

Enterovirus 71 infection induces apoptosis in Vero cells

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Abstract

The effects of Enterovirus 71 (HEV71) infection on African green monkey kidney cells (Vero) were investigated. It was found that the infected cells showed progressive cellular morphological changes characteristic in apoptotic cells within 10 hours post-infection. The number of apoptotic cells correlated significantly with the number of HEV71 antigen positive cells when cells were labeled using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) and stained for HEV71 antigen. Approximately 11, 26, 45 and 50 % of the infected cells were apoptotic at 12, 24, 48 and 72 hours post-infection, respectively. Internucleosomal DNA fragmentation, characteristic in the late stage of apoptosis was noted beginning on day 2 post-infection. The DNA fragmentation, however, was absent in cells treated with the heat- and ultraviolet light-inactivated virus inocula. These results demonstrate the capacity of HEV71 to induce apoptosis in the infected cells. The induction, however, requires high level of HEV71 infectivity and the presence of live virus particles, suggesting the need for the presence of specific viral proteins for apoptosis to occur.

Key words: Enterovirus 71, apoptosis, Vero cells, neurogenic pulmonary oedema

INTRODUCTION

Enterovirus 71 (HEV71), a member of the genus Enterovirus is an icosahedral, non-enveloped, positive single stranded RNA virus belonging to the *Picornaviridae* family. The virus was first isolated and described from a patient presenting with symptoms of neurological infection during an outbreak of the hand, foot and mouth disease (HFMD) in California in 1969.¹ Infection with the virus normally causes mild non life threatening disease and most of the affected patients recover uneventfully. Serious complications subsequent to an infection by the virus are rare. However, in some outbreaks patients with severe neurological illnesses have been reported; even so in most instances fatalities were rare.² Outbreaks of HEV71 infection involving a high number of deaths nonetheless, have been reported in Bulgaria in 1979³ and more recently in Malaysia^{4,5,6} and Singapore⁷ in 1997 and a year later in Taiwan⁸. In these later outbreaks, it was reported that most patients died within 24 hours of admission to the hospital suggesting a rapid deterioration of the patients' vital functions. Affected patients' chest examination revealed evidence of pulmonary oedema and pathological findings from autopsy showed evidence of brainstem

encephalomyelitis.^{4,8,9} Hence, it was suggested that most patients succumbed to the complications associated with HEV71 infecting the brainstem leading to pulmonary oedema and cardiac dysfunction.^{4,8} In support of this suggestion was the detection of HEV71 antigen positive cells and also evidence suggesting typical inflammatory responses in a few of the affected patients' tissues.^{4,9,10} However, in a number of cases no evidence of significant inflammatory responses or the presence of viral antigens was reported. These raised the possibility that perhaps in these tissues most of the infected cells were removed following activation of the apoptotic cellular defense pathways. To investigate this possibility, in the present study the effects of HEV71 infection on African green monkey kidney cells (Vero) cultured *in vitro* were investigated.

MATERIALS AND METHODS

Cells, Virus Infection and Propagation

HEV71 (EV71/7/97/UH1, accession number AJ238445) used in the present study was isolated from the brain of a patient who died of HEV71-associated neurogenic pulmonary oedema.^{4,11} The virus was propagated in Vero cells (American Type Culture Collection, Rockville, MD, USA)

and the crude virus inoculum was prepared by freeze-thawing the infected cells. The titer of the resulting virus inoculum was determined by performing virus plaque assays. Throughout the study, unless otherwise mentioned, Vero cells were infected with the virus inoculum to give an estimated multiplicity of infection (MOI) of ~ 1-5 plaque forming units per cell (PFU/cell). The development of cytopathic effects was monitored daily following the infection under an inverted microscope.

