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Resveratrol inhibition of varicella-zoster virus replication in vitro

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Abstract

Resveratrol was found to inhibit varicella-zoster virus (VZV) replication in a dose-dependent and reversible manner. This decrease in virus production in the presence of resveratrol was not caused by direct inactivation of VZV or inhibition of virus attachment to MRC-5 cells. The drug effectively limited VZV replication if added during the first 30 h of infection. Western blot analysis and real-time RT-PCR studies demonstrated that protein and mRNA levels of IE62, an essential immediate early viral protein, were reduced when compared to controls. These results demonstrate that VZV replication is adversely affected by resveratrol which is negatively impacting IE62 synthesis.

Keywords: Resveratrol; Varicella-zoster virus; Cell infection

1. Introduction

Varicella-zoster virus (VZV) is a member of the Herpesviridae family that is spread among humans through respiratory droplets and direct contact. Primary infection with VZV results in varicella (chickenpox) which begins as a viremia and is characterized by fever and vesicular rash. After the viremia has cleared, the virus enters a state of latency in the dorsal root, cranial and autonomic ganglia (Gilden et al., 2001). Viral reactivation of latent VZV is brought about by immunosuppression which can be caused by numerous factors including stress. The reoccurring virus infection results in herpes zoster (shingles) that forms chickenpox-like lesions that cause acute pain in the area innervated by the latently infected ganglia. This exanthematic eruption is characteristically unilateral and sharply limited in a band or patch-like distribution to the dermatome supplied by a specific dorsal root or extramedullary cranial nerve ganglion. Within the segmental area of localization, lesions may be scattered and few or so numerous as to form an almost confluent large plaque. Prior to the appearance of the lesions there may be pain, itching, and paresthesia in the involved segment. The lesions appear as crops and evolve and resolve as in varicella, but at a slower pace. A complication of herpes zoster is postherpetic neuralgia (PHN) which is persistent pain at the affected site that occurs after the lesions have healed.

Treatment of varicella-zoster virus disease is dependent on drugs such as acyclovir, valaciclovir, famciclovir and foscarnet. These are often coupled with corticosteroids for inflammation and analgesics for pain (Stankus et al., 2000). Treatment is started as early as possible, and a good outcome is considered to be a more rapid healing of lesions and pain reduction. In the 1970s, a live-attenuated VZV vaccine was developed and used in routine infant immunization in the United States beginning in 1995 (Krause and Klinman, 1995; Takahashi et al., 1975). Some older adults have received the vaccine in order to help reduce zoster pain. However, concern still exists for millions of people who did not receive the vaccine that are carrying latent VZV and are susceptible to shingles.

Since a large number of people are susceptible to the morbidity of zoster, and because of the lack of an effective topical treatment for this malady, we examined the effects of resveratrol on VZV replication. Resveratrol is an active compound of stilbene phytoalexins and is known to have several biological properties. This drug has been shown to have cardioprotective effects by reducing platelet aggregrates which are a predisposition to atherosclerosis (Fukao et al., 2004). Resveratrol also has chemoprotective effects be increasing cellular genes involved with tumor suppression (Baek et al., 2002) or inhibiting angiogenesis (Cao et al., 2005). This drug is also anti-inflammatory by inhibiting cell-signaling pathways, such as cyclooxygenase

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(Jang et al., 1997). Resveratrol was tested againt VZV because it has been shown previously to inhibit herpes simplex virus (HSV) (Docherty et al., 1999); and human cytomegalovirus (HCMV) (Evers et al., 2004); in vitro and HSV in vivo when topically applied (Docherty et al., 2004, 2005). Our results presented here demonstrate that resveratrol inhibits VZV replication in vitro and acts on an immediately early essential regulatory protein of VZV.

2. Materials and methods

2.1. Cells and virus

Human diploid lung cells (MRC-5) were obtained from the American Type Culture Collection (ATCC), Rockville, MD. The cells were grown and maintained in Eagle's basal medium supplemented with 10% fetal bovine serum, 0.075% $NaHCO_3$ and 50 μg/ml gentamycin sulfate. MRC-5 cells were used to produce virus pools, in plaque assays and all studies.

