·Clinical Research·

Apoptosis in the iris and trabecular meshwork of medically treated and untreated primary open angle glaucoma patients

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Abstract

• **AIM: To compare the trabecular meshwork (TM) and iris apoptosis of treated and untreated primary open angle glaucoma (POAG) patients.**

• **METHODS: Eight treatment-naive, newly diagnosed (group 1) and 11 medicaly treated (group 2) patients with POAG were included in the study. Each patient underwent a limbus-based trabeculectomy. The TM and peripheral iris specimens were dissected out and were snap-frozen in liquid nitrogen and stored at -80℃ until they were assayed. Apoptosis in each group was assesed by TUNEL method.**

• **RESULTS: The mean patient age was 60.6±5.8 years (53-68 years) *vs* 58.9±8.9 years (47-70 years) in group 1 and group 2 (P=0.859). The mean treatment time in group 2 was 22.2±7.3 months (12-34 months). Apoptotic indexes in TM and iris were significantly higher in POAG patients using medication (group 2) compared to treatment-naive POAG patients (group 1) (P=0.004, 0.015; respectively).**

• **CONCLUSION: Long term administration of topical antiglaucoma medications causes additional toxic effects on TM.**

• **KEYWORDS:** glaucoma; trabecular meshwork; apoptosis
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INTRODUCTION

Glaucoma is a chronic disease characterized by progressive loss of retinal ganglion cells and glaucomatous optic neuropathy leading to progressive loss of vision. Aqueous humor resistance is a main mechanism for the increased intraocular pressure (IOP) in primary open angle glaucoma (POAG). Trabecular meshwork (TM) is the principal site of this flow resistance in the aqueous outflow network [1]. The structural alterations in the TM of eyes with POAG increase aqueous outflow resistance. The most characteristic structural alterations involve the decrease in cellularity, an increase in the extracellular matrix (ECM) and thickened sheats of elastic fibers[2,3].

The loss of cells within the TM is the major histological finding in POAG but the exact mechanism is not known[2]. However, various mechanisms have been suggested; including wear and tear, phagocytosis, cell migration and cell death. Cell death may occur *via* apoptosis (type I), autophagy (type II), and necrosis (type III)[4,5]. Besides, IOP is also thought to increase mechanical stress and hypoperfusion within the TM[6].

Decreasing IOP *via* topical antiglaucomatous therapy is usually the first step in treatment of glaucoma. On the other hand, human and animal studies have shown that chronic topical antiglaucomatous therapy may lead to alterations in tear film, damage and remodeling of the corneal surface, an increase in inflammatory cytokines, as well as other deleterious effects [7-12]. Acute exposure models using animals or cell culture systems demonstrate significant damage/death in cornea and conjunctival cells either immediately after exposure or within 24h[13-16]. Some toxicity can be attributed to either of the active ingredients of the prostaglandin analog (PGA) and beta adrenergic receptor antagonist (BB) fixed combination therapy [17,18]. However, much of the ocular surface changes seen with chronic daily topical antiglaucomatous therapy are associated with the commonly used preservative, benzalkonium chloride (BAK)[10,11].

The goal of this study is to compare apoptotic indexes in iris and TM of advanced POAG patients who were conventinally treated with long term medical therapy before filtrating surgery and patients initially underwent surgery because of the presence of advanced disease and late diagnosis.

SUBJECTS AND METHODS

Subjects Human tissue was handled in accordance with the Local Committee of Ethics for experimentation with human tissues. Eight treatment-naive, newly diagnosed and 11 treated patients with POAG in Gazi University Ophthalmology Department were included in the study. Before surgery, all participants underwent a comprehensive ophthalmological examination, including slit-lamp biomicroscopy, best-corrected visual acuity (BCVA), IOP measurement by Goldman applanation tonometer, dilated fundoscopy, gonioscopy, central corneal thickness and visual field perimetry using the Humphrey Field Analyzer 750 (Humphrey-Zeiss Instruments, Dublin, California, USA). The eyes were divided into 2 groups, group 1 consisted of 8 eyes (8 patients) without any history of previous medication, group 2 consisted of 11 eyes (11 patients) using latanoprost/timolol fixed combination. All eyes in both groups underwent trabeculectomy. POAG was defined as the presence of at least 3 glaucomatous visual fields with reproducible glaucomatous defects and the apperance of the corresponding glaucomatous optic discs, together with a pretreatment IOP of at least 21mmHg and an open angle in gonioscopic examination.

