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Varicella zoster virus infection of highly pure terminally differentiated human neurons

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Abstract In vitro analyses of varicella zoster virus (VZV) reactivation from latency in human ganglia have been hampered by the inability to isolate virus by explantation or cocultivation techniques. Furthermore, attempts to study interaction of VZV with neurons in experimentally infected ganglion cells in vitro have been impaired by the presence of nonneuronal cells, which become productively infected and destroy the cultures. We have developed an in vitro model of VZV infection in which highly pure (>95 %) terminally differentiated human neurons derived from pluripotent stem cells were infected with VZV. At 2 weeks post-infection, infected neurons appeared healthy compared to VZV-infected human fetal lung fibroblasts (HFLs), which developed a cytopathic effect (CPE) within 1 week. Tissue culture medium from VZV-infected neurons did not produce a CPE in uninfected HFLs and did not contain PCR-amplifiable VZV DNA, but cocultivation of infected neurons with uninfected HFLs did produce a CPE. The nonproductively infected neurons contained multiple regions of the VZV genome, as well as transcripts and proteins corresponding to VZV immediate-early, early, and late genes. No markers of the apoptotic caspase cascade were detected in healthy-appearing VZV-infected neurons. VZV infection of highly pure terminally differentiated human neurons provides a unique in vitro system to study the VZV-neuronal relationship and the potential to investigate mechanisms of VZV reactivation.

Keywords Varicella zoster virus · Human neurons · Nonproductive infection

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Introduction

Varicella zoster virus (VZV) is a ubiquitous exclusively human neurotropic alphaherpesvirus. Primary infection usually produces varicella (chickenpox), after which virus becomes latent in ganglionic neurons along the entire neuraxis (Gilden et al. 2003). VZV often reactivates decades later to produce zoster (shingles), characterized by dermatomal distribution pain and rash. Zoster may be further complicated by chronic pain, as well as VZV meningoencephalitis, myelopathy, vasculopathy, and retinitis (Gilden et al. 2011). Prevention of these serious neurological and ocular complications requires a better understanding of the VZV–host relationship in infected neurons.

Unlike herpes simplex virus, which is readily recovered by explantation of latently infected human ganglia in tissue culture or by cocultivation of latently infected human ganglionic cells with indicator cells, VZV cannot be recovered from latently infected human ganglia by these techniques (Plotkin et al. 1977). Thus, attempts have been made to establish a nonlytic infection of human neurons in vitro that can be used to study virus reactivation. Unfortunately, such attempts have been hampered by the presence in these cultures of

nonneuronal cells, which become productively infected and destroy the entire culture. Herein, we report the successful nonproductive VZV infection of highly pure (>95 %) terminally differentiated human neurons (iCell neurons). iCell neurons are derived from induced pluripotent stem cells and are a mixture of post-mitotic neural subtypes, comprised primarily of GABAergic and glutamatergic neurons, with typical physiological characteristics and responses and positive staining with multiple neuronal markers. Weeks after VZV infection, analyses of healthy-appearing neurons for cell-free and cell-associated virus, as well as for the presence of VZV DNA, VZV transcripts, VZV protein, and early and late markers of apoptosis confirmed the nonproductive nature of the infection.

Materials and methods

Coating surfaces for neuronal cultures

Tissue culture plates (six wells) (Corning, Tewksbury, MA) were coated with 1 ml poly-L-ornithine solution (Sigma, St. Louis, MO) for 1 h, washed twice with sterile water followed by addition of 3 ml of 3.3 μg/ml laminin solution (Sigma) for 1 h at 37 °C, and used immediately.

Cells

iCell neurons (Cellular Dynamics International, Madison, WI) are derived from induced pluripotent stem cells and are a mixture of post-mitotic neural subtypes, comprised primarily of GABAergic and glutamatergic neurons, with typical physiological characteristics and responses and positive staining with multiple neuronal markers. iCell neurons obtained in frozen vials were thawed, seeded at 75,000–100,000 cells per cm onto coated surfaces and maintained in fresh iCell complete maintenance medium at 37 °C in 5 % CO_2. Tissue culture medium (75 % of the total volume) was changed three times per week. 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

To minimize any possibility of overwhelming infection, neurons ($\sim 1 \times 10^6$) were infected with 1,000 pfu attenuated virus (Zostavax, Merck, Whitehouse Station, NJ) in iCell complete maintenance medium. HFLs were similarly infected with 1,000 pfu VZV and maintained in DMEM supplemented with 2 % FBS.

