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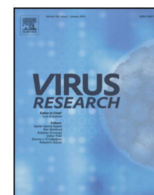

Robust antiviral responses to enterovirus 71 infection in human intestinal epithelial cells

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ABSTRACT

Enterovirus 71 (EV71) is a single-stranded RNA virus that belongs to *Picornaviridae* family. It causes the hand-foot-and-mouth disease and fatal neurological diseases in young children and infants. The mechanism of EV71 pathogenesis remains obscure. The intestinal tract is the initial site of EV71 replication, but no or only mild gastrointestinal symptoms are observed clinically, suggesting that host immune responses of the intestinal epithelium to EV71 may be unique, which, however, remains rarely investigated. In this study, we showed that human intestinal epithelial cells HT-29 were susceptible to EV71, and the infected cells exhibited cytopathic effects (CPEs) and were prone to apoptosis. TLR-7 and TLR-8 were induced significantly post infection and may be pivotal in the induction of IFN-β and host innate immune responses against EV71. Among proinflammatory responses in EV71-infected intestinal epithelial cells, IL-6, CCL5, and IP10 were up-regulated and may play a key role in intestinal pathogenicity. We examined extrinsic and intrinsic apoptotic pathways and found that both were activated in EV71 infection. The mitochondria-mediated intrinsic pathway may also be activated through Bid cleaved by active caspase-8. Robust induction of IFN-β in human intestinal epithelial cells contradicts the finding that IFN induction was suppressed in other types of the cells, suggesting that mild gastrointestinal symptoms may be the result of sufficient local antiviral inductions. Our study has demonstrated a unique way of antiviral responses in human gut different from other tissue cells in response to EV71, which may account for mild symptoms in intestinal tract. This finding will broaden our understanding of host defense mechanism and the pathogenesis of EV71 infection.

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1. Introduction

Enterovirus 71 (EV71) is a single-stranded, positive-sense RNA virus which belongs to the *Enterovirus* genus of the *Picornaviridae* family (Solomon et al., 2010; Wong et al., 2010). The genome of human enterovirus 71 is approximately 7500 nucleotides in length, with a single open reading frame that encodes a large polyprotein. The polypeptide is then proteolytically cleaved to individual proteins including structural proteins VP1, VP2, VP3, and VP4, and non-structural proteins 2A, 2B, 2C, 3A, 3B, 3C, and 3D, respectively (Solomon et al., 2010; McMinn, 2002). EV71 infection usually causes mild symptoms such as herpangina or exanthema, also known as hand, foot, and mouth disease (HFMD), in childhood. In severe cases, however, EV71 infection may cause systemic diseases and complications of the central nervous system (CNS), including encephalitis, aseptic meningitis, and brain stem encephalitis, and patients could rapidly developed fatal pulmonary edema and hemorrhage (Wong et al., 2010; McMinn, 2002; Chen et al., 2007a,b).

EV71 was first isolated in California in 1969 from the stool sample from an infant with encephalitis (Schmidt et al., 1974). Since the first report, there have been outbreaks of EV71 disease worldwide, especially in Asia-Pacific region (Chan et al., 2003; Gilbert et al., 1988; Ho et al., 1999a). The largest and most fatal outbreak occurred in Taiwan in 1998, with 405 severe cases and 78 deaths reported (Ho et al., 1999a). It has become endemic ever since with outbreaks occurring in 2000, 2001, and 2008, respectively (Lin et al., 2002). EV71 infection has become an emerging disease in mainland China since March 2008 (Zhu et al., 2010). Currently, neither vaccines nor specific antiviral drugs are available for prevention of EV71 or effective treatment clinically.

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The pathogenesis of HFMD has remained obscure. It is believed that immature or impaired immunity is associated with increased morbidity and mortality upon EV71 infection in younger children (Chang et al., 2007; Ho et al., 1999b; Huang et al., 1999). Interestingly, some precious reports have shown that neutralizing antibody levels were not correlated with HFMD severity (Yang et al., 2011; Chang et al., 2006). However, there were studies suggesting that cell-mediated immune (CMI) responses may play a significant role in immunity against EV71 infection and CMI was correlated irreversibly to clinical severity of HFMD (Chang et al., 2006). Innate immunity is the first line of defense against pathogen invasion, which also bridges to the adaptive immune responses to eventually clear the viruses. Activation of innate immunity is dependent on the pattern recognition receptors (PRR) as shown in EV71 infected newborn mice (Sasaki et al., 1986). Studies have shown that type I IFNs exerted a direct protective effect on EV71 and played an important role in controlling EV71 infection and replication (Liu et al., 2005). Various mechanisms have been employed by enteroviruses to modulate the IFN responses. Coxsackievirus B attenuates type I IFN via cleavage of MAVS and TRIF by a viral protease, $3C^{pro}$ (Mukherjee et al., 2011). In HeLa and RD cells, EV71 was capable of suppressing IFN induction through its 3C protein, which binds RIG-I, disrupts it from binding to IPS-1, and inhibits the activation and nuclear translocation of IRF3 (Lei et al., 2010). EV71 3C protein can also cleave the adaptor protein TRIF and impair type I IFN production in response to TLR3 activation (Lei et al., 2011).

