·Basic Research·

TGF−β₁ in retinal ganglion cells in rats with chronic ocular hypertension: its expression and anti − apoptotic effect

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Abstract

• AIM: To investigate the anti-apoptotic effect of transforming growth factor beta-1 (TGF-β ₁) on chronic ocular hypertension.

• METHODS: The expression of TGF-β ₁ in retinal ganglion cells (RCGs) was measured using the immunohistochemiscal S-P method and real-time PCR in the normally control group, the ocular hypertension group (experimental group A), the ocular hypertension plus antibody intervention group (experimental group B) and the ocular hypertension plus antigen intervention group (experimental group C) at 1, 2, 3 and 4 weeks postoperatively. The count of apoptotic RCGs was measured using the TUNEL method.

• RESULTS: The expression of TGF-β ₁ was significantly higher in experimental group C than that in other three groups (*P*<0.05). The expression was the lowest in experimental group B (4.17%). A statistically significant difference was noted between the four groups (*P*<0.01). The count of apoptotic RCGs was statistically significantly lower in experimental group C than that in the experimental groups A and B (*P*<0.01). A statistically significant difference was noted in the count of apoptotic RCGs between these three experimental groups (*P*<0.01).

• CONCLUSION: TGF-β ₁ can inhibit the apoptosis of RCGs in rats with chronic ocular hypertension.

• KEYWORDS: glaucoma nerve protect; TGF-β ₁; retinal ganglion cells; chronic ocular hypertension

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INTRODUCTION

I n patients with glaucoma, the vision deterioration results from injuries to optic nerve fiber and apoptosis of retinal ganglion cells (RCGs). Levkovitch-Verbin *et al* [1] reported that apoptosis occurred when intraocular pressure was high. Retrograde conduction block, lack of multiple nutrition factors, ischemia, and toxicities of excitatory amino acids are possible factors inducing apoptosis. Currently, one major therapy for glaucoma is to decrease the intraocular pressure. As intraocular pressure controlling cannot completely prevent injuring to optic nerve, nerve protection is more and more emphasized. Neural protection with conventional medications is an ideal technique.

TGF-β₁, as a polypeptide growth factor, is widely distributed in tissues and cells. It has regulatory effects on cell growth and differentiation, embryogenesis, tissue repair, and immune function. Previous study showed that TGF-β₁ was a mediator for cytoskeletal proteins in rebuilding of synapses which was involved in the development and growth of axons. TGF-β₁ also can inhibit neuronal apoptosis, promote axon growth and improve neuron survival and differentiation. In addition, TGF-β₁ may act with other nutrition factors to facilitate neutron survival. In cerebral injuries, TGF-β₁ can be of nerve protection and apoptosis inhibition factor.

In this study, we measured the expression of TGF-β₁ in RGCs and the apoptosis of RGCs challenged by antibody or antigen to investigate the anti-apoptotic effect of TGF-β₁ in rats with ocular hypertension.

MATERIALS AND METHODS

Materials Eighty-four (168 eyes) healthy Wistar male rats with average weight of 200±18g were included in this study. The rats were divided into four groups: the normally control group (*n* =12, 24 eyes), the ocular hypertension group (experimental group A), the ocular hypertension plus TGF-β₁ antibody intervention group (experimental group B)
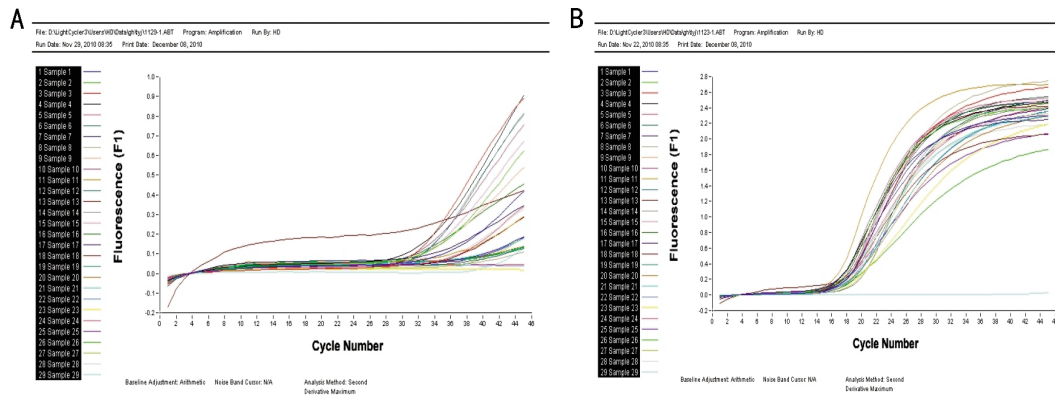
Figure 1 A: Amplification curve of TGF-β_1; B: Amplification curve of β-actin

