• Basic Research •

Effects of etanercept on the apoptosis of ganglion cells and expression of Fas, TNF-α, caspase-8 in the retina of diabetic rats

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Abstract

• AIM: To evaluate the effects of etanercept on the expression of Fas, tumor necrosis factor-alpha (TNF-α) and caspase-8 in the early stage of the apoptotic pathway in diabetic rats, and to explore the therapeutic effect of etanercept on diabetic retinopathy.

• METHODS: A total of 60 Sprague-Dawley (SD) rats were randomly and evenly divided into 3 groups with 20 rats each, including control group, and diabetic groups with or without treatment. Streptozotocin (STZ)-induced diabetic rats were established for diabetic groups. Blood glucose and body weight were measured weekly. All the rats were sacrificed at the 12wk after treatment. The expressions of Fas, TNF-α and caspase-8 in rat retina were quantitatively detected by PCR and Western blot. The leakage of Evan blue was adopted to measure the retinal vascular leakage quantitatively, and to compare it among different groups. TUNEL method was used to compare the amount of apoptotic bodies quantitatively in rat retina ganglion cells under electron microscope.

• RESULTS: The expressions of Fas, TNF-α and caspase-8 in each group were compared *via* PCR and Western blot, in which the diabetic group with treatment was lower than those without treatment (*P*<0.01), but all the diabetic groups were higher than the control group (*P*<0.01). Evans blue leakage in the diabetic treatment group was lower than those without treatment (*P*<0.01), but those in the control group was the lowest compared with the other two groups (*P*<0.01). TUNEL method showed that the apoptotic bodies of retina in the diabetic treatment group was lower than those without treatment (*P*<0.01), while those in the control group was the lowest compared with the other two groups (*P*<0.01).

• CONCLUSION: Etanercept can effectively reduce the expression of Fas, TNF-α and caspase-8, as well as the retinal leakage and retinal cell apoptosis in diabetic rats.

• KEYWORDS: etanercept; ganglion cells; Fas; tumor necrosis factor-alpha; caspase-8; apoptosis; diabetes; rat

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INTRODUCTION

Diabetic retinopathy (DR) is a common severe complication of diabetes mellitus (DM). With the increasing number of DM patients, DR has become a major issue threatening the vision of people in China. However, the pathogenesis of DR is complicated. To date, animal models of DM indicated the pathogenesis from the perspective of metabolism, hemodynamics, angiogenin and apoptosis. Researchers revealed that a variety of cytokines constitute a network of cytokines involved in the pathogenesis of DR, including vascular endothelial growth factor (VEGF), interleukin (IL) -2, tumor necrosis factor-alpha (TNF-α), IL-1, interferon-gamma (IFN-γ), *etc*[1-3]. Furthermore, Fas, TNF-α, and caspase-8 are important cytokines in the apoptotic signaling pathway, which taken together cause apoptosis of retinal cells and trigger a series of inflammatory responses in DR patients.

Etanercept is one of competitive inhibitors of cell surface TNF receptor, inhibiting the biological activity of TNF, *via* which further regulate the other downstream molecules (such as cytokines, adhesion molecules and proteases) to control the biological response. In the current study, we aimed to observe the effect of etanercept on the expression of Fas, TNF-α and caspase-8, as well as on the retinal leakage and cell apoptosis of retina in diabetic rats, to further understand the mechanism and provide supports and guidance for clinical treatment.

MATERIALS AND METHODS

Ethical Approval Experimental protocols were approved by the animal care and Ethics Committee at Fujian Medical University according to the Association for Research in Vision and Ophthalmology under the guidelines with the Animal Welfare Act (www.nal.usda.gov/awic/animal-welfare-act).

Establishment of Diabetic Animal Model The animal center of Fujian Medical University provided 60 male Sprague-Dawley (SD) rats aged 2mo and weighed 180-220 g, which were housed in the standardized animal centers without specific pathogens, and with ad libitum feeding. The 60 rats were randomly divided into control group, diabetic treatment group' and diabetic non-treatment group (model group), with 20 in each group. Rats of diabetic groups were adaptively fed for 1wk and then fasted for 12h. After that, intraperitoneal injection of 1% streptozotocin (STZ) solution (STZ solution in citrate buffer) with the dose of 60 mg/kg was given to those rats to establish the diabetic animal model. Venous blood from the tails of rats was collected to measure blood glucose after 72h. Diabetic rat model was successfully established if the blood glucose higher than 16.7mmol/L while normal blood glucose values in controlled rats were 5.2±0.3 mmol/L. On the third day after the establishment of the diabetic model, the treatment group was given subcutaneous etanercept (concentration 0.4 mg/kg) once a day from the third day after the successful establishment of the model till the end of the experiment.

Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction of Retina RNA was extracted from retinal tissue samples of 6 rats randomly selected from each group. RNA purity was determined through ultraviolet (UV) absorption method, cDNA was synthesized by reverse transcription, which was amplified through real-time quantitative polymerase chain reaction (RT-PCR). RT-PCR primers were designed by Primer Premier 5.0 shown in Table 1. The annealing temperature was 60℃, and the PCR reaction conditions were: 50℃, 2min; 95℃, 10min; 95℃, 15s; 60℃, 1min. Fluorescence melting curve was used to verify the amplification specificity of the target genes. The $2^{-\Delta\Delta Ct}$ method was adopted to analyze the relative changes in the target genes expression.

Western Blot Six rats were taken from each group and their eyeballs were collected under anesthesia. Subsequently, the anterior segments of the eye and the retinal tissues were removed from eyeballs. After the eyeballs from both eyes were weighed with a precision balance, a 10% homogenate was prepared in an ice bath with 0.05 mmol/L of ice-cold phosphate buffer [pH=7.8, containing 0.01 mmol/L of ethylene diamine tetraacetic acid (EDTA)] for detection. Frozen retinal tissues were taken out from refrigerator and added with lysate (containing 5% protease inhibitor). After being fully cracked, tissues were centrifuged for 5min with 12 000 rpm. And then the proteins were added by the loading buffer solution according to the ratio of 4:1. After 95℃ water bath for 5min, the mixtures of protein and buffer solution were centrifuged for 5min. After that, total cell proteins were obtained and then were reserved at 4℃. Electrophoresis was applied after loading and the voltage was adjusted to 40 V for 40min. After the bromophenol blue band entered the separation gel, the voltage was adjusted to 90 V for 80min. Electrophoresis was stopped when the bromophenol blue reached the bottom of the gel during electrophoresis. Transfer voltage was adjusted to 100 V for 1h. And after successful transfer, the polyvinylidene fluoride (PVDF) membrane was immersed in the blocking solution, placed on a shaker and sealed at room temperature for 2h. Next, primary antibodies were added (Fas concentration was 1:800, TNF-α concentration was 1:2000 and caspase-8 concentration was 1:1000) and incubation was carried out overnight at 4℃. And then, washing was conducted and secondary antibodies were added (concentrations of Fas, TNF-α and caspase-8 were 1:2000). The solution was placed on a shaker and incubated for 2h at room temperature. Finally, β-actin was taken as an internal reference, and developing by chemiluminescence method, imaging by gel imager and photographing and analyzing by gel analysis system were conducted.

Table 1 Real-time PCR primer sequences

Gene	Primer sequence
Fas	5'-CTACGGTTCTGCTCTATATGC-3'
	5'-GTTGGAGACTTTCTCCATCA-3'
TNF-α	5'-GGACGAAAGTCTTGGAGTGTC-3'
	5'-TGCCACAGGGCCATTAAGAG-3'
Caspase-8	5'-AGTCTGCATGCCATGGTTCT-3'
	5'-GGACCCAGCAGGTGGTACAA-3'
GAPDH	5'-CAACGGGAAACCCATCACCA-3'
	5'-ACGCCAGTAGACTCCACGACAT-3'

Evans Blue Leakage of Retina After anesthesia of 4 rats in each group, the amount of retinal Evans blue (EB) leakage was measured to understand the degree of damage of the blood-retinal barrier (BRB)[4]. The absorbance (OD) of samples at 620 and 740 nm was measured with a spectrophotometer. Each sample was measured 3 times and averaged. Net OD value =OD620-OD740. A standard curve for the concentration of EB dye in formamide was established. Statistical analysis showed a highly linear correlation between EB concentration and net OD (r=0.996, P=0.001). Y=0.0702X+0.0125; where Y represents the net OD value and X represents the EB concentration. Based on the OD of each group, the EB mass concentration was calculated, and the retinal EB leakage amount (ng) was obtained by multiplying the concentration by 300 μL. Finally, the EB leakage (ng) was normalized by the dry weight of the retina (mg) and the result was expressed as ng/mg.

