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IGF-1 affects the development of myocarditis in LDL-R knockout mice by inhibiting peritoneal infiltration of macrophages

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Abstract. – OBJECTIVE: Infiltration of the inflammatory cells, such as macrophages and myocardial necrosis, are involved in myocarditis. Insulin-like growth factor (IGF-1) exerts a variety of biological effects. However, the role of IGF-1 in myocarditis remains unclear.

PATIENTS AND METHODS: LDL-R knockout mice were randomly divided into the control group, myocarditis group, and IGF-1 siRNA group. Real Time-Polymerase Chain Reaction (PCR) and Western blot were used to measure IGF-1 expression in myocardial tissue. The myocardial tissue changes were analyzed by HE staining. The total cholesterol (TC) and low-density lipoprotein cholesterol (LDL) in each group were detected. Flow cytometry was used to analyze the number of macrophages. The secretion of TNF-α and INF-γ and macrophage migration inhibitory factor (MIF) were detected by enzyme-linked immunosorbent assay (ELISA).

RESULTS: Compared with the control group, IGF-1 expression, TC, and LDL in myocarditis group was significantly increased, along with decreased heart rate (HR), left ventricular end-systolic diameter (LVEDs), left ventricular end-diastolic diameter (LVEDd), and left ventricular mass index (LVMI). In addition, inflammatory cell infiltration, fibrosis, and macrophages number in the peritoneum were increased. Moreover, the secretion of TNF-α, INF-γ, and MIF was also significantly increased ($p<0.05$). However, IGF-1 siRNA treatment inhibited IGF-1 expression and reversed the changes in the myocarditis group with statistically significant differences compared with the myocarditis group ($p<0.05$).

CONCLUSIONS: IGF-1 expression is increased in myocarditis. The downregulation of IGF-1 expression inhibits macrophages infiltration, reduces the expression of MIF and inflammatory factors, and improves myocarditis injury.

Key Words:
IGF-1, Myocarditis, Macrophage, Inflammatory factor, MIF.

Introduction

Myocarditis is one of the common diseases in cardiology. It can be caused by bacterial, viral, and fungal infections, autoimmune disorders, drugs, and other factors. Myocarditis caused by viruses is more common[1,2]. With the accelerated pace of life and increased work pressure, the population's immunity is reduced, the incidence of viruses and other infections is increased, the incidence of myocarditis is significantly increased, which is more common in young people[3,4]. Myocarditis is mainly caused by localized or diffuse inflammatory cell infiltration in the myocardial interstitial, resulting in the degeneration and necrosis of myocardial cells, myocardial fiber necrosis or degeneration, which affects cardiac function, leading to the occurrence of severe arrhythmia, cardiogenic shock, heart failure, and even sudden death[5,6]. Viral myocarditis can be caused by a variety of cardiomyogenic infections, causing non-specific inflammatory reactions, which accounts for the vast majority of myocarditis. Among them, Coxsackievirus group B type 3 (CVB3) infection is an important infection virus, accounting for more than half of the causes of viral myocarditis[7,8]. Infiltration of inflammatory cells, such as macrophages and myocardial necrosis, are important features of viral myocarditis[9]. Due to the concealed symptoms of viral myocarditis, a lack of typical clinical symptoms leads to easy delay in treatment, and the pathogenesis of viral myocarditis is still unclear, leading to conservative treatment and poor treatment efficacy[10,11].

However, the role and mechanism of insulin-like growth factor (IGF-1) in myocarditis has not been reported. IGF-1 is a member of the insulin family, a highly similar family of insulins, a single-chain polypeptide consisting of 70 amino

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acid residues[12]. IGF-1 binds to its receptors and exerts a variety of biological effects, including regulation of metabolism, cell differentiation, and proliferation, as well as vasoconstriction and diastolic function[13,14]. IGF-1 is widely distributed in the cardiovascular system and can exert cardiac regulation through autocrine or paracrine. It is also called cardiogenic hormone, and IGF-1 is associated with the occurrence and development of various cardiovascular diseases[15,16]. However, the role and mechanism of IGF-1 in myocarditis has not been reported.