VZV (Ellen strain) was obtained from ATCC and used in all studies. Cell-associated virus pools were produced by passing the virus for 48 h in MRC-5 cells then trypsinizing the cells and resuspending the cells in media containing 10% DMSO before freezing at −80 °C. The average titer of the cell-associated virus pools were 1.0×10^5 pfu/ml. Cell-free virus pools were produced using the method of Schmidt and Lennette (1976). Briefly MRC-5 cells were infected with VZV. After 24 h, the cells were trypsinized, growth media containing 10% sorbital added, and the cells dispersed using glass beads. This cell suspension was sonicated, centrifuged and the supernantant frozen at −80 °C in 10% DMSO. The final titer of the cell-free virus pool used in this study was 3.2×10^3 pfu/ml.

2.2. Chemicals

Resveratrol (3,5,4′-trihydroxystilbene) was obtained from Royalmount Pharma Inc., Montreal, Que., Canada. Stock concentrations of resveratrol were prepared in DMSO and diluted to final concentrations in tissue culture media. The final concentration of DMSO was 0.2%, which did not interfere with viral replication and the highest concentration of resveratrol used in these studies was 219 μM. Toxicity studies utilizing trypan blue established that 219 μM resveratrol and 0.2% DMSO were not toxic to MRC-5 cells. MTT assays (Takeuchi et al., 1991) were also performed to determine cell viability at 24, 48 and 72 h. The CD_{50} of resveratrol based on the MTT assay on MRC-5 cells was >600 μM after 24 and 48 h of exposure to resveratrol and 384 μM after 72 h exposure to resveratrol. Acyclovir was obtained from Sigma–Aldrich Corporation and diluted to final concentrations in tissue culture media.

2.3. Viral replication studies

Cells in culture were infected with cell-associated VZV at a multiplicity of infection (moi) of 0.1. Each flask contained 1×10^6 cells and 1×10^5 pfu/ml of cell-associated virus was used to infect the cells. After 1 h of adsorption, media was added with or without resveratrol at the stated concentrations. At that time, 1 h samples were placed in the freezer at −80 °C in the flask containing the media. Samples were then taken every 24 h for 72 h by placing them at −80 °C. At the end of that period, samples containing cell-associated virus were thawed and titrated in duplicate by plaque assay on MRC-5 cells. After 7 days, the cells were stained with 0.5% crystal violet in 70% ethanol and viral plaques counted. These studies were repeated with acyclovir.

2.4. Attachment study

Cells in six well tissue culture plates were incubated in media with or without resveratrol for 1 h. The media was removed and fresh media with or without resveratrol was added along with cell-free VZV for 1 h. The cells were then washed with media twice and incubated with only media for 7 days. The cells were then stained with 0.5% crystal violet in 70% ethanol and viral plaques counted.

2.5. Direct inactivation

Cell-free VZV was mixed with media alone, media with 0.2% DMSO or media with resveratrol (219 μM in 0.2% DMSO) and incubated in a 37 °C waterbath. At 1, 30 or 60 min, representative samples were taken from each tube and titrated by plaque assay on MRC-5 cells as previously described.

2.6. Time of inhibition study

Confluent MRC-5 cells were infected with cell-associated VZV at a moi of 0.1. After 1, 3, 6, 9, 12, 24, 30 and 36 h of infection, the cells were washed with media and then media containing 219 μM resveratrol or 0.2% DMSO was added to the appropriate flasks. Each flask was incubated for a total of 48 h. The flasks containing cell-associated virus and media were frozen at −80 °C, thawed and titrated on confluent MRC-5 cells.

2.7. Reversal study

MRC-5 cells were infected with cell-associated VZV at a moi of 0.1 for 1 h. Media containing 219 μM resveratrol or 0.2% DMSO were added to the appropriate flasks. Each flask was incubated for up to 96 h. At 24, 48 or 72 h, the drug was removed by washing the cells twice and placing only media on the cells. After 24, 48, 72 or 96 h, the samples were frozen at −80 °C, thawed and titrated on MRC-5 cells.

2.7.1. Immediate early viral protein, IE62 synthesis

IE62 production was done as previously described by Kinchington et al. (1992). Briefly, MRC-5 cells were infected with cell-associated VZV and cultured for 24 h. The infected cells were then trypsinized and one-tenth of the total infected cell volume was placed on fresh MRC-5 cells and allowed to adsorb for 1 h. Additional media was added with or without resveratrol. After 24, 48 or 72 h of incubation, the cells were scraped from the flask, collected by centrifugation and resuspended in

cold tris buffered saline (TBS). The cells were pelleted by centrifugation, TBS removed, and the cell pellet frozen at −80 °C. The pellets were thawed, resuspended in cold RIPA buffer (Gilman et al., 1980) containing DNase (50 μg/ml) and incubated on ice for 30 min prior to clarification by centrifugation at 14,000 × *g*.

