Varicella-Zoster Virus infected human neurons are resistant to apoptosis

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ABSTRACT

Varicella-Zoster virus (VZV) is a pathogenic human herpesvirus that causes varicella (chickenpox) as a primary infection following which it becomes latent in ganglionic neurons. Following viral reactivation many years later VZV causes herpes zoster (shingles) as well as a variety of other neurological syndromes. The molecular mechanisms of the conversion of the virus from a lytic to a latent state in ganglia are not well understood. In order to gain insights into the neuron-virus interaction we studied virus-induced apoptosis in cultures of both highly pure terminally differentiated human neurons and human fetal lung fibroblasts (HFL). It was found that (a) VZV DNA did not accumulate in infected human neurons; (b) VZV transcripts were present at lower levels at all days studied post-infection in neurons; (c) Western blot analysis showed less VZV IE 63 and very little detectable VZV gE proteins in infected neurons compared with HFL; (d) lower levels of the apoptotic marker cleaved Caspase-3 protein were detected in VZV-infected neurons compared with HFL, and higher levels of the known anti-apoptotic proteins Bcl2 , Bcl-XL and also the mitochondrial MT-CO2 protein were found in VZV infected neurons compared to uninfected cells; (e) both the MT-CO2 protein and VZV IE 63-encoded protein were detected in infected neurons by dual immunofluorescence. These findings showed that neurons are resistant to VZV-induced apoptosis, which may have relevance to the switching of VZV from a lytic to latent ganglionic neuronal infection.

INTRODUCTION

Varicella-Zoster virus (VZV) is a human neurotropic alpha herpesvirus that causes varicella ('chicken pox') as a primary infection following which it is converted to a latent state within ganglionic neurons throughout the entire neuroaxis (Kennedy et al 1998, Gilden et al 2011, Nagel and Gilden 2013). After an interval that may be decades, the virus may reactivate, either spontaneously or after various triggering factors, to cause herpes zoster ('shingles') which is an extremely painful dermatomal rash that may be followed in some cases by post-herpetic neuralgia which is typically very refractory to treatment (Gilden et al 2011, Kennedy and Gershon 2018). VZV reactivation may also cause a wide variety of other acute, subacute, and chronic infections of the nervous system (Kennedy 2011, Nagel and Gilden 2013). An understanding of both the lytic and latent behavior of VZV in human neurons is therefore of clinical as well as biological importance.

One factor that could play a role in the shift between acute lytic viral infection of neuronal cells and the subsequent latent VZV infection of ganglionic neurons is the extent of apoptosis, or programmed cell death (Wyllie 1997), that the virus may induce in neurons during acute infection. Apoptosis may be induced by various viral infections resulting in disruption of normal cellular functions (Kennedy 2015). However, some viruses, such as Herpes simplex virus type-1 (HSV-1) have the capacity to inhibit host cell apoptosis so that virus infection is not limited by cell death, thereby allowing viral spread and either viral persistence or latency, thus favoring the virus rather than the host. Apoptosis may occur via the intrinsic pathway occurring in the mitochondrion with release of cytochrome c leading to activation of caspase 9, then caspase 3 with cleavage of DNA (Aubert et al 2007, Kennedy 2015), or via the extrinsic pathway which uses the Fas ligand (Fas L) leading to cleavage of procaspase-8 resulting in activation of caspase-3 and cleavage of cellular DNA (Thornberry 1997, Aubert et al 2007, Kennedy 2015). Thus, in both pathways the presence of caspase-3 may be utilized as a marker of apoptosis. There is evidence that VZV is able to induce apoptosis in susceptible cells via both the intrinsic and extrinsic pathways (James et al 2012). Since virally-induced phagocytosis of an apoptotic host cell has recently been recognized as anti-inflammatory (Kennedy 2015), it is also important to recognize that these various interacting cellular processes may be complex in terms of the eventual outcome of an acute viral infection.

