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Research article

Coxsackievirus B3 induces the formation of autophagosomes in cardiac fibroblasts both *in vitro* and *in vivo*

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ABSTRACT

Coxsackievirus group B (CVB) is one of the common pathogens that cause myocarditis and cardiomyopathy. Evidence has shown that CVB replication in cardiomyocytes is responsible for the damage and loss of cardiac muscle and the dysfunction of the heart. However, it remains largely undefined how CVB would directly impact cardiac fibroblasts, the most abundant cells in human heart. In this study, cardiac fibroblasts were isolated from Balb/c mice and infected with CVB type 3 (CVB3). Increased double-membraned, autophagosome-like vesicles in the CVB3-infected cardiac fibroblasts were observed with electron microscope. Punctate distribution of LC3 and increased level of LC3-II were also detected in the infected cardiac fibroblasts. Furthermore, we observed that the expression of pro-inflammatory cytokines, IL-6 and TNF-α, was increased in the CVB3-infected cardiac fibroblasts, while suppressed autophagy by 3-MA and Atg7-siRNA inhibited cytokine expression. Consistent with the *in vitro* findings, increased formation of autophagosomes was observed in the cardiac fibroblasts of Balb/c mice infected with CVB3. In conclusion, our data demonstrated that cardiac fibroblasts respond to CVB3 infection with the formation of autophagosomes and the release of the pro-inflammatory cytokines. These results suggest that the autophagic response of cardiac fibroblasts may play a role in the pathogenesis of myocarditis caused by CVB3 infection.

1. Introduction

Coxsackievirus group B (CVB) belongs to the *Enterovirus* genus within the *Picornaviridae* family [1]. CVB3 is a single stranded, positive sense RNA virus. It is among the prevalent etiologies of myocarditis that may develop to dilated cardiomyopathy, the leading cause of heart failure among young adults [2–4]. In spite of the extensive clinical and experimental investigations, the pathogenesis of CVB-induced myocarditis has not been completely elucidated. Based on the available data, acute myocarditis is the direct result of virus-induced cardiomyocyte damage or death, which in turn triggers the immune and inflammatory responses in the heart [1,2]. Although early inflammatory responses are critical for the resolution of viral infection, evidence implicated that inflammation also contributed to the severity of myocarditis and the dysfunction of the heart [2,5].

Autophagy is an evolutionarily conserved catabolic process in which eukaryotic cells remove intracellular components through lysosomal degradation to meet the energy need during starvation [6]. Autophagy also plays a central role in maintaining cellular homeostasis by recycling damaged organelles or proteins [6]. To maintain cellular homeostasis is of critical importance, especially for terminally differentiated cells such as neurons and cardiomyocytes [7]. In addition, autophagy provides an important mean for cellular defense by removing intracellular pathogens such as viruses, bacteria, and parasites [8,9]. However, evidence has also shown that autophagy is exploited by RNA viruses such as poliovirus and CVB3 to facilitate viral replication

Abbreviations: CVB, coxsackievirus B; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; ORF, open reading frame; MOI, multiplicity of infection; EGFP, enhanced green fluorescence protein; LC3, microtubule-associated protein 1light chain 3; PCR, polymerase chain reaction; 3-MA, 3-methyladenine; ERK, extracellular regulated kinase; MAVS, mitochondrial antiviral signaling protein; TRIF, Toll/IL-1 receptor domain-containing adaptor inducing interferon-beta

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[10,11].

CVB3 is a cardiotropic RNA virus that primarily targets cardiomyocytes [1,2]. CVB3 damages cardiomyocytes by inducing apoptosis, necrosis, or both [12]. The loss of cardiac muscle directly associated with the disturbed function of the heart [12,13]. Besides cardiomyocytes, heart also contains other resident cell types such as fibroblasts, endothelial cells, and macrophages [14]. Fibroblasts are the most abundant cells in human heart [15]. Studies have demonstrated that cardiac fibroblasts play a critical role in myocarditis elicited by CVB3 through secreting pro-inflammatory cytokines or chemokines [16,17]. Despite the strong association between the cardiac fibroblasts and the inflammation or remodeling of the virus-infected heart, it remains largely unknown that how CVB infection could directly impact cardiac fibroblasts.

This study showed that CVB3 infection induced a significantly increased assembly of autophagosomes in cardiac fibroblasts both *in vitro* and *in vivo*. CVB3 infection also increased the expression and release of pro-inflammatory cytokines, IL-6 and TNF-α. Moreover, down-regulation of autophagy suppressed the expression of cytokines in cardiac fibroblasts.

2. Materials and methods

2.1. Cell culture and mouse

Primary culture of cardiac fibroblasts was prepared as described previously with modifications [18]. Briefly, the hearts of 10 newborn Balb/c mice (within 24 h of birth) were harvested and fragmented into blocks in the size of 1 mm^3. The tissue blocks were digested with trypsin and collagenase II (Sigma-Aldrich) at 37 °C with repeated suspension. The separated cells in the supernatant were harvested and centrifuged at 1500 rpm for 2 min. Cell pellet was suspended with cold Dulbecco's modified Eagle's Medium (DMEM) (GIBCO) containing 15% heat-inactivated fetal bovine serum (FBS) (Biological Industries, Israel) and filtered through a 100 μm nylon strainer (BD Falcon). Cells were transferred to culture flasks (75 cm^2, Falcon) and cultured for 2 h to allow cells to adhere. The supernatant of the culture was discarded and DMEM supplemented with 15% FBS was added. Cells were cultured for 48 h at 37 °C with 5% CO_2 before passage. The second and third passage of the cardiac fibroblasts were used in this study. HeLa cells (ATCC CCL-2) were grown and maintained in DMEM supplemented with 10% FBS.

