

Fas/FasL induces myocardial cell apoptosis in myocardial ischemia-reperfusion rat model

X.-M. LIU¹, Z.-M. YANG², X.-K. LIU¹

¹Department of Cardiology, Tangshan Gongren Hospital, Tangshan, Hebei, China

²Department of Orthopedic Trauma, The Second Hospital of Tangshan, Tangshan, Hebei, China

Abstract. – **OBJECTIVE:** Myocardium ischemia reperfusion is easy to induce myocardial injury. Fas/FasL is an important signaling pathway mediating cell apoptosis. This study aims to analyze the cell apoptosis and Fas/FasL expression in myocardial cell ischemia reperfusion rat model.

MATERIALS AND METHODS: Coronary artery ligation method was used to establish myocardial ischemia reperfusion model. Rats were grouped according to different ischemia and reperfusion time: Group A, myocardial ischemia for 30 min and reperfusion for 24 h; Group B, myocardial ischemia for 30 min and reperfusion for 48 h; Group C, myocardial ischemia for 1 h and reperfusion for 24 h. Myocardial injury indicators were tested. Myocardial cell apoptosis was detected by transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) assay. Fas and FasL mRNA and protein expressions were evaluated by Real-time PCR (RT-PCR) and Western blot.

RESULTS: Creatine kinase (CK), lactic dehydrogenase (LDH), and malondialdehyde (MDA) significantly elevated, while superoxide dismutase (SOD) obviously declined in the experimental group compared with control and blank group ($p < 0.05$). CK, LDH, and MDA gradually upregulated, whereas SOD was reduced in experimental groups following the time extension of ischemia and reperfusion ($p < 0.05$). Apoptosis cell number was markedly higher in the experimental group compared with control and blank group ($p < 0.05$). Apoptosis cell number gradually increased in the experimental groups following ischemia and reperfusion time extension ($p < 0.05$). Fas/FasL mRNA and protein markedly upregulated in the experimental group compared with control and blank group ($p < 0.05$). Fas/FasL mRNA and protein expressions enhanced in experimental groups following the time extension of ischemia and reperfusion ($p < 0.05$).

CONCLUSIONS: Fas/FasL induces myocardial cell apoptosis in the process of myocardium ischemia reperfusion in rat model.

Key Words:

Fas, FasL, Ischemia reperfusion, Myocardium, Apoptosis.

Introduction

Numerous researches suggest that metabolic disorder may appear or aggravate in ischemic tissue even after timely blood reperfusion, named ischemia-reperfusion injury¹. Ischemia-reperfusion injury is based on the ischemic damage. Reperfusion aggravates the reversible ischemic damage, leading to the injury irreversible². The heart is the firstly discovered organ for ischemia-reperfusion injury. Further in-depth investigations showed that the whole body organ, such as brain, kidney, intestine, lung, and skin, can suffer from ischemia-reperfusion injury^{3,4}. Previous studies^{3,4} pointed out that the process of ischemia-reperfusion injury can cause myocardial cell death, which includes necrosis and apoptosis. Among them, cell apoptosis is an important process in myocardial cell ischemia-reperfusion injury. Inhibiting apoptosis can reduce the area of myocardial infarction⁵. A basic research proposed that during ischemia-reperfusion injury, a large number of oxygen free radicals appear, intracellular calcium level significantly increases, numerous neutrophils infiltrate, and mitochondria is damaged. All of these pathological changes promote the myocardial cell apoptosis⁶. The occurrence and development of cell apoptosis are regulated by multiple signaling pathways. Fas/FasL signaling pathway is close related to myocardial cell apoptosis. Fas, known as Apo1 or CD95, is a transmembrane glycoprotein that is widely distributed in a variety of cell surfaces. It is homologous with tumor necrosis factor receptor and nerve growth factor receptor. FasL, the ligand of Fas, is a kind of type II transmembrane glycoprotein⁷. It is proved that Fas/FasL system participates in the occurrence and development of various cardiovascular diseases⁸. This study analyzed cell apoptosis and Fas/FasL expression in myocardial cell ischemia reperfusion via constructing rat myocardial ischemia-reperfusion model.

Materials and Methods

Experimental Animals

A total of 50 male Wistar rats at 8-week old and 180±20 g were provided by Chinese Academy of Military Medical Sciences Laboratory Animal Center. Rats were raised under specific pathogen free (SPF) with standard fodder and water. Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Tangshan Workers' Hospital.