Proteins from the infected cell extract were separated in 5% SDS-PAGE for IE62 or 10% SDS-PAGE for β-actin and transferred to nitrocellulose. The IE62 protein was immunochemically detected by reacting the blotted proteins with goat polyclonal antibody to IE62 (Santa Cruz), then horseradish peroxidase conjugated donkey anti-goat antibody, and finally with ECL (GE Healthcare).

2.8. Real-time RT-PCR

MRC-5 cells were infected and treated with resveratrol containing media as described for the IE62 protein detection. At 24 and 48 h post-infection, RNA was isolated and purified by the Trizol method, Invitrogen (Carlsbad, California). One microgram of total RNA was subjected to DNAase1 digestion (Sigma AMP-D1). cDNA was synthesized by first annealing the RNA with random hexamer primers, Promega (Madison, WI), in the presence of 0.5 mM dNTP. Next, the mixture was incubated with 200 units of MMLV-reverse transcriptase (Sigma M1302) and 20 units of RNAse inhibitor (Sigma) for 1 h at 37 °C. The MMLV-reverse transcriptase was heat denatured at 90 °C for 10 min and the samples stored at −20 °C until use. Fifty nanograms of total cDNA was added to SYBR Green Ready Start TaqPolymerase mixture (Sigma) with 100 nM each of forward and reverse primers for IE62 (+) 5′-TTGGGGTGAGCATCGTGTCGGTGG-3′ and (−) 5′-GACGAGGACGAGGACAACAGC-3′ (Cohrs et al., 1996) or 18 s RNA (+) 5′-AGTCCCTGCCCTTTGTACACA-3′ and (−) 5′-GATCCGAGGGCCTCACTAAAC-3′ containing 3.5 and 1.5 mM $MgCl_2$, respectively. Samples were analyzed on an ABI 7700 sequencer (Applied Biosystems) with parameters of 94 °C, 2 min polymerase activation phase, followed by 35–40 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C (IE62) or 58 °C (18 s) for 30 s and elongation at 72 °C for 1 min. Primer efficiencies were validated using 10-fold serial dilutions of template and the $2^{-\Delta\Delta C_t}$ method (Lavik and Schmittgen, 2001) was employed to report the mean fold change in IE62 transcripts.

2.9. Stastical analysis

The data presented are the means + S.D. The significance of the data was calculated according to *t*-tests using Sigma Plot software (Systat Software, Point Richmond, CA). A *p*-value of <0.05 was considered significant.

3. Results

3.1. Resveratrol inhibits VZV replication

MRC-5 cells infected with cell-associated VZV were incubated in media containing various concentrations of resveratrol or 0.2% DMSO. The results in Fig. 1 demonstrate that over a period of 72 h resveratrol inhibited VZV replication in a dose dependent fashion. Resveratrol at 219 μM completely inhibited VZV replication at all time points tested (lower limit of plaque assay is 10 pfu/ml). Resveratrol at concentrations of 55 and 110 μM also inhibited VZV, but at a rate of 86 and 93%, respectively, at the 48 h time point which was the point of maximum virus production in controls. Based on these studies resveratrol was used at its most effective concentration of 219 μM. This was well below the CD_{50} of resveratrol for MRC-5 cells which was >600 μM at 48 h as determined by the MTT assay. For comparative purposes, these studies were repeated with acyclovir. At 48 h, the results gave an EC_{50} value for acyclovir of 4 μM compared to an EC_{50} value for resveratrol of 19 μM (data not shown).

