Original Article
Research on expression and effect of transforming growth factor β2 and vascular endothelial growth factor in filtering bleb after trabeculectomy

Yuan Wang1*, Yuebing Lu2*, Dongmei Zhu1, Xiaoping Sun1

1Department of Ophthalmology, Zhengzhou Central Hospital Affiliated to Zhengzhou University, Zhengzhou, Henan, P. R. China; 2Department of Ophthalmology, Children’s Hospital of Zhengzhou City, Zhengzhou City, Henan, P. R. China. *Co-first authors.

Received June 12, 2016; Accepted June 20, 2016; Epub September 15, 2016; Published September 30, 2016

Abstract: Objective: To observe the expression changes of Transforming growth factor beta-2 (TGF-β2) and Vascular endothelial growth factor (VEGF) in filtering bleb tissues after trabeculectomy on rabbit eyes, and to discuss their correlation with the post-operative scarring of filtering bleb. Methods: Chinchilla rabbits were selected for the experiment; each rabbit had one eye undergoing Glaucoma trabeculectomy and the other without surgery, the operated eyes were treated as the experiment group while the un-operated eyes were regarded as the control group. The dynamic expression of VEGF and TGF-β2 in the formation of filtering bleb scar after trabeculectomy in rabbit eyes was detected by ELISA and immunohistochemical staining. By subconjunctival injection of Bevacizumab, ELISA and immunohistochemical staining were adopted for detection of the expression changes of VEGF and TGF-β2 in Filtering bleb one week after the operation. Results: After trabeculectomy, the content of VEGF and TGF-β2 in the filtering bleb tissues changed, and both reached the peak one week after the surgery; the comparison of the contents between the experiment group and the control group was statistically significant, and the differences at different time points were also with statistical significance. Bevacizumab could inhibit fibrosis of the filtering bleb tissues one week after the operation, as well as the content of VEGF and TGF-β2 in filtration tissue. The postoperative contents of VEGF and TGF-β2 in filtration tissues were relevant to each other (0.775, P=0.012). Conclusions: VEGF and TGF-β2 had synergistic effects in the process of filtering bleb scarring after trabeculectomy operation. Both of them promoted the occurrence and development of scar together.

Keywords: Glaucoma trabeculectomy, vascular endothelial growth factor, transforming growth factor β2, filtering bleb

Introduction

Glaucoma trabeculectomy is the most common surgical procedure for the treatment of glaucoma at present [1]. However, the postoperative filtering bleb scarring obstructs the aqueous humor and increases intraocular pressure, which is one of the main reasons for the failure of trabeculectomy [2, 3]. Although, clinical application of anti-scarring drugs can effectively improve the early filtering function after glaucoma surgery, the side effects of these drugs cannot be ignored. Therefore, how to inhibit scar formation with high efficiency and low toxicity has become a hot spot in the research of glaucoma.

In recent years, the research has shown that the filtering bleb scarring is a complex process of the interaction between cell, extracellular matrix and Cytokines [3]. Usually, after trabeculectomy, granulation tissues formed at the edge of the cut on the postoperative 3rd day, then the secretion of collagen fibers increased and capillary de-generated one week after the operation, followed by granulation tissues gradually transforming into scar tissue. Cytokines play an important role in this process. Vascular endothelial growth factor (VEGF) is the most important positive regulator of angiogenesis, which plays an important role in the process of wound healing. VEGF can specifically act on vascular endothelial cells and cause the proliferation, and induce the formation of the blood vessels [4]. Transforming growth factor β2 (TGF-β2) is the most critical modulating factor in the repair process, and it has a strong effect on promoting the fibrosis [5]. However, at present, there is
no report about the expression and role of VEGF and TGF-β in the process of scar formation in filtering bleb tissues; by observing the dynamic expression of VEGF and TGF-β in process of filtering bleb scarring in rabbit eyes after trabeculectomy, we investigated the roles of VEGF and TGF-β in the scarring process, to provide experimental basis and theoretical foundation for clarifying the pathogenesis of filtering bleb scarring after Glaucoma trabeculectomy on molecular level, as well as to improve the successful rate of glaucoma surgery.

