Original Article
Protective mechanism of IL-37 in the regulation of Tregs in myocardial ischemia microcirculation reperfusion injury

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Abstract: IL-37 is a newly discovered natural inflammatory inhibitor in recent years. The role and mechanism of IL-37 in the inflammatory reaction of atherosclerosis in patients with coronary artery disease may be related to the abnormal blood lipid and regulatory T cells (Tregs). But the effect of IL-37 on myocardial ischemia reperfusion injury and its mechanism are still not clear. Therefore, this study will do the protection mechanism of IL-37 in myocardial infarction microcirculation reperfusion injury by regulating Treg. Rat myocardial microcirculatory reperfusion injury model was established. Rats were randomly divided into three groups, A. normal group; B. model group; C. IL-37 administration group. Serum lactate dehydrogenase (LDH) and creatine kinase isoenzyme (CK-MB), superoxide dismutase (SOD) in the myocardial tissue live, content of MDA, NOS activity, NO content, Tregs cell ratio, Foxp3 and CTLA-4 mRNA expression levels, protein in the spleen tissue expression level were examined. Serum LDH and CK-MB were lower in IL-37 group than that in model group. SOD activity, NOS activity and NO increased in myocardium while MDA decreased (LDH and CK-MB: P<0.05, SOD, MDA, NOS and NO: P<0.01). Tregs cells in IL-37 group were increased (P<0.05). FoxP3 and CTLA-4 was lower in the model. If we provided the IL-37, FoxP3 and CTLA-4 expression increased. In Tregs cells FoxP3 and CTLA-4 mRNA expression were decreased P<0.01). IL-37 group was versed model group, IL-6 and TGF-α decreased. IL-37 has protective effect on myocardial infarction microcirculation reperfusion injury, decrease serum LDH and CK-MB values, increase SOD activity, NOS activity, NO content, decrease MDA, and its mechanism may be to promote Tregs cells, inhibit inflammatory reaction, and the expression of CTLA-4 and FoxP3.

Keywords: IL-37, myocardial infarction, reperfusion injury, Tregs, apoptosis, SIRT4

Introduction

In the clinical research, the patients with ST segment elevation myocardial infarction (STE-MI) were received PCI in the emergency treatment and the coronary blood flow returned to normal, patients with coronary microvascular injury and dysfunction can lead to poor prognosis [1]. The full reperfusion of myocardial microcirculation is the key to improve the prognosis of blood vessels. No reflow is an independent predictor of long-term cardiovascular events in patients with acute myocardial infarction [2]. The patients are prone to malignant arrhythmia, heart failure, left ventricular remodeling, etc., or death in patients with acute myocardial infarction (AMI). IL-37, as a member of the IL-1 family, is a natural inflammatory inhibitor in recent years study finding, which can inhibit the inflammatory and immune responses. As a negative regulatory gene, IL-37 was not expressed in normal healthy people, but it was expressed in the inflammatory tissue [3, 4]. Studies show that IL-37 mediated inflammatory immune response may be involved in the pathogenesis [5], play an anti-inflammatory and protective effect of AMI. At the same time, it may be related to the decrease of IL-37 level, and the role and mechanism of IL-37 in the inflammatory reaction of atherosclerosis in patients with coronary heart disease may be related to the abnormal lipid and Tregs [6]. But the effect of IL-37 on myocardial ischemia reperfusion injury and its mechanism are still not clear. Therefore, this study has great theoretical and practical value to further study the protection mechanism of -37 in myocardial infarction microcirculation reperfusion injury by regulating Treg.

Materials and methods

Main reagents

Lactate dehydrogenase (LDH) determination kit, creatine kinase isoenzyme (CK-MB) determination kit, malondialdehyde (MDA) determination kit, superoxide dismutase enzyme activity kit, nitric oxide synthase activity kit were purchased from Nanjing Jiancheng Biological Engineering Research Institute. APC labeled mouse CD25, FITC anti mouse CD4 monoclonal antibody and anti mouse monoclonal antibody was purchased from American BD Company. Reverse transcriptase polymerase chain reaction (RT-PCR) kit and RT-PCR primers were purchased from Dalian Takara Company. RNA extracting reagent Trizol was purchased from Dalian Takara Company. Foxp3 rabbit antibody was purchased from American CST Company. Rat IL-10 and TGF-ELISA kit was purchased from the Perprotech Company.

