European Review for Medical and Pharmacological Sciences 2018; 22: 5614-5619

IL-10 targets Th1/Th2 balance in vascular dementia

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Abstract. – OBJECTIVE: The pathogenesis of vascular dementia (VD) is not fully elucidated. Th1/Th2 balance may change in VD, leading to numerous inflammatory cytokines secretion. Interleukin-10 (IL-10) is an immune suppressor, while its function in VD and correlation with Th1/Th2 balance are still unclear.

MATERIALS AND METHODS: The healthy male rats were randomly divided into three groups, including sham group, model group, and IL-10 group. Th1 and Th2 cytokines IL-2, IL-4, IL-6, and tumor necrosis factor-α (TNF-α) expressions in the serum were tested by enzyme-linked immunosorbent assay (ELISA). IL-10 expression in brain tissue and peripheral blood was detected by Real-Time PCR and ELISA. The correlation relationship between IL-10 and T helper cells 1/2 (Th1/Th2) cytokines was analyzed. Hippocampus cell apoptosis was determined by caspase 3 activity kit. Nuclear transcription factor 2 κB (NF-κB) expression was evaluated by Western blot.

RESULTS: IL-10 levels were decreased, caspase 3 activity was enhanced, NF-κB expression was declined, IL-2 and TNF-α secretion were up-regulated, while IL-4 and IL-6 secretion were reduced in hippocampus tissue and peripheral blood from VD model rat compared to sham group ($p<0.05$). IL-10 significantly attenuated caspase 3 activity, up-regulated NF-κB expression, reduced IL-2 and TNF-α secretion, and enhanced IL-4 and IL-6 secretion ($p<0.05$). IL-10 was negatively correlated with Th1 cytokines and positively correlated with Th2 cytokines ($p<0.05$).

CONCLUSIONS: IL-10 expression declined in VD and participated in regulating Th1/Th2 balance. IL-10 participated in VD incidence and development through regulating cell apoptosis and NF-κB expression.

Key Words

Vascular dementia, IL-10, NF-ÐB, Caspase 3, Th1/Th2, Inflammation.

Introduction

Vascular dementia (VD) is cerebral dysfunction caused by multiple cerebrovascular diseases and pathological changes, leading to learning and cognitive disorder. As the second common dementia disease after Alzheimer disease, VD seriously affects the quality of life[1,2]. Moreover, it is difficult to be differentiated from Alzheimer disease because of similar age of onset and symptom[3,4]. It is widely concerned in clinic because of its serious influence on quality of life, economic and mental pressure, and society burden[5,6]. VD prevention is an important problem needs to be solved in geriatrics and related disciplines[7,8]. Different from the progressiveness and uncontrollability of Alzheimer disease, VD shows the most hopeful prevention in dementia[9,10]. Multiple factors may lead to VD, and most of them are still unclear. It was found that oxidative stress injury and inflammation were closely related to VD[11,12]. The high incidence rate of atherosclerosis, hypertension, and cardiovascular and cerebrovascular disease in our country leads to high VD morbidity around the world[13]. Therefore, investigation of the pathogenesis of VD is of great significance to find a new molecular target for the treatment.

Immune and inflammation are the key factors of VD[14]. T helper cells (Th) could be divided into two groups according to different cytokines secretion. It was reported that Th1 and Th2 regulated each other via cytokines secretion. Maintaining Th1/Th2 balance is of great significance to sustain normal immune function[15,16]. IL-10 is a multifunctional negative regulatory factor that is generated by Th2 cells, active B cells, monocytes, and macrophages[17]. It plays a critical role in autoimmune disease, severe infectious disease, tumor, and transplantation immunity by involving in the regulation of immune cells, inflammatory cells, and tumor cells[18]. The IL-10 inhibited the apoptosis by suppressing the expression of the caspase 3[19]. Meanwhile, IL-10 inhibits the NF-kB production of pro-inflammatory mediators and inhibits the apoptosis[20]. However, the role of IL-10 in VD and its correlation with the Th1/Th2 balance still needs further elucidation.

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Materials and Methods

Experimental Animals

Healthy Wistar male rats aged two-months and weighted 250 ± 20 g were purchased from Jilin University Experimental Animal Center and raised in specific pathogen free (SPF) grade experimental animal center. The raising condition contained temperature at 21 ± 1°C, relative humidity at 50%-70%, and 12 h day/night cycle.