Materials and Methods

Experimental Animals

Healthy male LDL-R knockout mice, aged 6-8 weeks, weighed 20 g ± 5 g, SPF grade, were purchased from the experimental animal center of the unit and fed in SPF animal experiment center with a temperature of 21 ± 1°C and relative humidity of 50-70% under constant temperature and constant humidity conditions, ensuring a 12/day cycle every 12 h. Animal experiments are performed in strict accordance with the experimental design and performed by experienced technicians to minimize animal suffering. This study was approved by the Ethics Committee of our hospital.

Main Reagents and Instruments

Coxsackie virus CVB3 is supplied by the laboratory and stored in liquid nitrogen. The MIF ELISA test kit, INF-γ, and TNF-α ELISA test kits were purchased from eBioscience (San Diego, CA, USA). Rabbit anti-mouse F4/80 monoclonal antibody and rabbit anti-mouse CD11c antibody were purchased from American BD Company (San Jose, CA, USA). IGF-1 siRNA was designed and synthesized by the Shanghai Jikai Gene Company (Shanghai, China). The polyvinylidene difluoride (PVDF) membrane was purchased from Pall Life Sciences (Port Washington, NY, USA), the Western blot related chemical reagent was purchased from Shanghai Biyuntian Biotechnology Co., Ltd. (Shanghai, China), the enhanced chemiluminescence (ECL) reagent was purchased from Amersham Biosciences (Piscataway, NJ, USA), the rabbit anti-mouse IGF-1 monoclonal antibody, and the goat anti-rabbit horseradish peroxidase (HRP) labeled IgG secondary antibody was purchased from Cell signaling Corporation of the United States (Danvers, MA, USA). The Medlab-U automatic biochemical analyzer and M-type ultrasound system were purchased from Nanjing Meiyi Technology Co., Ltd (Nanjing, China). Other commonly used reagents were purchased from Shanghai Shenggong Biological Co., Ltd (Shanghai, China). ABI7900 HT real time-PCR was purchased from ABI (Foster City, CA, USA). Surgical microscopy equipment was purchased from Suzhou Medical Instrument Factory (Suzhou, China). The Labsystem Version 1.3.1 microplate reader was purchased from Bio-Rad Corporation (Hercules, CA, USA). The Melody C6 flow cytometer was purchased from BD Corporation (San Jose, CA, USA).

Grouping and Processing of Experimental Animals

Thirty experimental LDL-R knockout mice were randomly divided into 3 groups, with n=10 in each group, control group, myocarditis group, in which mice were infected with CVB3 virus according to the literature[17], followed by intraperitoneal injection of 0.2 ml 2×10^5 PFU of CVB3 virus PBS solution; IGF-1 siRNA group, in which, based on the myocarditis group model, IGF-1 siRNA 5×10^5 IU/ml was injected into the tail vein.

Evaluation of Heart Function Index of Each Group of Mice

M-mode ultrasound was used to evaluate cardiac function changes, left ventricular mass index, ventricular contraction, and diastolic diameter in each group after 48 hours of onset. The rats in the supine position were fixed, and the 15L8 ultrasound probe was placed in the right hand. The ultrasound probe was placed next to the sternum of the rat, and the horizontal section of the left ventricular short-axis papillary muscle was taken. After obtaining a clear 2D image, the M-mode echocardiogram was converted, and the trajectory was rotated to measure HR, LVEDs, LVED, and LVMI.

Specimen Collection

5 ml peripheral blood was collected at 48 h after onset, centrifuged at 3000 rpm for 15 min, and placed in a -80 °C refrigerator, and stored. The mice were sacrificed, the heart was removed, and part of the heart tissue was fixed with formaldehyde. Part of the heart tissue was quickly frozen in liquid nitrogen for 2 h, and then stored in a -80 °C refrigerator for later use.

ELISA Detection of TNF-α, INF-γ, MIF Secretion

The changes of TNF-α, INF-γ, and MIF secretion in the serum of each group were detected by ELISA according to the ELISA kit instructions. The linear regression equation of the standard curve is calculated according to the concentration of the standard product and the corresponding absorbance value (A value), and the corresponding sample concentration is calculated on the regression equation according to the A value of the sample.

