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Protective function of tocilizumab in human cardiac myocytes ischemia reperfusion injury

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ABSTRACT

Objective: To investigate the protective function of tocilizumab in human cardiac myocytes ischemia-reperfusion injury. **Methods:** The human cardiac myocytes were treated by tocilizumab with different concentrations(1.0 mg/mL, 3.0 mg/mL, 5.0 mg/mL) for 24 h,then cells were cultured in ischemia environment for 24 h and reperfusion environment for 1 h. The MTT and flow cytometry were used to detect the proliferation and apoptosis of human cardiac myocytes, respectively. The mRNA and protein expressions of Bcl-2 and Bax were measured by qRT-PCR and western blot, respectively. **Results:** Compared to the negative group, pretreated by tocilizumab could significantly enhance the proliferation viability and suppress apoptosis of human cardiac myocytes after suffering ischemia reperfusion injury ($P<0.05$). The expression of Bcl-2 in tocilizumab treated group were higher than NC group ($P<0.05$), while the Bax expression were lower ($P<0.05$). **Conclusions:** Tocilizumab could significantly inhibit apoptosis and keep the proliferation viability of human cardiac myocytes after suffering ischemia reperfusion injury. Tocilizumab may obtain a widely application in the protection of ischemia reperfusion injury.

1. Introduction

Acute myocardial infarction (AMI) is one of the most fateful diseases in China[1]. With the development of cardiac intervention therapy, percutaneous coronary intervention (PCI) has become a crucial treatment to restore the blood flow in early stage[2]. However, there are still 10%-30% of the patients that suffer ischemia-reperfusion injury (IRI) after PCI treatment. Consequently, the size of myocardial infarction was expanded due to the apoptosis[3] and autophagy[4] caused by IRI. Therefore, discovering more effective drugs for reducing ischemia-reperfusion injury and keeping the vitality of myocardial cells is necessary.

Interleukin-6 (IL-6) was originally identified as B-cell stimulatory factor 2 that promotes immunoglobulin synthesis by activated B cells[5]. IL-6 is not expressed in healthy persons, but when infections or tissue injuries occur, IL-6 is rapidly synthesized and contributes to host defense[6]. However, excessive production of IL-6 during this process has been implicated in the development of acute, severe complications, including systemic inflammatory response syndrome (SIRS) and cytokine-release syndrome (CRS)[7]. Recent studies showed that the expression of IL-6 and its receptor was significantly increased during Acute myocardial ischemia-reperfusion (AMI/R) period and appeared a prognostic relevance for patients who experienced AMI[8]. Tocilizumab(Trade name: ACTEMRA@) is a humanized monoclonal antibody directed against interleukin-6 receptor. The molecule was humanized by the grafting of the complementarity-determining regions of a mouse anti-human IL-6 receptor mAb onto human IgG1. It inhibits the binding of IL-6 to its

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receptors, and thus reduces the cytokines pro-inflammatory activity by competing for both the soluble and membrane-bound forms of the human IL-6 receptor[9]. Tocilizumab is marketed in Japan for Castleman disease and several types of arthritis[10,11]. In the European Union, the product is approved for treatment of moderate-to-severe rheumatoid arthritis[12]. However, the functions and molecular mechanisms of tocilizumab for protection myocardial cells ischemia-reperfusion injury after AMI are still poorly understood.

In this study, we demonstrated that tocilizumab displayed a protective effect for human cardiac myocytes after ischemia-reperfusion injury. Tocilizumab could suppress apoptosis and keep vitality of human cardiac myocytes after ischemia-reperfusion injury partly through down-regulating Bcl-2 and up-regulating Bax expression *in vitro*.

2. Materials and methods

2.1. Cell culture

The human cardiac myocytes were obtained from the ScienCell Research Laboratories (Carlsbad, California, USA). Cells were cultured in complete Dulbecco's modified Eagle medium (DMEM, Mediatech, USA) containing 10% fetal bovine serum (FBS, Gibco BRL, USA) in a humidified containing of 5% CO_2 incubator at 37 ℃. The logarithmic growth phase cells were harvested for further assays.