Patients with secondary open angle glaucoma, angle closure glaucoma, ocular disease other than glaucoma; a history of previous eye surgery, diabetes mellitus and hypertension were exluded from the study.

Methods

Surgical tecnique All surgeries were carried out under either general or retrobulbar anesthesia by a single experienced surgeon (Onol). After the local anesthesia; bulbus was stabilized with an atraumatic 4-0 silk traction suture suspending the superior rectus muscle. A limbus based conjunctival incision was performed 8-9mm posterior to the limbus and sclera was exposed after the blunt dissection of Tenon's capsule and conjunctiva. Conjunctiva was also undermined nasally and temporally via blunt dissection with Weskott scissors parallel to the limbus. Before scleral flaps were dissected, 4mm×4mm piece of sponge soaked with Mitomycin-C (MMC) were placed under the Tenon's capsule temporally and nasally in addition to scleral flap area as we previously described as large area MMC application[19]. MMC were applied for 2min with dose of 0.2g/mL. Then MMC application area was irrigated with balanced salt solution and a scleral flap of 5mm×5mm was dissected. When the entire corneoscleral limbus was exposed, trabeculectomy was performed with the excision of approximately 1mm×3mm trabecular block followed by a peripheral iridectomy. Scleral flap was repositioned with three 10/0 monoflament nylon sutures and adjusted in such a manner that anterior chamber kept its depth allowing slow leakage from the edge of the scleral flap. The Tenon's capsule was closed with three interrupted and the conjunctiva was then closed with running 7/0 vicryl suture, respectively. Topical dexamethasone and antibiotic drops were given in all cases four times daily for 3 weeks. Dexamethasone was tapered during this time.

The TM and peripheral iris specimens were dissected out and were snap-frozen in liquid nitrogen and stored at -80℃ until they were assayed.

Histopathological examination The histopathologist was masked to the patient groups. The biopsy specimens were immediately immersed in 10% buffered formalin solution and were fixed 3-4h at room temperature. The specimens were then embedded in paraffin, sliced to 4-μm sections, and mounted on poly-L-lysine-coated slides. Apoptosis was detected by labeling the 3'OH ends of DNA using digoxigenin incorporation by terminal deoxynucleotidyl transferase (TdT) (TUNEL method). Antidigoxigenin antibodies and immunoperoxidase staining were used to demonstrate digoxigenin-nucleotide incorporation with a commercially available in situ apoptosis detection system (Apop Tag, Chemicon International, CA, USA). Briefly, paraffin-embedded sections were deparaffinized and dehydrated in graded alcohol and treated with 20μg/mL proteinase K at room temperature for 15min. Endogenous peroxidase activity was quenched with 3% hydrogen peroxidase in phosphate-buffered saline. After digoxigenin-nucleotide was added catalytically, detection with anti-digoxigenin peroxidase was performed using 3-3'-diaminobenzidine for 6min. The tissue sections were visualized after counterstaining with 1% methyl green. The apoptotic index was determined as the ratio of positive cells to the total amount of epithelial cells in each section by counting the positively stained cells under light microscopy.

Statistical Analysis Data analysis was performed using SPSS software version 15 (SPSS, Inc., Chicago, Illinois, USA). The differences in apoptotic indexes, ages and mean deviation (MD) values between two groups were performed by using the Mann-Whitney U test. P values of less than 0.05 were considered as statistically significant.

RESULTS

The mean patient age was 60.6±5.8 years (53-68 years) and 58.9±8.9 years (47-70 years) in group 1 and group 2, respectively (P=0.859). The mean treatment period with latanoprost/timolol combination in group 2 was 22.2±7.3 months (12-34 months). The mean MD values were 18.7±1.4dB (17.3-21.3dB) and 17.6±1.6dB (14.5-20.3dB) in both groups, respectively (P=0.126).

Apoptotic indexes both in TM and iris specimens were higher in group 2 compared to group 1 as presented in Table 1 and both were statistically significant.