Table 2 Weight of each group at each time point in rats mean±SD, g

Groups	Sample size	Weights of rats at each time point			
		Before model establishment	4wk	8wk	12wk
Control	20	220.4±4.6	301.7±4.9	352.6±5.0	405.9±6.9
Treatment group	20	220.3±5.1	250.6±4.1	281.7±4.3	302.4±5.1
Non-treatment group	20	221.4±5.5	281.5±5.2	312.8±4.6	334.3±4.1
F		0.305	591.973	1181.246	1886.506
P		0.739	<0.001	<0.001	<0.001

Table 3 Blood glucose of each group at each time point in rats mean±SD, mmol/L

Groups	Sample size	Blood glucose at each time point			
		Before model establishment	4wk	8wk	12wk
Control	20	5.2±0.3	5.4±0.5	5.5±0.3	5.7±0.3
Treatment group	20	5.2±0.5	25.2±1.1	26.8±1.2	27.0±1.0
Non-treatment group	20	5.3±0.5	25.5±1.0	26.2±1.2	27.0±1.0
F		0.229	3109.582	2933.011	4247.192
P		0.796	<0.001	<0.001	<0.001

TUNEL Method: The Number of Apoptotic Cells TUNEL Assay Kit was used to detect the apoptosis (Roche, Switzerland). Four rats were taken from each group. After anesthesia, the eyeballs were removed for frozen sections, and the tissues sections were mounted on slide coated with polylysine and then fixed. The slide was immersed in trypsin for 40min, and washed with phosphate buffer saline (PBS) for 3min×3 times. Place the slides in 3% H_2O_2 and incubate in a wet box for 10min to eliminate endogenous peroxidase activity. Totally 50 μL of TUNEL reaction mixture was added to the sample, and reacted in a dark and wet box at 37℃ for 1h; PBS was washed for 3 min×3 times. Anti-quenching tablets were diluted with DAPI 1:500, stored in a -20℃ refrigerator, and photographs were taken by a fluorescence microscope. The apoptotic rate was calculated by analyzing the relevant parts of the sample by microscopy. A total of 200 cells were counted in the 200-fold field of view. The number of apoptotic cells was estimated, and its percentage was calculated.

Statistical Analysis All analyses were performed using SPSS 17.0 software (USA). The mean±standard deviation of PCR products, Western blot data and EB leakage in each group were calculated. One-way analysis of variance (ANOVA) and subsequent least significant difference (LSD) pairwise comparison tests were used to compare the difference among three groups. A *P*-value of 0.05 or less was considered statistically significant.

RESULTS

Changes of Fasting Blood Glucose and Weight in Rats Compared with the control group, the blood glucose of diabetic groups no matter treated or not was significantly increased, and the body weight was significantly decreased at each time point ($P<0.05$). After treated with etanercept, the weight of the treatment group significantly increased compared with non-treatment group ($P<0.05$; Table 2). However, there was no significant difference in decreasing of blood glucose between the treatment groups and non-treatment group ($P>0.05$; Table 3).

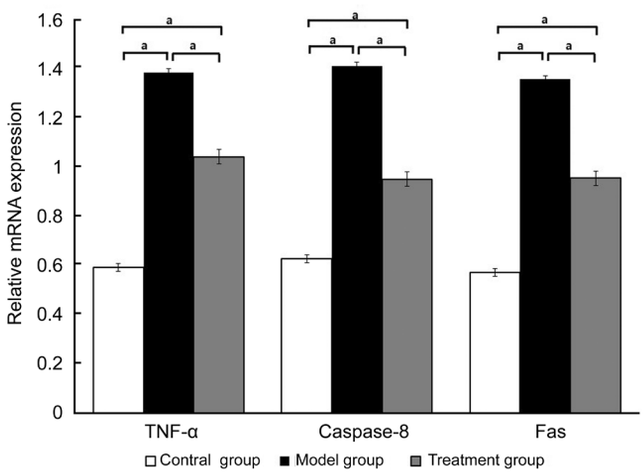

Figure 1 Relative expression levels of Fas, TNF-α and caspase-8 mRNA in the retina of rats There were significant differences in the mRNA of Fas, TNF-α and caspase-8 between different groups. [a]$P<0.01$.