Attempts to rescue virus from VZV-infected neurons

Two weeks after infection, tissue culture medium from infected neurons was added to HFLs and cells were monitored for CPE; in addition, 5 μl of 2.5 ml tissue culture medium was used in real-time PCR with VZV open reading frame (ORF) 9- and 63-specific primers. CPE was also monitored in HFLs cocultivated with infected neurons released by trypsin treatment and suspended in DMEM.

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 μl 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) (Table 1) on an Applied Biosystems Fast 7500 Real-Time PCR System and analyzed using Fast 7500 software. 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 a cellular 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 2,000 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 two portions with and without RT to control for any residual DNA. cDNA was stored at −80 °C. Both RT-positive and RT-negative samples were amplified along with wild-type VZV DNA (10^6 to 10^0 genome copies) to compare transcription levels to residual DNA levels. VZV primers (Table 1) and Taq Universal

Table 1 Primers/probes for real-time PCR

Name	Forward primer	Reverse primer	Probe (FAM)	Size (bp)
ORF 4	GTTGTCCGTGGCTCTAAATTTG	ACATTCAACACGACCACACTC	CCTCTAAAACACCGGCCAGACTGA	144
ORF 9	GGGAGCAGGCGCAATTG	TTTGGTGCAGTGCTGAAGGA	CAATTGCCAGCGGGAGACC	54
ORF 11	GAGCAAGACAGGATAGTTCAGG	AAGATTTTCGGCTAGGTCCAC	TCCGTTTTGTGGGATTGGAGACAT	117
ORF 28	CGAACACGTTCCCCATCAA	CCCGGCTTTGTTAGTTTTGG	TCCAGGTTTTAGTTGATACCA	62
ORF 29	GGCGGAACTTTCGTAACCAA	CCCCATTAAACAGGTCAACAAAA	TCCAACCTGTTTTGCGGCGGC	66
ORF 62	CCTTGGAAACCACATGATCGT	AGCAGAAGCCTCCTCGACAA	TGCAACCCGGGCGTCCG	79
ORF 63	GCTTACGCGCTACTTTAATGGAA	GCCTCAATGAACCCGTCTTC	TGTCCCATCGACCCCCTCGG	67
ORF 66	CCACGTTACCGAACAGATTTATACTG	GATCGCTGAATTGCTAAAATGTCA	CTAGCTGCAAAGCGCAACCTCCCC	83
ORF 68	GTACATTTGGAACATGCGCG	TCCACATATGAAACTCAGCCC	AAAACAAGAAACCCTACGCCCGC	140

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.

Immunocytochemistry

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 and secondary Alexa Fluor-conjugated (Invitrogen, Grand Island, NY) antibodies were sequentially added at dilutions listed in Table 2, followed by rocking for 1 h at room temperature after each addition. After three 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).

Results

Human neurons used for infection with VZV are highly pure

iCell neurons were obtained in frozen vials, plated onto six-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. After 1–3 days in tissue culture and before VZV infection (day0), neurons exhibited a homogeneous morphology (Fig. 1a), and more than 95 % cells stained positive with neuronal marker βIII tubulin (Fig. 1d).

VZV-infected neurons did not develop CPE

Neurons and HFLs were infected in parallel. A CPE developed in infected HFLs at 5–7 days post-infection, while

Table 2 Antibodies used for immunocytochemistry

Animal	Target protein	Dilution	Supplier
Mouse	VZV IE62	1:500	Novus Biologicals, Littleton, CO
Rabbit	VZV IE63	1:1,000	Mahalingam et al. 1996
Rabbit	VZV ORF 29	1:500	Cohrs et al. 2002
Goat	VZV thymidine kinase	1:200	Santa Cruz Biotech, Santa Cruz, CA
Mouse	VZV glycoprotein E	1:500	Santa Cruz Biotech
Mouse	VZV glycoprotein H	1:10	Stipan Jonjić, University of Rijeka, Croatia and Jürgen Haas, University of Edinburgh, UK
Rabbit	Human βIII tubulin	1:1,000	Cell Signaling Technology, Danvers, MA
Mouse	Human glial fibrillary acidic protein (GFAP)	1:300	Cell Signaling Technology
Rabbit	Human cleaved caspase-3	1:400	Cell Signaling Technology
Rabbit	Human cleaved caspase-9	1:400	Cell Signaling Technology