The current availability of highly pure (>95%) terminally differentiated human neurons in culture, as well as established cell biological techniques and robust markers of apoptosis, now allows a detailed analysis of the extent to which VZV has the capacity to induce apoptosis in human neuronal cells. We report here the results of our investigation of this process using these cellular and molecular tools.

MATERIALS AND METHODS

Cells

iCell neurons (Cellular Dynamics International, Madison, WI) were the same as previously described in detail (Yu et al 2013) and were derived from induced pluripotent stem cells. They were comprised of a combination of post-mitotic neural subtypes with typical physiological characteristics and responses, and which showed positive staining with multiple neuronal markers. Human fetal lung fibroblasts (HFLs) were cultured in Dulbecco's minimum essential medium (DMEM) containing 100 U/ml penicillin and 10 μg/ml streptomycin supplemented with 10% fetal bovine serum (FBS).

Virus Infection

This was carried out as previously described (Yu et al 2013). Neurons (~1×10^6) were infected with 1000 plaque forming units (pfu) of attenuated VZV (Zostavax, Merck, Whitehouse Station, NJ) in iCell complete maintenance medium. Human fetal lung fibroblasts (HFL) functioned as control cultures in these experiments and were similarly infected with 1000 pfu VZV and maintained in DMEM supplemented with 2% FBS.

Total nucleic acid extraction

Total nucleic acid was extracted using the mirVANA miRNA extraction kit (Ambion, Austin, TX). Briefly, cells were scraped in lysis buffer (Ambion) using a rubber policeman. If present, visible cell clumps were homogenized using a 17–26-gauge needle with a 1-ml syringe (12–20 strokes) and kept on ice for 10 min. Total nucleic acid was extracted with acid-phenol chloroform and applied to an affinity column, washed three times, and eluted in 100 ul of PCR-grade water (Teknova, Hollister, CA) at 95°C.

Quantitative real-time PCR

Total nucleic acid was examined by quantitative real-time PCR (RT-qPCR) with VZV-specific primers (IDT, Coralville, IO) on an Applied Biosystems Fast 7500 and analyzed using Fast 7500 software (Yu et al., 2013). Samples were denatured at 95°C and allowed to elongate and anneal for 40 cycles at 95°C for 15 s and at 60°C for 30 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers and probe were used as an expression control.

RNA extraction, cDNA synthesis and RT-qPCR

Total nucleic acid (200 ng) was DNase-treated using a Turbo-DNA free kit (Ambion) for 30 min at 37°C, followed by inactivation using inactivation buffer (Ambion) for 5 min at room temperature with mixing. Sequestered DNase was pelleted by centrifugation at 2000 rpm for 5 min and the supernatant was transferred to a new tube. cDNA synthesis was completed using the Transcriptor First-Strand cDNA synthesis kit (Roche, Indianapolis, IN). Anchored oligo-[dT] and random hexamer primers were added and annealed for 10 min at 65°C to ensure high conversion of mRNA to cDNA. After primer annealing, dNTPs, reverse transcriptase (RT) reaction buffer and RNase inhibitor were added. Samples were divided into 2 portions with and without RT to control for any residual DNA. cDNA was stored at 0°C. Both RT-positive and -negative samples were amplified along with wild-type VZV DNA (106 to 100 genome copies) to compare transcription levels to residual DNA levels. VZV primers and Taq Universal Probes superscript ROX mix, (Bio-Rad, Hercules, CA) were combined with 2 µl of cDNA and added to a MicroAmp 96-well plate. Quantitative real-time PCR was performed as described above. GAPDH primers and probe were used as a cellular control to confirm cDNA synthesis. VZV gene expression was presented as a Ct ratio to that of GAPDH.

Protein Extraction

VZV infected neurons at 14 DPI and HFL at height of CPE as well as uninfected cells were used for total protein extraction. The cell culture medium was removed, adherent cells were washed with 1X PBS and M-PER® Mammalian Protein Extraction Reagent (Thermo Scientific) was added. Cells were incubated for 5 minutes with gentle agitation, and then scraped with a cell scraper. The lysate was collected and mixed with 1X Complete Mini EDTA-Free Protease Inhibitor (Roche), then centrifuged at 14,000 x *g* for 5 minutes and the supernatant was collected for Western Blot analysis.

