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Therapeutic implications of new insights into the critical role of VP16 in initiating the earliest stages of HSV reactivation from latency

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Abstract

Reactivation of herpes simplex virus (HSV) is a leading cause of fatal encephalitis in the USA and recurrent herpetic keratitis is a major infectious cause of blindness. There is no effective vaccine and no cure for HSV latency. While current antiviral drugs reduce viral replication, none prevent the initiation of reactivation in the nervous system and, thus, chronic inflammatory damage proceeds. The discovery that HSV VP16 is necessary for the exit from latency represents the first potential target for preventing the chronic inflammatory insult associated with HSV reactivation. Blocking VP16 transactivation would reduce the spread of the virus in the population and, importantly, presumably reduce or prevent the pathological long term chronic inflammation in the nervous system.

The continuous infection of the human population at pandemic levels by the herpes viruses attests to the success of these viruses as 'pathogens'. Once consummated, the marriage between the infected host and the virus lasts until death. While many aspects of their evolutionary adaptations to the host account for this success, central and unique to the herpes viruses is a context-driven dual modality, productive lytic infection (on) or latent infection (off). Upon primary infection the virus enters the lytic replication cycle in certain cells and tissues, resulting in the geometric amplification and controlled dissemination of viral genetic information into the host. In other cellular contexts the viral genome is transcriptionally repressed and extrachromosomally maintained indefinitely within the cell. In response to certain stressors, viral genomes within a very small percentage of latently infected cells are derepressed, and transcriptional activity from the latent viral genome initiates. Ultimately, infectious virus is produced, amplified in permissive cellular environments, and shed to infect new hosts [1]. With respect to herpes simplex virus (HSV), recent clinical studies have revealed what appears to be an alarming rate of viral shedding not associated with lesions or other symptoms [2]. The significance of this viral shedding with respect to transmission is not fully understood but it has been suggested that it results in

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chronic inflammation in the genital track that may explain the link between HSV infection and the risk of acquiring HIV [3]. There is also a link between HSV infection and the apolipoprotein E4 allele as an important risk factor for Alzheimer's disease that may reflect the negative impact of the chronic inflammatory insult in the nervous system associated with HSV reactivation [4]. Thus, there is new urgency for developing strategies to block viral reactivation at its onset. Recent insights into the mechanism of HSV reactivation in sensory neurons provide a potential new antiviral target for blocking the earliest stages in reactivation and are the focus of this perspective.

Natural history of HSV infection

Herpes simplex virus is an enveloped virus with a large double-stranded DNA genome that encodes approximately 85 lytic phase proteins. Humans are the sole reservoir of this virus, which is transmitted by close physical contact and most primary infections are self-limiting. However, HSV is the agent of serious morbidity and mortality, including fatal encephalitis and blindness, and transmission to the neonate often results in disseminated infection of diverse organs, devastating disease, or death [1]. Considering that the vast majority of the world's population is currently, and will remain for the foreseeable future infected with HSV, its direct and indirect impact on human health is profound.

First proposed for HSV in 1929 by Goodpasture [5], it is now established that distinct lytic phase and latent phase programs characterize the natural history of herpes viruses (Figure 1). Lytic HSV infection of cultured cells and, by analogy, cells at the body surface results in a classic cascade of viral immediate early (IE), early (E) and late (L) gene expression that produces viral progeny and kills the host cell [6,7]. Virus enters the axons of sensory neurons innervating the site and, in these cells, can establish a latent state in which the expression of all lytic phase genes is suppressed and the latency-associated transcripts are uniquely actively transcribed [8]. The latent viral genome is maintained for the life of the host in thousands of neurons per ganglion [9–13] in a state that is capable of initiating the lytic-phase program in response to stressful stimuli [14]. Once the acute stage of infection has ended at 30 days postinfection, approximately 18,000 neurons remain in a mouse trigeminal ganglion, and of these, an average of 6000 are latently infected [15]. In the mouse *in vivo* model, spontaneous reactivation can be detected in one neuron per ten latently infected mice at any given time examined (one positive neuron/120,000 latently infected [11] and in an average of 2.2 neurons per trigeminal ganglion following stress (one positive/2700 latently infected) [10,11,16]. Viral reactivation can result in asymptomatic shedding or recurrent disease and spread to new hosts (Figure 1) [14].