Inactivation of virus

In experiments requiring inactivated virus, HEV71 inoculum was heat-inactivated by incubating it at 56° C for 30 minutes or boiling for 10 minutes. For ultraviolet (uv) light inactivation, the virus inoculum was placed in 60 mm² tissue culture dishes (Costar, USA) and irradiated using a uv-crosslinker (Spectrolinker XL-1500 UV Crosslinker, Spectronics Corporation, USA) on ice to give an estimated uv light dosages of 100, 500 and 2000 J/cm², respectively. Virus infectivity before and after inactivation was determined using virus plaque assay as mentioned above.

Detection of antigen and apoptotic cells

Mock-treated or virus-infected cells were scraped from the tissue culture flasks at selected intervals post-infection. Cells were placed on Teflon-coated slides and fixed in ice-cold acetone for 15 minutes. The presence of HEV71-infected cells was determined using monoclonal antibody specific against HEV71 (Cat. #3324, Chemicon, USA) and TRITC-conjugated anti-mouse IgG as the secondary antibody. Cells were then washed with phosphate-buffered saline and stained for apoptotic cells using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) following the protocol as recommended by the Promega Apoptosis Detection kit manufacturer (Promega, Madison, WI, USA). Cells were viewed under a Zeiss Axiolab fluorescence microscope (Zeiss, Germany). The number of cells stained positive for virus antigen or apoptosis alone or positive for both antigen and apoptosis were counted from ten randomly picked microscopic fields. The averages were determined and the percentages were calculated and used for ANOVA and Pearson's correlation statistical analyses.

DNA fragmentation assay

DNA fragmentation assay for the assessment of

apoptotic cellular DNA fragmentation was performed as previously described.¹² Treated cells were scraped, sedimented by centrifugation and lysed in 1 % N-laurylsarcosine, 0.2 % sodium deoxycholate and proteinase K (1mg/ml) in L-buffer with 0.01M Tris-HCl pH 7.6, 0.02M NaCl and 0.1M EDTA. Oligonucleosomal DNA was separated by agarose gel electrophoresis as previously described.¹³

RESULTS

HEV71 infection of Vero cells resulted in characteristic cellular morphological changes within 10 hours post-infection. In general, cells were rounded and refractile and evidence of fragmented cells were noted in the HEV71-infected cell cultures (Fig. 1a). At 48 hours post-infection, at least 50 % of the infected cells were rounded and refractile when viewed under the microscope (Fig. 2). Evidence of cellular membrane blebbing, formation of dendritic-like projections protruding from the cell membrane and fragmentation of the infected cells were noted (Fig. 1a). The percentage of cells manifesting these cellular morphological changes increased steadily with increasing number of days post-infection. These changes were observed only in the HEV71-infected Vero cells and not in the mock-treated cell cultures (Fig. 2). A significant correlation ($r = 0.965$, Pearson's) between the percentages of rounded cells with hours post-infection was demonstrated in HEV71-infected cell cultures but a poor correlation ($r = 0.081$, Pearson's) was obtained with the mock-treated samples. Hence, the percentage of rounded cells in the HEV71-infected cell culture could perhaps be used as an estimate for the extent of HEV71 infection *in vitro*.

The possibility that HEV71 infection stimulated apoptotic cellular responses was examined further using TUNEL. Using this detection method, the presence of HEV71 antigen positive cells was detectable as early as at 12 hours post-infection and increased till ~ 66 % of the cell population by 72 hours post-infection. It was also noted that at 12, 24, 48 and 72 hours post-infection, approximately 11, 26, 45 and 50 % of the infected cells were apoptotic, respectively (Fig. 3). Double staining of the HEV71-infected cells for the presence of specific virus antigens using immunofluorescence (TRITC) staining and TUNEL showed a significant correlation ($r = 0.957$, Pearson's) between the average percentage of HEV71

