Tocilizumab inhibits neuronal cell apoptosis and activates STAT3 in cerebral infarction rat model

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ABSTRACT

Cerebral infarction is a severe hypoxic ischemic necrosis with accelerated neuronal cell apoptosis in the brain. As a monoclonal antibody against interleukin 6, tocilizumab (TCZ) is widely used in immune diseases, whose function in cerebral infarction has not been studied. This study aims to reveal the role of TCZ in regulating neuronal cell apoptosis in cerebral infarction. The cerebral infarction rat model was constructed by middle cerebral artery occlusion and treated with TCZ. Cell apoptosis in hippocampus and cortex of the brain was examined with TUNEL method. Rat neuronal cells cultured in oxygen-glucose deprivation (OGD) conditions and treated with TCZ were used to compare cell viability and apoptosis. Apoptosis-related factors including B-cell lymphoma extra large (Bcl-xL) and Caspase 3, as well as the phosphorylated signal transducer and activator of transcription 3 (p-STAT3) in brain cortex were analyzed from the protein level. Results indicated that TCZ treatment could significantly prevent the promoted cell apoptosis caused by cerebral infarction or OGD ($P < 0.05$ or $P < 0.01$). In brain cortex of the rat model, TCZ up-regulated Bcl-xL and down-regulated Caspase 3, consistent with the inhibited cell apoptosis. It also promoted tyrosine 705 phosphorylation of STAT3, which might be the potential regulatory mechanism of TCZ in neuronal cells. This study provided evidence for the protective role of TCZ against neuronal cell apoptosis in cerebral infarction. Based on these fundamental data, TCZ is a promising option for treating cerebral infarction, but further investigations on related mechanisms are still necessary.

INTRODUCTION

Cerebral infarction is a severe hypoxic ischemic tissue necrosis in the brain, which is caused by blockage in brain vessels, including embolism and thrombosis. Various diseases and unhealthy living habits may lead to cerebral infarction, such as high blood pressure, diabetes mellitus, smoking, obesity and dyslipidemia, which are proved to positively correlated to the high mortality and disability of this disease [1]. Great efforts have been made to improve the therapeutic effect of cerebral infarction as to the application of thrombolytic drugs, recombinant tissue plasminogen activator [2] for example, and thrombectomy [3]. Studies are still seeking for effective strategies like multidisciplinary treatment to raise the outcomes [4].

Accelerated neuronal cell apoptosis is an important manifestation of cerebral infarction [5,6], which is supposed to be induced by the modulation of apoptosis-related factors, such as the Caspase family and the B-cell lymphoma 2 (Bcl-2) family. In addition, interleukin 6 (IL-6), a pro-inflammatory factor, seems to be involved in the modulation of diseases. However, its roles may be amphibolous, since evidence has been found supporting its relationship with both anti- and pro-apoptosis. For example, IL-6 inhibits apoptosis in myocardial infarction [7], but induces apoptosis in the variant pre-B-cell line 1A9-M [8] and melanoma cells [9]. Despite all this, IL-6 is up-regulated in the serum of acute cerebral infarction patients [10], having pivotal value in the diagnosis and treatment of cerebral infarction [11]. From this point of view, IL-6 and its agonists or antagonists are likely to play vital roles in the regulation of cerebral infarction.

Tocilizumab (TCZ) is a humanized monoclonal antibody against IL-6 receptor (IL-6R). It inhibits the signal transduction of IL-6 through binding to IL-6R [12], and thus being widely used as an immunosuppressive drug of IL-6-induced immune diseases, especially rheumatoid arthritis [13]. However, the function of TCZ in cerebral infarction is still elusive. This study aimed to uncover the roles of TCZ in regulating neuronal cell apoptosis during cerebral infarction. To achieve this, we constructed a rat model of cerebral infarction to compare

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cell apoptotic changes after TCZ treatment. Oxygen-glucose deprivation (OGD) was performed on cultured rat neuronal cells to imitate the infarction conditions *in vitro*. Cell apoptosis and viability in tissues and cultured cells were detected and compared. Apoptosis-related factors were analyzed to reveal the regulatory mechanism of TCZ. These data will offer evidence for new roles of TCZ in regulating neuronal cells and provide potential therapeutic strategies for treating cerebral infarction.