Main instruments

Flow cytometry (American BD, Calibur FACS), ELIASA (American Thermo, FC Multiskan), Fluorescence quantitative PCR and analysis software MXPro4.01 (Stratagene).

Experimental animals grouping

SD rats of SPF were selected, 8 rats in each group: A. normal group, B. model group (reperfusion group), C. IL-37+ reperfusion group (IL-37).

Establishment of the model of myocardial infarction microcirculation reperfusion injury

The model of Filippo was performed [7]. The experimental animals were anesthetized, and then the heart was opened, and the color of the myocardium was gradually changed to dark and then myocardial ischemia happened. Myocardial reperfusion after myocardial ischemia 30 min which can be judged by ECG ST segment decreased significantly. After 2 h reperfusion, the myocardial ischemia reperfusion injury model was successfully duplicated.

Determination of serum lactate dehydrogenase (LDH) and creatine kinase isoenzyme (CK-MB), and myocardial tissue SOD activity and the content of MDA, NOS activity and NO content

After 2 hours of reperfusion, 2 ml blood was removed from the apical portion, and then immediately at the low temperature of 4°C,

3000 r/min 15 min, and the supernatant was kept in the -20. The activity of LDH and CK-MB in serum was determined by automatic biochemistry analyzer and spectrophotometer. Subsequently clipping left ventricular apical ischemic myocardial tissue was prepared for 10% homogenate of myocardial tissue, determined the content of MDA and SOD of myocardial tissue. NOS activity and NO content in myocardium were detected. The specific operation is carried out according to the kit instructions.

Tregs cells were detected by flow cytometry

PBMC cells were separated by density gradient centrifugation. The cell samples were added to the corresponding reagent, and then were mixed evenly, at room temperature, 15 minutes after incubation: FITC CD4, APC CD25, PE Foxp3 three labeled cell surface antibody staining, BD FACSCalibur Cell Sorting System flow cytometry 88 nm, the emission wavelength 535 nm. RI gate: Lymphocyte cluster, RI gate: CD4+ cells, 5000 cells were collected using CellQuest software.

Expression of CTLA-4 and FoxP3 mRNA in peripheral blood Tregs cells of rats by RT-PCR

TRIzol method was used to extract the total RNA of peripheral blood PBMC, and the RNA was transcribed into cDNA. We took 1 μl as a template for RT-PCR reaction: CTLA-4 upstream primer 5'-ACCTTCAGTGGTGTTGGCTAG-3', 5'TA-AATCTGCGTCCCGTTGC-3', FoxP3 upstream primer 5'-TGTGAGGACTACCGAGCC-3', 5'-AGGAG-AAAGCGGATACCA-3'. β-actin upstream primer 5'-TTCGAGCAAGAGATGGCCA-3', 5'-TACATGGTG-GTGCCGCC-3', the length of the fragment was 270 bp. RT-PCR reaction conditions: the first pre-denaturalization 95°C, 5 min, and then 30 cycles, 95°C, 30 s, 55°C, 30 s, 72°C 1 min extend, finally 2°C 10 min, 4°C keep.

Expression differences of FoxP3 and CTLA-4 protein in the tissues were detected by western blot

The appropriate size of the spleen tissue implantation in mortar, adding liquid nitrogen grinding to collect tissues, adding the lysate of 1:100, placing on the ice, fully cracked 60 min; cracking implant EP tube, 4°C, 12000 rpm, centrifugal for 30 min, packing -80°C cryopreservation. Protein expression changes were detected by Western blot, SDS-PAGE gel electrophoresis was used to detect the expression,

Table 1. Comparisons between groups of lactate dehydrogenase and creatine kinase isoenzyme ($\bar{x}\pm S$, n=8)

Group	N	LDH (U/mL)	CK-MB (U/mL)
Normal	8	3.45±1.09	0.89±0.45
Model	8	5.09±1.65*	2.46±1.05**
IL-37	8	3.69±1.12#	1.40±0.87#

Note: comparison with normal group, *P<0.05, **P<0.01; comparison with model group, #P<0.05, ##P<0.05.