Material and methods

Laboratory animals

30 healthy male chinchilla rabbits were provided by the Experimental Animal Center of Zhengzhou University. The rabbits weighted about 2.5 kg, and were raised under room temperature. Slit-lamp microscope examination, fundus and intraocular pressure examination were performed to exclude eye disease; chloramphenicol eye drops was applied three times a day to prevent infection. Each of the 30 chinchilla rabbits had one eye undergoing trabeculectomy, and the other eye was used as blank control; then the 54 rabbits were randomly divided into three groups with 18 in each group: bevacizumab group with injection of 0.1 ml of bevacizumab (at the concentration of 100 mg/4 ml) under conjunctival filtering bleb after trabeculectomy; normal saline group with injection of 0.1 ml of normal saline under conjunctival filtering bleb after trabeculectomy; and control group without any treatment after operation.

Main reagents and instruments

Mouse anti rabbit VEGF monoclonal antibody and mouse anti rabbit TGF-β monoclonal antibody (Santa Cruz company, USA); Rabbit VEGF and TGF-β ELISA Kit (R&D company, USA); Rebound tonometer (TIOLATOY, Finland); Slit-lamp microscope (Zeiss company, Germany); RT-6100 type enzyme-labeled instrument (Shenzhen Rayto Life Science incorporated company, China), Bevacizumab (Genetech company, USA).

Methods

Operation method: The rabbits were anesthetized by intravenous injection of 30 g/L sodium pentobarbital (1 ml/kg), and one was randomly selected to undergo trabeculectomy, specific steps are as follows: the eye was surface anesthetized by using oxybuprocaine hydrochloride. A fornix based conjunctival flap and 4 mm × 4 mm scleral flap with 1/2 scleral thickness were made during the operation, to expose corneal limbus; deep flap sclera and 1.5 × 2 mm Trabecular tissue were cut off, as well as the corresponding peripheral iris; then scleral flap and conjunctival flap were restored and sutured. After surgery, TobraDex eye drops was applied 3 times a day and TobraDex eye ointment was applied every night. Experiment rabbits were sacrificed by air embolization at different time points (pre-operation and postoperative 1 day, 3 days, 1 week, 2 weeks and 3 weeks) to harvest the eyes; conjunctivas of filtering bleb area, subconjunctival tissues and scleral tissues were cut off for subsequent experiments (3 rabbits at each time point of each group).

Detection of VEGF and TGF-β content: the tissues were fully grinded to form homogenate, then centrifuged at 3000 r/min for 15 min, and supernatant was obtained for ELISA test according to the instructions of ELISA kit; finally, the absorbance value of each hole were determined at 450 nm wavelength. The measurement was carried by the same researcher in accordance with double blind principle.

Immunohistochemical staining: All specimens were paraffin embedded and sectioned, then de-waxed by gradient ethanol to water. The specimen were incubated at room temperature for 10 min with 3% H2O2 to eliminate the activity of endogenous peroxidase. After being washed with PBS, the specimens were then closed with 10% normal goat serum and incubated at room temperature for 10 min; gently removed serum and add Mouse anti rabbit VEGF monoclonal antibody or Mouse anti rabbit TGF-β monoclonal antibody to incubate in a wet box at 4°C for overnight; after washed with PBS for 5 min × 3 times, add HRP marked sheep-anti mouse IgG antibodies and incubated at 37°C for 30 min, then washed with PBS for 5 min × 3 times; DAB was used for color showing, which was controlled under light microscope; after completely colored, the specimens were rinsed with distilled water to terminate the color rendering, and re-dyed with Hematoxylin and mounted.

Statistical treatment

SPSS 17.0 software was used for statistical analysis. The measurement data was expressed
Research of TGF-β₂ and VEGF in filtering bleb

Results

To detect the expression of VEGF and TGF-β₂ by immunohistochemical staining

VEGF was expressed in the cytoplasm, and the nucleus was not stained. VEGF was expressed in the conjunctival epithelial cells in both control group and blank control group; and in experiment group, VEGF was post-operatively expressed in conjunctival epithelial cells, fibroblast cells and vascular endothelial cells, with the highest expression (showing dark brown) at postoperative 1 week. As time went on, the number of positive staining cells reduced gradually, as shown in Figure 1.

TGF-β₂ weakly expressed in the fibroblasts and conjunctival epithelial cells of the control group and blank group, located in cytoplasm. In experiment group, TGF-β₂ showed the highest expression in positive staining cells at postoperative 1 week, which representing bulky nuclear and various shape. As time went on, the number of TGF-β₂ positive staining cells reduced gradually, and the nucleus was elongated, and the shapes tend to be unified, as shown in Figure 2.