Balb/c mice were purchased from the Experimental Animal Center of Harbin Medical University, Harbin, China. All procedures involving in animals were approved by the Ethics Committee of Harbin Medical University in accordance with the Regulation for the Use of Laboratory Animals at Harbin Medical University.

2.2. Virus and viral infection

CVB3 Woodruff strain was propagated in HeLa cells and titrated by $TCID_{50}$. To infect cardiac fibroblasts, cells were seeded at 2×10^4 cells/well in a 24-well culture plate. CVB3, diluted with serum-free DMEM, was added to cells at a multiplicity of infection (MOI) of 10 and allowed to adhere for 1 h. After the adhesion, the supernatant was removed and cells were cultured in DMEM supplemented with 15% FBS. To infect mice, Balb/c mice (one-week-old) were intraperitoneally inoculated with 1×10^3 $TCID_{50}$ CVB3. The ventricular tissues were harvested at 0, 3, 5, and 7 days of post-infection (p.i.) and subjected to histopathological examination and the preparation of ultrathin section for electron microscopic observation.

2.3. Plasmids and transfection

pMKS1 was a kind gift of Professor J. Lindsay Whitton, The Scripps Research Institute, La Jolla, California, USA. pMKS1 carried the full-length genomic cDNA of coxsackievirus B3 (CVB3) Woodruff variant (GenBank U57056.1) with an artificial coding sequence of the 3C cleavage site at the beginning of viral open reading frame (ORF) [19]. pEGFP-CVB3 was constructed based on pMKS1 as described previously [20]. Plasmid pEGFP-LC3 was a kind gift of Dr. Xiaoning Si, Carestream Molecular Imaging (China). It contains the ORF of microtubule-associated protein light chain 3 (LC3) between *Bgl* II and *EcoR* I site in pEGFP-C1 (Clontech). Cardiac fibroblasts were transfected with 1 μg plasmid pEGFP-CVB3 mixed with 3 μl Lipofectamine 2000 (Invitrogen) per well in serum-free DMEM for 4 h at 37 °C. Cells were then cultured in fresh DMEM supplemented with 15% FBS. Control cells were transfected with pcDNA3.1-EGFP. Cardiac fibroblasts were transfected with Atg7 siRNA mixed with Lipofectamine 2000 for 48 h.

2.4. Real-time quantitative PCR

Total cellular RNA was extracted from cardiac fibroblasts cultured in 6-well plates by TRIzol reagents (Invitrogen). About 1 μl of RNA and the antisense primers were used for cDNA synthesis by reverse transcription using the PrimeScript RT Reagent Kit (TaKaRa, Dalian, China), and 2 μl of reverse transcription product was subjected to real-time quantitative PCR (RT-qPCR) with the SYBR Premix EX Taq II reagents (TaKaRa) in a final volume of 20 μl, following the instruction provided by the supplier. GAPDH mRNA was used as the intracellular RNA control. The cycle threshold (Ct) values were used to determine the relative levels of viral RNA and cytokine mRNA by the $2^{-\Delta\Delta Ct}$ method [21]. RT-qPCR was carried out in LightCycler 2.0 (Roche). Primers used were listed in Table 1.

2.5. Western blot

Cells were washed in PBS and lysed on ice for 15 min with Pierce RIPA buffer (Thermo) containing protease inhibitor cocktail PMSF (Beyotime, Beijing, China). Cell lysates were harvested and centrifuged at 12,000× *g* for 10 min at 4 °C. The protein concentration was determined by the Bradford assay with Bio-Rad Protein Assay Kit I (Bio-Rad, Hercules). Equal amounts of protein were loaded and separated on a 15% sodium dodecyl sulfate-polyacrylamide gel and then transferred to polyvinylidene fluoride membrane (Millipore). The membrane was blocked for 1 h with PBS containing 5% skimmed milk and 0.1% Tween 20 and then probed with primary antibodies overnight at 4 °C. After washing with PBS containing 0.1% Tween 20, the membrane was incubated with HRP-conjugated secondary antibody for 1 h at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence with SuperSignal West Pico chemiluminescent substrate (Thermo). β-Actin was used as loading control.

2.6. Immunofluorescence microscopy

Cells grown in the glass bottom culture dish (NEST, China) were washed and fixed with 4% paraformaldehyde. Cells were then permeabilized with 0.2% Triton X-100 in phosphate-buffered saline (PBS) and

Table 1
The primers for real-time PCR.

Primers	Sequences
CVB3 sense	5′-GCACACACCCTCAAACCAGA-3′
CVB3 antisense	5′-ATGAAACACGGACACCCAAAG-3′
IL-6 sense (mouse)	5′-TTCCATCCAGTTGCCTTCTT-3′
IL-6 antisense (mouse)	5′-ATTTCCACGATTTCCCAGAG-3′
TNF-α sense (mouse)	5′-GCCTCTTCTCATTCCTGCTT-3′
TNF-α antisense (mouse)	5′-CACTTGGTGGTTTGCTACGA-3′
GAPDH sense (mouse)	5′-AGGGCATCTTGGGCTACAC-3′
GAPDH antisense (mouse)	5′-CATACCAGGAAATGAGCTTGA-3′