Western Blot

Bio-Rad Mini-protean TGX 4-20% Gels (Bio-Rad) were used for SDS-PAGE analysis with 1X Tris/Glycine/SDS buffer (Bio-Rad). Protein samples (1-5ug/well) were denatured by incubation with 1X lane marker reducing sample buffer containing dithiothreitol (Thermo Scientific) at 95°C for 10 min. Gels were electrophoresed for 40 min under constant voltage of 150 V. The gel was then electro-blotted onto a PVDF membrane (Bio-Rad) for 50 min under constant voltage of 10 V using Trans-Blot® Semi-Dry Cell (Bio-Rad). After blocking with 6% milk in TBS/0.1% Tween 20 for 1 h, membranes were probed with primary antibodies (1:100 to 1:10,000) (Table 1) in 1% milk/TBS/0.05% Tween 20 for 1 hour or overnight, then washed once with 0.05% TBST for 10 min, followed by four more washes at 5 min each. Membranes were then incubated with secondary antibodies (1:2,000-1:20,000) in 1% milk/TBS/0.05% Tween 20 for one hour, washed five times as before, then incubated with SuperSignal® West Femto Maximum Sensitivity chemiluminescent substrate (Thermo Scientific) as recommended by the manufacturer. The FluorChem Q™ system was used to measure the band intensity of the western Blots.

Immunocytochemistry

Assays were carried out at 14 days post infection (DPI). Coated coverslips used for neuron propagation were obtained from BD Biosciences (San Jose, California). Cells were fixed in 4% paraformaldehyde for 20 min at room temperature, washed three times in PBS, and permeabilized in 0.3% Triton for 10 min. After three rinses with PBS, cells were either stained or stored at 4°C in PBS. Primary (anti MTCO2 and anti- VZV IE 63) and secondary Alexa-fluor-conjugated (Invitrogen, Grand Island, NY) antibodies were sequentially added followed by rocking for 1 h at room temperature after each addition. After 3 washes with PBS, coverslips were sealed using mounting media containing DAPI (Vectashield, Vector Labs, Burlingame, CA) and visualized on an Eclipse E800 microscope (Nikon, Melville NY) (Yu et al., 2013).

RESULTS

Highly pure human neurons were used for the study (Yu et al., 2013). iCell neurons were obtained in frozen vials, plated onto 6-well cell culture plates freshly coated with a base layer of poly-L-ornithine and a top layer of mouse laminin, and maintained in complete iCell neuron maintenance medium for up to 21 days. To ensure the purity of neurons used, we carry out immunocytochemistry of the cells with neuronal marker anti-βIII tubulin after each new vials of cells were plated. Figure 1 demonstrates the purity of neurons at day 14 in culture by dual immunofluorescence staining anti-βIII-tubulin antibody and anti-GFAP antibody.

After 1–3 days in tissue culture and before VZV infection (day 0), neurons exhibited a homogeneous morphology, and more than 95% cells stained positive with neuronal marker anti-βIII tubulin (Yu et al., 2013).

1) *VZV DNA (ORFs 9 and 63) did not accumulate in infected human neurons compared with human fetal lung fibroblasts (HFL).*

Using quantitative real-time PCR with DNA extracted from VZV-infected cells, we showed that both ORFs 63 and 9 did not accumulate in neurons whereas they did in HFL (see Figure 2). We carried out standard curves for each set of primers/probes used for qPCR. The PCR efficiency was between 92-95%. ORF 9 is a highly expressed VZV gene during acute lytic infection (Kennedy et al 2005), and ORF 63 is an immediate early gene that is the most highly expressed VZV gene during latency (Mitchell et al 2003, Kennedy et al 2015). At 7 DPI it can be seen from the figure that there was a large increase in both VZV ORFs in HFLs but not in neuronal cells. Specifically, for ORF9 there was more than a 7- fold change at 14 DPI in HFL compared with neurons. In the case of ORF 63 there was more than a 4-fold change at 12DPI in HFL compared with that seen in neurons. Further, as has been previously described (Baird et al 2014b) no VZV-induced cytopathic effects (CPE) were observed at any time point.

