Abstract. — OBJECTIVE: To investigate the effect of tacrolimus on the proliferation of fibroblasts after glaucoma surgery.

MATERIALS AND METHODS: Biopsy was applied in this study. Under aseptic conditions, tissues were collected from rabbits, cut into small pieces and cultured. Morphology of fibroblasts was observed under a microscope. Features of fibroblasts were identified via immunocytochemistry and reverse transcription-polymerase chain reaction (RT-PCR). Western blotting and RT-PCR were performed to detect the expressions of related proteins after treatment. Flow cytometry and cell counting kit-8 (CCK-8) assay were employed to examine the proliferation of Tenon’s capsule fibroblasts (TFs) after tacrolimus treatment.

RESULTS: Tacrolimus decreased the levels of survivin and α-smooth muscle actin (α-SMA) after transforming growth factor-β (TGF-β) treatment. Besides, it inhibited proliferation and induced apoptosis of TFs.

CONCLUSIONS: Tacrolimus reduces proliferation and promotes apoptosis of TFs by inhibiting the expression of survivin, which may be a strategy for treating hypertrophic scar after glaucoma surgery.

Key Words: Tacrolimus, Survivin, Scar Fibroblasts, Glaucoma Surgery.

Introduction

The filtering bleb scarring after glaucoma filtering surgery is the most primary cause of surgery failure. There are dense collagen fibrillar connective tissues abnormally thickened below the conjunctival epithelium of filtering blebs in the failed filtering surgery, accompanied by the active proliferation of fibroblasts, thus blocking the filtering passage, and losing the aqueous drainage function. At present, anti-metabolism drugs commonly used in surgery, such as fluorouracil and mitomycin, can reduce the scar formation of filtering passage after surgery and improve the success rate of surgery, but its anti-metabolic effects may also lead to a series of postoperative complications, such as ocular hypotension and filtering blebleakage. Therefore, exploring safer and more effective treatment methods of scarring after glaucoma surgery has important significance. Tenon’s capsule fibroblasts (TFs) play major roles in the scarring process of filtering passage after glaucoma surgery. Under conditions of surgery or injury, the increased level of transforming growth factor-β (TGF-β) activates local fibroblasts and transforms them into myofibroblasts (MFs), thus initiating the wound healing response. MFs play important roles in different stages of wound healing. Once the wound is healed, in general, MFs will be rapidly restored to fibroblasts or enter the apoptosis program. If MFs exist persistently, it will lead to excessive proliferation of cells, increased synthesis of extracellular matrix, scar formation, premature healing of filtering passage and blockage of aqueous drainage passage, ultimately resulting in surgical failure.

Survivin, as a member of the anti-apoptosis protein family, is involved in the regulation of mitosis and the inhibition of the activity of caspase-3, thereby promoting the abnormal proliferation of transformed cells and avoiding the cell apoptosis. Tacrolimus (FK506) is a kind of natural macroline immunosuppressant isolated from Streptomyces in 1984, whose immunosuppressive activity is 10-100 times that of cyclosporin A. FK506 is clinically used in the treatment of rejection reaction after organ transplantation, atopic dermatitis and some...
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Materials and Methods

Isolation and Culture of Rabbit Tenon’s Capsule Fibroblasts

Rabbits were fixed on an operating table under supine position, and the anesthetic was injected via ear vein. Under sterile conditions, Tenon’s capsule tissues were removed, immersed in sterile phosphate buffered saline (PBS) containing 300 μ/mL penicillin and 300 μ/mL streptomycin, and stored in an ice box. The above Tenon’s capsule tissue samples were washed with sterile PBS containing 100 μ/mL penicillin and 100 μg/mL streptomycin, (Yangtze River Pharmaceutical Group, Beijing, China)). Then, tissues were cut into small tissue blocks (0.5-1 mm²) using sterile corneal scissors, added with a small amount of DMEM complete culture solution (containing 10% fetal bovine serum, FBS, Gibco, Rockville, MD, USA) 100 μ/mL penicillin and 100 μg/mL streptomycin, (Yangtze River Pharmaceutical Group, Beijing, China)). Then, tissues were cut into small tissue blocks (0.5-1 mm²) using sterile corneal scissors, added with a small amount of DMEM complete culture solution, and mixed evenly. Tissue block suspension was evenly inoculated into a sterilized 90 mm glass culture dish using an aseptic dropper, with an interval of about 1 cm between every two drops. The culture dish was placed into an incubator with 5% CO₂ at 37°C. After being cultured for 24 h, tissue culture dish was placed into an incubator with 5% CO₂ at 37°C for 2-3 min. When microscopic observation showed that the intercellular space was increased and cells became round, the trypsin solution in the culture dish was discarded, and DMEM complete culture solution was added to terminate the digestion. The bottom of culture dish was repeatedly blown and beaten to blow exfoliated cells fully and evenly, followed by passage in a ratio of 1:4 or 1:5. Cells were inoculated into a culture flask or culture plate, and passage or relevant experimental research was performed again when cells covered 70%-80% of flask or plate.