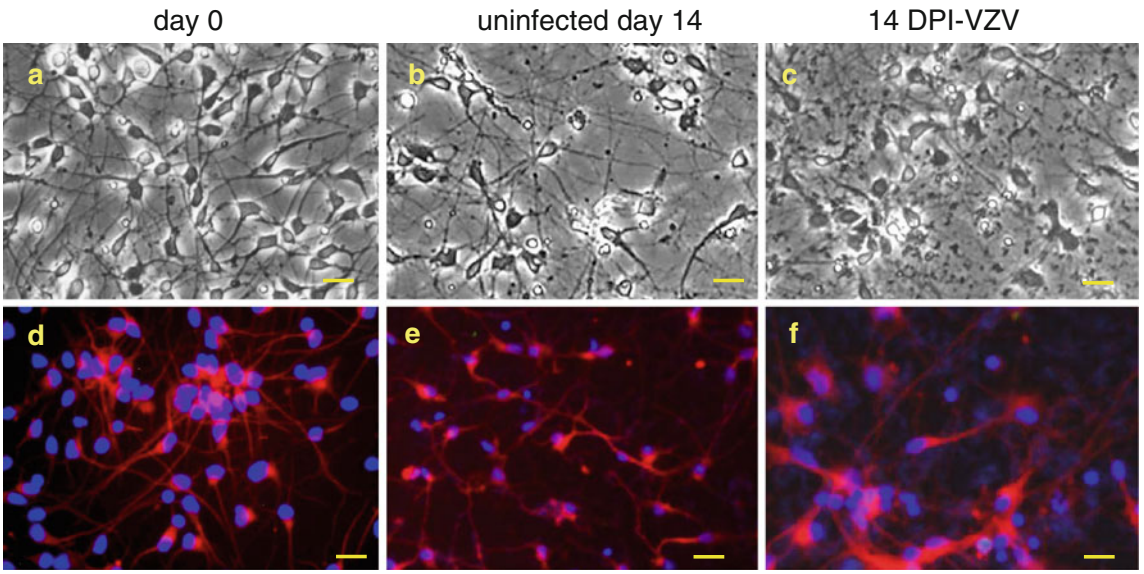

Fig. 1 VZV infection of highly pure human neurons did not produce a cytopathic effect. Terminally differentiated neurons were maintained in tissue culture for up to 21 days. Phase-contrast microscopy showed healthy-appearing neurons on day 0 (**a**) and day 14 in culture (**b**) as well as 14 days after VZV infection (**c**). Dual immunofluorescence staining (**d**–**f**) 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

VZV-infected neurons appeared healthy as did uninfected neurons 2 weeks later (Fig. 1b, c). Two weeks after infection, more than 95 % cells still stained positive with anti-βIII tubulin antibody (Fig. 1e, f).

Examination of VZV-infected neurons for cell-free and cell-associated virus

HFLs incubated with the tissue culture medium from VZV-infected neurons did not develop a CPE. Moreover, no VZV DNA was amplified from the tissue culture medium, indicating the absence of cell-free virus. In contrast, a CPE did develop in HFLs cocultivated with trypsinized neurons that had been infected 2 weeks earlier.

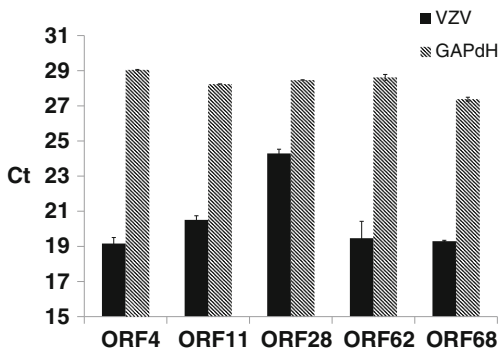

Fig. 2 VZV DNA was present in nonproductively infected human neurons. Two weeks after VZV infection, DNA was extracted from neurons and quantitated by real-time PCR. Multiple regions of the VZV genome were detected (*black* and *gray bars* are VZV and GAPdH DNA, respectively)

VZV DNA and transcripts were present in nonproductively infected human neurons

Two weeks after infection, multiple regions of the VZV genome were detected in nonproductively infected neurons (Fig. 2). A search for the presence of VZV transcripts corresponding to immediate-early (IE), early (E), and late virus genes revealed transcripts corresponding to VZV ORFs 62, 63, 28, 33, 68, and 31 in infected neurons; each VZV transcript in neurons was less abundant than in productively infected fibroblasts at the time of CPE (Fig. 3).