Entry into the lytic cycle

In a striking case of parallel evolution, most DNA viruses employ strong enhancers to promote the transcription of the earliest viral genes. HSV differs from other DNA viruses (including many, but not all other herpes viruses), in that its IE gene promoters are not principally dependent on classical enhancers responsive to host-cell factors. Rather, transcription of the IE genes is initiated by a protein component of the virion that is a potent transcriptional activator. This late gene protein (Virion protein 16 [VP16]) interacts with host-cell proteins including host-cell factor-1 (HCF-1), a cell-cycle regulator and octamer binding protein-1 (Oct-1), a POU domain transcription factor, to form the VP16-induced complex (VIC) that binds to TAATGARAT elements present in the five HSV-1 IE gene promoters (Figure 2) [7,17–19]. There is extensive literature identifying VP16 as the first example of a class of viral regulators that activate genes through a specific *cis*-acting sequence but do not themselves directly bind DNA [7,20]. With regard to reactivation from latency, the dependence on a viral structural protein produced late in the infectious cycle to initiate transcription from the viral genome presented a conundrum. How can the latent viral

genome initiate the transcription of lytic phase genes in the absence of the crucial transcriptional activator VP16? The dogma has been simply that VP16 is not involved in reactivation.

Regulation of latency & reactivation

The brass ring

The cycle of lifelong latent infection punctuated by periodic reactivation and recurrent disease lies at the heart of the ubiquity of infection by this virus. An understanding of the molecular mechanisms regulating HSV latency and reactivation is central to identifying novel drug targets to disrupt these processes. For those outside the field, it may seem confusing considering the known functional role of VP16 in initiating the lytic cycle that this protein was ruled out as a central and perhaps initiating player in reactivation from latency. VP16's expression as a late gene largely dependent on viral DNA replication during infection of cultured cells did suggest that its very early expression during reactivation would not be expected. In addition, early work by Sears and Roizman on the role of VP16 in reactivation utilizing transgenic mice expressing VP16 from the metallothionein (cadmium inducible) promoter seemed to rule out a role for VP16 for the initiation of reactivation [21]. It was also determined that the viral mutant in 1814 (in which the transactivation function of VP16 was ablated [22]) established latent infections [23,24]. In addition, these latent genomes were able to produce infectious virus in an explant reactivation assay in which latently infected mouse sensory ganglia were axotomized, placed into culture and sampled for infectious virus over a period of many days [23,25]. The authors concluded correctly that VP16 was not necessary for the reactivation from latent infection in the axotomy/explant model.

Since these studies, more refined approaches for investigating latency and reactivation have been developed; these include a well-characterized and physiologically relevant model of *in vivo* reactivation [16], PCR detection of viral DNA in latent ganglia [26,27], a method for quantifying at the single-cell level the number of neurons latently infected and the number of viral genome copies that individual neurons contain [9] and a method for detecting and quantifying individual neurons exiting latency that is not dependent on the production of infectious virus [11]. This last approach provides the ability to parse out stages in the process of reactivation from latency. It is now possible to distinguish between the exit from the latent state (the initiation of reactivation), abortive reactivation and the full completion of the virus replication cycle. Using these approaches we found that several viral proteins thought to be essential for the initiation of HSV reactivation from latency, including the IE proteins ICP0 [28,29] and ICP4 [30] and viral DNA replication and/or the viral thymidine kinase [31] were not required for the exit from the latent state [32–35]. To date, only viral mutants that lack the transactivation function of VP16 fail to exit the latent state *in vivo* and do not express detectable viral proteins during latency or following stress [35]. In addition, induced expression of VP16 during latency shifts the balance toward acute viral replication [Thompson, Sawtell, Unpublished Data]. Thus, there exists solid evidence that the stochastic derepression of VP16 in rare latently infected neurons is a very early and, perhaps, precipitating event during HSV reactivation from latency [35]. It is likely that the extreme changes in the proteome of explanted neurons such as the expression of cell cycle-related proteins including CdK 2 and 4, geminin and induction of apoptosis in axotomized and explanted neurons seen as early as 2 h post-explant obviate the need for the essential VP16 protein in the *ex vivo* reactivation model [12].

Silent danger: chronic inflammation in PNS & CNS accrues during the lifetime of the infected host

The chronic stimulation of the immune response by the periodic expression of viral proteins associated with reactivation results in persistent focal inflammation in the latently infected PNS and CNS [36–38]. However, the fact that most people harbor HSV in their nervous systems makes it extremely difficult to discern the health implications associated with this chronic inflammatory process. The potential that host genetics influence the characteristics of the immune response, including the onset, extent and resolution of inflammation is well recognised [39]. Thus, it is likely that individual differences in pathways regulating inflammatory responses will result in a spectrum of outcomes in response to the HSV reactivation cycles occurring in the nervous system.