and the ocular hypertension plus TGF-β_1 antigen intervention group (experimental group C) with 24 rats (48 eyes) for each. Expect for the normally control group, other three groups were divided into four subgroups (n=6 for each, 12 eyes). The rats in subgroups were killed at 1, 2, 3 and 4 weeks postoperatively.

Methods

Establishment of acute ocular hypertension The rats were anesthetized by 10% chloral hydrate 0.3mL/100g through peritoneal injection. Under the microscope, the bulbar conjunctiva was cut at 2mm from the corneal edge to expose the upper sclerotic and temporal episcleral vein. The episcleral vein was cauterized by the thermocoagulation device until the bleeding was stopped. 0.2mg/mL mitomycin cotton was placed on the sclerotic for 1 minute and flushed by 10mL normal saline. The bulbar conjunctiva was sutured by 10-0 nylon suture after reposition. The eyes were administered tobradex ointment. Ocular pressure was measured prior to operation, immediately after operation and before death using Tono-Pen XL tonometer for consecutive three times, and was averaged.

Injection of TGF −β_1 antibody and antigen in the vitreous cavity 50ng TGF-β_1 antibody and 10ng TGF-β_1 antigen was respectively injected using micropipettor connected to self-made capillary tube into the vitreous cavity from the ciliary body pars plana at 45° between superior rectus muscle and lateral rectus muscle. The injection wound was cauterized by the thermocoagulation device.

Sample preparation The left eye was harvested under sterile conditions, and then was fixed in polyoxymethylene. The cornea was excised at the corneal edge. After the lens was removed, the eye was fixed in 4% polyoxymethylene for the immunohistochemical S-P method and TUNEL method. The right eye was harvested similarly. The cornea was excised at the corneal edge. After the lens was removed, the scleral shell was turned over to remove the vitreous body. The retina was carefully harvested to be placed in the EP tube at -80℃ for real-time PCR.

The expression of TGF-β_1 proteins in RGCs was measured using the immunohistochemical S-P method in four groups. The S-P kit was purchased from Zhongshan Goldenbridge Biotechnology Co., Ltd (Beijing, China). Rabbit anti-rat TGF-β_1 multiclonal antibody and TGF-β_1 antigen were obtained from Beijing Biosynthesis Biotechnology Co., Ltd (Beijing, China).

Determination of immunohistochemical results Ten high-power fields were selected randomly under the microscope. One-hundred cells were taken into consideration in each field. The positive cell was graded according to the positive percentage: ≤5%, 0; 6-25%, 1; 26-50%, 2; and >50%, 3. The staining intensity was graded at four levels: no staining, 0; light yellow staining, 1; yellow-brown, 2; and dark brown, 3. The comprehensive score was the sum of the positive cell score and the staining intensity score. Positive expression (+) was considered if the comprehensive score was ≥4; and negative (-), <4.

The expression of TGF-β1mRNA in RGCs was measured using the real-time PCR in four groups. Quantitative RT-PCR was performed using the Quanti Tect SybrGreen RT-PCR system (Qiagen, Valencia, CA, USA). With established RNA as the standard, standard curves were plotted for TGF-β_1 and β-actin. TGF-β_1 and β-actin were quantified based on the standard curves. Corrected with β-actin, the relative levels of TGF-β_1mRNA were determined. TRIzol $^®$ reagent, real-time PCR kit, primers were purchased from TaKaRa Biotechnology (Dalian) Co., Ltd (Dalian, Liaoning, China). (Figures 1A and 1B).

The count of apoptotic RGCs was measured using the TUNEL in three experimental groups. The apoptosis detection kit and digestion reagents were purchased from Wuhan Boster Biological Technology Co., Ltd (Wuhan, Hubei, China).