The expressions of VEGF and TGF-β₂ in filtering bleb determined by ELISA

After the trabeculectomy, the content of VEGF and TGF-β₂ in the eyes of experiment group began to increase on the postoperative day 1.
Research of TGF-β2 and VEGF in filtering bleb

and reached the peak at postoperative day 7, then decreased gradually. The content change of VEGF and TGF-β2 showed in the peak pattern. Compared with the control group, the content of VEGF and TGF-β2 in the experiment group was significantly higher, and the difference was statistically significant (P<0.05). Compared with preoperative value, the content of VEGF and TGF-β2 was various at different time points, the differences between each time point were statistically significant (P<0.05), See Tables 1, 2.

Table 1. The contents of VEGF in filtering bleb tissues in two groups at different time points (pg/μL)

<table>
<thead>
<tr>
<th>Time</th>
<th>Experiment group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preoperative</td>
<td>0.343±0.012</td>
<td>1.365±0.030</td>
</tr>
<tr>
<td>Postoperative 1 d</td>
<td>4.345±0.270*</td>
<td>1.343±0.002</td>
</tr>
<tr>
<td>Postoperative 3 d</td>
<td>5.634±0.157*</td>
<td>1.356±0.001</td>
</tr>
<tr>
<td>Postoperative 7 d</td>
<td>7.524±0.352*</td>
<td>1.366±0.003</td>
</tr>
<tr>
<td>Postoperative 14 d</td>
<td>3.245±0.871*</td>
<td>1.376±0.001</td>
</tr>
<tr>
<td>Postoperative 21 d</td>
<td>3.035±0.452*</td>
<td>1.386±0.005</td>
</tr>
</tbody>
</table>

*P<0.05, compared with control group; *P<0.05, compared with preoperative.

Correlation analysis of content of VEGF and TGF-β2

Pearson Correlation analysis showed that the expressions of VEGF and TGF-β2 in filtering bleb was correlated with each other after trabeculectomy, and the correlation coefficient was 0.775, P=0.012, indicating the relevance between VEGF and TGF-β2 in scarring development of filtering bleb. See Table 3.

Table 2. The contents of TGF-β2 in filtering bleb tissues in two groups at different time points (pg/μL)

<table>
<thead>
<tr>
<th>Time</th>
<th>Experiment group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preoperative</td>
<td>1.343±0.120</td>
<td>1.365±0.030</td>
</tr>
<tr>
<td>Postoperative 1 d</td>
<td>4.345±0.270*</td>
<td>1.343±0.002</td>
</tr>
<tr>
<td>Postoperative 3 d</td>
<td>5.634±0.157*</td>
<td>1.356±0.001</td>
</tr>
<tr>
<td>Postoperative 7 d</td>
<td>7.524±0.352*</td>
<td>1.366±0.003</td>
</tr>
<tr>
<td>Postoperative 14 d</td>
<td>3.245±0.871*</td>
<td>1.376±0.001</td>
</tr>
<tr>
<td>Postoperative 21 d</td>
<td>3.035±0.452*</td>
<td>1.386±0.005</td>
</tr>
</tbody>
</table>

*P<0.05, compared with control group; *P<0.05, compared with preoperative.

Effect of bevacizumab on filtering bleb morphology and proliferation of fibroblasts

In saline injection group, most of the filtering blebs disappeared 1 week after the trabeculectomy, and the fiber cells were remarkable hyperplasia; however, in bevacizumab injection group, obvious follicular structure was observed, and fibrous tissue was mild hyperplasia. 2 weeks after the operation, a large number of collagen fibers were observed in the saline injection group, the collagen fibers were enlarged and in a disordered arrangement, and scars formed. But in bevacizumab injection group, only a small quantity of fibroblast cells were observed in filtering bleb, there was no large number of collagen fibers formed, and most of the filtration channels kept unobstructed, shown in Figure 3. Count the number of fibroblasts in filtering bleb under light microscope with an average of 200 field, it showed that compared with saline injection group, the fibroblast cells in bevacizumab injection group decreased significantly, and the difference was statistically significant, shown in Figure 4.

Table 3. Pearson correlation analysis on the correlation between VEGF and TGF-β2

<table>
<thead>
<tr>
<th>Variable</th>
<th>Correlation coefficient (r)</th>
<th>Statistic (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF and TGF-β2</td>
<td>0.775</td>
<td>0.012</td>
</tr>
</tbody>
</table>

Effect of bevacizumab on filtering bleb’s VEGF and TGF-β2

One week after surgery, the expressions of VEGF (0.212±0.020 pg/μL) and TGF-β2 (3.470±0.39 pg/μL) in Filtering bleb of bevacizumab injection group were lower than those of saline injection group (VEGF: 0.578±0.054 pg/μL, TGF-β2: 6.890±0.41 pg/μL), and the difference between the two groups was statistically significant (P<0.05), shown in Figure 5.