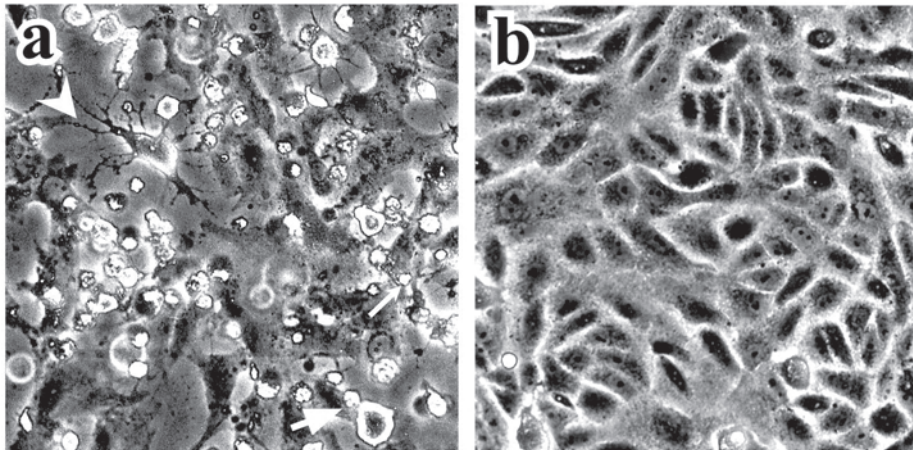


FIG. 1: Changes in Vero cells morphology upon infection with HEV71. At 48 hours post-infection, dendritic-like projections (arrowhead), membrane cell blebbing (big arrow) and fragmented cells (small arrow) were noted (a). More rounded and refractile cells were also observed in the HEV71-infected cells as compared to the mock-treated cells (b). Cells were viewed using an inverted microscope (Zeiss Televel, Zeiss, Germany) at 200 X magnification and photographed using a Nikon F3 (Nikon, Japan) SLR camera.

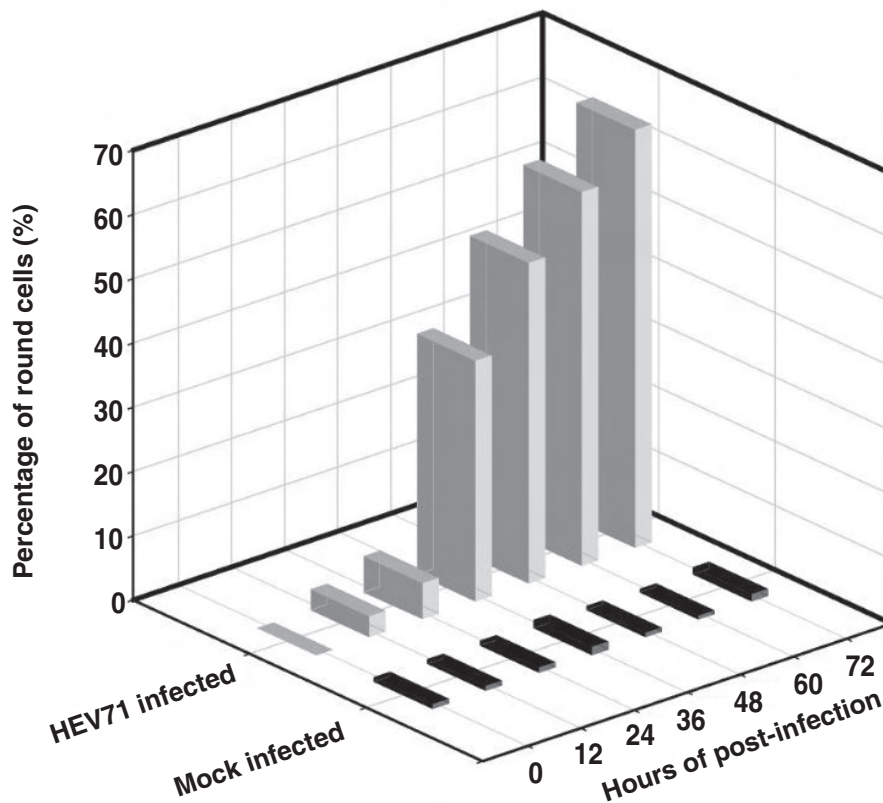


FIG. 2: Percentage of Vero cells showing cytopathologic effects of HEV71 infection. Cells were infected at ~ 5 PFU/cell. The average number of rounded cells per microscopic field was determined from the HEV71-infected cell culture and mock-treated cells. Data shown were cumulative averages from at least 10 microscopic fields.