Western Blot Analysis of IGF-1 Protein Expression

The myocardial tissue proteins of each group were extracted: the lysate was added, the cells were lysed on ice for 15-30 min, the cells were disrupted by sonication for 5 s × 4 times, centrifuged at 4°C, 10 000 × g for 15 min, and the supernatant was transferred to a new Eppendorf (EP; Hamburg, Germany) tube. The Bradford method was used to quantify the protein, which was stored at -20°C for Western blot experiments. The isolated protein was electrophoresed on a 10% SDS-PAGE, transferred to a PVDF membrane by a semi-dry transfer method at 150 mA for 1 h to remove the non-specific background, blocked with 5% skim milk powder for 2 h and incubated with 1:2000 dilution of IGF-1 monoclonal antibody at 4°C overnight. After PBST washing, 1:2000 diluted goat anti-rabbit secondary antibody was added and incubated for 30 min under the dark followed by washing with Phosphate-Buffered Saline with Tween 20 (PBST), chemiluminescence Color development for 1 min, and subsequent X-slice exposure imaging. X-film and strip density measurements were separately scanned using protein image processing system software and Quantity one software. The experiment was repeated four times (n=4).

Real Time-PCR Detection of IGF-1 mRNA Expression

The total RNA was extracted using TRIzol reagent, and DNA reverse transcription synthesis was performed according to the kit instructions. The primers were designed by PrimerPremier 6.0 according to each gene sequence and synthesized by Shanghai Yingjun Biotechnology Co., Ltd. (Shanghai, China) (Table I). Real time-PCR was performed for detection of the gene of interest with reaction conditions as follows: 92°C 30 s, 58°C 45 s, 72°C 35 s, a total of 35 cycles. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference. According to the fluorescence quantification, the starting cycle number (CT) of all samples and standards was calculated. Based on the standard CT value, a standard curve was drawn and then, the semi-quantitative analysis was carried out by the $2^{-\Delta Ct}$ method.

Analysis of Blood Lipid Index

The expression of TC and LDL in the blood lipid index of each group was analyzed by the automatic biochemical analyzer.

HE Staining Analysis of Myocardial Tissue Changes

The myocardial tissue of each group was prepared into 4 μm paraffin section specimens, dried at 37°C overnight, dewaxed with gradient xylene, dyed with hematoxylin staining solution for 4-8 min, differentiated with 1% hydrochloric acid aqueous solution for 5-10 s, 0.5% hematoxylin (water soluble) staining for 30 s, gradient ethanol dehydration, xylene transparent, neutral gum for sealing, and observation.

Flow Cytometry Analysis of Peritoneal Infiltration Macrophage Changes

The peritoneal tissue was collected, and the infiltrating cells were obtained by EDTA trypsinization and centrifugation. The adherent cells were washed once with pre-cooled PBS, digested, transferred to a centrifuge tube, centrifuged at 1000 g for 5 min, and the supernatant was discarded. The cells were resuspended in 100 μl Binding buffer, and we gently mixed the cells. After incubation at room temperature for 15 min under dark, 300 μl × Binding buffer was added, mixed, followed by the addition of 5 μl anti-F4/80

Table I. Primer sequences.

Gene	Forward 5′-3′	Reverse 5′-3′
GAPDH	ACCAGGTATCTTGGTTG	TAACCATGTCAGCGTGGT
IGF-1	AGCTTTGCAGTGCCTTG	CAGCAGACGTTTAGC

and 5 ul anti-CD11c antibody for 5 min and subsequent detection of macrophages by flow cytometry.

Statistical Analysis

Data were processed using the Statistical Product and Service Solution (SPSS Inc., Chicago, IL, USA) 19.0 software. Measurement data were expressed as mean ± standard deviation (SD), and the comparison of the multiple groups of samples was performed by One-way analysis of variance with Bonferroni post-hoc analysis. $p < 0.05$ indicates statistical difference.

Results

Expression of IGF-1 in Myocarditis

The expression of IGF-1 in myocarditis of LDL-R knockout mice was analyzed by real time-PCR and Western blot, respectively. The results showed that the expression of IGF-1 mRNA and protein was significantly increased in the model of myocarditis in LDL-R knockout mice compared with that in the control group ($p < 0.05$). Injection of the IGF-1 siRNA significantly inhibited IGF-1 mRNA and protein expression. Compared with the myocarditis group, the difference was statistically significant ($p < 0.05$) (Figure 1).