Reagents and Instruments

Transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) kit was purchased from Roche (Catalogue No. 11684817910; Basel, Switzerland). Rabbit anti-rat Fas polyclonal antibody (Catalogue No. sc-7886; 1:2000) and rabbit anti-rat FasL polyclonal antibody (Catalogue No. sc-6237; 1:2000), horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (Catalogue No. sc-2004; 1:2000), and RT-PCR kit (Catalogue No. sc-43500) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Super-cleavable worktable was from Formal (Shanghai, China). Centrifugal machine was from Flying Pigeon (Tianjin, China). Inverted microscope was from Olympus (Tokyo, Japan). Desktop thermostatic oscillator was from Jinghong (Shanghai, China).

Rat Myocardial Ischemia-Reperfusion Model Establishment

Coronary artery ligation method was used to establish myocardial ischemia reperfusion model [9]. The rat was anesthetized by urethane 800 mg/kg intraperitoneal injection. Next, the rat received endotracheal intubation and artificial ventilation under electrocardiogram monitoring. The chest was opened to expose the heart, and the thread was passed through the left coronary artery. Another two silk threads were drawn from the knot to loosen the ligation. After ligating the left coronary artery, electrocardiogram (ECG) showed ST-segment elevation and local myocardium appeared cyanosis. The ligation was loosened after a specified period to restore coronary permeability, leading to reperfusion. At last, the chest was closed to restore animal spontaneous breathing.

Grouping

Rats were randomly divided into 5 groups with 10 in each group. Experimental group: rats were divided into 3 groups according to different ischemia and reperfusion time, Group A, myocardial ische-

mia for 30 min and reperfusion for 24 h, Group B, myocardial ischemia for 30 min and reperfusion for 48 h, and Group C, myocardial ischemia for 1 h and reperfusion for 24 h. Control group: the rat was anesthetized by urethane to expose the heart. The thread was passed through the left coronary artery without ligation. Blank group: The healthy rat received conventional feeding.

Myocardial Damage Indicators Detection

Serum creatine kinase (CK) and lactic dehydrogenase (LDH) contents were tested by colorimetry assay on JH-752 ultraviolet and visible spectrophotometer. Superoxide dismutase (SOD) activity was evaluated by xanthine oxidase method. Malondialdehyde (MDA) content was determined by barbituric acid method.

TUNEL Assay

The rat was sacrificed and the heart was taken out. The tissue between infarction and non-infarction areas was fixed and cut into 1 mm × 1 mm × 1 mm. After dehydration, paraffin embedding, and section, the tissue was stained by TUNEL and rinsed by xylene. After ethanol gradient washing, the tissue was treated by proteinase K. Next, the tissue was added to 50 µl TUNEL reaction mixture to count the apoptotic cell. Then the tissue was added to 50 µl converter-POD, 50-100 µl diaminobenzidine (DAB) substrate, and hematoxylin or methyl green. Cells were photographed and counted.

Western blot

A total of 40 µg protein was separated by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blocked at room temperature for 1 h. Then the membrane was incubated with Fas and FasL antibodies (1:1200) at 4°C overnight. After a secondary antibody was added for 1 h, and the membrane was developed.

RT-PCR

Total RNA was extracted using TRIzol and quantified by D260 nm/D280 nm. The RNA was synthesized for the poly A tail of miRNA and reverse transcribed to cDNA. The primers used for PCR were listed in Table I. PCR reaction was composed of 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. U6 was selected as internal reference.

Statistical Analysis

SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was adopted for data analysis. Measurement

Table I. Primer sequence.

Gene		Sequence (5'-3')
Fas mRNA	Forward	5'-AGCCGTCAAGAGCAATAACGAA-3'
	Reverse	5'-GTGCAGGGTCCGAGGT-3'
FasL mRNA	Forward	5'-TGGTGGGCAGTATGTTGT-3'
	Reverse	5'-GCTATTGGCATTGGTGAA-3'
U6	Forward	5'-CTCGCTTCGGCAGCACACA-3'
	Reverse	5'-AACGCTTCACGAATTTGCGT-3'

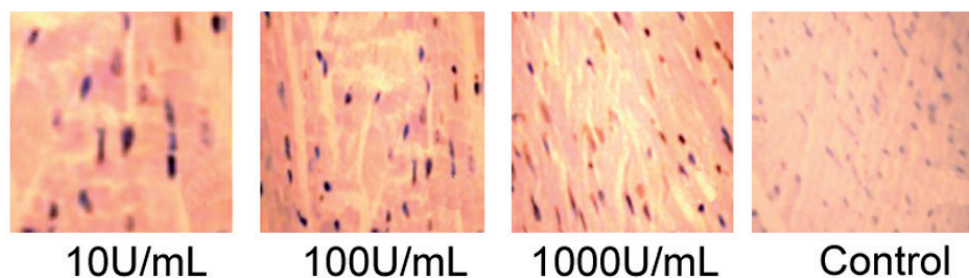


Figure 1. Myocardial cell apoptosis.

data was depicted as mean ± standard deviation and compared by analysis of variance (ANOVA). Enumeration data was compared by χ^2 test. $p < 0.05$ was considered as statistical significance.