Determination of apoptosis Positive cells in five

Table 1   The expression of TGF-β1 protein, relative expression of TGF-β1mRNA and the count of apoptotic RCGs in different groups   mean±SD

Groups	n	-	+	TGF-β_1 protein expression (%)	TGF-β_1mRNA expression	Count of apoptotic RCGs
Normal	12	8	4	33.33[a]	1.19±0.09[b]	
Experimental group A	24	6	18	75[a]	6.33±4.81[b]	43.58±24.30[d]
Experimental group B	24	23	1	4.17[a]	0.20±0.17[b]	57.47±23.25[d]
Experimental group C	24	0	24	100[a]	15.46±5.27[b]	15.52±9.01[d]

[a]$P<0.05$, [b]$P<0.01$ between four groups; [d]$P<0.01$ between three experimental groups

high-power fields (×400) for each section were taken into account. The labeling index was calculated as (the count of positive cells/ the total count of cells) ×100%.

Statistical Analysis Continuous variables were given as mean±standard deviation (SD) and categorical variables as percentages. In order to verify if there was a normal distribution, the quantitative variables were tested using the Kolmogorov-Smirnov test. In the cases of normal distribution and similar variances, the ANOVA test would be used to compare the results of real-time PCR and TUNEL between three or more groups. For the other cases, the Mann-Whitney test would be used to compare two groups. The results comparison on immunohistochemistry in the differential expression between different groups was using Square test. All analysis were performed with SPSS statistical software version 12.0. P value less than 0.05 was accepted as indicating statistical significance.

RESULTS
The intraocular pressure was 10.69 ±3.52mmHg in the normally control group. At preoperation, postoperation and before death, it was 11.54±3.31mmHg, 32.43±3.85mmHg and 29.09±4.38mmHg in experimental group A; was 10.85±3.24mmHg, 33.42 ±2.91mmHg and 30.57 ±3.69mmHg in experimental group B; was 10.85 ±3.24mmHg, 33.42 ± 2.91mmHg and 30.57±3.69mmHg in experimental group C. There was no statistically significant difference at the three time points between three experimental groups (P>0.05).

Immunohistochemical positive products of TGF-β_1 were yellow granules. They were mainly expressed in the membrane or cytoplasm of RGCs.

The expression rate of TGF-β_1 was 33.33%, 75%, 4.17% and 100% in four groups, respectively, and had statistically significant difference (P< 0.05, Table 1). The relative expression of TGF-β_1mRNA was 1.19 ±0.09, 6.33 ±4.81, 0.20±0.17 and 15.46±5.27 in four groups, respectively, with a statistically significant difference (P< 0.01, Table 1). The count of apoptotic RGCs was 43.58 ±24.30, 57.47 ±23.25 and 15.52±9.01 in three experimental groups, respectively, and there was a statistically significant difference between the three groups (P<0.01, Table 1).

Table 2   The relative expression of TGF-β_1mRNA in three experimental groups at different time points

Time (wk)	Experimental group A	Experimental group B	Experimental group C
1	7.16±1.14	0.13±0.07	16.67±5.29
2	11.78±2.68	0.33±0.09	24.32±6.17
3	3.73±0.79	0.18±0.10	9.27±2.34
4	2.66±0.48	0.16±0.07	5.32±2.12

At 1, 2, 3 and 4 weeks postoperatively, the relative expression of TGF-β_1mRNA was 7.16 ±1.14, 11.78 ±2.68, 3.73 ±0.79 and 2.66 ±0.48 in experimental group A; was 0.13 ±0.07, 0.33±0.09, 0.18±0.10, 0.16±0.07 in experimental group B; and was 16.67±5.29, 24.32±6.17, 9.27±2.34 and 5.32±2.12 in experimental group C. In three experimental groups, the relative expression of TGF-β_1mRNA was higher at 2nd week, then decreased. In experimental group B, the relative expression of TGF-β_1mRNA was not statistically significant at the 2nd week (P>0.05). In experimental group A and C, the relative expression of TGF-β_1mRNA was statistically significant at the 2nd week comparing with other time points (P<0.01, Table 2).