In situ labeling of apoptotic cells in paraffin sections of iris and TM of group 1 and group 2 (TUNEL method) are presented in Figures 1, 2. Note that apoptotic index was

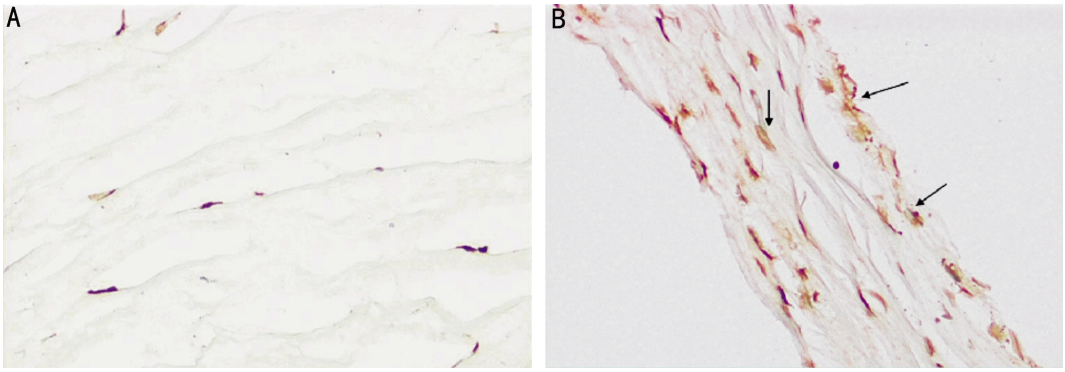

Figure 1 ***In situ*** **labeling of apoptotic cells in a paraffin sections of TM** A: Minimal TM apoptosis in Group 1 (TUNEL ×200); B: Intense TM apoptosis in Group 2 (TUNEL ×200).

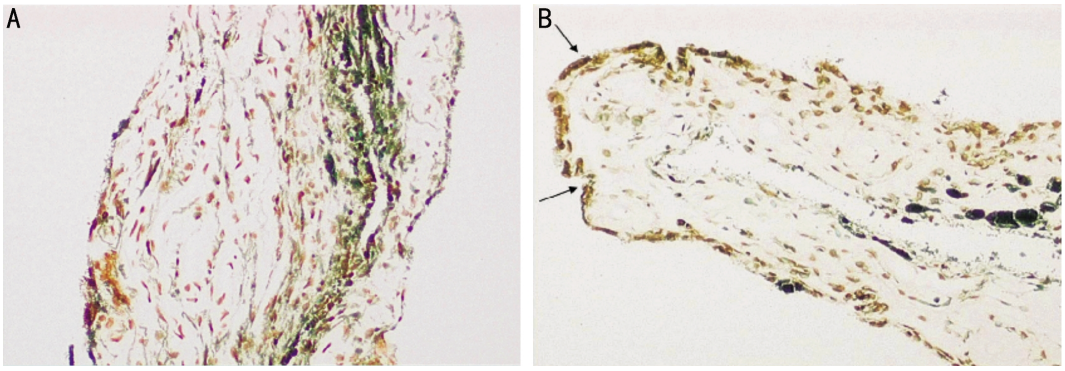

Figure 2 ***In situ*** **labeling of apoptotic cells in a paraffin sections of iris** A: Minimal iris apoptosis in Group 1 (TUNEL ×200); B: Intense iris apoptosis in Group 2 (TUNEL ×200).

Table 1 Apoptotic indexes in both groups

Apoptotic index (%)	Group 1	Group 2	*P*
Trabecular meshwork	5.3± 4.9 (0-13)	15.6±8.0 (8-31)	0.004
Iris	7.4±5.3 (0-15)	24.5-18.1 (4-61)	0.015

significantly higher in group 2 compared to that of group 1 (original magnification ×200).

DISCUSSION

The current study examined whether the programmed cell death occurs in the TM and iris cells of POAG patients who were treated and untreated with antiglaucomatous drops. This was achieved by developing a TUNEL method to stain TM and iris cells as a whole, facilitating the visualization and scoring of the relatively high number of apoptotic cells in these tissues.