Results

Myocardial Injury Indicators Changes

CK, LDH, and MDA significantly elevated, while SOD obviously declined in the experimental group compared with control and blank group ($p < 0.05$). CK, LDH, and MDA gradually upregulated, whereas SOD gradually reduced in experimental groups following the time extension of ischemia and reperfusion ($p < 0.05$) (Table II).

Myocardial Cell Apoptosis

A total of 1000 cells were counted in each group. Apoptosis cell number was significantly higher in experimental group compared with control and blank group ($p < 0.05$). Apoptosis cell number gradually increased in experimental groups following ischemia and reperfusion time extension ($p < 0.05$) (Table III, Figure 1).

Fas and FasL Protein Expressions

Fas/FasL protein markedly upregulated in the experimental group compared with control and blank group ($p < 0.05$). Fas/FasL protein expression enhanced in experimental groups following the time extension of ischemia and reperfusion ($p < 0.05$) (Table IV, Figure 2).

Table II. Myocardial injury indicators changes.

Group	Cases	CK (U/L)	LDH (U/L)	SOD (U/g)	MDA (nmol/g)
Experimental group	30				
Group A	10	3011±589 ^{*#}	2142±313 ^{*#}	11285±3054 ^{*#}	1962±336 ^{*#}
Group B	10	3568±612 ^{*#&}	2562±405 ^{*#&}	10024±2096 ^{*#&}	2204±341 ^{*#&}
Group C	10	3923±708 ^{*#&@}	2962±412 ^{*#&@}	9867±924 ^{*#&@}	2652±383 ^{*#&@}
Control	10	2026±783	1036±348	19242±3425	1785±235
Blank group	10	1912±205	924±134	19785±3156	1512±202

^{*} $p < 0.05$, compared with control. [#] $p < 0.05$, compared with blank group. [&] $p < 0.05$, compared with group A. [@] $p < 0.05$, compared with group B. CK: Creatine kinase, LDH: lactic dehydrogenase, MDA: malondialdehyde, SOD: superoxide dismutase.

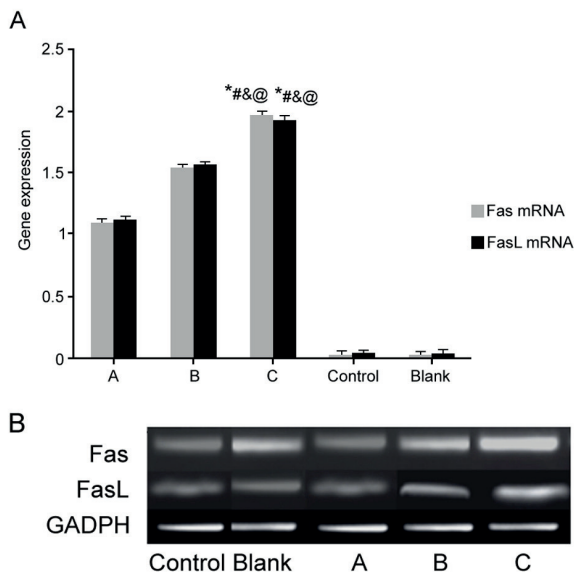


Figure 3. Fas and FasL mRNA expressions in myocardial cells. * $p < 0.05$, compared with control. # $p < 0.05$, compared with blank group. & $p < 0.05$, compared with group A. @ $p < 0.05$, compared with. with group B.

Fas and FasL mRNA Expressions

Fas/FasL mRNA markedly up-regulated in the experimental group compared with control and blank group ($p < 0.05$). Fas/FasL mRNA expression enhanced in experimental groups following the time extension of ischemia and reperfusion ($p < 0.05$) (Figure 3).

Discussion

Ischemia-reperfusion injury refers to the more serious and larger area damage of myocardial cells after ischemia and blood reperfusion¹⁰. Apoptosis, or programmed cell death, is a type of

Table III. Myocardial cell apoptosis detected by TUNEL assay.