DISCUSSION
Glaucoma is caused by optic nerve injury and resultant apoptosis of RGCs. Lack of multiple nutrition factors is a major cause of apoptosis of RGCs. Administration of nutrition factors combining with inocular pressure control becomes a major approach for treating glaucoma and inhibiting RGCs. Previous studies showed that the apoptotic RGCs reduced apparently after nutrition factors were given[2].

Transforming growth factor beta (TGF-β) is a super family that regulates cell growth and differentiation, immune function and apoptosis. There are four subtypes, TGF-β_1, TGF-β_2, TGF-β_3, and TGF-$\beta_1\beta_2$ in mammals. The content of TGF-β_1 is the highest among those four. It is the most active and representative. The homology of human and murine TGF-β_1 nucleotide sequence reaches 99%, so the rat model is established to research the function of TGF-β_1.

TGF-β_1 is widely distributed in different cells, including

epithelial cells, endothelial cells, hematopoietic cells, nerve cells and connective tissue cells. TGF-β_1 is mainly distributed in retinal inner limiting membrane, and weakly expressed in nerve fiber layer, ganglion cell layer, and internal granular layer in normal retinal tissues. TGF-β_1 is a multifunctional cytokine with multiple biological effects, including embryogenesis, cell growth and differentiation, angiogenesis, tumor differentiation, wound healing, inflammation, and immune regulation. In the nervous system, TGF-β_1 can inhibit neuron apoptosis, promote axon growth, and improve neuron survival and differentiation.

TGF-β_1 is also a strong cytokine for nerve nutrition and immune regulation, which can be expressed in the normal nervous system and upregulated when injuries occur in the nervous system [3]. *In vitro* research demonstrates that TGF-β_1 is a mediator for cytoskeletal proteins in rebuilding of synapses that is involved in the development and growth of axons. Latest research shows that TGF-β_1 is closely correlated with nervous system diseases and has protection effect for nerve injury. Consequently, the correlation between TGF-β_1 and cerebral ischemia is a hot spot in current research. Under different conditions of cerebral ischemia, observations of increased TGF-β_1 expression during ischemia and after reperfusion indicate that TGF-β_1 is involved in injury and repair of them after ischemia. Hardt *et al* [4] found that microglia could secrete TGF-β_1 with protection effect *in vitro* in the condition of ischemia using western blotting. In the ischemia-reperfusion procedure, endogenous TGF-β_1 expression can change, as part of the repair in ischemia-reperfusion injury and body self-repair and protection.