We have found that topical fixed combination therapies significantly decreased the number of TM and iris cells. However, loss of cells in untreated patients suggests that apoptosis could be one of the mechanisms by which TM and iris cells die in POAG. It has been suggested that intense phagocytic activity of trabecular cells could lead to cell death[20]. Furthermore, glaucoma itself could also produce apoptosis of TM cells through mechanical stress or trabecular hypoperfusion [6,21]. An increase in oxidative stress may also contribute to cell loss or alterations in the functioning of TM and iris cells[5].

In the current study, we compared medically treated and untreated POAG patients. The loss of TM and iris cells was found to be higher in group 2. Since we included the advanced POAG patients who were at the same stage of the disease according to visual fields, the only difference between two groups was the history of medical therapy. We thought that it might have been resulted from the toxic effects of topical drops. Cell loss detected also in medically untreated patients, supporting the idea that increased IOP could be one of the mechanisms in patients with glaucoma[6]. Baleriola *et al* [5] compared the trabecular apoptosis between patients with POAG and angle closure glaucoma in which topical antiglaucomatous therapy were given for years in both groups. In this study, there was no group consisted of initially treated with surgery, so it is impossible to know the cell loss was secondary to medical therapy in POAG patients. To the best of our knowledge, our study is the first one investigating the apoptosis in TM and iris secondary to medical therapy in POAG patients compared to those who were initially operated because of the presence of advanced disease and late presentation.

Identification of dying cells by labeling cells with TUNEL was originally described to identify apoptotic cells spesifically [22]. DNA fragmentation is a main feature of apoptotic cell death and TUNEL is a procedure in which 3'-OH DNA ends are enzymatically labeled with dUTP-fluorescein isothiocyanate using TdT. Although the spesificity of this method for labeling apoptotic cells was

questioned in some papers, it is one of the widely accepted methods for the detection of the apoptotic trabecular meshwork cells[5,23-25].

Finally, we have to mention the limitations of this study. One is the absence of the control group since it is impossible to obtain TM and iris specimens from healthy control subjects because of the ethical issues. The other one is the small number of subjects included in the study.

In conclusion, applying long term topical antiglaucoma medications containing preservatives could damage the structures within TM by reducing the number of healthy TM cells. Additional *in vitro* and *in vivo* animal studies are needed to determine the clinical implications of our findings for patients treated with fixed combination therapies.

REFERENCES

1 Johnson MC, Kamm RD. The role of Schlemm's canal in aqueous outflow from the human eye. *Invest Ophthalmol Vis Sci* 1983;24(3):320-325

2 Alvarado J, Murphy C, Juster R. Trabecular meshwork cellularity in primary open angle glaucoma and non-glaucomatous normals. *Ophthalmology* 1984;91(6):564-579

3 Hamard P, Sourdille P, Valtot F, Bourles-Dagonet F, Baudouin C.. Confocal microscopic examination of trabecular meshwork remowed during ab externo trabeculectomy. *Br J Ophthalmol* 2002;86(9):1046-1052

4 Krysko DV, Vanden Berghe T, D'Herde K, Vandenabeele P. Apoptosis and necrosis: detection, discriminaton and phagocytosis. *Methods* 2008;44 (3):205-221

5 Baleriola J, García-Feijoo J, Martínez-de-la-Casa JM, Fernández-Cruz A, de la Rosa EJ, Fernández-Durango R. Apoptosis in the trabecular meshwork of glaucomatous patients. *Mol Vis*2008;14:1513-1516

6 Grierson I. What is open angle glaucoma? *Eye (Lond)* 1987;1 (Pt 1): 15-28

7 Baudouin C, Liang H, Hamard P, Riancho L, Creuzot-Garcher C, Warnet JM, Brignole-Baudouin F. The ocular surface of glaucoma patients treated over the long term expresses inflammatory markers related to both T-helper 1 and T-helper 2 pathways. *Ophthalmology* 2008;115(1):109-115

8 Noecker RJ, Herrygers LA, Anwaruddin R. Corneal and conjunctival changes caused by commonly used glaucoma medications. *Cornea* 2004; 23(5):490-496

