wells. For cultures that were latently infected and then treated with TSA, media was collected at DIV 13, 3 days after removal of ACV and addition of TSA. Titers were determined by plaque assay on confluent Vero cell cultures.

RESULTS

GGN cultures

After two days in culture, geniculate ganglion neurons were readily identifiable using light microscopy. A typical litter (10 to 13 rat pups) yielded an average of 1,500 neurons. Using the culture conditions described, these cultures remained healthy for up to two weeks. In the absence of 5FU and aphidicolin, these cultures would become overrun with replicating cells, such as fibroblasts and Schwann cells (data not shown).

Primary lytic HSV1 infection

At DIV2, GGN cultures were exposed to HSV1:GFP for 1.5 hours at a low MOI. One day following exposure of the cultures to HSV1, typical cytopathological features of HSV1 infection were seen in cultures by DIC and GFP+ cells were detected using fluorescent microscopy (Fig. 3A and B). The presence of a productive infection was confirmed by plaque assays of culture media on Vero cells, with an average of 7.0×10^3 plaque forming

units (pfu)/ well (Fig. 4B, far left dataset). In subsequent experiments, primary lytic infection was used as a positive infection control and to confirm the titer of the viral stock.

Latent HSV1 infection

Following pretreatment with 100 μ M ACV on DIV 2, GGN cultures were exposed to HSV1:GFP for 1.5 hours. Following removal of virus, cultures were post-treated with ACV for 4 days. After a media change at DIV 8, most cultures retained a normal appearance by light microscopy and showed no evidence of GFP+ cells on fluorescent microscope (Fig. 3C, 3D). Only 9% of cultures demonstrated GFP+ cells on fluorescent microscopy (Fig. 4A, dashed line). In latently infected cultures, no infectious virus was shown by plaque assay in the absence of GFP+ cells (Fig. 4B, middle dataset).

Reactivation of latent HSV1 infection

GGNs were treated at DIV 2 with 100 μ M acyclovir (ACV; Calbiochem). At DIV 4, these neurons were infected with HSV1. Wells that demonstrated GFP-positive cells before ACV removal were not used to determine rates of viral reactivation. ACV was removed at DIV 8 and fresh media was added (Fig. 2).

On DIV 8, latently infected cultures were treated with 100 μ M trichostatin A (TSA; Sigma). In six wells of the 96-well plate, cells were plated in fresh cell culture media and carrier and observed for GFP fluorescence. These served as a baseline viral reactivation control (Fig. 4A, dashed line).

Treatment of latently-infected neurons with TSA resulted in HSV1 reactivation and GFP-positive neurons (Fig. 3E & 3F). TSA-induced reactivation occurred at a rate of 65% ($\pm$ 1.83%) in latently infected GGNs. Most of the GFP+ wells were detected within three days of addition of TSA (Fig. 4A, solid line). These results were confirmed by plaque assay of culture media on confluent Vero cells, (Fig. 4B, far right dataset). Media harvested from cultures at 3 days following TSA treatment showed that infectious HSV1 was produced in these cultures (3.99×10^3 pfu/well).

RT-PCR for viral transcripts

In order to assess levels of viral transcripts, total RNA was isolated from cultured GGNs from the lytically infected and latently infected cultures. cDNA was produced from each RNA sampled, and reverse-transcription polymerase chain reaction (RT-PCR) was performed. Primers used conformed to either the LAT transcript (which is produced in both latent and lytic infections), ICP27 (a late lytic HSV-1 gene), and GAPDH (a housekeeping gene) (23). GAPDH served as an RNA quantity control. An H₂O control was performed with each primer set to evaluate for cross-contamination. PCR reactions were run in separate wells on 1.25% agarose:TAE-ethidium bromide gels (Fig. 5). No viral transcripts were detected in uninfected cultures (data not shown).

DISCUSSION

We have shown that cultured GGNs are readily infected with HSV1, yielding a productive infection. Infection with a low amount of virus in the presence of pre- and post-infection ACV produces a latent infection. Latently infected GGN cultures can be induced to enter a productive, lytic infection following treatment with TSA, a histone deacetylase inhibitor and known HSV1 reactivator in other systems.

The premises underlying our experiments were established in many previous observations. In 1972, McCormick proposed that either trauma or metabolic insult results in HSV1

reactivation in latently infected facial neurons. He hypothesized that the resulting inflammation and swelling in the fallopian canal causes swelling and pressure on the facial nerve leading to paralysis (24). Subsequent human and animal studies supported the hypothesis that HSV1 infection plays a role in both BP and DFP.