2. *Time course experiments showed that VZV transcripts were present at lower levels at all days studied post-infection in neurons compared with HFL.*

The results of these experiments are shown in Figure 3 which shows the copy numbers over a 14 DPI period for IE 62 (A), ORF 33(B) and ORF 14 (C). For all three transcripts the copy numbers are lower in neurons compared with HFLs. The transcript increases occurred at 3DPI in HFLs whereas the increases occurred at 7 DPI in the case of infected neurons. In all three cases the copy numbers were less in HFL compared with neurons at the various time points with the final time of comparison being at 12 DPI.

3. *Western blot analysis of infected cultures showed less VZV IE 63 protein in neurons compared with HFL and lower levels of VZV gE in neurons compared with abundant levels in HFL*.

The results of these Western blot analyses are shown in Figure 4. It can be seen that when the signals for VZV IE 63 protein were detected on the blot for both neurons and HFLs, though this protein was detected in both cell types, nevertheless the abundance of this protein was much greater in HFL compared with neuronal cells. When the signals for VZV gE were examined there was a far greater abundance of this protein in HFLs than in neurons in which much lower levels detected. Notice that the total protein loaded for WB was 6 µg for neurons and 1 µg for HFL.

4. *Western blot analysis demonstrated lower levels of Caspase 3 protein in VZV-infected neurons compared with HFL, and higher levels of anti-apoptotic proteins in VZV infected neurons compared to HFL and uninfected cells.*

The results of this Western blot analysis are shown in Figure 5. Table 1 lists the primary antibodies and dilutions used. Infected (+) and uninfected (-) cell lysates were separated by SDS-PAGE, blotted, and detected with respective primary antibodies (top blots) with ß actin as loading control (lower blots). When the band intensity of the targeted proteins was measured & normalized with a ß actin control, the relative levels (infected to uninfected) were shown as bar graphs for each protein. It can be seen that Caspase-3, a marker of apoptosis, was barely detected in VZV-infected neurons but was detected in infected HFL, and this is quantified in the bar graph which shows the relative levels of caspase 3 to IE 63. By contrast, higher levels of the anti-apoptotic proteins Bcl2 (B), Bcl-XL (C) and also the

mitochondrial protein MT-CO2 (D) were detected in infected neurons vs uninfected neurons, compared with HFL.

5. *Immunofluorescence staining demonstrated the presence of both the mitochondrial protein MT-CO2 and VZV IE 63-encoded protein in infected neurons.*

In view of the above Western blot results in which three different anti-apoptotic proteins had been detected in higher levels of VZV-infected neurons (vs uninfected cells), it was important to confirm this by demonstrating the presence of MT-CO2 protein within infected neuronal cells co-expressing a key VZV-encoded protein IE63. Accordingly, dual immunofluorescence was used to demonstrate the co-expression of MT-CO2 and VZV IE 63-encoded protein in infected neurons (14 DPI), and this result is shown in Figure 6.

DISCUSSION

We have shown in this study that VZV-induced apoptosis does occur but is cell-type specific. Highly pure terminally differentiated human neurons derived from induced pluripotent stem cells were resistant to apoptosis, as evidenced by several established criteria, while human fetal lung (HFL) (control) cells were highly susceptible to virally-induced apoptosis. These observations may be biologically significant since following an acute infection VZV establishes a latent infection in human ganglionic neurons, a state that would be unlikely to develop if the initial infection of neurons led to the widespread cell death of these cells via apoptotic pathways. Thus, apoptosis of neurons would favour the host since the productive infection would be limited, whereas an apoptosis- resistant neuronal population would favour the virus and could be a key factor leading to viral latency (Kennedy 2015). VZV-induced apoptosis has also been shown to be cell-type specific as it has been shown, for example, in fibroblasts but not neurons in one previous study (Hood et al. 2003).