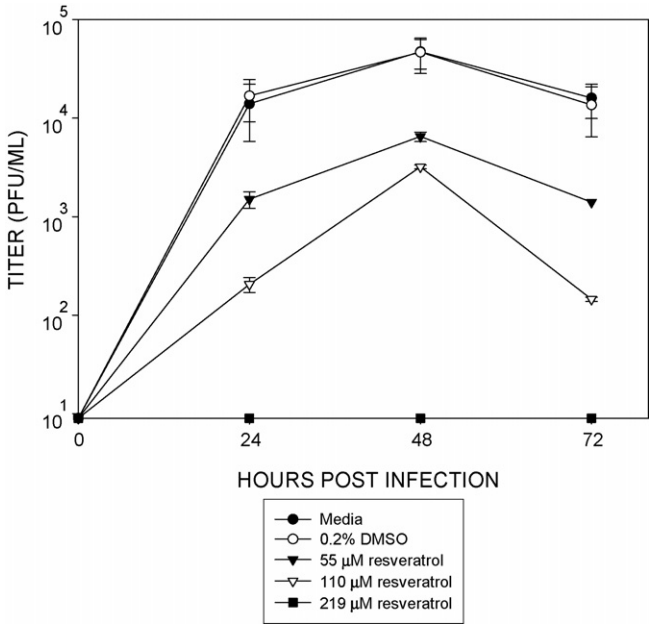

Fig. 1. VZV replication in the presence or absence of resveratrol. MRC-5 cells were infected with cell-associated VZV and incubated in the presence or absence of various concentrations of resveratrol. At 24 h intervals, samples were taken and titrated in duplicate by plaque assay.

3.2. Resveratrol does not block VZV attachment

To determine if attachment of VZV to MRC-5 cells was affected by resveratrol resulting in blockage of virus replication, MRC-5 cells were incubated with media containing 219 μM resveratrol or 0.2% DMSO prior to and during infection with cell-free VZV. After 1 h for attachment, resveratrol or 0.2% DMSO containing media was removed and replaced with media only. The infected cells were incubated for 7 days prior to counting the plaques. The results in Fig. 2 demonstrate that pretreatment and concurrent treatment with resveratrol does not affect attachment of the virus to the cells since all groups had a statistically indistinguishable number of plaques.

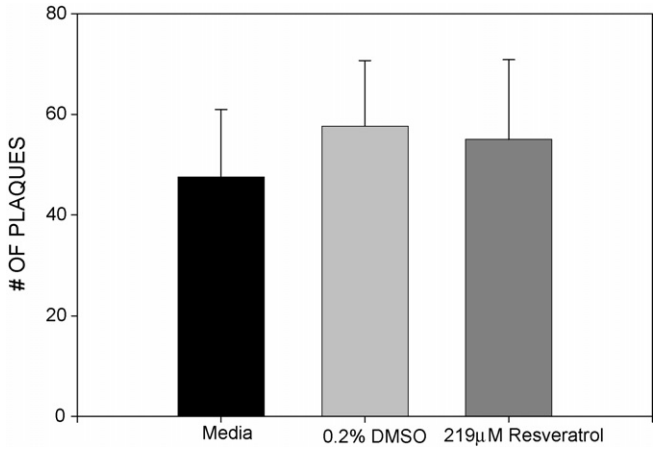

Fig. 2. The effect of resveratrol on virus attachment. MRC-5 cells were incubated in triplicate with control media or media containing 0.2% DMSO or 219 μM resveratrol for 1 h. The drug was removed and the cells immediately exposed to cell-free VZV for 1 h with fresh media or media containing 0.2% DMSO or 219 μM resveratrol. The cells were washed with media and incubated with only media for 7 days then stained and counted for viral plaques.

3.3. Resveratrol does not significantly inactivate VZV

To determine if resveratrol was capable of directly inactivating VZV resulting in reduced virus yields, cell-free VZV was mixed with media alone, media containing 0.2% DMSO or 219 μM resveratrol and incubated in a 37 °C waterbath. Samples were taken at various times for 60 min and assayed on MRC-5 cells. The results in Fig. 3 revealed a reproducible trend of resveratrol to directly inactivate VZV with time. However, statistically there was no significant inactivation of VZV by resveratrol at any of these time points ($p > 0.05$) when compared to controls.