Rats were used for all experiments, and all the procedures were approved by the Animal Ethics Committee of The First Affiliated Hospital of Harbin Medical University, China.

Main Materials and Instruments

10% chloral hydrate was purchased from Zhpharma (Shanghai, China). The IL-1β monoclonal antibody was bought from Sigma-Aldrich (St. Louis, MO, USA). IL-10, IL-2, IL-4, IL-6, and tumor necrosis factor-α (TNF-α) enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D Systems Inc. (Minneapolis, MN, USA) polyvinylidene difluoride (PVDF) membrane was derived from Pall Life Sciences (Covina, CA, USA). Enhanced chemiluminescence (ECL) reagent was obtained from Amersham Biosciences (Piscataway, NJ, USA). Rabbit anti-mouse nuclear transcription factor 2 κB (NF-κB) monoclonal antibody and goat anti-rabbit horseradish peroxidase (HRP) labeled IgG secondary antibody were provided by Cell Signaling Technology (Beverly, MA, USA). RNA extraction kit and reverse transcription kit were purchased from ABI (Foster City, CA, USA). Caspase 3 activity detection kit and Western blot related reagents were provided by Beyotime Biotechnology (Shanghai, China). ABI 7500 Real-Time PCR amplifier was derived from ABI (Foster City, CA, USA). Microscopic surgery instrument was purchased from Suzhou Medical Apparatus Factory (Suzhou, China). Multi-Parameter animal physiological monitor, electroencephalograph (EEG) recorder, and YC-2 stimulator were bought from Yuyanbio (Shanghai, China). DNA amplifier was obtained from PE Gene Amp PCR System 2400 (PE Gene Applied Biosystems, Foster City, CA, USA). Labsystem Version 1.3.1 microplate reader was provided by Bio-Rad Laboratories (Hercules, CA, USA). Other reagents were purchased from Sangon Biotech. Co. Ltd. (Shanghai, China).

Experimental Animal Grouping and Treatment

The healthy male rats were randomly divided into three groups, including sham group, VD model group established by bilateral common carotid artery ligation, and IL-10 group treated by IL-10 monoclonal antibody intracerebroventricular injection based on the model group.

Rat VD Model Establishment

Rat VD model was established by bilateral common carotid artery ligation[21]. The rat was anesthetized by 0.35 ml/100 g 10% chloral hydrate abdominal injection and fixed on stereotaxic apparatus. Then, the neck skin was disinfected and an incision was made on neck midcourt line. The muscle and connective tissue were separated to isolate bilateral common carotid artery for ligation. The vagus nerve was protected to avoid damage. Rat breathes and heart rates were observed. 10^4 U gentamycin was used for three days after surgery to prevent infection. The rats in the sham group received same treatment without bilateral common carotid artery ligation.

Sample Collection

A total of 2 ml blood was extracted from the rat caudal vein and centrifuged at 2000 r/min for 5 min. The supernatant was stored at -20°C. The hippocampus tissue was extracted and stored at -80°C.

Real-Time PCR

Total RNA was extracted from hippocampus tissue by TRIzol and reverse transcribed to complementary DNA (cDNA). The primers were designed using PrimerPremier 6.0 software (Table I) and synthesized by Sangon. Real-time PCR was performed at 56°C for 1 min, followed by 35 cycles of 92°C for 30 s, 58°C for 45 s, and 72°C for 35 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected as internal reference. The relative expression of mRNA was calculated by $2^{-\Delta Ct}$ method.

Table I. Primer sequences.

Gene	Forward 5′-3′	Reverse 5′-3′
GAPDH	AGAGTACCTTGCTTCTGGG	TAATGATAGGTGACCCCTGGT
IL-10	CACTCCCTGCATTACAATC	CAATGATGGTATTATAGGATCCC

ELISA

ELISA was used to test IL-2, IL-4, IL-6, and TNF-α contents in the serum. A total of 50 μl diluted standard substance were added to each well to establish a standard curve. Next, the plate was added with 50 μl sample and washed for five times. Then, the plate was incubated in 50 μl conjugate reagent at 37°C for 30 min. After washed five times, the plate was treated with 50 μl color agent A and B at 37°C avoid of light for 30 min. At last, the plate was added with 50 μl stop buffer to stop the reaction and tested at 450 nm to obtain the optical density (OD) value. The OD value of standard substance was used to prepare the linear regression equation, which was adopted to calculate the concentration of samples.