TGF-β_1 can inhibit apoptosis [5]. Dhandapani *et al* [6] showed that TGF-β_1 inhibited the apoptosis of neurons in the *in vitro* experiment. Sheng *et al* [7] found that the apoptotic neurons decreased apparently in the rats with middle cerebral artery occlusion following administration of TGF-β_1. TGF-β_1 can inhibit the apoptosis of stem cells in the cartilage tissues[5, 8]. Hendriks *et al*[9] reported that TGF-β_1 enhanced synthesis of Bcl-2 protein and inhibited cell apoptosis induced by cerebral ischemia for synergic effect. Chu *et al* [10] exhibited that TGF-β_1 inhibited anti-apoptotic protein Bcl-2 for neutron protection. TGF-β1 increased the Bcl-2/Bax ratio and thus inhibited apoptosis of HL-60 cells in leukemia[11]. In the research on colorectal cancer, TGF-β_1 increased the Bcl-2/Bax ratio, inhibited cancer cell apoptosis, and promoted cancer cell growth [12]. Currently, this mechanism was thought to involve Ca^{2+} concentration decrease, stability of intracellular Ca^{2+}, and Bad phosphorylation to decrease
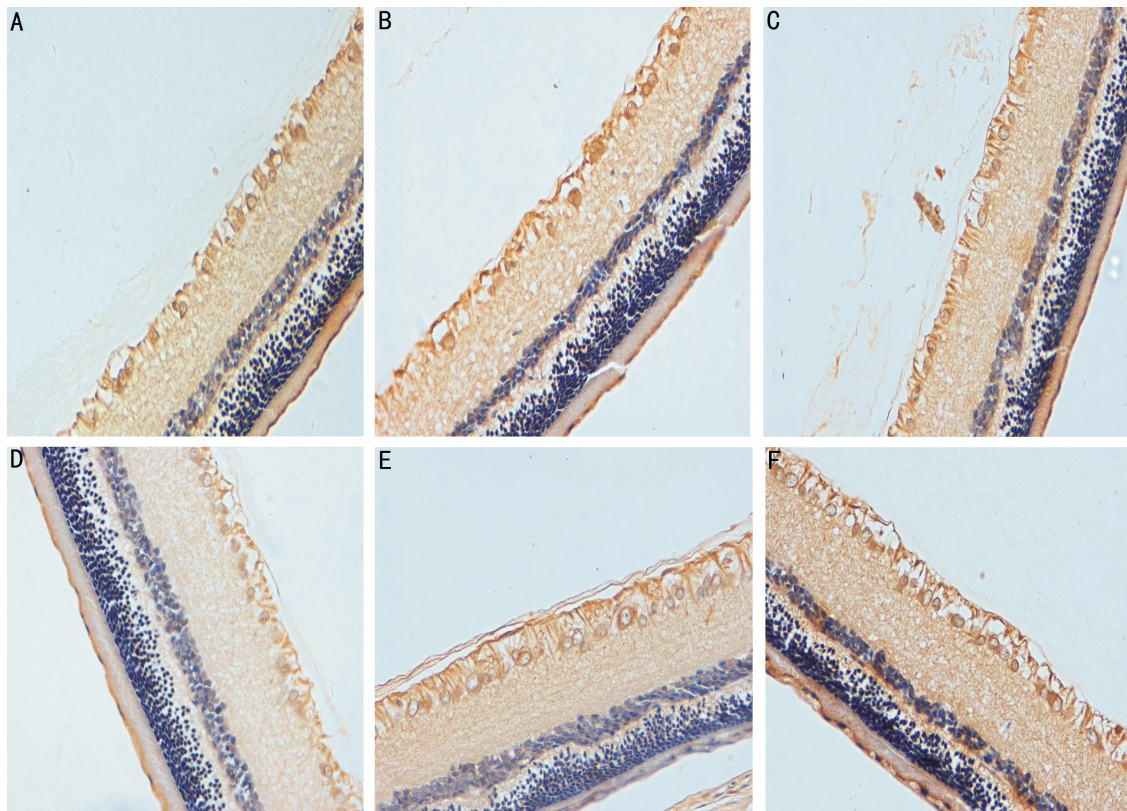
Bad protein expression[13].

In our study, the results showed that TGF-β_1 was lowly expressed in RGCs in the normally control group. In the ocular hypertension group, when the inocular pressure increased, endogenous TGF-β_1 was also upregulated. In the first two weeks, the expression was especially high.(Figures 2A and 2B) As a nutrition factor and anti-apoptotic factor, this is a self-protection mechanism for apoptosis of RGCs. As the period of high inocular pressure is prolonged, the expression of TGF-β_1 is weakened and the apoptosis of RGCs deteriorates. (Figures 3A and 3B).

In the ocular hypertension plus antibody intervention group, the expression of TGF-β_1 was inhibited due to use of anti-TGF-β_1 antibody. (Figures 2C and 2D) The apoptosis of RGCs in this group was high. Pathology of retinal tissues showed that RGCs decreased at 2 weeks and the ganglion cell layer became loose with expanded perinuclear cistern. The inner nuclear layer was with limited nuclear condensation, deep staining and limited vacuoles. At 4 weeks, the inner nuclear layer became atrophied with disappearance of RGCs and remaining irregular nuclear condensation. In the ocular hypertension plus antibody intervention group, as the expression of TGF-β_1 was lacked, the nerve nutritional effect and anti-apoptotic effect were lost, leading to higher count of apoptotic RGCs than other groups. (Figures 3C and 3D).

In the ocular hypertension plus antigen intervention group, the expression of TGF-β_1 was significantly higher than that of other two experimental groups. (Figures 2E and 2F) The count of apoptotic RGCs was significantly lower than that of the other groups, indicating that upregulated TGF-β_1 inhibits apoptosis of RGCs in the rat with ocular hypertension. (Figures 3E and 3F).

In this study, chronic ocular hypertension was successfully established. The expression of TGF-β_1 was detected at both the protein and the gene level using immunohistochemical SP method and real-time PCR. Similar findings were obtained from the two methods. The TUNEL method also showed corresponding apoptosis of RGCs in different groups at different time points.