2.2. Tocilizumab intervention process

The Tocilizumab (Roche, Switzerland) was diluted with 1 ×DMEM medium and adjusted the density of 1.0 mg/mL, 3.0 mg/mL and 5.0 mg/mL, respectively. human cardiac myocytes were seeded in 6-well plates at the concentration of 1×10^6/well, and divided into four groups, describing as negative control group (NC Group, DMEM only), Test A group (Tocilizumab 1.0 mg/mL), Test B group (Tocilizumab 3.0 mg/mL), Test C group (Tocilizumab 5.0 mg/mL). Cells were intervened with different densities of tocilizumab or DMEM, and cultured in a humidified containing 5% CO_2 incubator at 37 ℃. Cells were harvest after 24 h intervention, and then used for further experiments.

2.3. Establishment of model of acute ischemia and reperfusion of human cardiac myocytes

The simulated ischemia reagent and reperfusion reagent were prepared according to the manufacture, that Ross *et al* previously reported[13]. The intervened human cardiac myocytes were washed by PBS for twice. Cells were added with 3 mL simulated ischemia reagent and cultured in a humidified containing 5% CO_2 and 95% N_2 incubator at 37 ℃ for 24 h. 3 mL simulated reperfusion reagent was used to replace the simulated ischemia reagent, and cultured the cells in normal environment for 1 h.

2.4. MTT assay

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Roche, USA) assay was used to determine the proliferation viability. Cell viability was calculated at 24 h, 48 h and 72 h after ischemia simulation and reperfusion intervention. The absorbance of the samples was measured using a model 550 microplate reader (Bio-Rad Laboratories, USA), at a wavelength of 490 nm. Three independent experimental replicates were performed.

2.5. Cell apoptosis detection

Annexin-V-FLUOS Staining Kit (Roche, USA) was purchased to evaluate cell apoptosis after 48h intervention. Briefly, the samples were analyzed by BD FACS Canto Ⅱ Flow Cytometer (Becton Dickinson, USA). Three independent experimental replicates were performed.

2.6. qRT-PCR

Total RNA was isolated from human cardiac myocytes, which underwent simulated ischemia and reperfusion intervention, by TRIZOL® reagent (Invitrogen, USA) according to the manufacturer. The following primers were synthesized: Bcl-2 sense primer 5′-CCTTTGTGTAACTGTACGGCC-3′ and anti-sense primer 5′-CTTTGGCAGTAAATAGCTGATTCGAC-3′, Bax sense primer 5′-TCCACCAAGAAGCTGAGCGAG-3′ and anti-sense primer 5′-GTCCAGCCCATGATGGTTCT- 3′, β-actin sense primer 5′-CTCCATCCTGGCCTCGCTGT-3′ and anti-sense primer 5′-GCTGTCACCTTCACCGTTCC -3′. Reverse transcription was performed by the Revertid™ First Strand cDNA Synthesis Kit (Fermentas, USA). Synthetized cDNA was amplified and quantified by Real-time PCR using SYBR® Premix Ex *Taq*™ Ⅱ (Tli RNaseH Plus, Takara, Japan). The human β-actin gene was served as an internal control. Relative gene expression was calculated with the $2^{-\triangle\triangle C_t}$ method. Three independent experimental replicates were performed.

2.7. Western blot assay

Human anti-rabbit Bcl-2(SC-7382, Santa Cruz, USA) (1:1 000), Bax(SC-7480, Santa Cruz, USA) (1:1 000) and human

anti-mouse β-actin (Santa Cruz, USA) (1:5 000) antibodies were used for western blot assay. Secondary horseradish peroxidase-conjugated goat anti-mouse or -rabbit antibody (Bio-Rad, USA) were used at a 1:5 000 dilution. The relative protein concentration were measured by gray value, which was displayed by the Enhanced Chemiluminescence Regent (Millipore, USA).

2.8. Statistic analysis

All date are presented as (Mean±SD). Two-tailed Student's *t* test or ANOVA was used to evaluate statistical significant using GraphPad Prism 5 software (GraphPad Software, Inc). $P<0.05$ was considered as statistically significant different.

3. Results

3.1. Proliferation viability of tocilizumab intervened human cardiac myocytes after IRI

Compared to the NC group, cells intervened by tocilizumab appeared a higher proliferation viability after underwent IRI *in vitro* ($P<0.05$). Moreover, a significant proliferation difference was observed between test A group and test B group ($P<0.05$), but not in test B group and test C group ($P>0.05$). Data was shown in Figure 1.