A variety of studies and techniques show that HSV1 can cause BP in humans, and that reactivation of HSV1 in the geniculate ganglion is a significant cause of BP in adults. HSV1 DNA was identified in 86% of geniculate ganglia (25), while another study found 75% of their postmortem temporal bones had LAT transcripts but no evidence of lytic viral transcripts (26). In human geniculate ganglia, co-localization of LAT and elevated levels of CD8 transcripts has been identified by PCR, suggesting that active immune surveillance is activated when these neurons are latently infected with HSV1 (27). A postmortem study of a patient who had developed BP 6 weeks prior to death from unrelated causes showed infiltration of the facial nerve with immune cells and HSV1 DNA by PCR, which was not present in controls (28). MRI studies have shown geniculate ganglion enhancement in 70% -- 91% and frequent labyrinthine segment enhancement in patients with BP (29–32). Several studies have noted presence of HSV1 DNA in saliva and tears of patients with BP at higher rates than in nonaffected controls (30–50% v. 0–19%) (33–36).

Critically, intraoperative specimens taken from patients undergoing facial nerve decompression have supported the theory that active HSV1 infection underlies many cases of BP. Murakami et al. found HSV1 DNA in the endoneural fluid in 11 of 14 (79%) patients with Bell's palsy and in none of the controls or their patients with Ramsay-Hunt syndrome (37). Culture of facial epineurium in one of two patients with BP revealed HSV1 (13).

Similar involvement of HSV1 reactivation appears to underlie the pathogenesis of DFP. Viral titers following acoustic neuroma resection and stapedectomy show an elevation consistent with HSV1 reactivation in a significant percentage of patients with DFP compared with unaffected patients (4,8). Pre- and postsurgical treatment of patients undergoing acoustic neuroma resection with famciclovir significantly decreased DFP incidence (38).

Several *in vivo* animal studies have lent support to the theory that HSV1 causes facial paralysis. Inoculation of HSV1 in the stylomastoid foramen of the rabbit induces a permanent facial paralysis in most injected animals. Microscopic examination of infected rabbit facial nerves showed early facial nerve edema early with dissolution of the nerve sheath by 13 days following injection and presence HSV1 virions by electron microscopy (20). Intracerebral or intralabyrinthine inoculations of HSV1 in hamsters led to characteristic pathologic changes around the geniculate ganglion in 57% of injected animals (19). In mice, inoculation of HSV1 in the anterior tongue or postauricular area yielded a transient facial paralysis in 56% of treated animals (21). Subsequent elevation of antiviral antibody titers was noted 6–9 days following inoculation (15). All of the *in vivo* animal studies elicit an acute facial palsy following lytic (primary) HSV1 infection. These studies more closely resemble HSV1-related BP in pediatric patients, who develop their facial palsy in the setting of a primary HSV1 infection (39,40). However, they are less relevant to cases of BP and DFP in adults, who have experienced primary HSV1 infection many years prior to onset of facial palsy (17).

While other groups have developed model systems to show that lytic HSV1 infection can cause facial nerve paralysis, none have previously demonstrated that reactivation of a latent HSV1 infection can occur in the geniculate ganglion. Using our *in vitro* culture system, GGNs can be latently infected with HSV1 and induced to re-enter the lytic part of the HSV1 lifecycle, yielding infectious virus. The use of an *in vitro* model system removes neurons

from their *in situ* environment of supporting cells, nutrients and intracellular connections. Additionally, contributions to disease processes other systems (and particularly in the case of facial paralysis, the immune system) cannot be directly assessed. Despite these limitations, an *in vitro* model is useful for studying intracellular mechanisms and specific inhibitors of latency.

Acknowledgments

Source of funding: NIH 1K08 DC009288-01A1

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FIG. 1. HSV1 lifecycle. Following harvest of GGNs from young rats, cultures are pretreated with ACV and then infected with HSV1. Treatment with ACV is continued for 4 additional days. Following withdrawal of ACV, cultures are treated with TSA to induce viral reactivation. Cultures are monitored with fluorescent microscopy for the expression of GFP.

FIG. 2.

Time course of latent HSV1 infection and reactivation experiments.

FIG 3. Light microscopy (A) and fluorescent microscopy (B) of lytically infected GGN cultures. Light microscopy (C) and fluorescent microscopy (D) of latently-infected GGN cultures (D). Light microscopy (E) and fluorescent microscopy (F) of latently infected GGN cultures treated with TSA. Size bar = 30 microns.

FIG. 4.

(A) HSV1 reactivation in latently-infected GGN cultures measured as cumulative percentage of GFP-positive wells. Open circles = baseline reactivation, black squares = TSA treated. (B) HSV1 titer measured by plaque assay on media from lytically infected (lytic), latently infected (latent), and latently-infected, TSA treated cultures (+TSA). pfu = plaque-forming units.

FIG. 5. End-point reverse transcription polymerase chain reaction of total RNA extracted from GGN cultures lytically (lytic) and latently (latent) infected with HSV1.