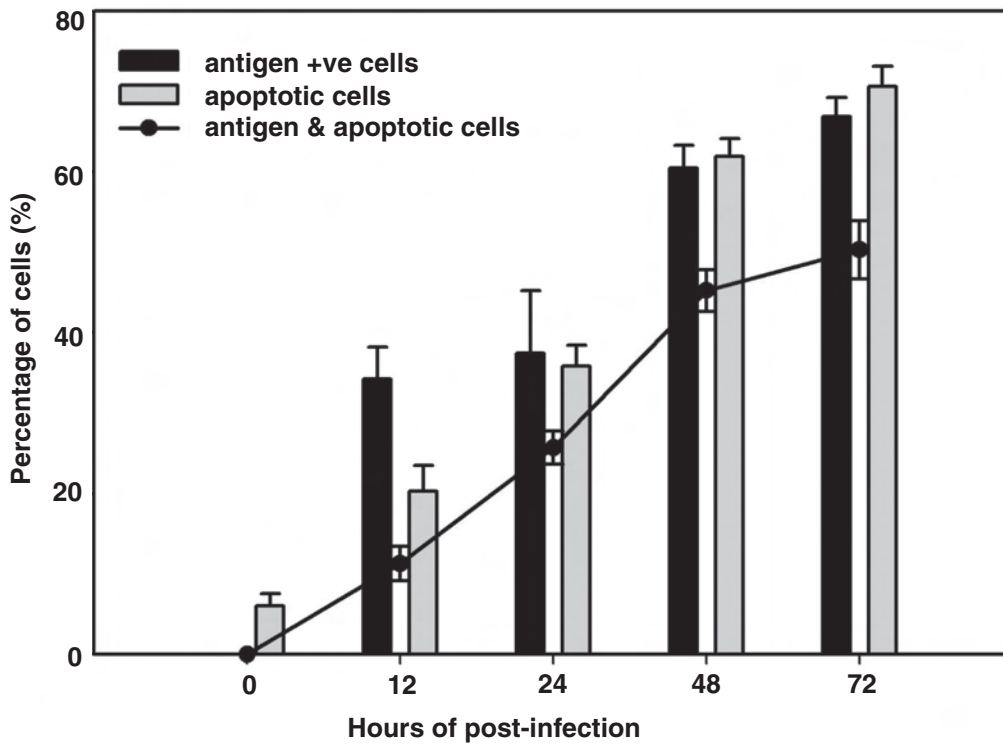


FIG. 3: Induction of apoptosis in HEV71-infected Vero cell cultures. Cells were infected at MOI of ~5 PFU/cell. The percentages of cells positive for the presence of HEV71, positive for apoptotic cells or positive for both antigen and apoptosis were determined from at least ten microscopic fields and used for the statistical analysis.

