Enterovirus 71 Infection of Human Immune Cells Induces the Production of Proinflammatory Cytokines

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A proinflammatory cytokine storm has been proposed to explain the pathogenesis of enterovirus 71 (EV71)-induced fatalities; however, the mechanism to induce these cytokines during EV71 infection remains unclear. Since most of the proinflammatory cytokines are produced by immune cells, we tested whether EV71 infects human immune cells and induces cytokine production. EV71 infection of a human T cell line (Jurkat), macrophage cell line (THP-1), and freshly isolated human peripheral blood mononuclear cells was demonstrated using RT-PCR and immunofluorescence assays in vitro. In addition, live but not UV-inactivated EV71 increased the secretion of two proinflammatory cytokines, tumor necrosis factor-α (TNF-α) and macrophage migration inhibitory factor (MIF) by immune cells, which was inhibited in the presence of actinomycin D. RT-PCR further confirmed that EV71 infection of immune cells triggered the de novo synthesis of MIF mRNA. Therefore, our results suggest that the production of TNF-α and MIF induced by EV71 infection of immune cells may contribute to the proinflammatory cytokine storm during EV71 infection.

Key words: inflammation; cytokine; infection

Introduction

Enterovirus 71 (EV71) is a positive-stranded RNA virus transmitted from person to person primarily via the fecal-oral route [1]. After it is replicated in the mucosal system, the virus may enter the circulation (viremia) and finally find its way to the central nervous system (CNS) [2]. Even though the spinal cord and brain stem are the main targets of EV71 in fatal cases, the virus can be detected in several different types of tissue, including heart and pancreas tissue, and in throat swabs, blood samples, and stool [2, 3].

The clinical manifestations caused by EV71 infection vary from mild hand-foot-and-mouth disease or herpangina to aseptic meningitis, encephalitis, pulmonary edema, and death [4, 5]. Genomic comparisons of the strains isolated from fatal and benign cases have shown an overall amino acid homology greater than 97% [3, 6], which indicates that host response rather than virus virulence is more important in the determination of the disease severity of EV71 infection.

In accordance with the importance of the host immune response in the pathogenesis of EV71 infection, a significant increase of cytokines (a cytokine storm) is associated with EV71 patients with encephalitis and pulmonary edema, the leading causes of death. Pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 in sera as well as IL-6 in cerebral spinal fluid (CSF) are more elevated in EV71 patients with pulmonary edema than in EV71 patients with encephalitis alone [7, 8]. In addition, both TH1 cytokine (interferon (IFN)-γ) and TH2 cytokine (IL-10) are elevated in EV71 patients with encephalitis and pulmonary edema [9], indicating that immune activation is involved in the pathogenesis of EV71 infection.

Macrophage migration inhibitory factor (MIF) is a 12.5-kD protein important in the modulation of inflam-
matory and immune responses [10]. MIF was originally described as a T-lymphocyte protein that inhibited the random migration of macrophages. More recently, however, MIF was found to be released by different cells in many different types of tissue in response to a variety of stimuli, including different viruses [11-13]. Once released, MIF can augment the secretion of TNF-α and has the potential to override the anti-inflammatory action of glucocorticoids in alveolar cells from patients with acute respiratory distress syndrome [14]. Therefore, MIF is important as a mediator that sustains the inflammatory response in many different diseases [14-16].

We studied the effects of EV71 infection of human immune cells on the production of TNF-α and MIF using a clinical isolate of EV71 (4643) to infect a human T cell line (Jurkat), a monocytic cell line (THP-1), and freshly isolated human peripheral blood mononuclear cells (PBMC). Our results indicated that EV71 infection of immune cells triggered TNF-α and MIF synthesis, both of which may contribute to the pathogenesis of EV71 infection.

Materials and Methods

Reagents

The following reagents were purchased from the indicated sources: fetal calf serum (FCS), Dulbecco’s modification of Eagle’s minimum essential medium (DMEM), RPMI medium 1640, penicillin-streptomycin-glutamine, Hank’s balanced salt solution (HBSS) (Gibco, Invitrogen, Carlsbad, CA); actinomycin D, 4’,6’-diamidino-2-phenylindole (DAPI), Histopaque-1.077 lymphocyte separation medium, lipopolysaccharide (LPS) (Sigma-Aldrich, St. Louis, MO); FITC-conjugated goat-polyclonal anti-mouse IgG antibody (Jackson ImmunoResearch, West Grove, PA); mouse-monoclonal anti-EV71 VP1 antibody (Chemicon, Temecula, CA); Alexa 594-conjugated goat-polyclonal anti-Rabbit IgG antibody (Molecular Probes, Inc., Eugene, OR); rabbit anti-human MIF antibody (Santa Cruz Biotechnology, Santa Cruz, CA); and cytokine ELISA assays (R&D Systems, Minneapolis, MN).