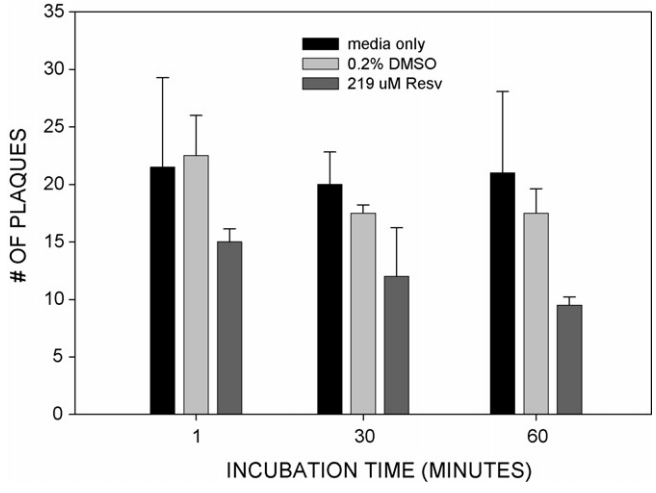

Fig. 3. Lack of direct inactivating effects of resveratrol on VZV. To determine if resveratrol is capable of directly inactivating VZV, cell-free virus was incubated with control media or media containing 0.2% DMSO or 219 μM resveratrol for 1, 30 and 60 min at 37 °C. At the indicated intervals, an aliquot was removed and the virus titrated in duplicate by plaque assay. Results are expressed as number of plaques.

3.4. Time of exposure necessary for resveratrol inhibition of VZV replication

To approximate the time period in the VZV replication scheme that resveratrol was effective, the drug was added at various times after MRC-5 cells were infected. The data in Fig. 4 reveal that resveratrol was effective when added up to 30 h after infection with cell-associated VZV reducing virus yields by >99.9%. There was no difference in the level of inhibition if the drug was added 1 or 30 h after infection. However, this effect was lost when the drug was added 36 h after infection.

3.5. Inhibitory effects of resveratrol on VZV replication are reversible

The inhibition of VZV replication by resveratrol was examined to determine if the effect was reversible. VZV-infected cells were exposed to 219 μM resveratrol for a period of 72 h. At 24 h intervals, a set of infected cells had resveratrol containing media replaced with non-resveratrol containing media. From the data presented in Fig. 5, it can be seen that in the presence of resveratrol, VZV replication was severely inhibited. However, when the resveratrol was removed at 24 h, the virus immediately proceeded to replicate. If resveratrol was present for 48 h, replication of VZV was delayed an additional 24 h after the drug was removed before it began to replicate.

3.6. Resveratrol limits IE62 production

In an attempt to define the point at which resveratrol inhibits VZV infection, we examined IE62, an essential immediate early gene transactivating protein. Western immunoblots were used to detect IE62 in cell extracts from VZV infected cells that were incubated in the presence or absence of resveratrol. The results in Fig. 6 demonstrate that limited amounts of IE62 were detectable

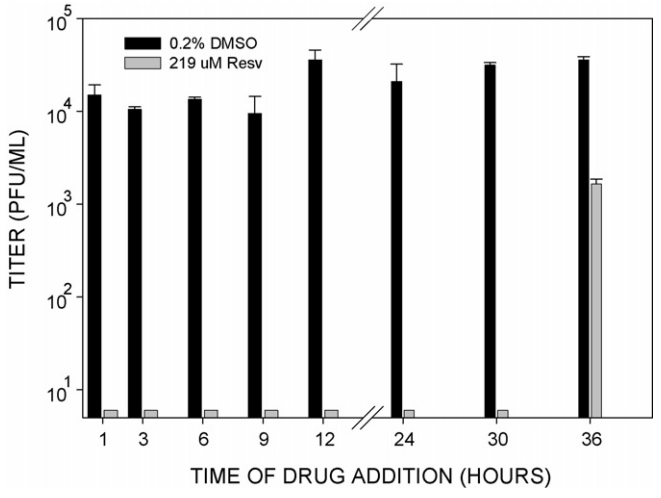

Fig. 4. Time of addition of resveratrol necessary to inhibit VZV replication. MRC-5 cells were infected with cell-associated VZV and incubated with media containing 0.2% DMSO or media containing 219 μM resveratrol beginning at 1, 3, 6, 9, 12, 24, 30 and 36 h after infection. All samples were frozen at 48 h and titrated in duplicate by plaque assay.