Group	Cases	Apoptotic cell number
Experimental group		
Group A	10	11.18±1.02*#
Group B	10	17.97±1.44*#&
Group C	10	21.24±2.45*#&@
Control	10	3.01±1.28
Blank group	10	3.12±1.23

* $p < 0.05$, compared with control. # $p < 0.05$, compared with blank group. & $p < 0.05$, compared with group A. @ $p < 0.05$, compared with group B. TUNEL: transferase-mediated deoxyuridine triphosphate-biotin nick end labeling.

physiological phenomena during body development. Insufficient or enhanced apoptosis under some pathological conditions may result in tissue damage and dysfunction^{11,12}. Fas/FasL signaling pathway is one of the signaling pathways that directly trigger apoptosis. It is closely related to the occurrence, development, treatment, and prognosis of various diseases¹³. This study explored the impact of Fas/FasL system on myocardial cell apoptosis via constructing rat myocardial ischemia-reperfusion model. Coronary artery ligation method was used to establish myocardial ischemia reperfusion model. Rats were grouped according to different ischemia and reperfusion time: Group A, myocardial ischemia for 30 min and reperfusion for 24 h; Group B, myocardial ischemia for 30 min and reperfusion for 48 h; Group C, myocardial ischemia for 1 h and reperfusion for 24 h. Myocardial injury indicators were tested. CK, LDH, and MDA significantly elevated, while SOD obviously declined in the experimental group compared with control and blank group. CK, LDH, and MDA gradually upregulated, whereas SOD gradually reduced in experimental

Table IV. Fas and FasL protein expressions in myocardial cells.

Group	Cases	Fas	FasL
Experimental group			
Group A	10	5.02±1.13*#	4.62±1.69*#
Group B	10	7.36±1.57*#&	6.23±2.08*#&
Group C	10	9.15±2.89*#&@	8.76±2.54*#&@
Control	10	1.13±0.15	1.12±0.48
Blank group	10	1.07±0.25	1.08±0.67

* $p < 0.05$, compared with control. # $p < 0.05$, compared with blank group. & $p < 0.05$, compared with group A. @ $p < 0.05$, compared with group B.

groups, following the time extension of ischemia and reperfusion. It suggested that myocardial injury indicators obviously increased in ischemia-reperfusion rat. The heart tissue was obtained to test myocardial cell apoptosis. Apoptosis cell number was markedly higher in the experimental group compared with control and blank group. Apoptosis cell number gradually increased in experimental groups following ischemia and reperfusion time extension. Myocardial cell apoptosis is rare in normal rat. Myocardial cell apoptosis increased in ischemia-reperfusion rat, and mainly distributed in apex cordis. Numerous myocardial cell apoptosis led to cell loss, thus affecting myocardial contraction and resulting in cardiac dysfunction^{14,15}. Our results indicated that myocardial cell apoptosis participated in the occurrence and development of ischemia-reperfusion heart injury. *In vitro* cardiac ischemia-reperfusion model revealed that myocardial cells appeared apoptosis at 10 min after ischemia and reached peak at 30 min. It suggested that myocardial ischemia may induce cell apoptosis¹⁶. *In vivo* cardiac ischemia-reperfusion model demonstrated that both sustained ischemia for 2 h and ischemia for 45 min followed by reperfusion for 1 h can lead to cell apoptosis. Apoptotic cell number increased following reperfusion time extension¹⁷, which was similar to our results. To explore the role of Fas/FasL signaling pathway in myocardial ischemia-reperfusion, this work tested Fas and FasL expressions in myocardium. Fas/FasL protein markedly upregulated in the experimental group compared with control and blank group. Fas/FasL protein expression enhanced in experimental groups following the time extension of ischemia and reperfusion. Further gene analysis showed that Fas/FasL mRNA markedly upregulated in the experimental group compared with control and blank group. Fas/FasL mRNA expression enhanced in experimental groups following the time extension of ischemia and reperfusion. It confirmed that Fas/FasL upregulated in the myocardial cells under ischemia-reperfusion. Fas/FasL overexpressed in myocardial cells of *in vitro* ischemia-reperfusion model¹⁸. It was found that apoptotic cells accounted for 90% when myocardial infarction sustained for 2 h, and their level and Fas protein expression gradually increased following time extension¹⁹. Apoptotic index (AI) elevated in ischemia-reperfusion rat compared with in lymphedema rats. Apoptotic cells mainly exist in the marginal zone of infarction. Fas plays a critical role in the process of myo-

cardium ischemia-reperfusion²⁰⁻²³, which is in accordance with our study.

Conclusions

Myocardial cell apoptosis enhanced in cardiac ischemia-reperfusion following ischemia and reperfusion time extension. Cell apoptosis and Fas/FasL system involved in the pathogenesis of cardiac ischemia-reperfusion. Blocking cell apoptosis or Fas/FasL system may be a new approach for the prevention and treatment of cardiac ischemia-reperfusion.

Conflict of interest

The authors declare no conflicts of interest.

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