Preparing Virus Stocks and Virus Titration

EV71 (strain 4643), which was originally isolated from the throat swabs of an 18-month-old patient with encephalitis, was kindly provided by Dr. J. R. Wang [17]. The virus was propagated in Vero cells with DMEM supplemented with 10% FCS and antibiotics. Briefly, monolayers of Vero cells were inoculated with the virus at a multiplicity of infection (MOI) of 0.01 and the virus culture medium was harvested after it had incubated for 36 h. Cell debris was removed using centrifugation at 1000 × g for 10 min and filtrated using a 0.22-µm membrane filter (Millipore, Billerica, MA), and the supernatant was aliquoted and stored at −70°C until used. The virus titer was determined using a plaque assay. Briefly, a 10-fold serial dilution of viral supernatant was added to Vero cells and then cultured under 0.8% methylcellulose nutrient agarose for 3-5 days. Plaques were counted after they had been stained with 1% crystal violet and expressed as plaque-forming units per milliliter (PFU/ml). EV71 was inactivated using ultraviolet (UV) radiation (UV-EV71) 5000 mJ/cm², 20 min on ice (Stratalinker 1800 UV Crosslinker, 120 V; Stratagene, La Jolla, CA). A mock-infected control was prepared in exactly the same manner as the EV71 preparation with the exception that the Vero cells were not infected with EV71.

EV71 Infection of Immune Cells

A human T cell line (Jurkat) and a monocytic cell line (THP-1) were grown at 37°C in 5% CO₂ in RPMI 1640 medium with 10% FCS. Human peripheral blood mononuclear cells (PBMC) were isolated from normal blood donors using Ficoll-Hypaque gradient sedimentation. Approximately 2 × 10⁵ cells were incubated with EV71 at different MOI as indicated and allowed to absorb for 2 h at 37°C. Unbound viruses were removed by washing them with medium. Infected cells and culture supernatants were collected at different time intervals as indicated.

Immunofluorescent Staining

Immune cells were incubated with EV71, UV-EV71, or mock-infected controls for 24 h, washed and stained with 1000x diluted mouse anti-EV71 antibody at 37°C for 1 h. After they had been washed, the cells were incubated with DAPI (1 µg/ml) and 1500x diluted FITC conjugated goat anti-mouse IgG and observed under a fluorescent microscope (Olympus, Tokyo, Japan) or viewed under a confocal microscope (Leica TCS SP2; Leica Mikrosysteme Vertrieb GmbH, Bensheim, Germany).
Proinflammatory cytokines induced by EV71

Flow Cytometry

Cells were infected with EV71 at MOI of 5 for 24 h before being fixed with 4% paraformaldehyde for 30 min, then permeabilized with 0.5% Triton X-100 for 10 min. The cells were then washed with PBS and blocked with 0.05% BSA in PBS. The fixed cells were stained with mouse-monoclonal anti-EV71 antibody at 4°C for 1 h. After they had been washed, the cells were incubated with FITC-conjugated goat-polyclonal anti-mouse IgG antibody and analyzed using flow cytometry (FACSCalibur; BD Immunocytometry Systems, San Jose, CA).

Cytokine Quantization

The levels of TNF-α and MIF in patient sera and the supernatants of immune cells co-cultured with EV71 or UV-EV71 were assessed using commercially available ELISA kits (R&D Systems) according to the manufacturer’s instructions.

RT-PCR of MIF RNA and EV71 Negative-strand RNA

Total RNA was extracted from EV71-infected cells using a commercial reagent (REzol; PROtech Technology Ent. Co., Ltd., Taipei, Taiwan). One microgram of total RNA was reverse transcribed into cDNA using a kit (Advantage RT-for-PCR; BD Biosciences Clontech, Palo Alto, CA) according to the manufacturer’s protocol. Two microliters of cDNA were combined with a PCR mixture containing 1 unit of Taq polymerase, 0.1 mM of dNTPs, and 1 mM of primers and then subjected to PCR amplification in a PCR thermal cycler (Perkin-Elmer, Foster City, CA). The PCR conditions were 94°C for 5 min; 35 cycles at 94°C for 50 s, 50°C for 1 min, and 72°C for 2 min; and, finally, 1 cycle at 72°C for 7 min. The PCR products were analyzed in ethidium bromide-stained agarose gels. A housekeeping gene encoding GAPDH was used as an internal control. The primers used to amplify GAPDH, VP1 (of EV71), and MIF [18, 19] are listed in Table 1.

Statistical Analyses

Data are expressed as mean ± standard error (SE). Differences between the test and control groups were analyzed using the Mann-Whitney test. Significance was set at P < 0.05.