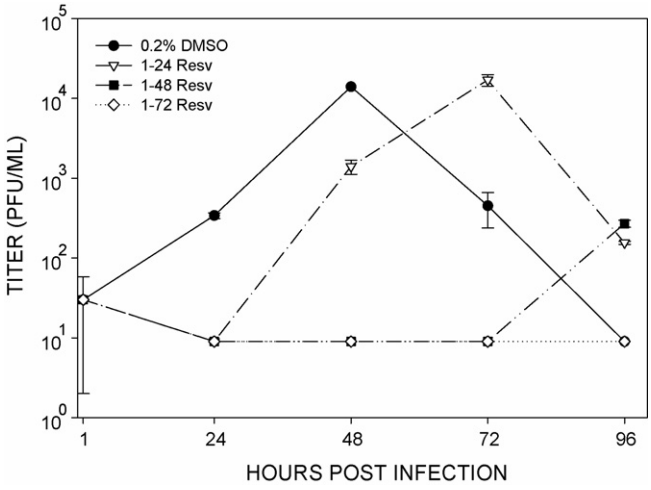

Fig. 5. Inhibitory effects of resveratrol on VZV replication are reversible. MRC-5 cells were infected with VZV and incubated in control media containing 0.2% DMSO or media containing 219 μM resveratrol. At 24, 48 and 72 h, resveratrol containing media was replaced with non-resveratrol containing media. Samples were taken every 24 h and titered by plaque assay in duplicate.

at 24 and 72 h after infection. Maximum amounts of IE62 were reproducibly detected at 48 h after infection. But if resveratrol was present, the amount of IE62 was severely diminished at 48 h, the point of maximum IE62 production.

To complement the above studies, the transcript levels of IE62 in the presence and absence of resveratrol at 24 and 48 h after infection were examined by real-time RT-PCR. A 7.2-fold reduction in ie62 mRNA was observed in the presence of 219 μM resveratrol compared to untreated control at 24 h after infection. At 48 h post-infection, resveratrol treatment resulted in a 2.1-mean fold decrease of ie62 mRNA (Fig. 7). These data support the apparent lack of IE62 protein in resveratrol treated VZV-infected cells due to the inhibition of ie62 gene activation.

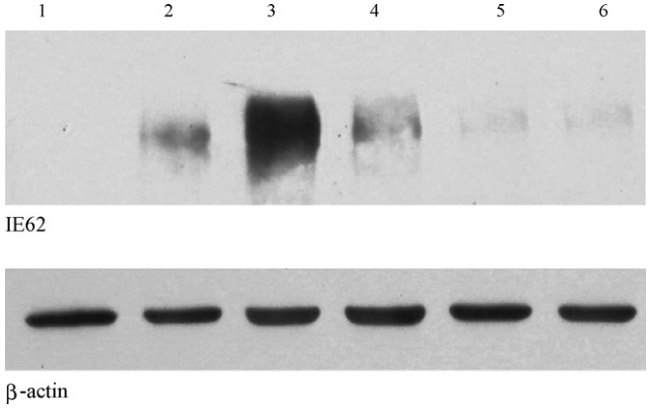

Fig. 6. The effect of resveratrol on IE62 formation. Cell-associated VZV infected MRC-5 cells were incubated in the presence or absence of resveratrol for 24, 48 or 72 h. The cells were solubilized, proteins separated on SDS-PAGE, transferred to nitrocellulose and reacted with polyclonal antibodies to IE62. Lane 1: mock infection; lane 2: 24 h VZV infection; lane 3: 48 h VZV infection; lane 4: 72 h VZV infection; lane 5: 48 h VZV infection with resveratrol; lane 6: 72 h VZV infection with resveratrol.

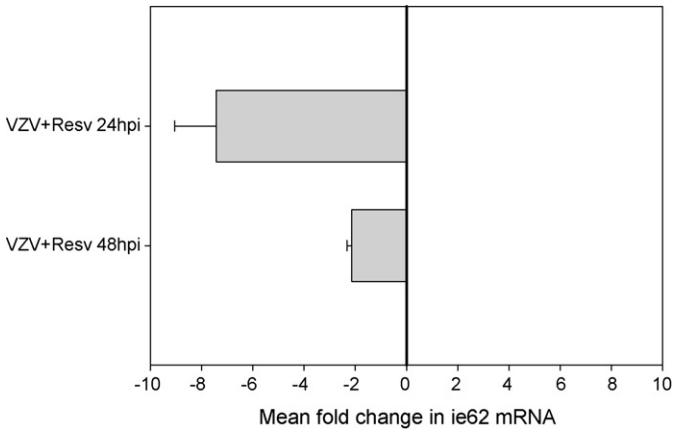

Fig. 7. Effect on VZV ie62 transcription by resveratrol. Real-time RT-PCR was used to determine mean fold change (±S.D.) of ie62 mRNA at 24 and 48 h post-infection using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001).