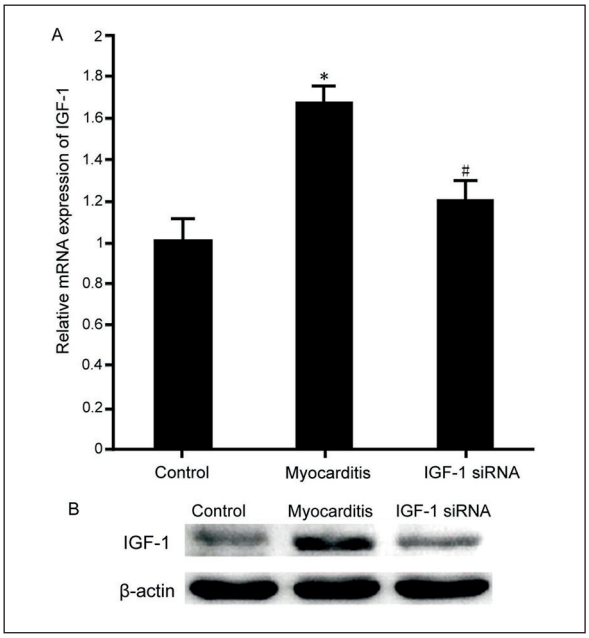

Figure 1. Expression of IGF-1 in myocarditis of LDL-R knockout mice by IGF-1. **A**, Real time-PCR analysis of IGF-1 expression of IGF-1 mRNA in myocarditis of LDL-R knockout mice, compared with the control group, $^{*}p < 0.05$; compared with myocarditis group, $^{\#}p < 0.05$. **B**, Western analysis of the IGF-1 protein expression in myocarditis of LDL-R knockout mice by IGF-1.

Effect of IGF-1 on Myocardial Tissue

The changes in the myocardial tissue of each group of mice were analyzed by HE staining. The results showed that compared with the control group, the myocarditis group showed inflammatory cell infiltration, disordered myocardial fiber arrangement, and swelling and fracture. In the IGF-1 siRNA group, the myocardial damage was significantly improved in mice, with reduced inflammatory cell infiltration, alleviated myocardial fiber arrangement and myocardial fibrosis (Figure 2).

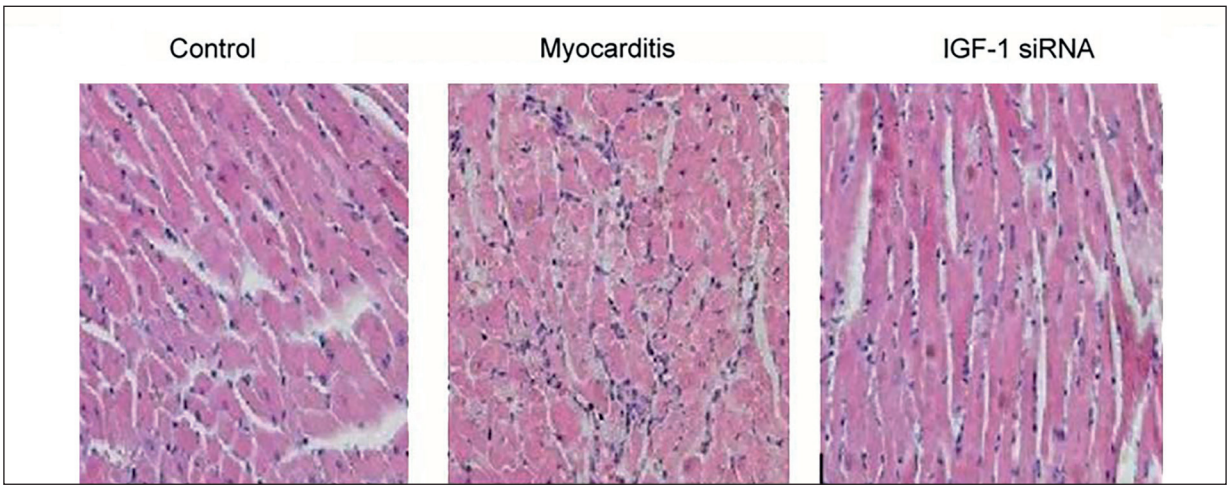

Figure 2. Effect of IGF-1 on myocardial tissue of myocarditis in LDL-R knockout mice (×100).

Table II. Effect of IGF-1 on cardiac function of myocarditis in LDL-R knockout mice.

Group	HR (times/min)	LVEDD (mm)	LVESD (mm)	LVMI
Control	123.00 ± 11.00	0.61 ± 0.05	0.41 ± 0.06	3.32 ± 0.02
Myocarditis	86.00 ± 9.00*	0.43 ± 0.08*	0.29 ± 0.06*	2.29 ± 0.11*
IGF-1 siRNA	101.00 ± 12.00*#	0.55± 0.09*#	0.35 ± 0.07*#	2.79 ± 0.07*#

Compared with the control group, *$p < 0.05$; compared with the myocarditis group, #$p < 0.05$.

