



# Fas-FasL expression and myocardial cell apoptosis in patients with viral myocarditis

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**ABSTRACT.** The aim of the current study was to investigate Fas and FasL expression and myocardial cell apoptosis in viral myocarditis patients. Human heart specimens were selected from patients who were autopsied between February 2012 and February 2015; of these, 25 patients were diagnosed with viral myocarditis. Another 15 cases with no diagnosis of myocarditis were selected for the control group. All tissue specimens were divided into two parts, one for reverse transcription-polymerase chain reaction analysis and the other for immunohistochemical and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) analyses. *In situ* detection of apoptosis was performed by the TUNEL method, which revealed that myocardial cells from the viral myocarditis group exhibited significant apoptosis, whereas no apoptotic cells were observed in the control group. The number of cells staining positive for Fas and FasL protein in the viral myocarditis group was significantly higher than that in the control group ( $P < 0.05$ ). There was also a correlation between Fas and FasL protein

expression levels and scores ( $r = 0.92$ ,  $P < 0.05$ ). The mRNA expression of Fas and FasL was significantly higher in the viral myocarditis group than in the control group ( $P < 0.05$ ). In conclusion, the Fas-FasL system may be involved in the pathogenesis of viral myocarditis. Furthermore, cytotoxic T lymphocytes may mediate cardiac muscle cells apoptosis via Fas-FasL signaling, and thus participate in the pathogenesis of viral myocarditis.

**Key words:** Fas; FasL; Viral myocarditis; Apoptosis

## INTRODUCTION

Viral myocarditis is a common infectious form of acute or chronic heart muscle inflammation, which can be characterized as either localized or diffuse inflammatory lesions, and is highly related to viral infection (Galenkamp et al., 2015). Generally, more than 5% of individuals with a viral infection will experience some form of myocarditis during the infection period with different clinical manifestations. Though the specific cellular mechanisms underlying viral myocarditis are yet to be determined, some studies have shown that they may involve impaired myocardial cell function due to direct damage by the virus, or damage caused by the body's immune response (Abu-Dahab et al., 2014; Yang et al., 2015). Currently, treatments for viral myocarditis are mainly non-specific and focus on clearing the viral infection and reducing myocardial inflammation. Additionally, even with proper treatment, there are still patients who die due to a combination of severe arrhythmia, acute heart failure, and cardiogenic shock (Yan et al., 2015). To date, there are no clearly defined molecular mechanisms in viral myocarditis. However, it has been suggested that the Fas-FasL signaling pathway is one of the main contributors in target cell damage. Therefore, the aim of the current study was to investigate the function and possible mechanisms of cytotoxic T lymphocytes (CTLs) in Fas-FasL-mediated apoptosis during viral myocarditis. To accomplish this, apoptosis of myocardial cells from the heart tissues of patients with viral myocarditis at different periods of infection was assessed, in addition to the evaluation of Fas-FasL gene and protein expression.

## MATERIAL AND METHODS

### Patient specimens

Selected human heart specimens were obtained from the Weifang Yidu Central Hospital that were autopsied between February 2012 and February 2015. Written informed consent was provided by patient family members for all included tissue samples. In total, samples from 25 patients diagnosed with viral myocarditis (11 males and 14 females) with ages ranging from 23 to 53 years (average age of  $32.4 \pm 10.1$  years) were included. All patients met the "diagnostic criteria of acute viral myocarditis in adults" when they were admitted to the hospital according to the 1995 National Myocarditis and Cardiomyopathy Symposium. After reviewing the symptoms before death and the myocardial tissue pathological characteristics, the ventricular muscle cells were evaluated under microscopy, which revealed that the myocardial cells

showed necrosis and/or appeared degenerated, and that the interstitial space had inflammatory cell (lymphocyte or monocyte) infiltration with or without interstitial space fibrosis. Samples with diseases such as viral hepatitis, AIDS, or other autoimmune diseases were excluded. All patients had not received hormonal or immune modulators within 3 months prior to inclusion into the viral myocarditis group. In this group, 6 patients experienced myocardial infarction on the 7th day after the onset of infection, 6 patients on the 14th day after the onset of infection, 6 patients on the 21st day after the onset of infection, and 7 patients on the 28th day after the onset of infection. Samples from another 15 patients diagnosed as viral myocarditis free (8 males and 7 females) were included in the control group with patient ages ranging from 21 to 52 years (average age of  $31.6 \pm 12.8$  years). The selected patients in the control group all suffered head injuries without any heart disease or viral myocarditis related symptoms or diagnosis. There were no significant differences in age or gender between the case and control groups ( $P > 0.05$ ), which indicates strong compatibility.