Real-Time Quantitative Reverse Transcriptase-Polymerase Chain Reaction of Retina The results of RT-PCR were shown in Figure 1. There were significant differences in the expression of Fas, TNF-α and caspase-8 between different groups (*F* value of Fas=67.626, $P<0.01$; *F* value of TNF-α=113.645, $P<0.01$; *F* value of caspase-8=72.136, $P<0.001$). The expression of diabetic groups no matter treated or not was significantly higher than the control group ($P<0.01$), however, the expression of treatment group was lower than the non-treatment group ($P<0.01$).

Results of Quantitative Western Blot The results of Western blot quantitative detection are shown in Figure 2. There

were significant differences in the expression levels of Fas, TNF-α and caspase-8 between groups (F value of Fas=6.726, P<0.01; F value of TNF-α=12.695, P<0.01; F value of caspase-8=25.039, P<0.01). The expression levels of the non-treatment group and the treatment group were stronger than those of the control group (P<0.01), and the expression level of the treatment group was lower than that of the non-treatment group (P<0.01).

Retinal Evans Blue Leakage Results The average leakage of EB in the control group was 2.43±0.24 ng/mg, the average leakage of EB in the non-treatment group was 7.93±0.31 ng/mg, and the average leakage of EB in the treatment group was 4.87±0.24 ng/mg. There was a statistically significant difference in the average leakage of EB between the groups (F=854.966, P<0.01). The average leakage of the non-treatment group was significantly higher than that of the control group (P<0.01), while the average leakage of the treatment group (P<0.01) and the non-treatment group (P<0.01) were higher than that of the control group.

Detection of Number of Retinal Apoptotic Cells by TUNEL Method The number of retinal apoptotic cells in control group was 4.6%±0.5%, in the non-treatment group was 16.3%±1.1%, and in the treatment group was 8.6%±0.7%. There was a statistically significant difference in the number of apoptosis between the groups (F=417.326, P<0.01). Compared with the control group, the number of apoptotic cells in the non-treatment group (P<0.01) and treatment group (P<0.01) was significantly higher. However, the number of apoptotic cells in the treatment group was significantly reduced than in the non-treatment group (P<0.01).

The number of apoptotic cells in the treatment group and non-treatment group was significantly higher than that in the control group, and it was lower in the treatment group than the non-treatment group (Figure 3). The number of apoptotic cells in the model group and the treatment group were significantly higher than those in the control group, and the number of apoptotic cells in the treatment group was significantly lower than that in the model group.

DISCUSSION

In this study, the diabetic rat model was successfully established by intraperitoneal injection of STZ. The blood glucose of the modeled rats all met the modeling requirements of DR. All DR model rats had symptoms of polydipsia, polyphagia, polyuria and weight loss. Rats had obvious signs of white opacity of the lens at 10wk, indicating that metabolic cataracts caused by hyperglycemia have occurred. At the 4th, 8th and 12th week, the fasting blood glucose of the non-treatment group and the treatment group were significantly higher than the control group, but there was no significant difference in fasting blood glucose between the non-treatment group and the treatment group at each time point, indicating that etanercept had no effect on lowering blood glucose.