The results in this study demonstrate that TGF-β_1 as a nutrition factor and anti-apoptotic factor can inhibit the apoptosis of RGCs in the rat with ocular hypertension. The mechanism for apoptosis of RCGs may need further exploration though TGF-β_1 may involve in the apoptosis in rat with ocular hypertension. Use of nutrition factors and anti-apoptotic factors may protect for RCGs, and this is a new area in the treatment of glaucoma.

Figure 2 Positive expression of TGF−β₁ protein (SP×400) in different groups at different time points A: 2 weeks postoperatively in experimental group A; B: 4 weeks postoperatively in experimental group A; C: 2 weeks postoperatively in experimental group B; D: 4 weeks postoperatively in experimental group B; E: 2 weeks postoperatively in experimental group C; F: 4 weeks postoperatively in experimental group C
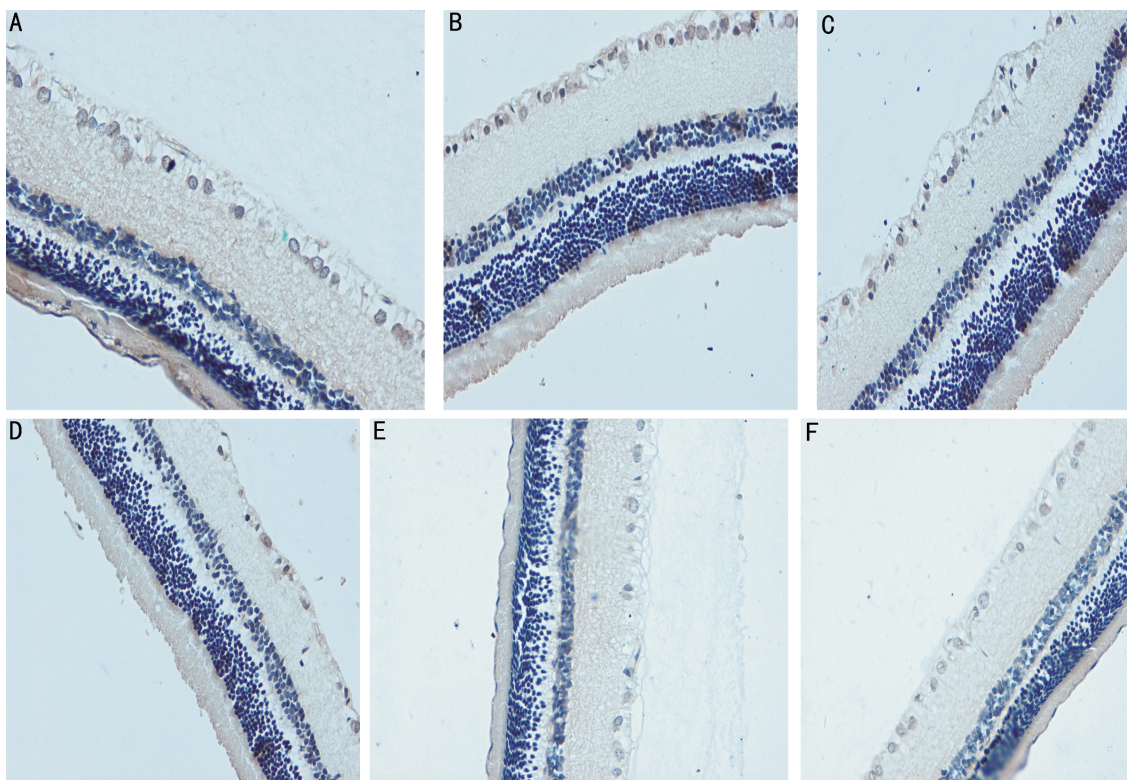
Figure 3 Positive expression of apoptotic RCGs (TUNEL ×400) in different groups at different time points A: 2 weeks postoperatively in experimental group A; B: 4 weeks postoperatively in experimental group A; C: 2 weeks postoperatively in experimental group B; D: 4 weeks postoperatively in experimental group B; E: 2 weeks postoperatively in experimental group C; F: 4 weeks postoperatively in experimental group C

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