Table 2. Comparison SOD activity and MDA content of myocardium in each group

Group	N	SOD (U/mg)	MDA (nmol/mg)
Normal	8	210.45±9.09	10.89±0.45
Model	8	155.09±6.65**	17.46±1.05**
IL-37	8	193.69±10.12##	11.40±0.87##

Note: comparison with normal group, *P<0.05, **P<0.01; comparison with model group, #P<0.05, ##P<0.05.

the membrane was closed, and the FoxP3 and CTLA-4 protein antibody was cultured and kept overnight 4°C. Re-temperature was cultured bi-antibody for 1 hour. The bands were putted on the gel imager, chemical luminescence method exposure bands were used, and reference β-actin gray value of the ratio was used to reflect the relative content.

ELISA used to detect the serum levels of IL-6 and TGF-α

The concentration of IL-6 and TGF- in serum of each group was detected by ELISA. ELISA detection method is according to the company manual operation. Draw standard curve, the specimens of OD can be found its concentration in the standard curve.

Statistic analyses

All data were entered into the computer by SPSS18.0 statistical software, and the statistical analysis was carried out. The analysis of variance was used in the two groups, the two groups were compared by t test, and the data were analyzed by rank sum test.

Results

Serum lactate dehydrogenase (LDH) and creatine kinase isoenzyme (CK-MB) determination

The rats in normal group were sensitive to external response, fur clean shiny, normal water diet. Model group rats were unrespon-

sive to the outside world, fur dirty dull, eating and drinking water greatly reduced. IL-37 group rats were more sensitive to the outside world than the model group, and the skin was clean, and the drinking water and urine were improved. Compared with the normal group, the LDH and CK-MB in the model group were significantly higher (P<0.05, P<0.01), and the LDH and CK-MB content in IL-37 against the WT+PBS group were significantly different (P<0.05). IL-37 could improve the LDH and CK-MB in the model of myocardial infarction microcirculation reperfusion (**Table 1**).

SOD activity and MDA content, NOS activity and NO content in myocardial tissue

The activity of SOD and MDA in model group were higher than those in normal group (P<0.01). After IL-37, the SOD activity and MDA content of IL-37 group were decreased (P<0.01). The SOD activity and MDA content in rats with IL-37 were improved (**Table 2**).

Comparison NOS activity and NO content in myocardium

NOS activity and NO content in model group were higher than those in normal group (P<0.01). After IL-37, the NOS activity and NO content of IL-37 group were found to be increased (P<0.01. IL-37 could improve NOS activity and NO content of myocardial infarction microcirculation reperfusion (**Table 3**).

Proportion of spleen Tregs cells in each group

Flow cytometry to detect the percentage of Tregs cells in each group was shown in **Figure 1**. The results showed that the proportion of Tregs cells in the model group was lower than that of the normal group (P<0.05), and the proportion of Tregs cells in IL-37 group was higher than that in IL-37 group (P<0.01), which indicated that IL-37 could promote the increase of Tregs cells in rats with ischemia reperfusion injury.
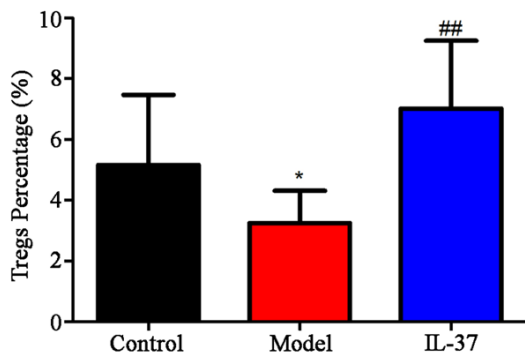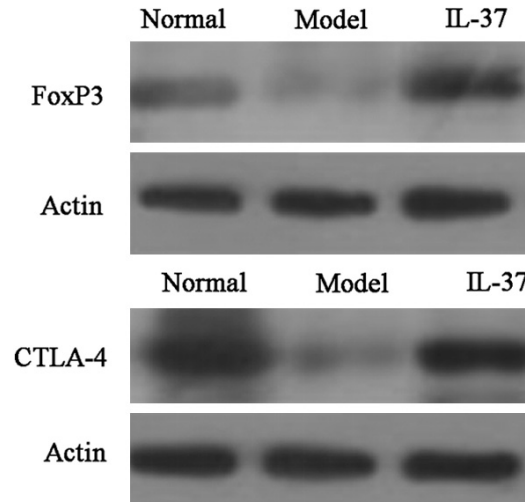
Expression of FoxP3 and CTLA-4 protein in the spleen tissues