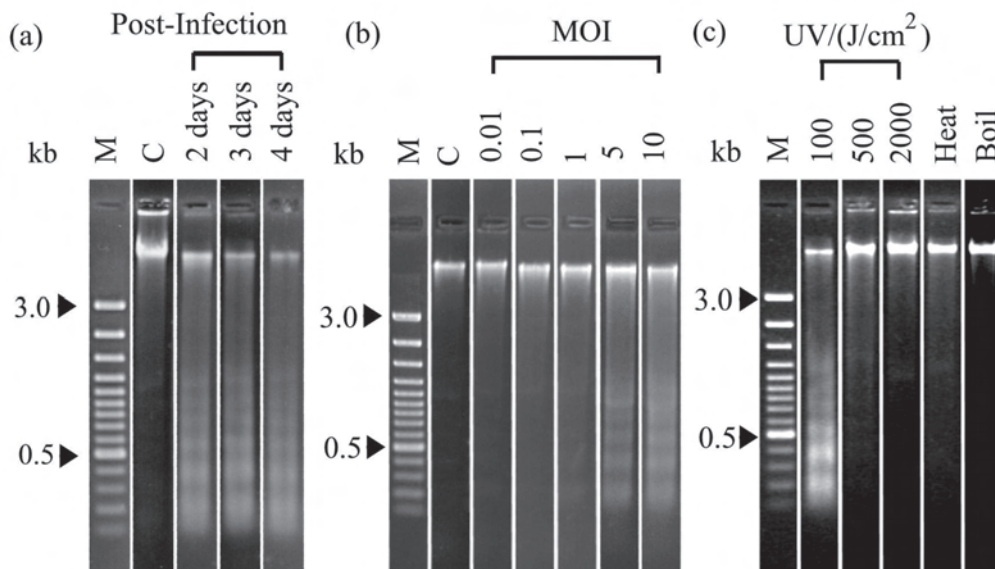


FIG. 4: Influence of virus infectivity on HEV71 induced apoptosis. Infected and mock-treated cells DNA were prepared at selected intervals post-infection and separated by agarose gel electrophoresis. Cellular DNA fragmentation was detected in HEV71-infected cells DNA samples beginning from day 2 post-infection (a). The influence of input multiplicity of infection (b), uv light-inactivated virus inocula and heat inactivation at 56° C for 30 minutes or boiling for 10 minutes of the HEV71 inocula (c). No cellular DNA fragmentation was detected in mock-infected cells (C). The molecular mass markers (M) shown are in kilobase (kb).

antigen positive cells and the apoptotic cells. These findings suggested that there was a direct correlation between HEV71 infection and the induction of apoptosis in Vero cells.

The presence of fragmented apoptotic cellular DNA in HEV71-infected cells was demonstrated further using agarose gel electrophoresis of the infected cells DNA. Oligonucleosomal DNA fragments observed as DNA ladders differing by repeating unit of about 180-200 bp were observed in HEV71-infected cells DNA prepared on day 2, 3 and 4 post-infection (Fig. 4a). No DNA ladders were noted in the mock-treated cells samples prepared in parallel even after 4 days following the treatment with the mock infecting fluid. The DNA ladders were detected in the infected cells samples at 16 hours post-infection only when cells were infected at MOI of > 5 PFU/cell (Fig. 4b) suggesting that the induction of apoptosis by HEV71 was dependent on at least the presence of sufficient number of virus particles. To examine if live virus was necessary for the induction of apoptosis, Vero cells were treated with inactivated and partially inactivated HEV71 inocula and the effects of the treatment were examined using agarose gel electrophoresis of the infected cells DNA. From this study, it was observed that uv irradiation of the virus inoculum at 100 J/cm^2 did not abolish the capacity of HEV71 to induce the formation of the apoptotic cellular DNA ladders. However, exposure of the virus inoculum at 500 and 2000 J/cm^2 which effectively inactivated the virus infectivity, rendered the HEV71 inocula ineffective in inducing apoptosis (Fig. 4c). Similarly, no DNA ladders were detected in samples treated with either heat-treated or boiled HEV71 virus inoculum (Fig. 4c), suggesting that infectious HEV71 was necessary for the induction of apoptosis to occur in Vero cells.