Results

Confirming EV71 Infection of Immune Cells

To understand whether immune cells were permissive to EV71 infection, freshly isolated PBMC, as well as Jurkat and THP-1 cells, were infected with EV71 in vitro. One day after infection, immunofluorescence staining with anti-EV71 antibody showed EV71 antigen in the cytoplasmic region of all three types of EV71-infected cells (Fig. 1a). Jurkat cells were the most susceptible cells to EV71 infection, which showed greater than 50% of infectivity. PBMC, on the other hand, were the least susceptible cells among these three different cell types. RT-PCR further confirmed the virus replication in PBMC from EV71 negative-strand RNA detected 6 h post-infection (Fig. 1b). In addition, flow cytometry showed that both monocytes and lymphocytes in PBMC were positive for EV71 antigen, indicating that they were both susceptible to EV71 infection (Fig. 2).

Table 1. Primers used for RT-PCR

<table>
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<th>Gene (bp)</th>
<th>Sequence (5’ → 3’)</th>
<th>Product size</th>
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<td>MIF</td>
<td>TCCTTCTGCCATGCGCA</td>
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</tr>
<tr>
<td></td>
<td>TGGGCTCTTTAGGGCAGGT</td>
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</tr>
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</tr>
<tr>
<td></td>
<td>CCAATTTCACGGGTAGCGA</td>
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</tr>
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<td>GAPDH</td>
<td>CCCCCATTAGACCTCAACTA</td>
<td>400</td>
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Fig. 1. Enterovirus (EV71)-infected immune cells demonstrated using immunofluorescence and RT-PCR. (a) EV71 antigen in EV71-infected immune cells detected using immunofluorescence. Jurkat, THP-1, and human peripheral blood mononuclear cells (PBMC) (1 × 10^6 each) were incubated with EV71 at a multiplicity of infection (MOI) of 5 or with virus-free control preparations (Mock). After 24 h of incubation, the cells were stained with anti-EV71 antibody, as described in Materials and Methods, and observed under a fluorescent microscope at 400× magnification. (b) EV71 negative-strand RNA detected in EV71-infected PBMC. PBMC were infected with EV71 at an MOI of 5 for 24 h at 37°C. RNA was extracted and the expression of EV71 negative-strand RNA was analyzed using semi-quantitative RT-PCR with specific primers for EV71, as described in Materials and Methods, using 30 amplification cycles. Lane 1: Mock (uninfected), lane 2: EV71 infected PBMC.

Fig. 2. Detection of EV71 antigen in (a) monocytes and (b) lymphocytes from PBMC using flow cytometry. PBMC (1 × 10^6) were incubated with EV71 at a multiplicity of infection (MOI) of 5 or with a virus-free control medium (Mock). After 24 h of incubation, the cells were stained with anti-EV71 antibody and fluorescein isothiocyanate conjugated (FITC) secondary antibodies, as described in Materials and Methods. The lymphocyte and monocyte populations were gated and analyzed using flow cytometry.
**EV71-Infection of Human PBMC Induced TNF-α and MIF Production**

To determine whether EV71 infection of PBMC induces TNF-α and MIF production, freshly isolated human PBMC were incubated with or without EV71 (live or UV-inactivated) and the levels of TNF-α and MIF in the supernatants was measured at different time points as indicated (Fig. 3). Levels of both substances in EV71-infected PBMC increased as early as 6 h post-infection, peaked at 18 h and 24 h, respectively, and then gradually declined (Fig. 3). UV inactivation of EV71 completely abolished TNF-α production and reduced the induction of MIF production compared to live EV71 (Figs. 3 and 4). LPS stimulation, on the other hand, induced only TNF-α but not MIF production in PBMC (Fig. 4). Both TNF-α and MIF production induced in EV71-infected PBMC were inhibited in the presence of transcription inhibitor, actinomycin D (Act).

![Fig. 3](image)

**Fig. 3.** Kinetic of tumor necrosis factor (TNF)-α and macrophage migration inhibitory factor (MIF) secretion of enterovirus (EV71)-infected human peripheral blood mononuclear cells (PBMC). PBMC (1 × 10⁶) were incubated with or without EV71 (live or UV-inactivated) at a multiplicity of infection (MOI) of 5. The concentrations of TNF-α (a) and MIF (b) in the culture supernatants after different periods of incubation were assayed by ELISA as described in Materials and Methods.

**EV71 Infection of Immune Cells Induced the de novo Synthesis and Release of MIF**

To further substantiate that EV71 infection of immune cells (monocytes and lymphocytes) induced *de novo* synthesis of MIF mRNA expression, the MIF levels in the supernatants of EV71-infected Jurkat and THP-1 cells were measured using ELISA. There was a significant time-dependent increase in the levels of MIF in the supernatants of both EV71-infected Jurkat and THP-1 cells (Fig. 5a). Twenty-four hours post-infection, however, a significant amount of MIF was detected in Jurkat but not in THP-1 cells (Fig. 5a), which indicated that MIF production in Jurkat cells was faster and stronger than in THP-1 cells, even though the replication rates of EV71 in Jurkat cells and THP-1 cells were similar (data not shown).