Caspase 3 Activity Detection

Caspase 3 activity was tested according to the manual. The cells were digested by trypsin and centrifuged at 600 ×g and 4°C for 5 min. Next, the cells were added with 2 mM Ac-DEVD-pNA and detected at 405 nm to calculate caspase 3 activity.

Western Blot

The hippocampus tissues were added with radioimmunoprecipitation assay (RIPA) containing protease inhibitor and cracked on ice for 15-30 min. Next, the tissues were treated by ultrasound at 5 s for 4 times and centrifuged at 10000 ×g for 15 min. The protein was transferred to new Eppendorf (Ep) tube and stored at -20°C. The protein was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membrane at 100 mA for 1.5 h. After blocked by 5% skim milk for 2 h, the membrane was incubated in NF-κB monoclonal antibody (1:2000) at 4°C overnight. Then, the membrane was incubated in goat anti-rabbit secondary antibody at room temperature for 30 min. Next, the membrane was treated by the developer for 1 min and exposed to observe the result. The film was scanned by Quantity One software and analyzed by the protein image processing system. Each experiment was repeated four times.

Statistical Analysis

All data were presented as mean ± standard deviation (SD). All data analyses were performed on SPSS11.5 software (SPSS Inc., SPSS for Windows, Chicago, IL, USA). The student's *t*-test was used to compare the differences between two groups. Tukey's post-hoc test was used to validate the ANOVA for comparing measurement data between groups. $p<0.05$ was considered as statistical significant.

Results

IL-10 mRNA Expression in Rat Hippocampus Tissue

Real-time PCR was adopted to test IL-10 mRNA expression in rat hippocampus tissue. IL-10 mRNA significantly increased in rat VD model compared with sham group ($p<0.05$). IL-10 injection obviously down-regulated IL-10 mRNA expression in VD model ($p<0.05$) (Figure 1).

IL-10 Content in Rat Serum

ELISA was applied to test IL-10 content in rat serum. IL-10 content markedly elevated in the serum from rat VD model compared with sham group ($p<0.05$). IL-10 injection apparently elevated IL-10 level in rat VD model ($p<0.05$) (Figure 2).

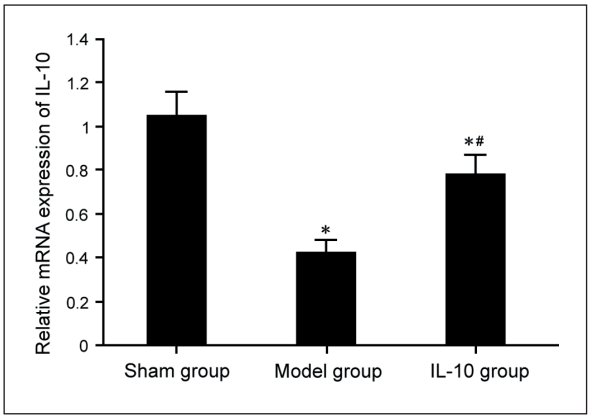

Figure 1. IL-10 mRNA expression in rat hippocampus tissue. *$p<0.05$, compared with sham group; #$p<0.05$, compared with model group.

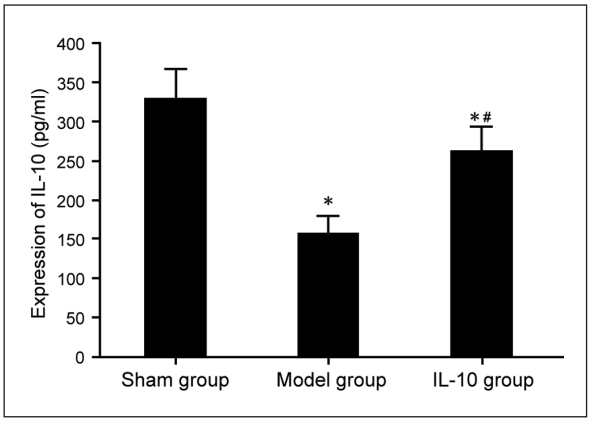

Figure 2. IL-10 expression in rat serum. *$p<0.05$, compared with sham group; #$p<0.05$, compared with model group.