Immunofluorescence Assay

Immunofluorescence assay was performed as previously described (29164574). The primary antibodies of vimentin, keratin, surviving and α-SMA were purchased from Abcam (Cambridge, MA, USA).

Western Blotting

Tissues and cells were added with radioimmunoprecipitation assay (RIPA) buffer, and various protease inhibitors, such as phenylmethanesulfonyl fluoride (PMSF), were also added, followed by incubation on ice for 30 min. During this period, the sample was repeatedly blown and beaten to avoid air bubbles, and transferred to a centrifuge tube for centrifugation at 15000 rpm and 4°C for 15 min. According to instructions of the bicinechonic acid (BCA) protein quantification kit (Thermo Fisher Scientific, Waltham, MA, USA), the sample concentration was determined. The loading quantities of all samples were adjusted equally. An equal volume of sodium dodecyl sulfate (SDS)-loading buffer was added, and the mixture was mixed evenly and boiled at 100°C for 10 min. The sample solution and standard substance prepared were loaded, the standard substance was added into the first well, and the protein was separated through the horseradish peroxidase oxidation reaction, followed by detection. Then, the membrane was washed again with TBS-T for 4 times (5 min each time). When the appropriate enzyme substrate was added, the complex would be converted into the substance with chemiluminescence property through the horseradish peroxidase oxidation reaction, followed by detection. Then, the membrane was washed again with TBS-T for 4 times (8 min each time). Exposure: the substrate was added, and the signal was collected using films, followed by color development.
Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RNA of TFs was extracted according to the manufacturer’s instructions (Yifeixue Bio Tech, Nanjing, China). The corresponding RNA sample was added, followed by reverse transcription to obtain complementary Deoxyribose Nucleic Acid (cDNA). The relative expression level of mRNA was analyzed using the Bio-Rad C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA).

Cell Viability Assay

TFs were inoculated on a 96-well plate with a density of $5 \times 10^4$ mL (100 μL each well). After various treatments were applied to these wells, cell counting kit-8 (CCK-8, TaKaRa, Tokyo, Japan) solution (10 μL) was added to each well, and cells were incubated for 2 h at 37°C. Absorbance was measured at 450 nm.

Annexin V/Propidium Iodide Double Staining

Annexin V/propidium iodide double staining was employed according to the previous study.

Statistical Analysis

Statistical product and service solutions (SPSS) 11.5 software (Chicago, IL, USA) was used for statistical analysis of data. The comparison between groups was done using One-way ANOVA test followed by Post Hoc Test (Least Significant Difference). $p<0.05$ suggested that the difference was statistically significant.

Results

Identification of TFs

Primary cells were in fusiform shape and arranged in bundles, a small number of cells were also in irregular shape, and they connected each other through long and thin synapses and covered the whole culture dish after about 2 weeks. If a small number of epithelial cells were mixed in the culture dish, cells were cultured using the differential culture method, and they were completely composed of single-layer long fusiform cells after subculture, which was consistent with the morphological characteristics of fibroblasts (Figure 1A). Vimentin is a kind of constitutive protein produced by fibroblasts, as well as a characteristic marker of fibroblasts, and keratin is a surface marker of epithelial cells. To eliminate the possibility of mixing conjunctival epithelial cells in cells cultured, the characteristics of fibroblasts cultured were detected using vimentin and keratin immunostaining technique. Fibroblasts interacted with vimentin antibodies, so cells showed

Figure 1. A, Morphological characteristics of fibroblasts. B-C, Increased level of vimentin produced by fibroblasts.
positive response and green fluorescence. After interaction with keratin antibodies, cells showed negative response without green fluorescence (Figure 1B). The levels of vimentin gene VIM and keratin gene KRT3 in cornea and TFs were detected via Real-time fluorescence quantitative PCR (Figure 1C), respectively.