MATERIALS AND METHODS

Animal model

Male Sprague-Dawley rats (Vital River Laboratories, Beijing, China) of SPF degree (250-280 g) were randomly divided into three groups, namely Sham, Operation and TCZ groups, 15 individuals in each group. The animal experiments were approved by a local animal ethics committee and our institution, and performed according to the rules of our laboratory.

Before the middle cerebral artery occlusion (MCAo) operation, rats of TCZ group were intraperitoneally injected with TCZ (8 mg/kg) daily for seven days. Then rats of Operation and TCZ groups underwent the complete process of MACo. Briefly, 10% chloral hydrate was intraperitoneally injected at 3.5 mL/kg for anesthetization. The skin was cleaned with iodine solution and a cervical midline incision was made. The common carotid artery and external carotid artery were found and ligated with a suture, and the internal carotid artery was clipped with a vascular clamp. Then an incision was made in the common carotid artery and a silicone-coated suture was inserted, followed by a ligation. The reperfusion was performed after 2 h. After the rats were recovered from anesthetization, symptoms like standing instability and left hemiparesis indicated the successful construction of cerebral infarction model. Rats of Sham group underwent all the procedures except the ligation. After the model was induced, rats of the three groups were anesthetized and sacrificed for brain sampling. The hippocampus and cortex were separately sampled for further analysis.

Cell culture and OGD

Five newborn rats (1 d old) were anesthetized and sacrificed for neuronal cell culture. Briefly, the hippocampal or cortical tissue was dissected and digested in 0.05% trypsin (Gibco, Carlsbad, CA) for 30 min. After centrifugation, the cell pellet was resuspended in Dulbecco's modified eagle medium supplemented with 5% fetal bovine serum (Gibco). Then the cells were cultured at a density of 1×10^6 cells/mL, and the medium was replaced by Neurobasal with B-27 (Gibco). The

cells were divided into three groups, namely Control, OGD and TCZ group. For TCZ group, TCZ (10 μg/mL) was added to the medium. After five days, OGD was performed. Briefly, the medium of OGD group was gradually replaced by glucose-free medium and the final glucose concentration was lower than 1 mM. Both OGD and TCZ groups were cultured in 95% N_2 and 5% CO_2, while Control group continued to be cultured in atmosphere with 5% CO_2. After 4 h, all the cells were cultured in the original conditions for further analysis.

TUNEL assay

The rat hippocampus and cortical were paraffin-embedded, cut into slices, dewaxed and rehydrated. DNA fragmentation in tissue cells was detected with the TdT-mediated dUTP nick-end labeling (TUNEL) method using In Situ Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany) according to the manuals. The tissue slices were incubated in TUNEL reaction mixture in dark for 60 min at 37°C. Signals were observed using a fluorescence microscope (Olympus, Tokyo, Japan). Ten visual fields were randomly selected for each sample to count the cells with positive signals.

Cell viability assay

After OGD, cells were adjusted to 2×10^4/mL, plated in 96-well plates, and cultured for another 0, 24 or 72 h, and collected for 3-(4,5-dimethylthiazol-2-yl)-2,5-dephenyltetrazolium bromide (MTT) assay using MTT Cell Proliferation and Cytotoxicity Assay Kit (Beyotime, Shanghai, China) according to the manuals. Briefly, 10 μL MTT solution was added to each well and the cells were cultured for 4 h, after which 100 μL Formanzan solution was added. The cells were incubated until formanzan was dissolved. The optical density was measured at 570 nm using a microplate reader (Bio-Rad, Hercules, CA).

Cell apoptosis assay

Cell apoptosis was analyzed using Annexin V: FITC Apoptosis Detection Kit (BD Biosciences, San Jose, CA) and a flow cytometer (BD Biosciences). Briefly, cells were collected at 24 h post OGD, washed using cold phosphate buffer saline, and resuspended in binding buffer. Annexin V-FITC was added and the cells were incubated in dark for 15 min. Before detection, prodium iodide (PI) and binding buffer were added. The percent of apoptotic cells were indicated as the Anenxin V-positive and PI-negative in quadrant 3.