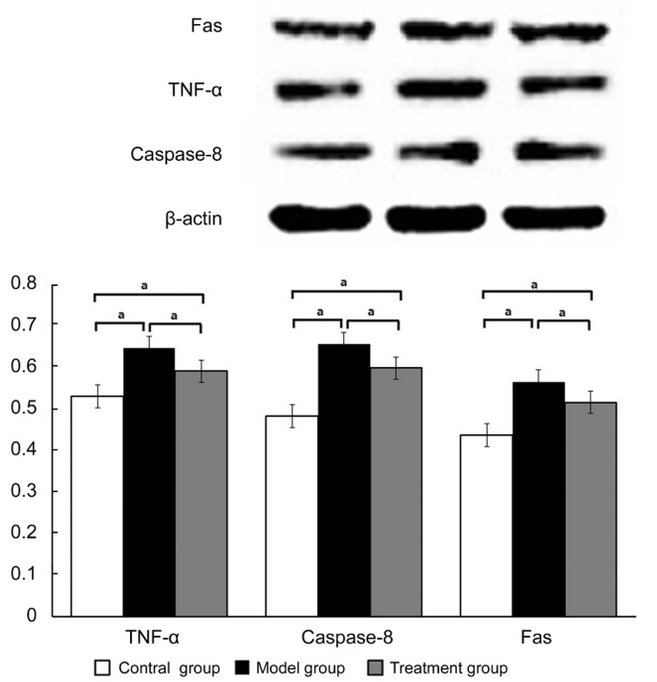

Figure 2 The relative protein expression levels of Fas, TNF-α and caspase-8 in the retina of each group There were significant differences in the protein expression of Fas, TNF-α and caspase-8 between different groups. ^{a}P<0.01.

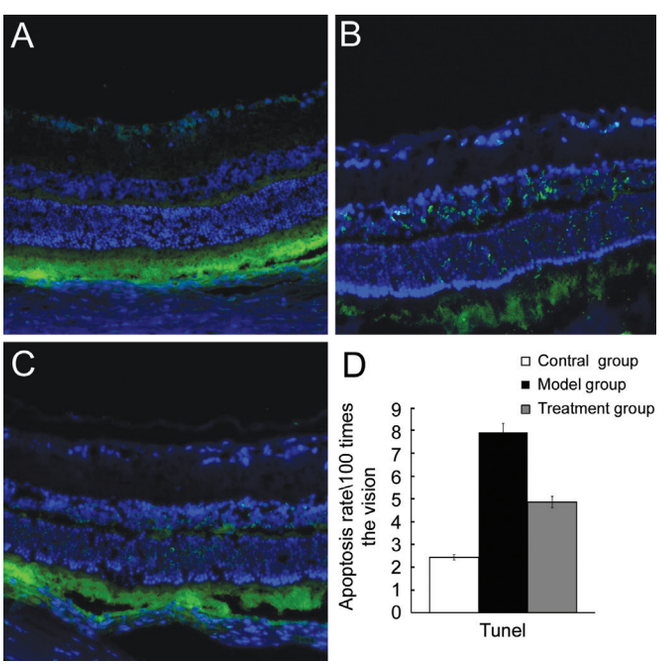

Figure 3 Detection of retinal apoptotic cells by TUNEL assay in each group A: Control group; B: Model group; C: Treatment group; D: The apoptosis rate in each group.

Current study showed that the expression levels of the three cytokines Fas, TNF-α, and caspase-8 in the apoptotic pathway were significantly higher in the non-treatment group and the treatment group than in the control group, indicating that the apoptotic effect plays a major role in diabetes retinopathy in

rats. Fas, TNF-α and caspase-8 were significantly lower in the treatment group than in the non-treatment group, suggesting that etanercept can block the apoptotic pathway and reduce the apoptosis effect. Akash *et al*[5] reported a chronic increase in serum TNF-α activity in the non-insulin-dependent rats. Tavares Ferreira *et al*[6] showed that the serum levels of TNF-α in DR patients were significantly higher than in the normal subjects. Moreover, With the use of the enzyme-linked immunosorbent assay, Zhang *et al*[7] found that the levels of TNF-α in serum and vitreous of DR patients were significantly higher than the normal subjects. All these findings were consistent with our current result.

In this study, the EB leakage measurement method was used to evaluate the retinal leakage in each group. The EB leakage in the non-treatment group and the treatment group was significantly higher than that in the control group, indicating an increase of leakage in the late stage of DR in rats. The EB leakage in the treatment group was significantly lower than that in the non-treatment group, indicating that etanercept can reduce the retinal leakage of DR caused by the apoptosis and inflammation, thereby reducing the damage of retina. The abnormal cellular metabolism in the hyperglycemic environment would lead to ischemia and hypoxia of the retina. It would break the balance between retinal vascular factors and inhibitors, which increases the distribution of TNF-α on the surface of DR vascular endothelial cells, and destroys the tight junction between the cells and the pigment epithelial cells resulting in the destruction of the BRB[3,8], which further increase the vascular permeability. Moreover, TNF-α can increase the expression of vascular endothelial VEGF which would interfere with the interaction between peripancreatic cells and capillary endothelial cells, causing neovascularization and increasing the capillary permeability, and inducing a series of pathological changes, such as an increase of retinal leakage in DR patients. In addition, TNF-α can increase the reactivity of target cells to VEGF, TGF-β, IGF-1 and *etc.*, so as to increase the expression of VEGF, leading to retinal neovascularization and BRB destruction. TNF-α also can directly damage BRB and improve the permeability of retinal blood vessels[9]. Furthermore, TNF-α can also activate microglia, which would also involve in the destruction of the BRB[10].