### **Diagnostic criteria of viral myocarditis**

The clinical diagnostic criteria included the following: cardiac insufficiency, cardiogenic shock, or cardio-cerebral syndrome; heart enlargement; electrocardiographic changes not less than 2 ST-T wave changes in I, II, aVF, and V5 leads last longer than 4 days; creatine kinase-MB (CK-MB) increases; and/or positive cardiac troponin I (cTnI or cTnT). The etiological diagnostics included the following: utilization of the biopsy and pathology study to evaluate the endocardium, myocardium, and pericardium; use of pericardial fluid to evaluate the isolated virus; utilization of viral nucleic acid probes to check for viral nucleic acids; and/or positive specific viral antibodies.

### **Laboratory reagents**

Fas-FasL rabbit anti-human polyclonal antibody was purchased from Zhongshan Biologic Company (Beijing, China). Avidin-biotin complex (ABC) immunohistochemistry kits were purchased from Zemai Biologic Company (Shanghai, China).

### **Techniques**

All samples were divided into two parts, of which one was stored at  $-80^{\circ}\text{C}$  for later use in a reverse transcription-polymerase chain reaction (RT-PCR) assay and the other was fixed in 4% paraformaldehyde and processed for pathology assessment via hematoxylin and eosin ( $H_{\text{E}}$ ) staining, as well as for immunohistochemical analysis of protein expression and the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method for apoptosis detection.

### **TUNEL assay**

*In situ* detection of myocardial cell apoptosis was performed via the TUNEL method. Five high magnification (400X) fields of vision were imaged for each sample, and the percent (%) of myocardial cell apoptosis was calculated as the number of positive myocardial cells/total number of cardiac muscle cells x 100%.

## Immunohistochemical analysis

Immunohistochemistry methods were adopted to detect Fas-FasL antigens. Specifically, the ABC method was used for immunohistochemical detection of Fas-FasL. PBS was used as the negative control, and 3,3'-Diaminobenzidine (DAB) staining was observed under light microscopy. For each sample, three fields of visions were imaged at 400X magnification, and the absorbance of positive staining cells was measured.

## RT-PCR

TRIzol Reagent was purchased from Thermo Fisher (Carlsbad, CA, USA) to extract total RNA, and RT-PCR was performed in accordance with the manufacturer instructions. The primer sequences were as follows: Fas: 3'-ACGTGAACCATAAGACCCAG-5', 3'-GGTTCTG TGTCGTCTTGTCT-5'; FasL: 3'-TGGTCTCGGTGGTCTTGGTA-5', 3'-CGTTGTTGGTCG GGGATTTG-5'; and  $\beta$ -actin reference gene: 3'-CAAGTACCTACGGTGTCTTAAGGT-5', 3'-GCCTGACAATGACTCGACGCAAAT-5'. Amplification conditions were as follows: 95°C for 5 min, followed by 25 cycles of 95°C for 30 s, 55°C for 30 s, and 70°C for 1 min. The reaction products were then subjected to electrophoresis for 60 min in 1X Tris/Borate/EDTA (TBE) buffer at 80 V, and the bands were then observed under UV light. A DNA ladder was used as a reference standard for PCR product identification. The resultant band-densities of the PCR products were quantified with a thin layer scanner, and the relative Fas expression was calculated as the Fas product electrophoresis band-density/ $\beta$ -actin electrophoresis band-density  $\times$  100%.

## Statistical methods

The SPSS 18.0 (SPSS Inc., Chicago, IL, USA) software package was used to analyze all data, and the data are reported as means  $\pm$  standard deviation. Analysis of variance was used to process repeated measurement data, and the Fisher LSD test was used for paired comparisons. Data expressed as rates were analyzed with the  $\chi^2$  test. Linear correlation methods were used to investigate correlations.  $P < 0.05$  was considered statistically significant.