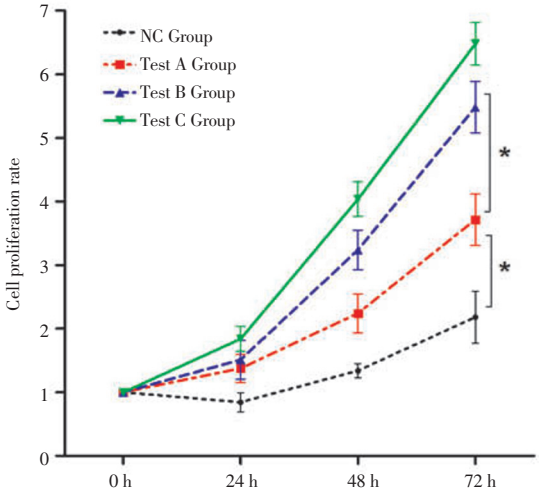

Figure 1. Tocilizumab protects proliferation viability of human cardiac myocytes after AMI/R.
The relative cell proliferation rate of each group at different time points was determined by MTT assay. Cell proliferation curves were plotted. $n=3$. *$P<0.05$.

3.2. Apoptosis of tocilizumab intervened human cardiac myocytes after IRI

As shown in Figure 2, tocilizumab could effectively protect human cardiac myocytes to resistance apoptosis caused by IRI in test groups compared to NC group ($P<0.05$). The medium concentration of tocilizumab (3 mg/mL) could play a more effective apoptotic resistance than minimal concentration (1 mg/mL) ($P<0.05$), but there was no significant changes at a more higher concentration (5 mg/mL) ($P>0.05$).

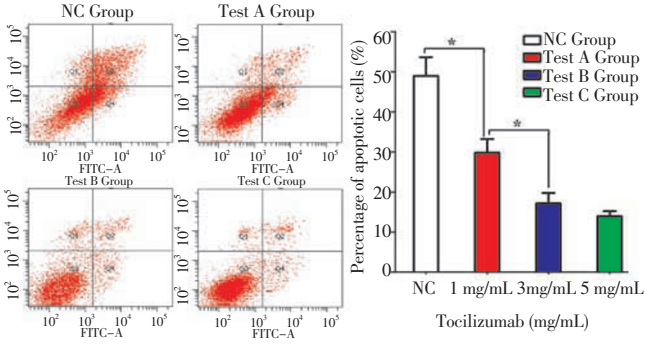

Figure 2. Tocilizumab inhibits apoptosis of human cardiac myocytes after AMI/R.
The cell apoptosis of each group was detected by flow cytometry. $n=3$. Data show representative results of repeat experiments. *$P<0.05$.

3.3. Expression changes of Bcl-2 and Bax in tocilizumab intervened human cardiac myocytes after IRI

According to the results shown above, tocilizumab could keep proliferation viability and resist apoptosis of human cardiac myocytes after IRI. To investigate the possible molecular mechanisms, the mRNA and protein levels of Bcl-2 and Bax were detected, which were two classic genes in cell growth regulation, in each test or NC group cells by qRT-PCR and western blot. Both mRNA and protein levels of Bcl-2 were more significantly up-regulated in test groups than in NC group ($P<0.05$, Figure 3A and 3C), in the meanwhile, the expression levels of Bax were more significantly down-regulated in test groups than in NC group ($P<0.05$, Figure 3B and 3C). Furthermore, within the test groups, the mRNA and protein levels of Bcl-2 were higher, while Bax were lower, in 3 mg/mL group than in 1 mg/mL group ($P<0.05$), but there was no significant change between 3 mg/mL group and 5 mg/mL group ($P>0.05$).

4. Discussion

AMI is the most common complications of coronary heart disease. Recently, the incidence of AMI rises every year in our country. Restoring myocardial blood flow as quickly as possible is one of the most crucial process to improve the prognosis of AMI patients[14]. At the same time, how to