TUNEL is a common pathological method for detecting apoptosis. In this study, frozen sections were cut to assess the apoptosis. We found that the number of apoptotic cells in the non-treatment group and the treatment group was significantly higher than that in the control group, indicating a serious apoptotic in the retina of diabetic rats. It suggests that apoptosis plays a crucial role in the pathogenesis of DR. The number of apoptotic cells in the treatment group was significantly lower than that in the non-treatment group, indicating that etanercept can effectively reduce the retinal apoptosis response in diabetic rats. Previons studies found that the apoptosis of retinal ganglion cells (RGC) appeared at 4wk of STZ rats established, the mean of the initials RGC followed by the apoptosis of photoreceptor cells and neuronal apoptosis[11-15]. The potential mechanisms of apoptosis were as follows: 1) TNF-α binds to its receptor, activates the death receptor pathway, and trigger the apoptosis[16-17], which would also induce the death of ganglion cell death[18]. 2) Death factors such as FasL and TNF bind to their related receptors such as Fas and TNFR, and then form a death-inducing signaling complex through the death domain structural protein and apoptosis promoters including caspase-8, caspase-12 *etc*., resulting in caspase cascade reaction and causing the apoptosis. 3) TNF-α can also alter the expression of vascular adhesion molecules, allowing lymphocytes and macrophages to reach the target site, stimulate the inflammatory cycle, and induce apoptosis by releasing cytotoxic substances. 4) TNF-α induces nuclear factor-κB (NF-κB) activation, increases the expression of apoptotic bodies and causes apoptosis of perivascular cells in the retina, leading to retinal microcirculatory disorders[19]. 5) The interaction of vascular endothelial apoptotic factor (Fas) and apoptotic factor ligand (FasL) adhering to the surface of leukocytes triggers a series of pathological changes such as vascular endothelial cell injury and apoptosis[19].

The pathogenesis of DR is complicated and chronic inflammation and immune response play a crucial role in the whole process of DR. Fas, TNF-α and caspase-8 are three important factors in the apoptotic signaling pathway. They work together and cause apoptosis of retinal cells and trigger a series of inflammation in DR. Etanercept is a TNF-α inhibitor that is a human tumor necrosis factor receptor p75Fc fusion protein produced by the expression system of Chinese hamster ovary cell. The extracellular ligand binding site of tumor necrosis factor receptor 2 (TNFR2/p75) is linked to the Fc fragment of human IgG1, which form the dimer. Etanercept is a competitive inhibitor of TNF receptors on the cell surface that inhibits the biological activity of TNF and thereby blocks the cellular response of TNF. It may also be involved in the regulation of biological responses controlled by other downstream molecules (such as cytokines, adhesion molecules or proteases) induced or regulated by TNF. Some researchers have studied the therapeutic effect of etanercept on experimental autoimmune uveitis in rats, confirming that etanercept can effectively relieve the ocular inflammation of uveitis, delay the onset time, and reduce the TNF-α and IL-6 levels in the eye of rats. The role of TNF-α-mediated apoptosis in the early stage of DR and the long-term histopathological changes, confirming that etanercept can reduce apoptosis and reduce risk of DR[20].

In summary, the mRNA and protein levels of Fas, TNF-α and caspase-8 in the retina of diabetic rats are higher, while etanercept can effectively reduce their levels in diabetic rats. Furthermore, etanercept can reduce the retinal leakage and apoptosis of retinal cells, which can effectively protect the retina of diabetic rats, relieve the retinal damage in diabetic rats. Therefore, this study may provide a new insight of treatment for DR.

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