Our results are broadly consistent with several previous studies of VZV infection of neuronal cells, the findings of which we have now extended. We noted a lack of accumulation of VZV DNA in neuronal cells (Baird et al 2014b) as opposed to control HFL. This may contribute to

the lack of neuronal susceptibility to VZV infection as well as the absence of an observable cytopathogenic effect. A similar lack of susceptibility of neurons to acute infection has also been documented in the case of HSV-1 infection of human neural cells (Kennedy et al 1983). In the current study, during kinetic analysis of the VZV transcripts, IE 62, ORF 33, and ORF 14 were all detected in VZV-infected neurons, consistent with previous studies, but the copy numbers of these transcripts were lower than in HFL at all time points analysed following virus infection. Although there is a difference in the transcripts tested (ORF 62, ORF33, ORF 14) between the infected neurons and HFL, there is no statistical significance was found. Perhaps one can reach a statistical difference if larger group of VZV transcripts are tested in multiple replicates over more time points. However, it is known that all VZV genes are transcribed and VZV proteins from all kinetic classes are translated in neurons during VZV infection, so defective virus transcription does not account for the lack of cell death in VZV-infected neurons in *vitro* (Baird et al 2014a).

It was also important to study VZV translation following viral infection in neuronal and HFL cultures. Western blot analysis was therefore used to achieve this, and as expected VZV IE 63-encoded protein was detected in neurons (Mahalingam et al 1996), though in lower abundance than was found in HFL, consistent with the more productive acute infection of the latter cells. This protein is of particular importance as ORF 63 is the most consistently transcribed VZV gene in VZV latency (Kennedy et al 2000, Mitchell et al 2003). It was perhaps surprising that the early-late VZV gE glycoprotein (ORF68 translation) was barely detected in infected neurons, though it was easily detected in HFL, since this protein has been previously described as being abundant in VZV-infected neurons (Grose et al 2013). Possible explanations for this discrepancy, which are not mutually exclusive, include the low levels of VZV DNA in infected neurons, the detection limits of the assay and a partial viral post-transcriptional block in neuronal cells.

Western blot analysis also demonstrated low levels of caspase-3, a reliable marker of apoptosis in virally infected neurons, though it was present in the more susceptible HFL. This was key evidence for the inferred resistance of neurons to VZV-induced apoptosis. This absence of apoptotic markers confirms the results of previous studies in such purified VZV-infected neuronal cultures (Pugazhenthi et al 2011, Yu et al 2013). It was also important to show that neurons expressed anti-apoptotic markers to strengthen our underlying interpretation of our data. Indeed, the two known anti-apoptotic proteins studied , Bcl-2, Bcl-

XL as well as MT-CO2 (mitochondrially encoded cytochrome c oxidase II, a key subunit of cytochrome c oxidase, which is the terminal enzyme involved in the oxidative phosphorylation complexes) were found to be upregulated in VZV-infected neurons compared with uninfected cells and HFL in which all of these apoptotic proteins were downregulated. In a previous study of VZV infection of MeWo cells it was also found that the anti-apoptotic protein BCL-2 was downregulated while apoptotic markers such as cleaved caspase-3 were present (Brazeau et al 2010). These results were strengthened by our observation using two-fluorochrome immunofluorescence that mitochondria in infected neurons co-expressed the presumed anti-apoptotic mitochondrial protein MT-CO2 and VZV IE 63-encoded protein demonstrating that the neuronal cells known to be virally infected also had a key marker of resistance to apoptosis.