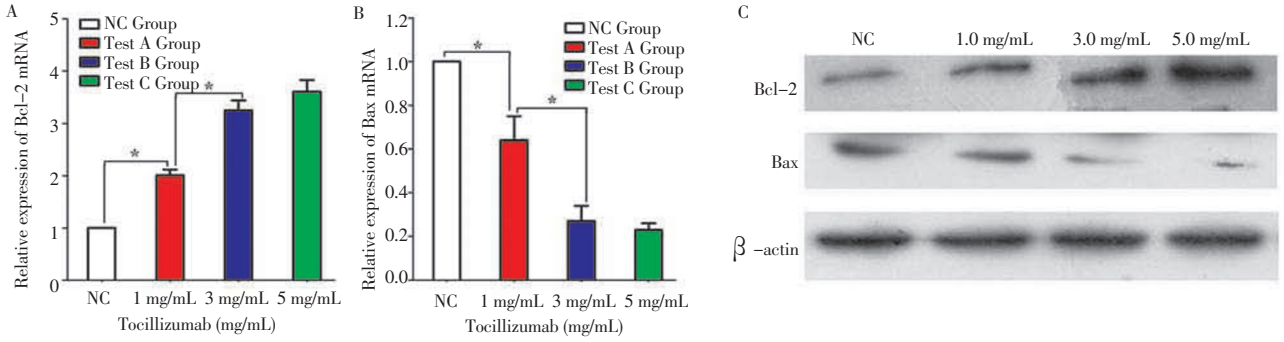

Figure 3. Tocilizumab regulates the expression of Bcl-2 and Bax in human cardiac myocytes after AMI/R.
The Bcl-2 (A) and Bax (B) mRNA levels of each group were determined by qRT-PCR. n=3. Data show representative results of repeat experiments. *P<0.05. The Bcl-2and Bax protein levels of each group were determined by western blot (C).

reduce or avoid the IRI becomes an overarching concern for all doctors. Nowadays, many of interleukins such as IL-1[15], IL-8[16], IL-10[17] and IL-17A[18], has been demonstrated to activate and participate in IRI of myocardium after AMI/R. IL-6 serve as an important factor in several inflammatory processes, and it can lead to a so called "waterfall effect" of inflammation[19], and eventually induce apoptosis and disintegration of certain cells. Recently, studies found that IL-6 of myocardium was significant elevated in IRI group compared to ischemic preconditioning group and positive associated with the infarct size in rat AMI/R model[20]. Zhao *et al* reported that atorvastatin could reduce myocardium necrosis area in a rabbit model of AMI/R through down-regulating the serum IL-6 level[21]. These evidences suggest that IL-6 could serve as a therapeutic target to prevent IRI of myocardium. This led to the development of tocilizumab, a humanized monoclonal antibody, with the CDR of a mouse anti-IL-6R grafted on to human IgG1 molecule. Tocilizumab can block both classic and trans-signaling pathways by inhibiting IL-6 binding to transmembrane IL-6R and soluble IL-6R.

In this study, we treated human cardiac myocytes with different concentrations of tocilizumab, and detected the tolerance of human cardiac myocytes to AMI/R. Firstly, we used MTT assay to detect cell proliferation viability, and found that tocilizumab could prominently keep the proliferation activity of human cardiac myocytes after AMI/R. Furthermore, the results of flow cytometry demonstrated that tocilizumab also could inhibit human cardiac myocytes apoptosis after AMI/R. Interestingly, we found that cells treated with 3 mg/mL tocilizumab could gain a better protective effect than 1mg/mL, but no more improvement when the concentration evaluate to 5 mg/mL. This means that the protective effect has a character of saturability, and the reason may be that the binding of antigen to antibody has reached saturation. In order to realize the molecular mechanisms of these effects, we detected two classic growth regulation genes, Bcl-2 and Bax. The expression of Bcl-2 was up-regulation, while the expression of Bax was down-regulation, in test groups compared to the NC group. In accordance with the functional results, these changes could be enlarged with the increasing concentrations of tocilizumab from 1 mg/mL to 3 mg/mL, but no more expansion observed when the concentrations of tocilizumab continuously increased to 5mg/mL. It has been proved that IL-6 combined with IL-6R could phosphorylate STAT1 to p-STAT1 in Ser-727 position, and p-STAT1 served as a co-transactivator for enhancing the transcription of Bax[22,23]. Tocilizumab could competitive bind with IL-6R and block the phosphorylation of STAT1, then regulating the expression of Bax and Bcl-2. That may partly explain the results we obtained.

In conclusion, tocilizumab could significantly suppress the apoptosis and keep the proliferation viability of human cardiac myocytes after AMI/R.tocilizumab has wide application prospects in the protection of myocardium ischemia reperfusion

Conflict of interest statement

We declare that we have no conflict of interest.

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