Western blot

Western blot was performed to detected the protein expression changes of B-cell lymphoma extra large (Bcl-xL),

cleaved Caspase 3, signal transducer and activator of transcription 3 (STAT3) and phospho-STAT3 (p-STAT3). The rat brain cortex tissues were lysed in protein lysis buffer (Beyotime) for protein extraction according to the manuals. Protein samples of the same amount (20 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to a polyvinylidene fluoride membrane. The membrane was blocked in 5% skim milk for 2 h at room temperature and incubated in the primary antibodies, including anti-Bcl-xL (ab77571), anti-active Caspase 3 (ab2302), anti-STAT3 (ab119352) and -anti STAT3 (phosphor Y705, ab76315) (Abcam, Cambridge, UK), at 4°C overnight. Then the membrane was incubated in the horse reddish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Anti-beta Actin antibody (ab8229, Abcam) was used to label the endogenous reference. Signals were developed using ECL Plus Western Blotting Substrate (Thermo Scientific, Carlsbad, CA), and analyzed using ChemiDoc XRS System (Bio-Rad).

Statistical analysis

All the experiments were repeated five times unless otherwise specified. Results were indicated as the mean ± standard deviation. *t* test and one-way analysis of variance were performed using SPSS 20 (IBM, New York, USA). $P < 0.05$ was considered to be statistically significant.
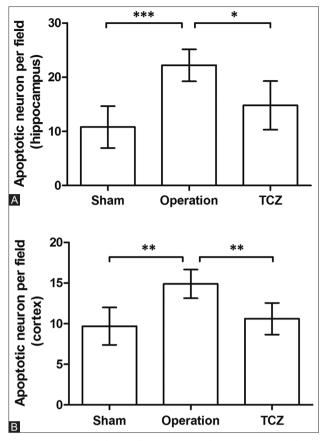
RESULTS

TCZ inhibits neuronal cell apoptosis in brain of cerebral infarction rat model

The function of TCZ was analyzed in cerebral infarction rat model by TUNEL assay to reveal its influences on neuronal cell apoptosis, in both hippocampus (Figure 1A) and cortex (Figure 1B). Results showed that in hippocampus, MCAo operation increased apoptotic neuronal cell number significantly ($P < 0.001$), while TCZ-treated rats possessed relatively less number of apoptotic neuronal cells compared to Operation group ($P < 0.05$). Similar results were also observed in cortex, with significant differences between Operation group and Sham or TCZ group ($P < 0.01$). These phenomena suggested the potential anti-apoptotic role of TCZ in neuronal cells of cerebral infarction rat model, which prompted further analysis in this study.

TCZ inhibits cultured neuronal cell apoptosis

Then the anti-apoptotic role of TCZ was verified in primarily cultured rat neuronal cells by MTT assay and Annexin V-PI dual-staining assay. OGD was performed on these cells to imitate the ischemic and anoxic state. MTT assay indicated that cell viability did not varied significantly when detected
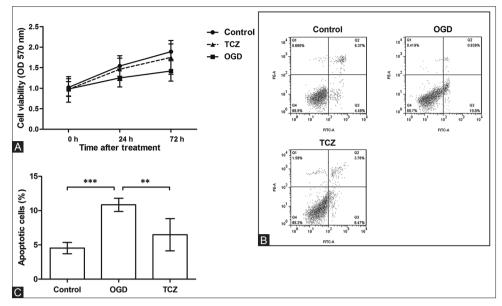

FIGURE 1. TCZ inhibits cell apoptosis in hippocampus and cortex of the cerebral infarction rat model. Sham, sham-operated group. Operation, middle cerebral artery occlusion (MCAo) operation group. TCZ, tocilizumab. *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$. Ten visual fields are randomly chosen for each sample. (A) MCAo increases the apoptotic cell number in hippocampus per visual field, and TCZ can reduce apoptotic cells. (B) MCAo increases the apoptotic cell number in cortex per visual field, and TCZ can reduce apoptotic cells.

immediately after OGD (Figure 2A), but during the 72 h post OGD, viability of cells undergone OGD was lower than Control group, and that of TCZ-treated cells was increased faster than OGD group, implying TCZ might help to promote neuronal cell viability after OGD, though no significant difference was detected between groups. Consistently, cell apoptosis detected by flow cytometry indicated that OGD greatly increased the percent of apoptotic neuronal cells ($P < 0.001$), while TCZ treatment abrogated this increase ($P < 0.01$, Figure 2B and 2C), suggesting that TCZ could inhibit cultured neuronal cell apoptosis caused by OGD. Together with the above results in brain tissues, it could be deduced that TCZ possessed anti-apoptotic roles in neuronal cells both in the rat model and in cultured cells, which implied the protective function of TCZ in cerebral infarction.