Blot western method was used to detect the expression of FoxP3 and CTLA-4 protein in the spleen tissues, as shown in **Figure 2**. The expression of CTLA-4 and FoxP3 in the model group was relatively low, and the expression of CTLA-4 and FoxP3 were increased after IL-37

Table 3. Comparison NOS activity and NO content of myocardium in each group

Group	N	NOS (U/g)	NO (µmol/g)
Normal	8	3.45±0.29	1.89±0.05
Model	8	2.09±0.65**	0.46±0.05**
IL-37	8	3.29±0.12##	1.40±0.07##

Note: comparison with normal group, *P<0.05, **P<0.01; comparison with model group, #P<0.05, ##P<0.05.

Figure 1. The proportion of spleen Tregs cells in each group. Note: comparison with normal group, *P<0.05, **P<0.01; comparison with model group, #P<0.05, ##P<0.05.

Figure 2. The expression of FoxP3 and CTLA-4 protein in each group.

treatment, indicating that IL-37 can promote the expression of FoxP3 and CTLA-4 in the spleen tissues of rats.

Expression levels of CTLA-4 mRNA and FoxP3 in spleen Th17 cells

The results of agarose gel electrophoresis showed clear 18 S and 28 S bands. RNA values

of PCR A260/A280 were 1.8-2.0, indicating that the total RNA samples were extracted from the samples with no obvious degradation of total purity. The expression of Tregs and mRNA FoxP3 in the model group was significantly higher than that in the normal group (CTLA-4) (P<0.01). After IL-37 treatment, the expression of CTLA-4 and mRNA FoxP3 in Tregs cells decreased (P<0.01) (**Figure 3**).

Content of cytokines in the culture supernatant of spleen in each group

In the model group, the IL-6 and TGF- were higher than the normal group (P<0.01). After IL-37 treatment, the IL-6 and TGF-α of IL-37 group were decreased (P<0.01), which indicated that IL-37 could improve the secretion of IL-6 and TGF-α (**Table 4**).

Discussion

Early intervention therapy is the main treatment for acute myocardial infarction, as soon as possible to open the relevant vessels in the infarct area, restore the blood flow in the infarct area, save the myocardium, and improve the prognosis, the quality of life and the survival rate [8]. However, we found that 30~40% patients had a slow flow or no reflow after the opening of the infarct related blood vessels, which result in the lack of myocardial microcirculation, and the area of myocardial infarction is not improved [9, 10]. The pathogenesis of this disease is closely related to ventricular remodeling, which is closely related to the quality of life and survival rate of patients. Ndrepepa et al. found that the myocardial microcirculation disturbance and reperfusion injury after PCI, which is a predictor of malignant arrhythmia, infarct area extension, ventricular remodeling, cardiac dysfunction and other serious complications. It can predict the short-term and long-term survival rate of patients. Therefore, it is a hot research topic to explore the therapeutic method of microcirculation reperfusion injury.