Discussion

Glaucoma is one of the major causes of blindness in the world [6, 7]. Trabeculectomy plays an important role in the treatment of glaucoma, but the filtration bleb scarring is one of the main reasons for the failure of trabeculectomy [8, 9]. The result of the research shows that postoperative fiber scarring is a complex bio-

Research of TGF-β₂ and VEGF in filtering bleb

A logical process that involves various cytokines [10, 11]. VEGF and TGF-β₂ play a very important role in this process [12, 13].

During the operation, many factors may promote the local chemotaxis of inflammatory cells, stimulate the migration and proliferation of fibroblasts, as well as blood vessel growth and the synthesis of the extracellular matrix; these factors include: blood vessel injury, outflow of aqueous humor, and a large quantity of growth factors and various inflammatory cytokines emerged in the surgical area, in which VEGF and TGF-β₂ are the most important factors [14-16]. Therefore, the role of VEGF and TGF-β₂ in the formation of scarring after trabeculectomy attracts more and more attention [17, 18]. In the present study, we used enzyme linked immunosorbent assay (ELISA) to detect the expression of VEGF and TGF-β₂ in the filtration bleb of rabbits that underwent trabeculectomy. The results of the present study showed, with the formation of filtering bleb scars, the expression of VEGF in the filtration bleb obviously changed - it highly expressed in the following 21 days after operation with a peak at postoperative 1 week. On the first day after operation, TGF-β₂ increased significantly, showing high expression in the postoperative 3 weeks with a peak at postoperative 1 week. In the control groups, the contents of VEGF and TGF-β₂ in the conjunctiva and scleral tissues were very low. Foreign experimental results showed that the most active period of the migration and proliferation of fibroblasts was 4-7 d, which is consistent with our experimental results in the peak of VEGF and TGF-β₂ in filtration bleb [19, 20].

Bevacizumab is a humanized VEGF monoclonal antibody, which can bind to all isomers of VEGF to inhibit VEGF function. In this study, the results showed that bevacizumab can restrain...
Research of TGF-β₂ and VEGF in filtering bleb fibrosis after trabeculectomy, and can inhibit the expression of VEGF in filtration bleb. At the same time, bevacizumab has inhibitory effect on TGF-β₂. So, bevacizumab can inhibit not only the postoperative filtration bleb scarring but also the hyperplasia of fibroblast after operation, by inhibiting the expressions of VEGF and TGF-β₂, respectively.

In this study, the content of VEGF and TGF-β₂ were detected simultaneously in the same filtration bleb tissues. The results showed that the content of TGF-β₂ was correlated with the expression of VEGF, promoting the formation of scars. The possible mechanisms are as follows [21-23]: (1) triggering an immune response: after tissue trauma, VEGF can combine with endothelial cells, which release a variety of growth factors and cytokines with biological activity, inducing the migration of neutrophil and mononuclear cells to the traumatized part and promoting the formation of granulation tissue; (2) promoting the formation of neovessels: vascular endothelial cells could proliferate, differentiate, and migrate under the action of VEGF to form new blood vessels; (3) increasing the vascular permeability: VEGF is one of the strongest known vascular permeability agents; it can enhance the permeability of postcapillary venules, and macromolecular substance in the blood, such as plasma protein and white blood cells, can migrate to the traumatized site to form fibrin gel, which is conducive to the growth of angiogenesis and stromal cells; (4) maintaining vascular structure.

In summary, VEGF and TGF-β₂ have synergistic effect in the process of filtration bleb scarring; they co-promote the occurrence and development of scarring. To inhibit the process of scarring, combined treatment should be applied in order to achieve a better effect.

Disclosure of conflict of interest

None.

Address correspondences to: Xiaoping Sun, Department of Ophthalmology, Zhengzhou Central Hospital Affiliated to Zhengzhou University, No. 195 Tongbai Road, Zhongyuan District, Zhengzhou 450000, Henan Province, P. R. China. Tel: +86-0371-55966339; E-mail: xiaopingsun2016@163.com

References

Research of TGF-β and VEGF in filtering bleb


