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Susceptibility of human tonsillar epithelial cells to enterovirus 71 with normal cytokine response

Guang-Cheng Xie [a], Ni-Jun Guo [a,b], Reidar Grénman [c], Hong Wang [a], Ying Wang [a,d], Minna Vuorenmma [c], Qing Zhang [a], Shuang Zhang [a], Hui-Ying Li [a], Li-Li Pang [a,e], Dan-Di Li [a], Miao Jin [a], Xiao-Man Sun [a], Xiang-Yu Kong [a], Zhao-Jun Duan [a,*]

[a] National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing, China
[b] Hunan Provincial People's Hospital, the First affiliated Hospital of Hunan Normal University, Changsha, China
[c] Department of Otorhinolaryngology-Head and Neck Surgery, Turku University and Turku University Hospital, Turku, Finland
[d] School of Basic Medical Sciences, Inner Mongolia Medical University, Hohhot, China
[e] Institute of Microbiology, Chinese Academy of Sciences, Beijing, China

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ABSTRACT

A recent histopathologic study implicated human tonsillar crypt epithelium as an important site for EV71 replication in EV71-caused fatal cases. This study aimed to confirm the susceptibility of human tonsillar epithelium to EV71. Two human tonsillar epithelial cell lines (UT-SCC-60A and UT-SCC-60B) were susceptive to EV71, and PI3K/AKT, p38, ERK1/2, and JNK1/2 signal pathways were activated. Interferon-α, IL-8, IL-1β, IL-6 and IL-12p40 were induced and regulated by PI3K/AKT, p38, ERK1/2, and JNK1/2 signal pathways. PI3K/AKT pathway activation appeared to suppress the induction of TNF-α, which induced cell survival by inhibiting GSK-3β. The activation of NF-κB was observed but inhibited by these pathways in EV71 infection. Furthermore, ERK1/2 and JNK1/2 were essential for efficient EV71 replication. Human tonsillar epithelial cells support EV71 replication and display innate antiviral immunity *in vitro*, indicating that human tonsillar epithelial cells may be novel targets for EV71 infection and replication *in vivo*.

1. Introduction

Hand, foot and mouth disease (HFMD)—a common infectious disease of global concern—circulates mainly in children under 5 years of age. Two RNA viruses of the *Enterovirus* genus of the *Picornaviridae* family, enterovirus 71 (EV71) and coxsackievirus A16 (CVA16), are the major agents of HFMD (Chang et al., 1999). EV71 was first isolated in 1969 in California and recognized as a causative agent of HFMD in Japan in 1973 (Ishimaru et al., 1980; Solomon et al., 2010). Large HFMD outbreaks with numerous deaths occurred in Bulgaria in 1975 and Hungary in 1978 (Nagy et al., 1982; Shindarov et al., 1979). In the 2000s, Taiwan, Japan, Malaysia, Singapore and Vietnam experienced cyclical epidemics (Weng et al., 2010). Subsequently, the largest epidemic to date occurred in mainland China in 2008, with approximately 490,000 reported cases and an estimated case-fatality rate ranging from 0.03% to 0.3% (Tan et al., 2011; Xing et al., 2014).

Humans are the only natural reservoir of EV71, as is the case with poliovirus. Person-to-person transmission is usually through fecal–oral or oral–oral routes (Ong and Wong, 2015). Viral isolation and detection from throat swabs appear to be significantly higher than that from rectal swabs or feces, suggesting that the oral–oral route may be more important than the fecal–oral route (Hu et al., 2015; Ooi et al., 2007a, 2007b). Studies showed that EV71 genome and antigen were distributed in neurons and microglia cells in the central nervous system (CNS) in EV71 fatal cases (Yu et al., 2014, 2015). It is hypothesized that virus first enters and replicates in the human body, which leads to viremia; finally, neuroinvasion occurs, similar to the poliovirus infection model (Bodian, 1955). However, the mechanism of EV71 entry into the body remains poorly understood. Detection of viral antigens and RNA in tonsillar crypt squamous epithelium, but not in other parts of the gastrointestinal tract, suggest that the palatine tonsil and oral mucosa may be the major source of viral shedding into the oral cavity, and likely in the feces as well (He et al., 2014; Ong and Wong, 2015).