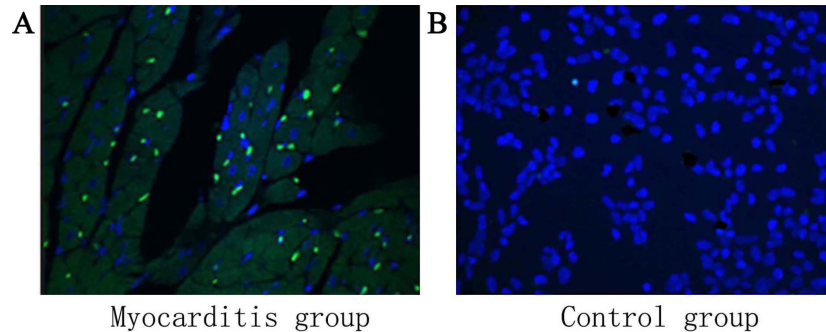
## RESULTS

### Myocardial cell apoptosis

Figure 1 shows *in situ* myocardial cell apoptosis detection via the TUNEL method, where evident cell apoptosis was observed in the cardiac muscle cells from the myocarditis group (seen as green fluorescence in Figure 1A). Cardiac muscle cell apoptosis was largely observed in the endocardium, epicardium, and around inflammation foci, whereas apoptotic cells were not seen in the control group, in which all cardiac muscle cells had normal morphology (Figure 1B).

### Immunohistochemical detection of Fas and FasL protein expression in myocardial tissue

The immunohistochemical results showed that the numbers of Fas- and FasL-positive



**Figure 1.** Myocardial cell apoptosis detection *in situ* by the TUNEL method. **A.** Myocardial cells from a patient with viral myocarditis. Green fluorescence indicates apoptosis. **B.** Myocardial cells from a patient without myocarditis. No apoptosis is observed. Magnification = 400X.

cells in the myocarditis group were significantly higher than those in the control group ( $P < 0.05$ ). Furthermore, the numbers of Fas- and FasL-positive cells were significantly higher on the 7th day after the onset of infection compared with those in controls, peaked on the 14th day, and then began to decrease thereafter with statistically significant differences among the groups ( $F = 35.214$ ,  $P < 0.01$ ;  $F = 55.021$ ,  $P < 0.01$ ). There was also a correlation between Fas and FasL protein expression levels and score ( $r = 0.92$ ,  $P < 0.05$ ) (Table 1).

**Table 1.** Immunohistochemical analysis of relative Fas and FasL protein expression in myocardial tissue.

Group		Fas	FasL	Score
Control group		$0.054 \pm 0.002$	$0.035 \pm 0.001$	$0.000 \pm 0.000$
Myocarditis group	7th day	$0.107 \pm 0.001^*$	$0.106 \pm 0.002^*$	$2.143 \pm 0.002$
	14th day	$1.320 \pm 0.002^*$	$1.024 \pm 0.001^*$	$2.952 \pm 0.001$
	21th day	$0.145 \pm 0.002^*$	$0.136 \pm 0.002^*$	$2.165 \pm 0.002$
	28th day	$0.112 \pm 0.001^*$	$0.107 \pm 0.001^*$	$2.031 \pm 0.002$
F value		35.214	55.021	
P value		<0.01	<0.01	

\* $P < 0.05$ : compared to the control group.

### RT-PCR detection of Fas and FasL mRNA expression in myocardial tissues

The results revealed that Fas and FasL mRNA expression were significantly higher in the myocarditis group than those in the control group ( $P < 0.05$ ). Furthermore, Fas and FasL mRNA expression were significantly higher on the 7th day after the onset of infection compared with those of controls, peaked in the 14th day, and then began to decrease thereafter with statistically significant differences among the groups ( $F = 25.13$ ,  $P < 0.05$ ;  $F = 27.78$ ,  $P < 0.05$ ) (Table 2).

**Table 2.** RT-PCR analysis of relative Fas and FasL mRNA expression in myocardial tissues.

Group		Fas mRNA	FasL mRNA
Control group		$0.31 \pm 0.01$	$0.21 \pm 0.00$
Myocarditis group	7th day	$0.40 \pm 0.00^*$	$0.33 \pm 0.00^*$
	14th day	$0.63 \pm 0.02^*$	$0.52 \pm 0.01^*$
	21th day	$0.42 \pm 0.02^*$	$0.44 \pm 0.03^*$
	28th day	$0.41 \pm 0.00^*$	$0.35 \pm 0.00^*$
F value		25.13	27.78
P value		<0.05	<0.05

\* $P < 0.05$  compared to the control group.