Ischemia reperfusion injury can cause aseptic inflammatory reaction, which involves the activation of Toll like receptor signaling pathway, activation of innate immunity and aggregation of monocytes, dendritic cells [12, 13]. At the same time, in microcirculation reperfusion injury area, the accumulation of CD8+ T and CD4+ cells was involved in the inflammatory reaction, and the activation of antigen specific T cells
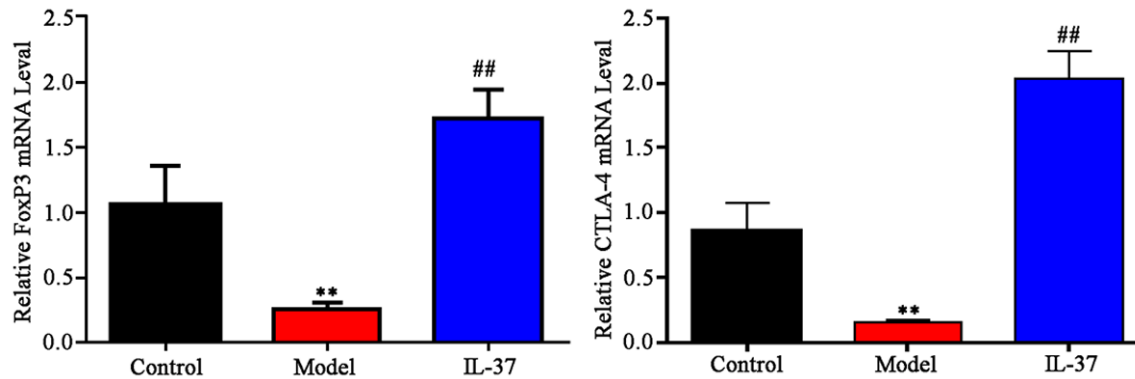
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Figure 3. Expression levels of CTLA-4 and mRNA FoxP3 in each group. Note: comparison with normal group, *P<0.05, **P<0.01; comparison with model group, #P<0.05, ##P<0.05.

Table 4. The content of cytokines in the culture supernatant of spleen in each group

Group	N	IL-6	TGF-α
Normal	8	0.66±0.09	395.68±30.57
Model	8	1.28±0.22**	631.45±36.66**
IL-37	8	0.68±0.05##	474.48±35.78*,##

Note: comparison with normal group, *P<0.05, **P<0.01; comparison with model group, #P<0.05, ##P<0.05.

[14]. Therefore, the study of immune imbalance in ischemia reperfusion injury may provide a promising treatment for the treatment.

IL-37 as a negative regulator plays main role in the inhibition of the inflammatory response and immune response, in a variety of mechanisms involved in the regulation of inflammation related diseases. Research shows that the induction of IL-37 macrophages or epithelial cells, IL-1 alpha, IL-1 beta and TNF alpha proinflammatory factor is almost completely inhibited, and can inhibit inflammation and atherosclerosis role [15, 16].

At present, the mechanism of anti inflammation effect of IL-37 has not been fully elucidated. There are two main points: one is that the IL-37 is released into the cell, and the other is to inhibit the activity of [17] by directly acting on the inflammatory cytokines or acting on the related receptors; the other is that the IL-37's mature body may form a IL-37/Smad3 complex with the nuclear Samd3, which regulates gene transcription and exerts an anti-inflammatory effect [18, 19].

This study found that the expression of LDH and CK-MB decreased, and the SOD activity and MDA content in rats increased after myocardial infarction microcirculation reperfusion injury IL-37 model rats. NO activity and NOS content were increased by IL-37, and IL-37 could improve myocardial infarction microcirculation reperfusion injury. Moreover, IL-37 can increase the proportion of Tregs cells in the peripheral blood of rats, and increase the expression of CTLA-4 mRNA, FoxP3 and protein in Tregs cells, which indicate that IL-37 plays a protective role in myocardial infarction microcirculation reperfusion injury by regulating Tregs cells. The expression of CTLA-4 and FoxP3 are closely related to IL-37.

In recent years, studies have shown that adoptive transfer of Tregs can relieve reperfusion injury ischemia in mice and in a dose dependent manner, which may inhibition of proinflammatory cytokine TNF-a, IL-6, the secretion of the anti-inflammatory cytokine IL-10 [20]. In this study, we found that IL-6 and TGF-α were decreased in IL-37 group. IL-37 can inhibit the secretion of IL-6 and TGF-α, and has the protective effect of Tregs on myocardial infarction microcirculation reperfusion injury by inhibiting inflammatory reaction and IL-37 cells.

The protective effect of IL-37 on myocardial infarction microcirculation reperfusion injury, decrease serum LDH and CK-MB, increase SOD activity, NOS activity and NO content, reduce MDA. Its mechanism may be the promotion of Tregs cells, and expression of CTLA-4 and FoxP3, which is closely related to IL-37. This study reveals the mechanism of immune regulation function of IL-37, and can give the clinical theory supporting.

Disclosure of conflict of interest

None.

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