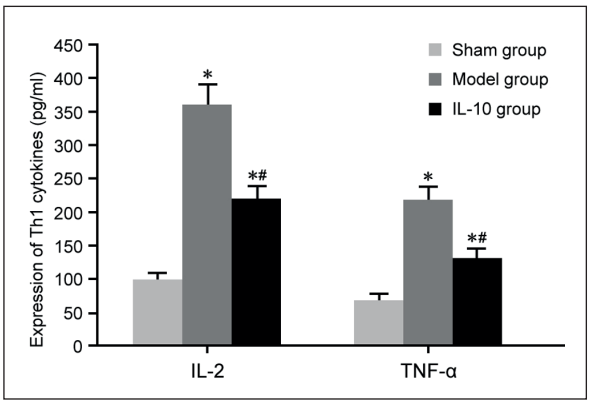

Figure 3. Th1 cytokines expression in rat serum. *p<0.05, compared with sham group; #p<0.05, compared with model group.

Th1 Cytokines Expression in Rat Serum

ELISA was selected to detect Th1 cytokine IL-2 and TNF-α expressions in rat serum. IL-2 and TNF-α levels significantly increased in VD model group compared with sham group (p<0.05). IL-10 markedly reduced IL-2 and TNF-α levels in the serum of VD rats (p<0.05) (Figure 3).

Th1 Cytokines Expression in Rat Serum

ELISA was selected to detect Th2 cytokine IL-4 and IL-6 expressions in rat serum. IL-4 and IL-6 levels markedly decreased in VD model group compared with sham group (p<0.05). IL-10 apparently elevated IL-4 and IL-6 levels in the serum of VD rats (p<0.05) (Figure 4).

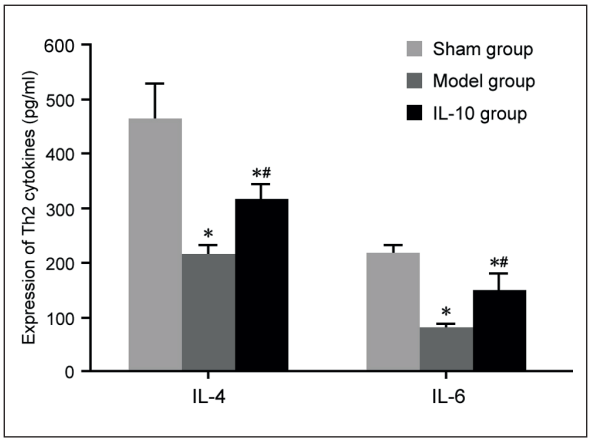

Figure 4. Th2 cytokines expression in rat serum. *p<0.05, compared with sham group; #p<0.05, compared with model group.

Table II. Correlation between IL-10 and Th1/Th2 balance in VD rat.

	IL-2	TNF-α	IL-4	IL-6
r-value	-0.683	-0.721	0.843	0.521
p-value	0.036	0.025	0.021	0.015

Correlation Between IL-10 and Th1/Th2 Balance in VD Rat

Effects of IL-10 on Th1/Th2 balance in VD rat was analyzed. IL-10 was negatively correlated with Th1 cytokines IL-2 and TNF-α, and positively correlated with Th2 cytokines IL-4 and IL-6 (p<0.05) (Table II).

Effects of IL-10 on Caspase 3 Activity in Rat Hippocampus Tissue

Caspase 3 activity detection kit was used to determine the impact of IL-10 on caspase 3 activity in rat hippocampus tissue. Caspase 3 activity markedly enhanced in VD rat hippocampus tissue compared with control (p<0.05). IL-10 significantly weakened caspase 3 activity in rat hippocampus tissue compared with model group (p<0.05) (Figure 5).

Effects of IL-1β on NF-κB Expression in Rat Hippocampus Tissue

Western blot was adopted to analyze the impact of IL-10 on NF-κB expression in rat hippocampus tissue. NF-κB protein significantly reduced in VD rat model compared with sham group (p<0.05). It apparently increased in VD rat treated by IL-10 (p<0.05) (Figure 6).