4. Discussion

These studies demonstrate that resveratrol, a polyphenol compound present in numerous edible plants, reversibly inhibits VZV replication in vitro in a dose- and time-dependent manner. A non-cytotoxic concentration, 219 μM resveratrol, completely blocked VZV replication when added up to 30 h post-infection. The chemical did not block attachment nor directly inactivate VZV. Instead, the data suggest that resveratrol treatment interferes with the first stage of VZV replication, the immediate-early phase, as evidenced by suppression of ie62 gene activation and absence of IE62 protein.

It was previously demonstrated that resveratrol has anti-viral activities to viruses. The chemical inhibited replication of HSV-1 and HSV-2 (Docherty et al., 1999, 2004, 2005) and HCMV (Evers et al., 2004). Influenza A, an orthomyxovirus, was also inhibited by resveratrol (Palamara et al., 2005). Additionally, resveratrol acted synergistically with nucleoside analogs to inhibit replication of HIV-1, a retrovirus (Heredia et al., 2000). Cumulatively, these reports suggest that resveratrol has a broad spectrum of anti-viral activities and that resveratrol may selectively target the host, rather than the virus as a mode of action for inhibiting viral replication.

Two lines of evidence suggest that resveratrol may target the cell, as a mechanism to inhibit VZV replication. First, our attempts to generate a resveratrol resistant VZV strain using classical methods were unsuccessful (data not reported). Secondly, resveratrol has previously shown extensive effects on cellular factors (Aggarwal et al., 2004). In particular, resveratrol has demonstrated suppressive effects on transcription factor NF-kappaB, (Takada et al., 2004), which is activated during VZV replication (Taylor et al., 2004; Wang et al., 2005). Additionally, Taylor et al. (2004) demonstrated that chemical inhibitors of cellular cyclin dependent kinases inhibited VZV replication and reduced expression of IE62.

IE62 is an essential regulatory protein of VZV, derived from the open reading frame 62 (ORF62) of the viral genome and expressed as an immediate-early product. IE62 is structurally and functionally homologous to ICP4 from HSV and demon-

strates DNA binding activity and regulates viral gene expression (Disney and Everett, 1990; Moriuchi et al., 1994; Tyler and Everett, 1993). Studies in which IE62 was reduced by chemical treatment or deleted from the viral genome resulted in little to no replication of VZV in vitro or in vivo (Sato et al., 2003; Taylor et al., 2004). In the studies reported here, resveratrol affected IE62 production by reducing levels of mRNA, as determined by real-time RT-PCR analysis. Previously, it was determined that resveratrol treatment in vitro resulted in the reduction of HSV-1 ICP4 protein (Docherty et al., 1999) and two immediate-early viral proteins from HCMV, IE1-72 and IE2-86 (Evers et al., 2004). Cumulatively, these findings indicate that resveratrol negatively affects the immediate-early phase of herpes virus replication, including VZV, by blocking/suppressing expression of essential viral proteins.

Since the introduction of the live-attenuated VZV vaccine in 1995, newly diagnosed infections are on the decline (Grose, 2005). In addition, a vaccine to prevent zoster and PHN in older adults was recently described and is reported to reduce the burden-of-illness by 61% (Oxman et al., 2005). However, a large group of older adults will not benefit from the vaccine and zoster is also seen in younger (<60 years of age) individuals. Therefore, novel and effective treatments will continue to be important until overall immunity is achieved. Resveratrol, a natural product, has demonstrated that it is effective in inhibiting VZV replication at non-cytotoxic doses in vitro. Recently, cream formulations of resveratrol have demonstrated efficacy in reducing morbidity and mortality in mice infected cutaneously or vaginally with HSV (Docherty et al., 2004, 2005). Similar to HSV lesions, varicella and herpes zoster lesions are the direct result of two factors, the destruction of tissue by the virus and the inflammation resulting from the host immune-system responses (Arvin, 2001). Since resveratrol inhibits VZV replication and has also proven to be an anti-inflammatory molecule (Chen et al., 2005; Donnelly et al., 2004; Marier et al., 2005), the topical application of resveratrol could be an ideal agent to treat both aspects of VZV cutaneous infections. Thus, further study of chemicals, such as resveratrol, that may affect the host as a means of inhibiting virus replication increases treatment options for VZV.

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