TCZ regulates apoptosis-related factors

TCZ was found to significantly inhibit neuronal cell apoptosis from the existing results, thus its function mechanism was analyzed based on the detection of apoptosis-related factors,
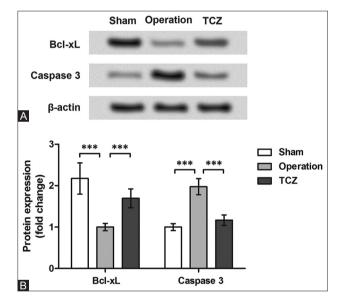
FIGURE 2. TCZ promotes cell viability and suppresses cell apoptosis of cultured neuronal cells. Control, cells without OGD or TCZ treatment. OGD, oxygen-glucose deprivation. TCZ, tocilizumab. **$P < 0.01$. ***$P < 0.001$. (A) Cell viability measured at 0, 24 and 74 h post OGD. (B) Cell apoptosis detected by flow cytometry. (C) Histogram indicating the percent of apoptotic cells in quadrant 3 (Q3).

Bcl-xL and Caspase 3, the former being an anti-apoptotic factor and the latter a pro-apoptotic one. Western blot showed Bcl-xL protein was inhibited and Caspase 3 was promoted in brain cortex of rat model, while TCZ treatment could weaken these changes (Figure 3A). The histogram showing the density of bands indicated significant differences between Operation group and Sham or TCZ group ($P < 0.001$).

Numerous studies have proved that IL-6 can regulate the phosphorylation levels of STAT3, which was thus detected by western blot to compare the proportion of phospho-STAT3 (p-STAT3) in the three groups. Western blot results showed that the total STAT3 protein levels hardly changed, but the p-STAT3 level, detected by the antibodies recognizing the phosphorylation at Y705, was reduced after MCAo operation, and was recovered to some extent in TCZ group (Figure 4A). Significant changes could be reflected after digitization ($P < 0.01$, Figure 4B). These data indicated that activation of STAT3 was inhibited in cerebral infarction rat model, but TCZ could promote the phosphorylation of STAT3. Taken together, TCZ could regulate the protein expression of Bcl-xL and Caspase 3, and the activation of STAT3, which might contribute to its anti-apoptotic role in neuronal cells after cerebral infarction.
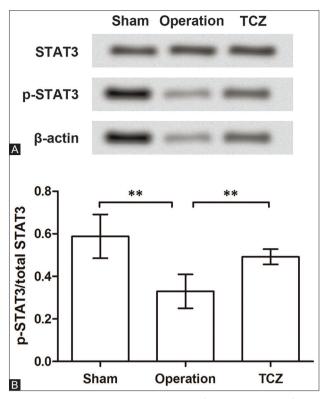
DISCUSSION

Based on the results of cerebral infarction rat model and cultured neuronal cells in this study, TCZ is proved to suppress neuronal cell apoptosis in hippocampus and cortex of the rat model, as well as in the cultured cells. Protein expression analysis reveals that TCZ up-regulates Bcl-xL, inhibits Caspase 3, and induces the activation of STAT3.

FIGURE 3. TCZ promotes Bcl-xL and inhibits Caspase 3 protein expression in cortex of cerebral infarction rat model. Sham, sham-operated group. Operation, middle cerebral artery occlusion (MCAo) operation group. TCZ, tocilizumab. Bcl-xL, B-cell lymphoma extra large. ***$P < 0.001$. (A) Western blot showing the expression changes of Bcl-xL and Caspase 3, with β-actin being the endogenous reference. (B) Histogram showing the relative density of bands in western blot.

The anti-apoptotic role of TCZ in rat cerebral infarction was proved both *in vivo* and *in vitro*. In hippocampus and cortex regions of model rat brain samples, TCZ treatment was capable of reducing the increase of apoptotic cells caused by MCAo operation. Besides, in cultured rat neuronal cells, the inhibited cell viability and the induced cell apoptosis after OGD were both reversed, to some extent, by TCZ treatment. Though the alleviating effects of TCZ might not bring cell apoptosis down to the original levels (Figure 1A and 2C), TCZ

FIGURE 4. TCZ induces the activation of STAT3 in cortex of cerebral infarction rat model. Sham, sham-operated group. Operation, middle cerebral artery occlusion (MCAo) operation group. TCZ, tocilizumab. STAT3, signal transducer and activator of transcription. p-STAT3, phosphorylated STAT3. **$P < 0.05$. (A) Western blot showing the expression of total STAT3 and p-STAT3, with β-actin being the endogenous reference. (B) Histogram showing the ratio between p-STAT3 and total STAT3.

did significantly mitigate the injury in tissues and cells after cerebral infarction in rats, implying its protective functions against cerebral infarction.