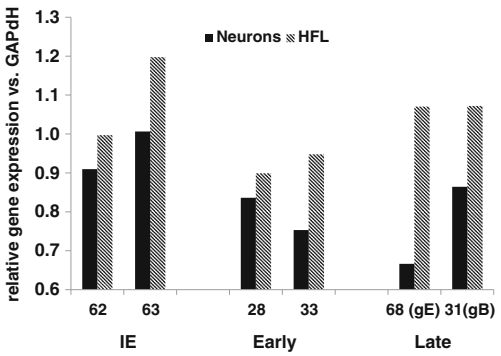

Fig. 3 Multiple VZV transcripts were present in nonproductively infected neurons. Two weeks after infection of neurons, total RNA was extracted, treated with DNase, and cDNA was synthesized. Transcripts corresponding to VZV immediate-early, early, and late genes were quantified by qPCR as compared to RNA obtained from VZV-infected fibroblasts at the height of a cytopathic effect (*black* and *gray bars* are neurons and HFL, respectively)

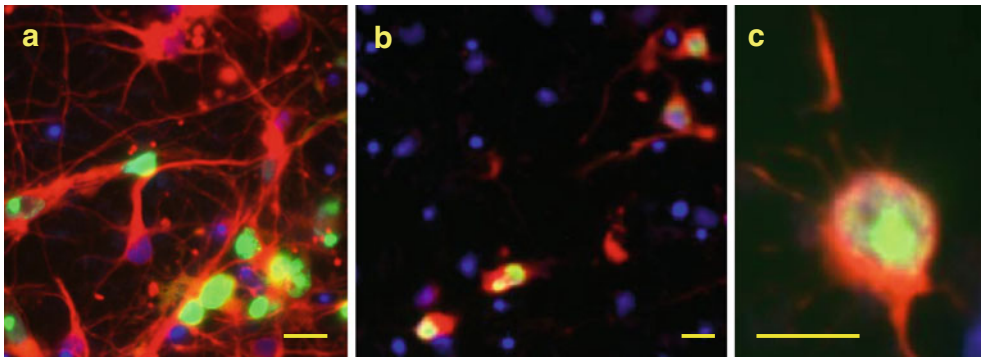

Fig. 4 VZV immediate-early proteins were present in nonproductively infected human neurons. Two weeks after VZV infection, neurons attached to laminin-coated coverslips were fixed and immunostained with anti-tubulin antibody and anti-VZV IE 62 antibody. Highly pure, βIII-tubulin-positive human neurons (*red*, **a**) contain VZV IE62 in the nucleus (*green*). **b**, **c** Dual-immunostained neurons: VZV IE62 protein was seen in the nucleus (*green*) and VZV IE 63 in the cytoplasm (*red*). *Scale bar* 20 μm

Multiple VZV proteins were detected in nonproductively infected neurons

Two weeks after VZV infection of neurons, two VZV IE proteins (Fig. 4), two VZV E proteins (Fig. 5), and two late VZV proteins (Fig. 6) were detected in neurons that stained positive for βIII tubulin (Fig. 4a, red). VZV IE 62 was present in the nucleus of neurons (Fig. 4a, green). VZV IE63 was present in both the nucleus and cytoplasm of infected neurons (Fig. 4b, c, reddish orange). Dual staining with DAPI (blue) revealed VZV early ORF 29 protein in the nucleus of neurons (Fig. 5a, green), while dual staining with DAPI (blue) revealed

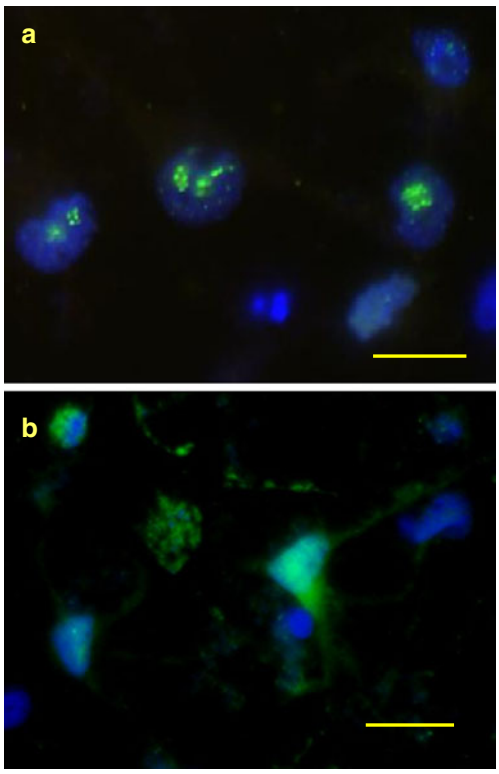

Fig. 5 VZV early proteins were present in nonproductively infected human neurons. Two weeks after VZV infection, neurons attached to laminin-coated coverslips were fixed and immunostained with anti-VZV ORF 29 antibody and anti-VZV thymidine kinase (TK) antibody. **a** Dual immunostaining of DAPI and early VZV ORF 29 protein in the nucleus (*green*). **b** Dual immunostaining of DAPI and anti-VZV TK antibody. VZV TK was seen in the cytoplasm of infected neurons. *Scale bars* 20 μm