We have begun to test this hypothesis using genetically distinct mouse strains. In experiments designed to determine whether periodic reactivation over time results in accrued damage in the CNS (Figure 3), we have clear evidence that stimuli that induce HSV reactivation in the PNS, can also result in reactivation in the CNS. As in the PNS, virus production is limited to a few detectable plaque-forming unit (PFU). Following repeated reactivation stimuli over periods of many weeks, increasing areas of focal reactive changes are observed in mice of select genetic backgrounds (Figure 3). Although these mice appear to be normal and healthy, damage in the CNS is accruing slowly. Such models will be useful to test how spontaneous or induced virus reactivation impacts CNS damage through time.

Current prevention & treatment strategies

To date there are no licensed vaccines for HSV, and there are formidable barriers to the generation of an effective and safe vaccine [40]. Herpes cannot be cured because neither the immune system nor antiviral drugs work against the latent virus in the nervous system. However, there are very safe and effective treatments against actively replicating HSV in the form of antiviral drugs. These drugs all block viral DNA replication and include acyclovir, famciclovir and valacyclovir. In people with normal renal function these drugs are generally safe and well tolerated [41]. Unfortunately, resistance to this class of drugs is becoming more prevalent [14]. While these drugs can effectively block the replication of the viral genome required for the production of infectious virus during reactivation, they do not prevent the expression of viral proteins, which occurs at the onset of this process [13] and, likewise, presumably do not prevent the chronic inflammation in the nervous system engendered by long term HSV infection. A recent report suggests that monoamine oxidase inhibitors can block viral reactivation in explanted mouse TG before viral proteins are expressed [42], suggesting that specific drug treatments that can block viral protein expression in latently infected tissues might be developed.

Anti-VIC therapeutics

To date, three different moieties are thought to disrupt the formation of the VP16 induced complex with HCF-1 and Oct-1. O'Hare and colleagues showed that a peptide spanning amino acids 360–390 of the VP16 sequence could block VIC assembly *in vitro* and found that 6 amino acids in the core of this peptide were particularly important [43]. As shown in Figure 4, this region is highly conserved between HSV-1 and HSV-2. Within this region are sites important for binding of HCF-1 and Oct-1 [44]. In addition, two moieties found in herbal extracts are thought to block VIC formation, yatein from *Chamaecypari obtusa* [45] and samarangenin B from *Limonium sinense* [46,47]. These two molecules have been shown to inhibit viral replication in cultured cells. These findings suggest it will be possible to identify small molecules that safely and efficiently block the VP16 transactivation function.

Such molecules would be a valuable new line of defense against HSV strains resistant to current therapies. In addition, such drugs would be expected to block virus reactivation from latency prior to significant viral protein expression. This property would limit or eliminate the chronic inflammatory response present in neural tissues latently infected with HSV.

Future perspective

The awareness of the risk of accrued damage to the human CNS of long-term HSV infection in the nervous system, especially in certain genetic backgrounds, will elevate the importance of preventing infection of future generations with HSV. As with vaccination against varicella zoster virus, an appropriately attenuated vaccine strain of HSV could be developed and safely administered to protect against primary infection. Strategies to maintain immunity in the vaccinated population will be needed if a vaccine is utilized that does not periodically reactivate or exit latency. It is extremely unlikely that a strategy for eliminating the latent HSV reservoir from the human population will be forthcoming. As our understanding of the multilayered mechanisms by which the viral genome is repressed in the nervous system increases, it may become possible to induce the reactivation of many latently infected neurons simultaneously. However, the risk of attempting a coordinated reactivation of all latently infected neurons, both PNS and CNS in an attempt to eradicate latency would seem considerable. Ideally, well-tolerated approaches to block reactivation at its onset will evolve along with our increasing understanding of the interactions between the neuron and the virus.

Executive summary

- Herpes simplex virus (HSV) establishes life-long latent infections in the PNS and CNS of humans.
- The majority of humans are infected with HSV and reactivation from latency occurs much more frequently than indicated by the typical skin lesions.
- Virion protein 16 is a viral late gene protein that is packaged into the virion tegument and transactivates the five viral immediate early genes starting the lytic cycle.
- VP16 is also required for initiating the lytic cycle from the latent viral genome.
- Chronic inflammation is associated with latently infected tissues most likely a result of periodic re-stimulation of the immune response by the expression of viral proteins during a reactivation event.
- Blocking reactivation by inhibiting viral DNA synthesis does not block the expression of viral proteins in neurons entering the lytic cycle (starting to reactivate).
- Blocking reactivation by inhibiting viral DNA synthesis does not block the chronic inflammatory response.
- Chronic inflammation in the CNS underlies several neurodegenerative disorders.
- It has been proposed that HSV infection combined with the apolipoprotein E4 allele significantly increases the risk of Alzheimer's disease.
- Thus blocking reactivation at the very earliest stages, prior to viral protein production and restimulation of the immune system is an important goal.