The following protein expression analysis in this study also suggested the anti-apoptotic role of TCZ. Bcl-xL is a member of Bcl-2 family, which is composed of various factors involved in cell apoptosis regulation, such as the pro-apoptotic factors Bax and Bak, as well as the apoptosis suppressors like Bcl-2 and Bcl-xL [14]. A great pile of studies have demonstrated Bcl-xL as the anti-apoptotic factor, which is distinguished from its shorter isoform Bcl-xS [15,16], and applied it in the identification of cell apoptotic changes [17,18]. Likewise, Caspase 3 is an acknowledged indicator of promoted cell apoptosis [19,20]. In this study, the anti-apoptotic role of TCZ demonstrated in brain tissues of rat model and cultured neuronal cells is consistent with the up-regulated Bcl-xL and down-regulated cleaved Caspase 3 levels, validating the function of TCZ, and implying the modulated Bcl-xL and Caspase 3 by TCZ as well, which might be the potential mechanism of TCZ in regulating neuronal cell apoptosis.

Furthermore, TCZ was capable of promoting the phosphorylation of STAT3 in rat brain and neuronal cells. STAT3 is activated by the phosphorylation of tyrosine 705, and thus inhibits cell apoptosis. The suppression of its activation is a potential therapeutic target of tumors such as breast cancer [21,22]. In this study, we used a p-STAT3 (Tyr 705)-specific primary antibody to detect the activation of STAT3 by comparing it to the total STAT3 level. Results indicated the activated STAT3 level was up-regulated by TCZ, which was in accordance with the inhibited cell apoptosis. In addition, the activation of STAT3 is involved in the regulation of Bcl-xL, contributing to the enhanced cell survival, as is proved in neck squamous cell carcinomas [23]. So it could be deduced that in cerebral infarction rat model, TCZ activated STAT3/Bcl-xL pathway, which might be a potential reason for the suppressed neuronal cell apoptosis.

Another important regulatory relationship regarding STAT3 is that IL-6 plays vital roles in activating STAT3, which is crucial for the blocked apoptosis during inflammatory processes, as well as the pathogenesis of diseases [24], such as lung adenocarcinoma and malignant pleural effusion [25]. STAT3 is phosphorylated at tyrosine 705 in response to IL-6, which seems, however, contradicting with the promotive effects of TCZ on p-STAT3. We speculated that one likely reason might be the complicated regulation of STAT3, because the activation of STAT3 can be modulated by various growth factors and cytokines besides IL-6. For example, the tyrosine phosphorylation of STAT3 is inhibited by p53 [26], and induced by IL-4 [27] and janus kinase 1 [28], amongst others. In addition, there is studies showing the dose-dependent effect of IL-6 in human primary melanoma cell line WM35 [9]. Studies in cerebral infarction also indicate both the anti-apoptosis role of IL-6 and its up-regulation in patient serum [10,29], which implies the dose-dependent effect and the complex regulation of IL-6. Though we did not detect IL-6 level changes in this study since its monoclonal antibody TCZ was used, it could be deduced that the regulation of STAT3 by the inhibited IL-6 in the rat model, if any, might be abrogated by other modulators promoting STAT3. This possibility needed to be verified by further studies. It also alerted us that TCZ, whose side effects have been found in some cases [30], should be prudently applied to disease treatment due to its impacts on factors besides IL-6.

In summary, our findings indicate the protective role of TCZ, a monoclonal antibody against IL-6, in preventing neuronal cell apoptosis in cerebral infarction rat model, as well as in cultured rat neuronal cells. Application of TCZ to the rat model and neuronal cells leads to the activation of STAT3 by tyrosine phosphorylation, and the up-regulated Bcl-xL and down-regulated Caspase 3, which may constitute the potential regulatory mechanism of TCZ in protecting neuronal cells. This study offers fundamental information for the possible usage of TCZ in treating cerebral infarction, but further research is still necessary to investigate the interrelated mechanisms.

DECLARATION OF INTERESTS

The authors declare no conflict of interests.

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