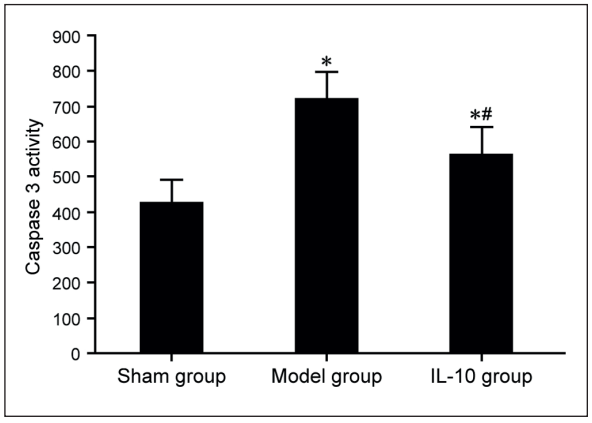

Figure 5. The impact of IL-10 on Caspase 3 activity in rat hippocampus tissue. *p<0.05, compared with sham group; #p<0.05, compared with model group.

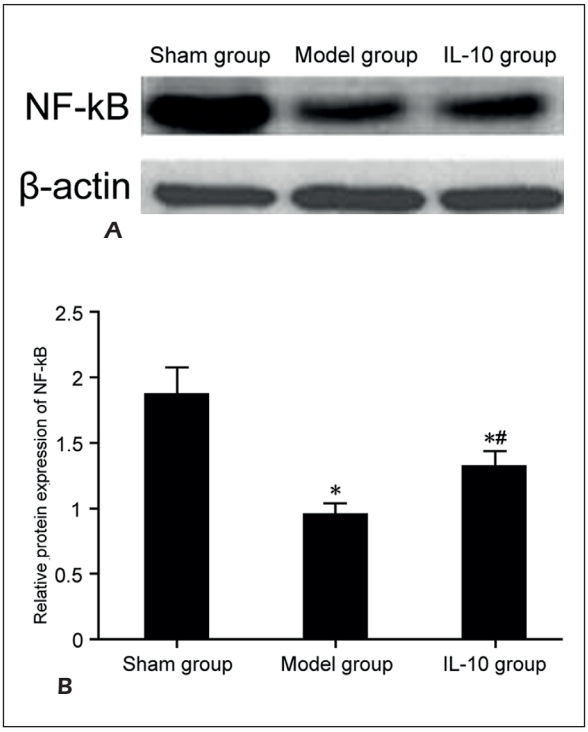

Figure 6. The impact of IL-10 on NF-κB expression in rat hippocampus tissue. **A**, Western blot detection of NF-κB protein expression. 1, sham group; 2, model group; 3, IL-1β group. **B**, NF-κB expression analysis. *p<0.05, compared with sham group; #p<0.05, compared with model group.

Discussion

Immune response and inflammation are key factors in VD occurrence and development[14]. Th1 and Th2 regulate each other by secreting cytokines to maintain Th1/Th2 balance. It plays an important role in sustain normal immune function[15,16]. Th1 mainly secretes IL-2 and TNF-α, while Th2 mainly secretes IL-4 and IL-6[21]. Th1/Th2 can keep homeostasis and play a crucial in cellular and humoral immunity through self-regulation and co-adjustment[22,23].

As a negative regulatory factor, IL-10 mainly inhibits proinflammatory cytokines secretion to alleviate inflammation and antagonist inflammatory medium. It plays a key role in tumor, organ transplantation, and inflammation[24]. However, it is still unclear about whether IL-10 is involved in regulating VD. This study established VD model using bilateral common carotid artery ligation and found that IL-10, IL-4, and IL-6 declined, while IL-2 and TNF-α up-regulated in VD occurrence. IL-10 treatment decreased IL-2 and TNF-α expressions, whereas elevated IL-4 and IL-6 levels. IL-10 was negatively correlated with IL-2 and TNF-α, and positively correlated with IL-4 and IL-6, indicating that IL-10 can regulate Th2 transforming to Th1. NF-κB is a member of transcriptional factor family that promotes cell transcription and proliferation. Caspase-3 activation may induce cell apoptosis[25,26]. In this study, caspase 3 activity enhanced, NF-κB expression declined, IL-2 and TNF-α secretion up-regulated, while IL-4 and IL-6 secretion reduced, and neuron apoptosis enhanced in rat VD model. IL-10 protects neurons by attenuating caspase 3 activity and up-regulating NF-κB expression.

Conclusions

We demonstrated that IL-10 expression declined in VD and participated in regulating Th1/Th2 balance. IL-10 participated in VD occurrence and development through regulating cell apoptosis and NF-κB expression.

Conflict of Interests:

The Authors declare that they have no conflict of interests.

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