Signal transduction pathways are essential for normal innate viral immunity and for virus replication. PI3K/AKT and mitogen-activated protein kinases (MAPKs)/ERK signaling pathways were activated at the early entry step of EV71 infection (Wong et al., 2005). The MEK1-ERKs signal cascade and ERK pathway, as well as

* Corresponding author.
E-mail address: zhaojund@126.com (Z.-J. Duan).

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the JNK1/2 and p38 MAPK pathways, were required for EV71 efficient replication (Peng et al., 2014; Wang et al., 2012; Zhu et al., 2015). MAPK pathways also had bivalent roles in the regulation of host responses and EV71 replication (Wang et al., 2015). Our previous studies showed that excessive proinflammatory cytokine and chemokine responses were induced in human monocyte-derived macrophages, and the levels of interleukin (IL)-1β, IL-6 and IL-8 were increased in EV71-infected patients (Gong et al., 2012; Ye et al., 2015). Previous studies were conducted in RD, HeLa and HT29 cells; however, it is unknown whether human tonsillar epithelial cells are susceptive to EV71 with normal activation of cytokine responses.

In the present study, we first investigated the susceptibility of two human tonsillar epithelial cell lines UT-SCC-60A and UT-SCC-60B to EV71 and further explored the signal transduction and cytokine responses of these cell lines to EV71 infection.

2. Results

2.1. Cellular morphology and cytokeratin characterization of human tonsillar epithelial cells, UT-SCC-60A and UT-SCC-60B

The cellular morphologies of the human tonsillar epithelial cells, UT-SCC-60A and UT-SCC-60B, revealed cobblestone appearances. The cell nucleus and cytoplasm were stained with mazarine blue and pink, respectively. Both UT-SCC-60A and UT-SCC-60B cells contained abundant quantities of sialomucin and sulfomucin (blue) and neutral-mucoprotein (purple) (Fig. 1A). UT-SCC-60A and UT-SCC-60B cells were positive for pan-cytokeratin and cytokeratin-5. UT-SCC-60A cells showed higher levels of cytokeratin-5 expression than UT-SCC-60B cells (Fig. 1B).

2.2. Susceptibility and replication cycle of EV71 in human tonsillar epithelial cells

Both cell lines showed CPE at 12 h p.o.i, with significant CPE appearing at 24 h p.o.i. at a MOI of 1. EV71 infection was more apparent in UT-SCC-60A cells (Fig. 2A). The kinetics of EV71 RNA synthesis in the infected cell lines was detected using qPCR at various time points (3, 6, 12, and 36 h). Viral RNA increased from 3 to 12 h p.o.i, with the mean viral RNA number greater than 7.82 Log_{10} EV71 genome copies/μL in the two cell lines; however, the amount of viral RNA in UT-SCC-60A cells was higher than in UT-SCC-60B cells (Fig. 2B). Expression of EV71 VP1 protein in infected cells was detected at 0.5 h p.o.i. in both cell lines (Fig. 2C). The titers of viable viral particles in the total lysates and culture supernatants were quantified and expressed as $TCID_{50}$. The results showed the viral titer in UT-SCC-60A cells reached 6.3 and 7.17 $Log_{10}TCID_{50}$/mL at 12 and 24 h p.o.i., respectively. This titer was 1.4- and 1.3-fold higher than that observed in UT-SCC-60B cells (4.5 and 5.4 $Log_{10}TCID_{50}$/mL at 12 and 24 h p.o.i, respectively) (Fig. 2D).

2.3. Up-regulation of SCARB2 not PSGL-1 in EV71-infected human tonsillar epithelial cells

To evaluate the known receptors of EV71 in human tonsillar epithelial cells, we determined the expression of SCARB2 and PSGL-1 in EV71-infected human tonsillar epithelial cells. The results showed that the expression levels of PSGL-1 in human tonsillar epithelial cells UT-SCC-60A and UT-SCC-60B were very weak from 0.5 h to 24 h, however, the expression levels of SCARB2 were up-regulated in a time-dependent manner, the peak expression of SCARB2 in UT-SCC-60A and UT-SCC-60B occurred at 6 h and 12 h, respectively (Fig. 3A). Furthermore, we performed double immunofluorescence staining for SCARB2 and viral antigen. In both two human tonsillar epithelial cells UT-SCC-60A and UT-SCC-60B, all viral antigen-positive cells expressed SCARB2 (Fig. 3B).