**α-SMA, Collagen I and Survivin Were Increased When Treated with TGF-β1**

After TFs were induced by 1-40 ng/mL TGF-β1, the expression of α-SMA was increased, among which it was expressed most significantly in TFs induced by 10 ng/mL TGF-β1 (Figure 2A). α-SMA, collagen I and survivin were increased when TFs were cultured with TGF-β (Figure 2B).

**FK506 Inhibited TFs Proliferation**

According to the results of the CCK-8 assay, TFs treated with FK506 at various concentrations (5 nM, 10 nM, 20 nM, and 40 nM) for 24 h exhibited significant reduction of cell viability (Figure 3).

**FK506 Induced Apoptosis in TFs**

Annexin V/propidium iodide double staining results showed that TFs treated with 10 nM, 20 nM, and 40 nM FK506 for 24 h exhibited significant apoptosis (Figure 4A). At the same time, Western blotting also showed that the expression level of apoptosis-specific protein Caspase-3 was significantly increased compared with that in the control group (Figure 4B).

**FK506 Treatment Decreased Expression of Survivin Induced by TGF-β1**

The results of Western blotting, q-PCR, and immunofluorescence demonstrated that FK506 (20 nM) inhibited the expression of survivin, thus inhibiting proliferation and inducing apoptosis (Figure 5).

**Discussion**

Glaucoma filtering surgery is one of the most commonly-used surgical methods for controlling the intraocular pressure, whose main principle is to artificially establish the aqueous outflow passage18,19. The functional filtering bleb is a main visual indicator to determine whether the aqueous outflow passage is established successfully or not. There are two kinds of failed filtering blebs: a) Filtering blebs disappear completely and fibrous scars are formed; b) the limited and hypertrophic encapsulated filtering blebs wrapped with Tenon’s fascia are formed around the filtering mouth. The latter one is similar to the functional bleb in appearance, but there is an abnormal proliferation of dense collagen fibers around the subconjunctival filtering mouth, hindering the aqueous drainage. From the perspective of histopathology, the process of wound repair after filtering surgery is divided into three phases: a) Deposition of fibrinoid materials and fibroblast proliferation at 6 d after surgery; b) Proliferation and migration of fibroblasts at 7-9 d after surgery; c) Formation of granulation tissues and wound closure at 10-14 d after surgery. In wound repair, a large number of fibroblasts proliferate through mitosis, synthesize and secrete a lot of collagen fibers and matrix components, and form granulation...
tissues together with new capillaries, thus filling the wound tissue defects. The mechanism of scar formation in filtering passage after surgery is not fully understood, and it is generally believed that the proliferation of a large number of fibroblasts, main repair cells, leads to the release of a series of cytokines, and the imbalance between generation and degradation of collagen fibers in extracellular matrix, which is a biological basis of pathological scar formation.

Survivin gene is the smallest one with an independent structure in the anti-apoptosis protein family, and its N-terminal baculovirus anti-apoptosis protein repetitive sequence structure has very important apoptosis-inhibiting amino acids (Trp67, Pro33, and Cys64 residue). Survivin inhibits the activity of caspase-3, etc., mainly through these amino acids, thereby exerting an anti-apoptotic effect. The main function of survivin gene is to inhibit cell division and apoptosis, and regulate cell mitosis through binding to the tubulin of spindle apparatus, thus promoting abnormal cell proliferation and avoiding apoptosis.

FK506 is currently found to be an efficient immunosuppressive agent, which can inhibit the activation of T cells and inactivate T cells through inhibiting the passage of the T cell receptor complex to the membrane. FK506 binds to FKBP-12 and inhibits the formation of the T cell receptor complex, thereby inhibiting the activation of the T cell receptor complex.

Figure 4. Annexin V/propidium iodide double staining suggested that surviving induced apoptosis.
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bitting the activity of calcineurin. Inactivated T cells cannot release pro-fibrotic inflammatory factors, such as interleukin-2 (IL-2), IL-4, IL-5, TGF-β and tumor necrosis factor-α (TNF-α). A large number of previous studies have shown that FK506 plays an important inhibitory role in the process of scar formation, such as epidural fibrosis. This experiment confirmed that FK506 can inhibit the proliferation of fibroblasts after glaucoma surgery.

**Conclusions**

FK506 can inhibit proliferation and promote apoptosis of TFs through inhibiting the expression of survivin, which can be used as a treatment method for the regulation of scar hypertrophy after glaucoma surgery.

**Conflict of Interest**

The Authors declare that they have no conflict of interest.

**References**