DISCUSSION

The potential involvement of apoptosis in HEV71 infection was first eluded during an investigation of the causal agent for an outbreak of HFMD-associated fatal childhood viral infection in Sarawak, Malaysia in 1997.^{14,15} In that outbreak at least 31 deaths were reported and the inocula derived from some of the patients' specimens were shown to induce apoptosis *in vitro*. Though, the causal agent of the fatal outbreak was never really identified, with reports of isolation of a novel Adenovirus from some of the fatal cases in addition to HEV71,^{16,17} the high prevalence of HEV71 isolated from the population at that time

and the confirmation of HEV71 as the causal agent of a subsequent fatal HFMD-associated outbreak in Malaysia strongly suggests the involvement of HEV71 in the Sarawak's outbreak.⁴ In the outbreak that occurred in Peninsular Malaysia, deaths were attributed to neurogenic pulmonary oedema possibly as a result of HEV71 infection of the brainstem.⁴ Evidence of apoptosis, however, was reported from the lung tissue of one of the deceased patient.¹¹ Attempts to demonstrate the presence of HEV71 directly in samples showing apoptotic cellular responses, however, were successful in only one of the patient's sample,¹⁴ raising initial doubt that apoptosis noted were caused by HEV71 infection. Nonetheless, it was argued that perhaps the inability to detect HEV71 positive cells in the patients' tissues was due to the induction of apoptosis that prevented further replication of the virus. Furthermore, the manifestation of apoptosis detected by the DNA fragmentation assay, was actually the terminal event in the apoptosis pathway in which possibly all virus replication have been terminated. Isolation of HEV71 from the single positive patient's sample was made only after the initial patient's specimen were passaged several times, suggesting a very low presence of HEV71. To demonstrate if HEV71 can indeed cause apoptosis, in the present study, an isolate from one of the fatal case^{4,11} was used to infect Vero cells.

It was found that HEV71 infection of cultured Vero cells resulted in characteristic cellular morphological changes similar to that previously described for poliovirus-infected apoptotic cells.¹⁸ Additionally, in agreement with these observations was the presence of characteristic apoptotic cellular DNA fragments demonstrated indirectly using TUNEL and directly by agarose gel electrophoresis of the HEV71-infected Vero cells DNA. The presence of specific viral antigens in cells expressing apoptotic features, added further support to the possible involvement of apoptosis in the pathogenesis of HEV71 infection. As the manifestation of apoptosis was detected by TUNEL and agarose gel electrophoresis only when cells were infected with live virus at ~ 5 PFU/cell; it also suggests that a relatively high virus infectivity was necessary for apoptosis to be detected in cell cultures. These may also help to explain the inability to detect the presence of HEV71 antigen or apoptotic cells in tissues of patients who succumbed to the HEV71-associated encephalitis; since in these patients it is very

likely that the infectivity could be significantly much lower than that used in the present study.

The relationship between the ability to induce apoptosis and the manifestation of the disease severity in HFMD is as yet has not been established for HEV71 infection. In other virus infections, apoptosis is thought to play an important role in the manifestation of the disease by directly affecting the neurons such as in rabies virus,¹⁹ poliovirus²⁰ and Sindbis virus²¹ infections of mice or as a consequence of the host immune responses involving cytotoxic T cells²² and the various cytokines²³ as noted in human immunodeficiency virus infection. As apoptosis has been shown to result in discrete removal of the diseased or infected cells in contrast to necrosis in which there is a significant involvement of inflammatory responses,²⁴ it is speculated here that the lack of detectable neuronal damage reported in most patients who succumbed to HEV71 infections^{9,10} was probably due to the effective removal of the infected cells through apoptosis. The removal of these infected neurons, however, indirectly resulted in damage to the vasomotor center hence resulting in the HEV71-associated neurogenic pulmonary oedema.^{4,9}

In summary, results presented here demonstrate the capacity of HEV71 to induce apoptosis. The induction, however, requires high level of HEV71 infectivity and the presence of live virus particles suggesting the need for the presence of specific viral proteins for apoptosis to occur. Apoptosis is also suggested as a possible mechanism whereby the virus indirectly affects hosts and caused death.

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