- Developing therapeutics that block VP16 function either at the stage of its expression or its ability to transactivate the immediate early genes should not only block reactivation but also the chronic inflammation associated with this event.

Key Terms

Lytic replication	Productive infection that yields infectious progeny.
Reactivation	Re-entry into lytic replication from the latent state.
Latency	Dormant state of limited transcription in neurons in which no viral proteins are produced.
Virion protein 16	Transactivates HSV immediate early genes.

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Figure 1. The natural history of HSV-1 infection

(A) HSV-1 is spread by close interpersonal contact, preferentially infecting at mucotaneous junctions around the lips, nose and eyes. Most of us are infected before puberty and the most frequent manifestation of a primary infection is gingivostomatitis (an infection of the lips and gums), which resolves in about 10 days. (B) During this period of acute infection the virus is transported up the axons of innervating sensory neurons. (C) When the virus reaches a neuronal nucleus one of two events ensues. The virus replicates, kills the neuron and the progeny travel to the brain (D) or back to body surface. Alternatively, a latent infection is established in which the viral genome is maintained indefinitely in the neuronal nucleus in what is thought to be an extrachromosomal episome.

Figure 2. Herpes simplex virus entry into the lytic cycle

(A) Entry into the lytic cycle during acute infection. (1) The HSV virion consists of a double-stranded DNA genome packaged within a icosododecahedral capsid, which is surrounded by a bilayered lipid envelope. The tegument resides between the capsid and the envelope and contains several proteins including VP16. **(2)** The viral envelope fuses with the cell membrane and the capsid and tegument proteins enter the cell. **(3)** The capsid is transported to the nuclear envelope, docks at the nuclear pore and **(4)** the viral genome is ejected into the nucleus **(5)** where the genome circularizes. **(6)** VP16, which has been transported to the nucleus interacts with HCF-1 and Oct-1 and this VP16-induced complex binds to the TAATGARAT motifs shared by the five IE genes in the viral genome. Activation of these IE genes starts off the viral lytic cycle [1]. **(B) Latency/reactivation. (1)** During latency, the viral genome resides within the neuronal nucleus in a repressed state, associated with histones [48]. **(2)** Following a reactivation stimulus changes in the modifications of the tails of histones associated with the viral genome can be measured [49]. We have found that histones associated with the genome in the region of the VP16 promoter adopt marks associated with more open chromatin within 45 min after reactivation stimulus is applied. The significance of this finding remains to be determined, since the vast majority of latent viral genomes remain transcriptionally silent. **(3)** In those rare neurons that exit latency, VP16 is expressed as a pre IE gene, that is, VP16 is expressed *de novo* and independent of normal constraints on late gene expression [35]. **(4)** Once expressed, VP16 is available to transactivate the five IE genes and initiate the viral lytic cycle. Importantly, VP16 has the ability to transactivate gene expression in genes within silenced chromatin [50].
HCF: Host-cell factor; IE: Immediate early; Oct: Octomer binding protein; VP: Virion protein.

Figure 3. Inflammatory changes in the CNS result from chronic reactivation of latent herpes simplex virus

We have begun studies to define the host genetics that underlie the long-term outcomes of chronic reactivation in the CNS. In order to test the effect of repeated reactivation in the CNS, we set up experiments in which latently infected mice were regularly exposed to our standard reactivation stimulus. Because this stimulus is not harmful, simulating a brief high fever (core body temperature elevated to 42°C for 6 min), the effect of repeated reactivation in the CNS can be tested. Mouse strains resistant to encephalitis during primary HSV infection and exhibiting no signs of disease (latent infections were confirmed in the CNS by PCR) were exposed to our standard reactivation stimulus periodically over 10 weeks starting 40 days after primary infection. Following variable numbers of reactivation stimuli, brains were examined using standard glial fibrillary associated protein staining to detect glial cells. The extent of pathology in the CNS ranged from focal discrete reactive glial cells to focal areas of gliosis, correlating with the number of reactivation stimuli received supporting the hypothesis that repeated reactivation in the CNS can lead to significant focal reactive lesions. Examples of the lesions observed in sectioned brain tissue harvested from latently infected mice after repeated reactivation stimuli are shown, as detailed above.

Figure 4. Primary amino acid sequences of the VP16 proteins from herpes simplex virus-1 (top) and herpes simplex virus-2 (bottom)

Nonidentical amino acids are highlighted. The amino acids between 328 and 390 on the herpes simplex virus-1 sequence have been implicated in the formation of the VP16 induced complex. Regions particularly important for binding HCF-1 and Oct-1 are shown as black bars. The dotted line represents a peptide capable of blocking VIC formation, with the core amino acids delineated by a double headed arrow.

HCF: Host cell factor; Oct-1: Octomer binding protein-1; VIC: VP16-induced complex.