2.4. IFN and cytokine expression in EV71-infected human tonsillar epithelial cells

IFN-α was induced in UT-SCC-60A and UT-SCC-60B cells by 12 h p.o.i. IFN-α peaked at 24 h p.o.i. in UT-SCC-60A cells, while INF-α maintained a similar level at 24 h compared to that observed at 12 h p.o.i. in UT-SCC-60B cells (Fig. 4A). Chemokine IL-8 was also induced in the cell lines, peaking at 24 h p.o.i. (Fig. 4B). Pro-inflammatory cytokines, including IL-1β, IL-6 and IL-12p40 were activated in both cell lines. IL-6 and IL-12p40 peaked at 12 h p.o.i. in UT-SCC-60A cells, but peaked at 24 and 12 h p.o.i, respectively in UT-SCC-60B cells. IL-1β was induced at 12 h p.o.i. and peaked at 24 h p.o.i. in both cell lines (Fig. 4C–E). TNF-α was induced only in UT-SCC-60B cells at 12 h p.o.i, but showed no

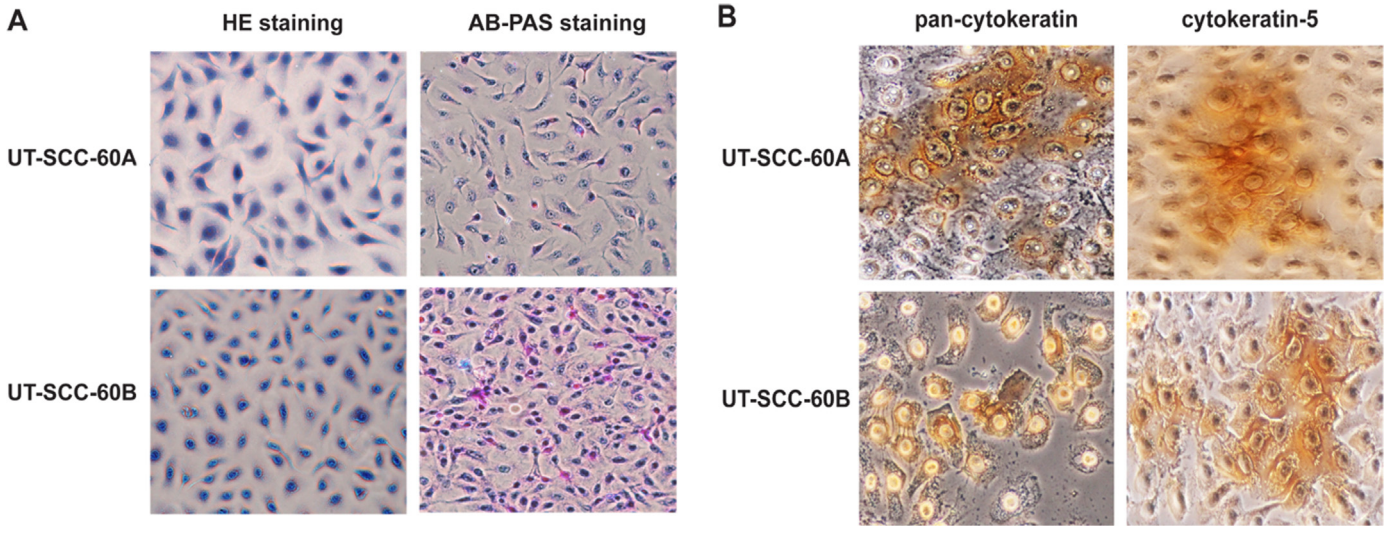

Fig. 1. Analysis of cellular morphology and evaluation of cytokeratin expression in human tonsillar epithelial cells, UT-SCC-60A and UT-SCC-60B. (A) Cellular morphology and cell membrane surface-associated protein assays of UT-SCC-60A and UT-SCC-60B cells by hematoxylin–eosin (HE) and Alcian blue–periodic acid–Schiff (AB–PAS) staining. (B) Pan-cytokeratin and cytokeratin-5 expression of human tonsillar epithelial cells, UT-SCC-60A and UT-SCC-60B, by immunocytochemistry. Original magnification, × 100 (A), × 200 (B).