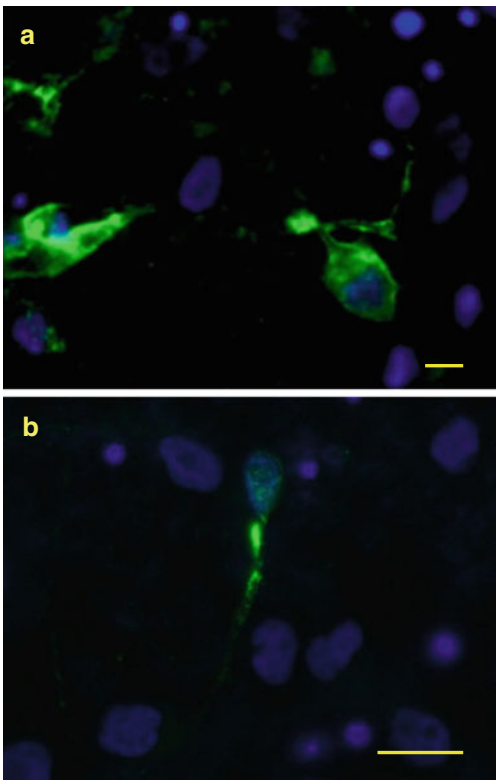

Fig. 6 VZV late proteins gE and gH were detected in nonproductively infected human neurons. Two weeks after VZV infection, neurons attached to laminin-coated coverslips were fixed and immunostained with anti-VZV gE (**a**) or anti-VZV gH (**b**). Dual immunostaining revealed late VZV gE in the cytoplasm (*green*, **a**) and VZV gH in the cytoplasmic membrane (*green*, **b**). *Scale bars* 20 μm

VZV early ORF 36 protein (TK) in the cytoplasm of infected neurons (Fig. 5b, green). Dual staining with VZV late ORF 68 protein (gE) was seen in the cytoplasm of infected neurons (Fig. 6a, green) and VZV ORF 37 protein (gH) in the cytoplasmic membrane of infected neurons (Fig. 6b, green).

VZV did not induce apoptosis in neurons

Immunostaining analysis of VZV-infected neurons and fibroblasts for the active form of caspase-3 and caspase-9 revealed both caspases in VZV-infected fibroblasts, but neither caspase in healthy-appearing neurons at 2 weeks after VZV infection (Fig. 7).

Discussion

Herein, we report nonproductive VZV infection of highly pure terminally differentiated human neurons, with purity verified by positive βIII tubulin and negative glial fibrillary acidic protein (GFAP) staining at the time of VZV infection as well as 2 and 3 weeks later. Two weeks after VZV infection, neurons appeared healthy and no CPE developed compared to HFLs infected at the same dose. Tissue culture medium removed from neurons 2 weeks after infection did not produce a CPE in HFLs, and the absence of cell-free VZV was confirmed by real-time PCR which did not amplify DNA with VZV-specific primers. In contrast, a CPE developed in HFLs after cocultivation with VZV-infected neurons. Rescue of VZV from these neurons is analogous to the isolation of measles virus in tissue culture by cocultivaton of brain cells from a patient with subacute sclerosing panencephalitis with Vero cells (Payne et al. 1969) and isolation of JC virus by cocultivation of brain cells from a patient with progressive multifocal leukoencephalopathy with human fetal brain cells (Padgett et al. 1971). Overall, the detection of VZV DNA corresponding to multiple regions of the VZV genome, together with the presence of transcripts and proteins corresponding to immediate-early, early, and late VZV genes in VZV-infected neurons that never showed CPE confirms the nonproductive nature of the infection. The absence of both early and late apoptotic markers indicated that the apoptotic cascade was not operative in neurons nonproductively infected with VZV.