EV71 infects human through GI system and intestines are the entry portal of the viral infection. Few studies have been conducted to examine how human intestinal epithelium responds to EV71 infection, and whether the host responses in GI epithelium have any impact on EV71 pathogenesis in humans. In this report, we demonstrated that the human intestinal epithelial cells were susceptible to EV71. EV71 infection induced proinflammatory cytokines production and apoptosis in human intestinal epithelial cells. Significantly, anti-viral IFN induction was robust, which differs from the previously observed in studies carried out in RD and HeLa cells (Lei et al., 2010, 2011). Our study suggests that host responses in guts may have significant influence on EV71 replication and the potential for subsequent viral spread and a systemic infection in patients.

2. Materials and methods

2.1. Cells, viruses and reagents

African green monkey kidney Vero (ATCC CCL-81) cells were maintained in DMEM medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (SΛFC Biosciences, Lenexa, KS) supplemented with 1 mM sodium pyruvate (Amresco, Solon, OH) and 1% antibiotic–antimycotic solution (Invitrogen). Human colorectal adenocarcinoma HT-29 (ATCC HTB-38) cells were cultured in RPMI-1640 medium (Invitrogen) containing 10% fetal bovine serum supplemented with 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 1% antibiotic–antimycotic solution and 55 μM 2-mercaptoethanol (Amresco, Solon, OH). Cell cultures were incubated at 37 °C in a 5% CO_2 humidified atmosphere. Enterovirus 71 (EV71) Fuyang strain was kindly provided by Dr. Wu Bin, Jiangsu Provincial Centers of Disease Control. Rabbit anti-EV71 structural protein VP1 antibody (bs-0852R) was purchased from Beijing Biosynthesis Biotechnology Co. (Beijing, China). Rabbit anti-cytochrome c (sc-7159), goat anti-procaspase-3 (sc-1226), mouse anti-poly (ADP-ribose) polymerase (PARP) (sc-365315), and mouse anti-β-actin antibodies (sc-130300) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-cleaved caspase-6 (#9761), rabbit anti-cleaved caspase-7 (#9491), rabbit anti-cleaved caspase-8 (#9496), and rabbit anti-cleaved capase-9 antibodies (#9501) were products of Cell Signaling Technology (Boston, USA).

2.2. Virus infection and titration

The viruses were inoculated in Vero cells at a multiplicity of infectivity (m.o.i.) of 1 in DMEM medium free of fetal bovine serum. Following two-hour viral attachment, the inoculum was replaced with fresh medium containing 2% fetal bovine serum. The EV71 virus stocks were collected from the supernatants of infected Vero cells at day 3 post infection. The titers of the virus stocks were determined by a $TCID_{50}$ assay, and the stocks were aliquoted and stored at −80 °C. As of the $TCID_{50}$ assay, serially diluted viruses from 10^{-2} to 10^{-9} in DMEM were inoculated to Vero cells in 96-well plates, and the cells were incubated for seven days at 37 °C. $TCID_{50}$ were calculated by counting the wells with cytopathic effect (CPE) in infected Vero cells using the formula: $\log TCID_{50} = L - d(s - 0.5)$, where L is the log of the lowest dilution, d is the difference between dilution steps, and s is the sum of the proportion of positive wells.

2.3. ELISA assay for cytokines

Tissue culture supernatants were harvested from uninfected and infected HT29 cells at indicated time points, followed by centrifugation at 1000 × g for 15 min at 4 °C. A cytokine ELISA kit (Bio-Rad) was used following the manufacturer's protocol, which starts with the preparation of the 8 point standards dilution series (IL-8 and IP10) and dilution of the samples at 1:4. 50 μl of beads were added to the assay plate for incubation in the dark at RT with shaking at 300 rpm for 30 min and subsequent washes. After an incubation with 25 μl of biotin-conjugated detection antibody and washes, 50 μl of strepavidin-PE was added. Following the final addition of 125 μl assay buffer and shake at 1100 rpm for 30 s, the plates were read and data analyzed with Bio-Plex manager software. The assay was repeated twice.

2.4. Quantitative real-time PCR

Total RNA prepared from uninfected and infected HT29 cells with RNeasy kit (Qiagen, Hilden, Germany), were used for reverse transcription with the Primescript RT reagent kit (TAKARA, Shiga, Japan) following the manufacturer's instruction. Real-time PCR was conducted with 1 μl cDNA in total volume of 10 μl with the SYBR Premix Ex Taq II (TAKARA, Shiga, Japan) according to the manufacturer's instructions. The transcripts of GAPDH were used as an internal control for assay of an expressed gene. Fold changes of each gene expression level was calculated based on the formula: $2^{(\text{Ct of gene} - \text{Ct of GAPDH})}$. Reactions were implemented in duplicate and repeated three times for each sample, and the mean values and standard deviations were calculated.

2.5. Subcellular protein extraction and western blot analysis

To obtain proteins in subcellular fractions, cells were lysed in pre-cooled 1% NP-40 lysis buffer containing 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 2 mM PMSF, 2 mM NaF, 1 mM Na_3VO_4, 1 μg/ml aprotinin, and 1 μg/ml leupeptin on ice for 20 min. The supernatant was harvested as a cytosolic fraction after centrifugation (500 × g, 5 min at 4 °C). For the mitochondrial fraction, we performed the preparation using KaiJi mitochondrial protein extraction kit (Keygentec, Nanjing, China) following the manufacturer's instructions. The lysates were separated by SDS-PAGE gels and transferred to Immuno-Blot PVDF membrane (Millipore, Billerica, MA, USA). The membrane was blocked with TBS-Tween 20 (TBST) containing 5% nonfat milk for 40 min at room temperature and incubated with appropriate primary antibody diluted in TBST at 4 °C overnight. After the incubation with primary antibodies, the membrane was washed three times with TBST, followed by further incubation with alkaline phosphatase

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