9 Pisella PJ, Debbasch C, Hamard P, Creuzot-Garcher C, Rat P, Brignole F, Baudouin C. Conjunctival proinflammatory and proapoptotic effects of latanoprost and preserved and unpreserved timolol: an *ex vivo* and *in vitro* study. *Invest Ophthalmol Vis Sci* 2004;45(5):1360-1368

10 Kahook MY, Noecker RJ. Comparison of corneal and conjunctival changes after dosing of travoprost preserved with sofZia, latanoprost with 0.02% benzalkonium chloride, and preservative-free artificial tears. *Cornea* 2008;27(3):339-343

11 Martone G, Frezzotti P, Tosi GM, Traversi C, Mittica V, Malandrini A, Pichierri P, Balestrazzi A, Motolese PA, Motolese I, Motolese E. An *in vivo* confocal microscopy analysis of effects of topical antiglaucoma therapy with preservative on corneal innervation and morphology. *Am J Ophthalmol* 2009;147(4):725-735.e1

12 Leung EW, Medeiros FA, Weinreb RN. Prevalence of ocular surface disease in glaucoma patients. *J Glaucoma*2008;17(5):350-355

13 Yee RW, Norcom EG, Zhao XC. Comparison of the relative toxicity of travoprost 0.004% without benzalkonium chloride and latanoprost 0.005% in an immortalized human cornea epithelial cell culture system. *Adv Ther* 2006;23(4):511-519

14 Liang H, Baudouin C, Pauly A, Brignole-Baudouin F. Conjunctival and corneal reactions in rabbits following short- and repeated exposure to preservative-free tafluprost, commercially available latanoprost and 0.02% benzalkonium chloride. *Br J Ophthalmol* 2008;92(9):1275-1282

15 Yu AL, Fuchshofer R, Kampik A, Welge-Lüssen U. Effects of oxidative stress in trabecular meshwork cells are reduced by prostaglandin analogues. *Invest Ophthalmol Vis Sci* 2008;49(11):4872-4880

16 Ammar DA, Kahook MY. The effects of combination glaucoma medications on ocular surface epithelial cells. *Adv Ther* 2009;26 (10): 970-975

17 Wu KY, Wang HZ, Hong SJ. Cellular cytotoxicity of antiglaucoma drugs in cultured corneal endothelial cells. *Kaohsiung J Med Sci* 2007;23 (3): 105-111

18 De Saint Jean M, Debbasch C, Brignole F, Rat P, Warnet JM, Baudouin C. Toxicity of preserved and unpreserved antiglaucoma topical drugs in an *in vitro* model of conjunctival cells. *Curr Eye Res* 2000;20:85-94

19 Onol M, Aktas Z, Hasanreisoglu B. Enhancement of the success rate in trabeculectomy: large-area mitomycin-C application. *Clin Experiment Ophthalmol* 2008;36(4):316-322

20 Hamard P, Blondin C, Debbasch C, Warnet JM, Baudouin C, Brignole F. In vitro effects of preserved and unpreserved antiglaucoma drugs on apoptotic marker expression by human trabecular cells. *Graefe's Arch Clin Exp Ophthalmol* 2003;241(12):1037-1043

21 Johnson DH, Richardson TM, Epstein DL. Trabecular meshwork recovery after phagocytic challenge. *Curr Eye Res* 1989;8(11):1121-1130

22 Gavrieli Y, Sherman Y, Ben Sasson SA. Identification of programmed cell death *in situ via*spesific labeling of nuclear DNA fragmentation. *J Cell Biol* 1992;119(3):493-501

23 Charriaut-Marlangue C, Ben-Ari Y. A cautionery note on the use of the TUNEL stain to determine apoptosis. *Neuroreport* 1995;7(1):61-64

24 Wood JP, Plunkett M, Previn V, Chidlow G, Casson RJ. Rapid and delayed death of cultured trabecular meshwork cells after selective laser trabeculoplasty. *Lasrs Surg Med* 2010;42(4):326-337

25 Cao Y, Wei H, Pfaffl M, Da B, Li Z. Apoptosis of human trabecular meshwork cells induced by transforming growth factor-beta2 *in vitra J Huazhong Univ Sci Technolog Med Sci* 2004;24(1):87-89