## DISCUSSION

Viral myocarditis is a complex virus-induced non-specific interstitial myocardial inflammatory disease that is associated with a variety of pathogenic factors (Ebsen et al., 2015). In recent years, there has been a marked increase in the incidence of the disease, and the symptoms are sometimes undetectable, which may result in sudden cardiac death and increase the level of harm (Kong et al., 2014; Chiu et al., 2015; Li et al., 2015). Studies investigating the pathogenesis of viral myocarditis and its underlying mechanisms may lead to effective control of its occurrence and provide a basis for improved treatments. However, the pathogenesis of viral myocarditis has yet to be fully defined. Previous studies have determined that the main factors involved in the pathogenesis may be impairment of myocardial cell functions due to direct viral damage, altered immune cell biochemistry, or microvascular injury. However, to date, there have been few reports regarding the specific pathogenesis of viral myocarditis. The clinical manifestations of viral myocarditis usually occur after acute viral infection (Cohen et al., 2014; Lee et al., 2015; Zhong et al., 2015). The main manifestation of viral myocarditis during the first 7 days of viral infection is myocardial infiltration by NK cells and macrophages. Though they are meant to control the early stages of viral infection, infiltrating NK cells release perforin, which damages myocardial cells (Li et al., 2014; Lutz et al., 2014; Hsiao et al., 2015; Künkele et al., 2015; Peng et al., 2015). Between 7 and 14 days after the initial onset of the viral infection, most of the infiltrated cells are T-cells, which become CTLs when activated, and are programmed to kill target cells (Chen et al., 2014; Grygorczuk et al., 2015; Shi et al., 2015; Tao et al., 2015). There are two primary means by which CTLs kill target cells: one is perforin/granzyme-mediated and the other involves the Fas-FasL signaling pathway (Arai et al., 2014; Gmeiner et al., 2015; Liu et al., 2015; Nallapalle et al., 2015; O'Donnell et al., 2015; Saigusa et al., 2015). Through the presentation of Fas by CTLs to FasL on target cells, Fas-FasL signaling passes programmed cell death signals to myocardial cells, which leads to apoptosis in a relatively short time frame (Fernandes et al., 2014; Nabhani et al., 2014; O'Reilly et al., 2015; Zhang et al., 2015). Additionally, Fas-FasL signaling also has an associated negative feedback mechanism, which showed no sustained damage to myocardium (Estlack et al., 2014; Zheng et al., 2014).

The current study investigated Fas and FasL expression and apoptosis in myocardial cells from patients with viral myocarditis at different time points after the onset of infection. The results showed that the numbers of cells staining positive for Fas and FasL protein in the viral myocarditis group were significantly higher than those in the control group ( $P < 0.05$ ). The numbers significantly increased on the 7th day after the onset of infection, peaked on the 14th day, and then decreased thereafter, with statistically significant differences seen among the groups. Furthermore, there was a correlation between both Fas and FasL protein expression levels and scores. These results indicate that there is a correlation between the expression of Fas and FasL protein in cardiomyopathies, and specifically that the Fas-FasL system may be largely involved in the pathogenesis of viral myocarditis. In agreement with these results, the expression of Fas and FasL mRNA in the viral myocarditis group was significantly higher than that in the control group ( $P < 0.05$ ). Similar to the protein, mRNA expression was significantly increased on the 7th day after the onset of infection, peaked on the 14th day, and then began to decrease with statistically significant differences among the groups. Taken together, the results herein indicate that myocardial cell apoptosis may be significantly influenced by the



combination of Fas on myocardial cell surfaces and FasL on infiltrating immune cell surfaces, and that CTLs may mediate cardiac muscle cell apoptosis through Fas-FasL signaling, thus contributing to viral myocarditis pathogenesis.

To summarize, the Fas-FasL system may be involved in the pathogenesis of viral myocarditis. CTLs may mediate cardiac muscle cell apoptosis via Fas-FasL signaling; thus, they may participate in the pathogenesis of viral myocarditis.

### Conflicts of interest

The authors declare no conflict of interest.

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