The ability of another herpes virus, HSV-1, to inhibit neuronal apoptosis is highly relevant to our current findings with VZV. Thus, it was first shown in 2000 that the latency-associated transcript (LAT) gene of HSV-1 promotes neuronal survival after HSV-1 infection by reducing apoptosis in the trigeminal ganglia of latently infected rabbits (Perng et al 2000). Following this seminal observation, further understanding of the actual mechanism of HSV-1 inhibition of apoptosis has been gained, particularly from the laboratories of Jones and Wechsler. For example, it was shown that the HSV 1 LAT inhibited apoptosis and also promoted neurite sprouting in neuroblastoma cells following serum starvation by maintaining protein kinase B (AKT) levels (Li et al 2010). These findings were then followed by the observation that the HSV-1 LAT protects cells against cold-shock-induced apoptosis by maintaining phosphorylation of protein kinase B (AKT) (Carpenter et al 2015). Further, two small RNAs encoded within the first 1.5 kilobases of the HSV-1 latency-associated transcript were shown to inhibit productive viral infection and cooperated to inhibit apoptosis(Shen et al 2009). An elegant study which used an HSV-1 mutant containing an unrelated antiapoptosis gene in place of the LAT also strongly supported the notion that LAT's anti-apoptosis activity is the dominant function that enhances HSV-1's spontaneous reactivation phenotype (Jin et al 2007). Furthermore, the viral anti-apoptotic function is not limited to human HSV-1 since both Bovine Herpes Virus 1 (BHV-1) and HSV-1 promote survival of latently infected sensory neurons, in part by inhibiting apoptosis (Jones 2013).

While the definite significance of neuronal resistance to VZV-induced apoptosis can only be inferred at present, we consider it likely that this observation has much relevance to the

cascade of molecular events that result in VZV latency. Further experiments will clearly be required to resolve this issue unequivocally.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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FIGURE LEGENDS

Fig. 1. Highly pure human neurons at day 14 in culture. Dual immunofluorescence staining with anti-βIII-tubulin antibody and anti-GFAP antibody revealed positive staining for the neuronal marker tubulin (red), but not for GFAP (green). Nuclei stained blue with DAPI.

Fig. 2. **VZV DNA did not accumulate in infected neurons.** Human neurons and human fetal lung fibroblasts were infected with cell-free VZV at time point indicated, and total DNA was extracted and detected by qPCR for ORF 63 (A) and ORF 9 (B). Relative quantities of VZV DNA, normalized to cell DNA were shown to have consistent levels compared DNA detected in HFL.

Fig. 3. Time course of VZV transcripts in infected neurons and HFL.
A. IE62, B, ORF33, C, ORF14.

Fig. 4. **Western Blots detecting IE63 and gE proteins in VZV infected neurons**. Total protein of infected neurons (6 µg) and HFL (1 µg) were loaded for SDS-PAGE; Western blotting was performed, and respective VZV proteins were detected.

Fig. 5. Western blots demonstrating higher levels of anti-apoptotic proteins in VZV infected neurons compared to uninfected cells. VZV infected (+VZV) and uninfected (-VZV) cell lysates were separated and detected with respective primary antibodies (top blot) with ß actin as control (lower blot). Band intensity of the targeted proteins was measured and normalized with ß actin control, and the relative levels (infected to uninfected) were shown as bar graph. A. Caspase-3 (~ 17 kDa) was detected at very low levels in both VZV-infected neurons and uninfected neurons, but in abundance in infected HFL. Bar graph shows relative level of caspase 3 to IE 63 (see Fig.3). Similar results were shown in our previous report (Yu 2013) B-D: Higher levels of anti-apoptotic proteins Bcl-2 (B, ~26 kDa), Bcl-XL (C, ~30 kDa) and MT-CO2 (D, ~22 kDa) are detected in infected neurons compared to uninfected neurons, but in HFL an opposite result is shown that higher levels of anti-apoptotic protein were present in uninfected HFL. The bar graphs next to the western blots show the percentage change between infected and uninfected cells.

Fig. 6. Dual immunofluorescence staining showing mitochondria and VZV IE63 in infected neurons (14 DPI). IE63 (A, red) in cytoplasm, mitochondria protein (MT-CO2, B, green), C: DAPI staining (nucleus), D: merged image.