Numerous attempts have been made to establish a model of VZV infection both in vitro and in vivo. For example, infection of partially purified human fetal sensory neurons with cell-associated VZV showed more CPE in nonneuronal cells than in neurons (Wigdahl et al. 1986). Analysis of explanted human fetal dorsal root ganglia cocultivated with human fetal fibroblasts revealed that neurons were resistant to apoptosis (Hood et al. 2003). In a study using human neural stem cells from fetal brain transplanted into nonobese diabetic SCID mouse brain and allowed to differentiate in vivo, VZV was found in both neurons and glial cells after infection (Baiker et al. 2004). The same group demonstrated productive VZV infection of human fetal dorsal root ganglionic explants containing neurons and nonneuronal cells; VZV infection peaked at day 4, but dropped dramatically by day 5 (Gowrishankar et al. 2007). In a study using human dorsal root ganglia engrafted under the kidney capsule of SCID mice and infected with VZV, electron microscopy revealed VZ virions in neuronal cell nuclei and cytoplasm, but not in satellite cells, and infectious virus was recovered 14 days after infection; however, 4–8 weeks later, no infectious virus was released from cells, no virion assembly was detected, and the number of VZV genome copies was

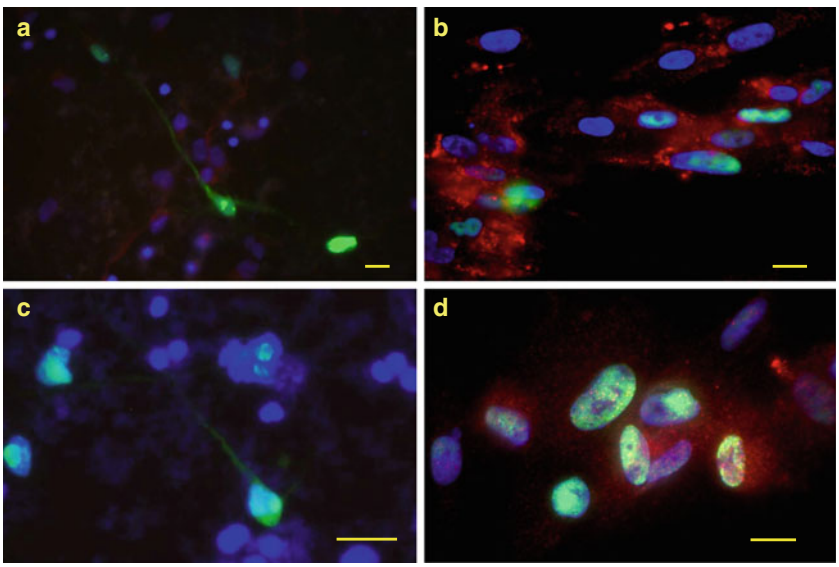

Fig. 7 Caspase-3 and caspase-9 proteins were not detected in VZV-infected neurons. Two weeks after VZV infection, neurons attached to laminin-coated coverslips were fixed and dual immunostained with anti-caspase-3 (*red*) and anti-VZV 62 (*green*) or with anti-caspase-9 (*red*) and anti-VZV 62 (*green*) antibodies. **a**, **c** VZV 62 protein (*green*) but not caspase-3 (*red*) or caspase-9 (*red*) in nonproductively infected neurons. **b**, **d** Extensive caspase-3 (*red*) and caspase-9 (*red*) and VZV IE 62 (*green*) staining in VZV-infected fibroblasts

markedly reduced (Zerboni et al. 2005). Infection of neurons derived from human embryonic stem cells with cell-associated VZV-expressing green fluorescent protein yielded productive infection although the percent of neurons in the heterogeneous culture was not provided (Markus et al. 2011), and VZV infection of differentiated neuroblastoma cells also induced productive infection (Christensen et al. 2011). In addition, VZV was shown to infect human embryonic stem cell-derived neurons and neurospheres, but not pluripotent embryonic stem cells or early progenitors (Dukhovny et al. 2012). Overall, definitive conclusions cannot be drawn since none of the studies above was performed in sufficiently pure neuronal cultures. Meanwhile, our findings herein verify our earlier demonstration of nonproductive infection of differentiated neurons without evidence of apoptosis (Pugazhenthi et al. 2011). However, because generation of cultures containing over 90 % neurons was inconsistent, we began infecting iCell neurons with VZV to show that infection of highly pure cultures of differentiated neurons with VZV produces a nonproductive infection in the absence of apoptosis. This model will allow molecular analysis of virus–neuronal interaction and studies of the mechanisms of VZV reactivation.

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