![Fig. 4](image)

**Fig. 4.** Comparison of tumor necrosis factor (TNF)-α and macrophage migration inhibitory factor (MIF) production induced by live enterovirus (EV71), ultraviolet (UV)-inactivated EV71, and LPS. Human peripheral blood mononuclear cells (PBMC) (1 × 10⁶) were incubated with EV71 with or without actinomycin D (Act), ultraviolet (UV)-inactivated EV71 at a multiplicity of infection (MOI) of 5 for 24 h. Control cells were incubated with medium alone (C) or LPS (200 ng/ml) for 24 h. The concentrations of TNF-α and MIF in the culture supernatants after incubation were assayed using ELISA, as described in Materials and Methods.
To further understand whether MIF production was caused by a de novo synthesis of MIF RNA or the release of pre-formed cytokines stored inside cells, we analyzed the MIF mRNA in EV71-infected Jurkat cells using RT-PCR. A steady-state of MIF mRNA levels was detected in the mock-treated cells (Fig. 5b). In contrast, the expression of MIF RNA was readily induced 24 h after EV71 infection, peaked at 48 h, and declined after 72 h.

**Discussion**

The pathogenesis of EV71-induced fatalities remains unclear, even though neurogenic inflammatory response has been proposed to explain the pulmonary edema and cardiovascular collapse in these fatal cases [20]. However, extensive lymphocyte activation and cytokine production are also found in EV71 patients with pulmonary edema [9], and EV71 can be detected in their blood.

Therefore, to understand the possible function of EV71 infection of immune cells in the pathogenesis of EV71-induced fatalities, we must understand the effects of that infection. Previous studies [21] have shown that EV71 can infect human Jurkat T cells and induced apoptosis. However, whether primary immune cells are susceptible to EV71 infection is unclear. In the present study, we demonstrated that EV71 can infect both human monocyctic cell (THP-1), Jurkat T cell and fresh isolated PBMC.

EV71 infection of human immune cells induced the production of both TNF-α and MIF. Unlike TNF-α, MIF is generally constitutively expressed and stored in intracellular pools; therefore, MIF does not require de novo protein synthesis before secretion [18, 22]. In the present study, however, we found that the transcription inhibitor Actinomycin D blocked EV71-induced MIF production in PBMC. Results of RT-PCR further supported the increase of MIF RNA synthesis in EV71-infected Jurkat cells. Taken together, these results indicated that EV71 infection induced the de novo synthesis of MIF produc-
tion in human immune cells. It is known that activation of MIF gene transcription requires the participation of several transcription factor complexes such as NF-κB [22]. Whether EV71 infection-induced MIF gene expression in immune cells is NF-κB dependent requires further study.

It is well known that both TNF-α and MIF are important mediators of bacterial infection induced lethality [23, 24]. However, little is known about the roles of these two proinflammatory cytokines during viral infection. Recently, it is found that abrogation of MIF can limit West Nile virus neuroinvasion and decreases its lethality [25]. Furthermore, there is a correlation of serum levels of MIF with disease severity and clinical outcome in dengue patients [26]. Therefore, TNF-α and MIF production induced by EV71-infected immune cells may also contribute to the pathogenesis of EV71 infection. Therapeutic approaches that interfere with TNF-α and MIF production may provide an effective way to prevent disease progress in EV71 patients [27, 28].

Acknowledgments

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References


腸病毒 71 型感染人類免疫細胞引起發炎細胞激素的產生

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發炎細胞激素風暴被認為和腸病毒 71 型感染引起死亡的致病機轉有關，但對於腸病毒 71 型感染引起發炎細胞激素的機制仍不清楚。因為大多數的發炎細胞激素是由免疫細胞產生，因此在本篇我們測試腸病毒 71 型是否可以感染人類免疫細胞並產生發炎細胞激素，我們使用 RT-PCR 及免疫熒光法證實腸病毒 71 型可以感染人類 T 細胞株 (Jurkat)、巨噬細胞株 (THP-1) 和新鮮分離的周邊血液單核性細胞，並引起發炎細胞激素腫瘤壞死因子及巨噬細胞移動抑制因子的產生。經紫外線照射後不活化的腸病毒 71 型及加入 actinomycin D 皆會抑制這些發炎細胞激素的產生，RT-PCR 也進一步證實這些發炎細胞激素的產生需要新合成的 mRNA；從上述結果我們認為腸病毒 71 型感染人類免疫細胞並產生發炎細胞激素可能和腸病毒 71 型感染引起的發炎細胞激素風暴有關。

關鍵詞：發炎、細胞激素、感染

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