Effect of IGF-1 on Cardiac Function of Myocarditis in LDL-R Knockout Mice

M-mode ultrasound was used to compare the effects of IGF-1 on myocardial function changes in myocarditis of LDL-R knockout mice. The results showed that HR, LVEDs, LVEDd, and LVMI were significantly decreased in myocarditis mice ($p < 0.05$). In the IGF-1 siRNA group, the HR, LVEDs, LVEDd, and LVMI were increased. Compared with the myocarditis group, the difference was statistically significant ($p < 0.05$) (Table II).

Effect of IGF-1 on Blood Lipids in Myocarditis Model of LDL-R Knockout Mice

The expression of TC and LDL was increased in the mouse model of myocarditis. Compared with the control group, the difference was statistically significant ($p < 0.05$). The expression of TC and LDL in the IGF-1 siRNA group was decreased. Compared with the myocarditis group, the difference was statistically significant ($p < 0.05$) (Table III).

Effect of IGF-1 on the Secretion of Inflammatory Factors

The effect of IGF-1 on the secretion of inflammatory factors in myocarditis of LDL-R knockout mice was analyzed by ELISA. The results showed that the secretion of the cytokines TNF-α and INF-γ in the serum of myocarditis mice was significantly increased, compared with that in the control group ($p < 0.05$). The secretion of cytokines TNF-α and INF-γ in the serum of the IGF-1 siRNA group was significantly decreased, compared with that in the myocarditis group ($p < 0.05$) (Figure 3).

Table III. Effect of IGF-1 on blood lipids in myocarditis model of LDL-R knockout mice.

Group	TC (mmol/L)	LDL (mmol/L)
Control	9.61 ± 0.92	6.41 ± 0.32
Myocarditis	17.32 ± 3.12*	15.69 ± 1.29*
IGF-1 siRNA	11.21 ± 2.18*#	9.56 ± 1.57*#

Compared with the control group, *$p < 0.05$; compared with the myocarditis group, #$p < 0.05$.

IGF-1 Affects the Development of Myocarditis in LDL-R Knockout Mice by Inhibiting Peritoneal Infiltration of Macrophages

Flow cytometry was used to analyze the effect of IGF-1 on peritoneal infiltration of macrophages in myocarditis of LDL-R knockout mice. The results showed that the peritoneal infiltration of macrophages increased in mice with myocarditis, and the difference was statistically significant ($p < 0.05$). The peritoneal infiltration of macrophages in the IGF-1 siRNA group was significantly lower than that in the myocarditis group ($p < 0.05$) (Figure 4).

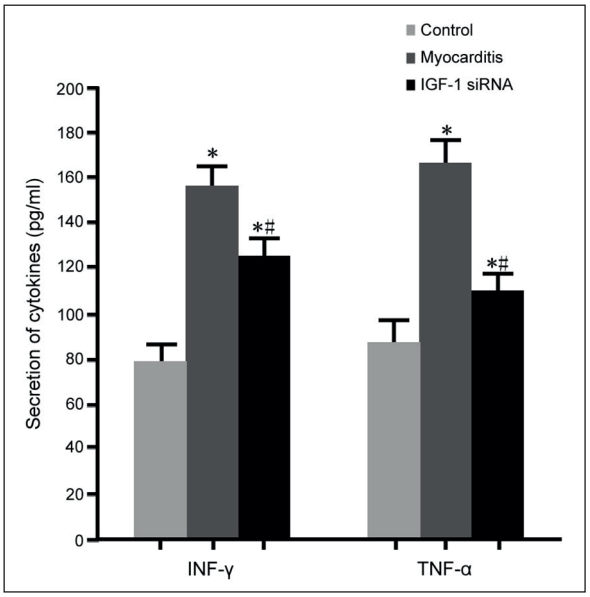

Figure 3. Effect of IGF-1 on the secretion of inflammatory factors in myocarditis of LDL-R knockout mice. Compared with the control group, *$p < 0.05$; compared with the myocarditis group, #$p < 0.05$.

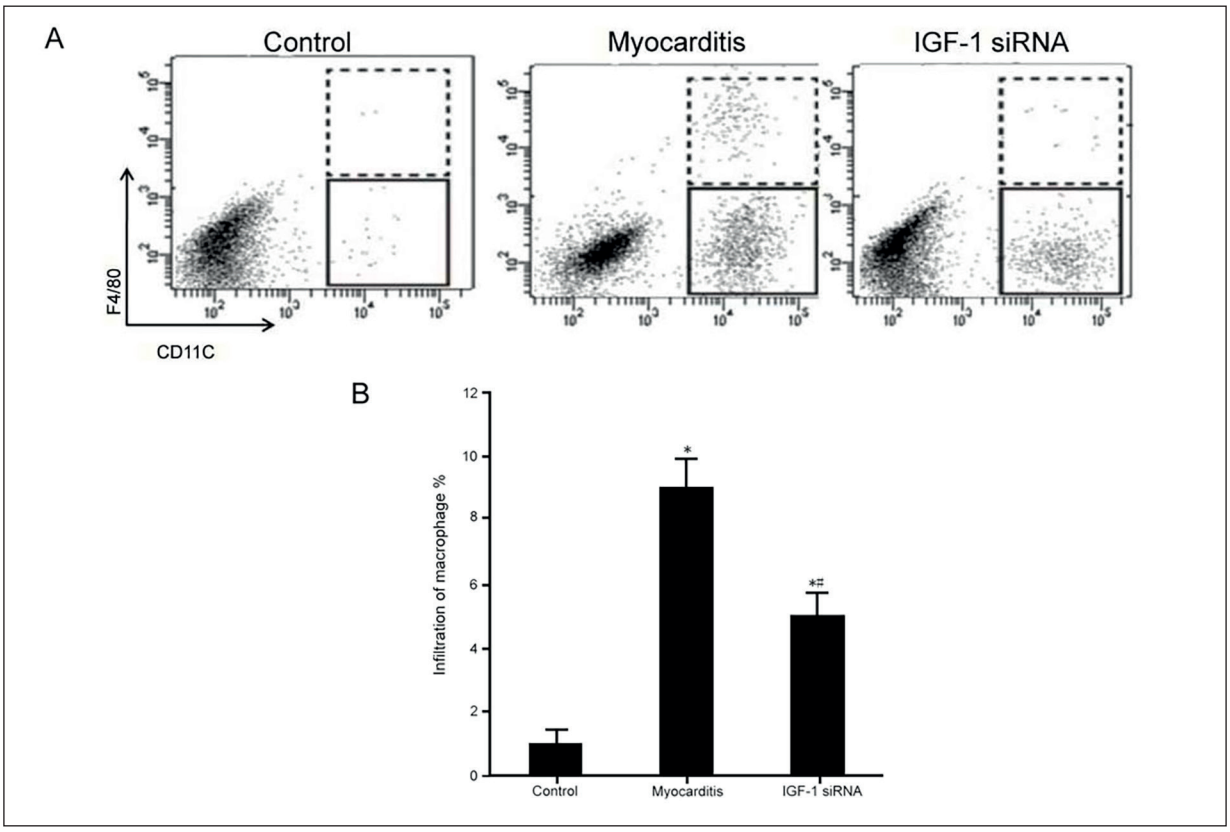

Figure 4. IGF-1 affects the development of myocarditis in the LDL-R knockout mice by inhibiting peritoneal infiltration of macrophages. **A**, Flow cytometry analysis of the effect of IGF-1 on peritoneal infiltration of macrophages in myocarditis of LDL-R knockout mice. **B**, Statistical analysis of peritoneal infiltrating macrophages compared with the control group, $*p < 0.05$; with the myocarditis Group comparison, $^{\#}p < 0.05$.

Effect of IGF-1 on MIF Secretion of Myocarditis in LDL-R Knockout Mice

The secretion of MIF in the serum of mice with myocarditis was significantly increased, compared with that in the control group ($p < 0.05$). The secretion of MIF in the serum of the IGF-1 siRNA group was decreased, compared with the myocarditis group; the difference was statistically significant ($p < 0.05$) (Figure 5).

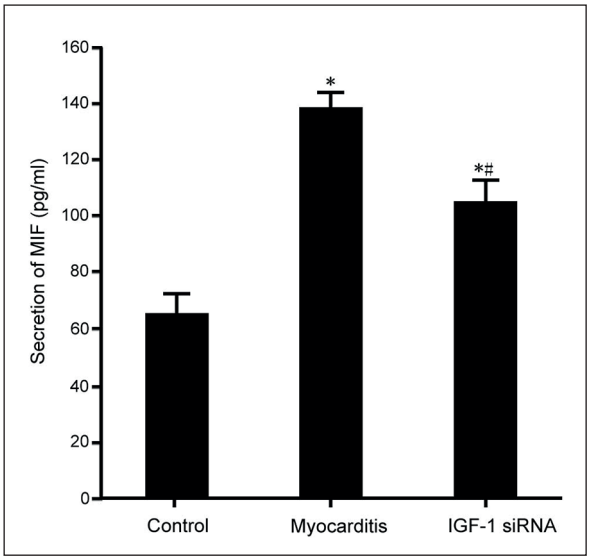

Figure 5. Effect of IGF-1 on MIF secretion of myocarditis in LDL-R knockout mice. Compared with the control group, $*p < 0.05$; compared with the myocarditis group, $^{\#}p < 0.05$.

Discussion

Viral myocarditis can cause sudden death and heart failure in young adults, and CVB3 infection is an important cause of myocarditis[3]. The pathogenesis of viral myocarditis and the sustained replication of the virus are associated with loss of myocardium and associated with increased release of inflammatory factors, following viral infection. After viral replication, myocardial necro-

sis, intracellular antigen exposure, and activation of the host immune system, and infiltration of immune cells, such as T cells and macrophages, lead to the damage of the target organs. The activation of cytokines, such as TNF-α and INF-γ, and myocardial proteins accelerates myocardial and systolic damage[6,18]. LDL-R knockout mice are commonly used animal models of atherosclerosis. Due to LDL-R gene knockout, abnormal blood lipid levels are caused, and abnormal blood lipid levels are more likely to cause viral infection and induce myocarditis[11,19]. Therefore, this study used LDL-R knockout mice, and CVB3 virus-infected mice to prepare a model of myocarditis, and confirmed the heart rate, left ventricular end-systolic diameter (LVEDs), left ventricular end-diastolic diameter (LVEDd), left ventricular mass index (LVMI) decreased, and blood lipid levels increased with inflammatory cell infiltration, fibrosis, and increased secretion of TNF-α and INF-γ inflammatory factors in typical myocardial myocarditis.

As a cardiogenic hormone, IGF-1 not only regulates metabolism, but also promotes the proliferation of vascular smooth muscle cells (VSMC) by regulating the tyrosine-protein kinase pathway, and may also promote the mitosis of cardiomyocytes and the expression of protooncogenes and protein synthesis, stimulate fibroblast or myocardial interstitial growth, leading to increased left ventricular hypertrophy, myocardial fibrosis, and early cardiac dysfunction[16,20]. This study indicated that the expression of the IGF-1 gene and protein in the mouse model of myocarditis was increased, suggesting that IGF-1 might play an important role in the development of myocarditis. Macrophages can cause myocardial damage by secreting INF-γ, participate in myocardial remodeling and atrial fibrillation, and lead to increased secretion of IGF-1[21,22]. The macrophage can secrete macrophage migration inhibitory factor (MIF), which is a cytokine and growth factor multifunctional protein molecule involved in the pathogenesis and development of atherosclerosis, myocardial infarction, ischemia-reperfusion progress in cardiovascular disease[18,23]. This study demonstrates that the downregulation of the IGF-1 expression in mice with LDR-L knockout myocarditis by siRNA not only regulates blood lipid levels, but also inhibits peritoneal macrophage infiltration, thereby inhibiting MIF secretion and reducing the secretion of the inflammatory factors TNF-α and INF-γ, thereby improving myocardial inflammatory cell infiltration, inhibiting myocardial fibrosis, promoting HR, LVEDs, LVEDd, LVMI cardiac function indicators, and thus improving cardiac function. In further research, it is proposed to further analyze the mechanism of IGF-1 in the regulation of peritoneal macrophage infiltration and provide a reference for the clinical analysis of the pathogenesis and effective treatment of myocarditis.

Conclusions

The expression of IGF-1 is increased in myocarditis. The downregulation of IGF-1 expression can inhibit the infiltration of peritoneal macrophages, reduce the expression of MIF and inflammatory factors, regulate blood lipid levels, and improve myocarditis injury.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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