Arsenic Trioxide Inhibits Proliferation of Rabbit Tenon’s Capsule Fibroblasts After Trabeculectomy by Downregulating Expression of Extracellular Matrix Proteins

Ying Su, Chenggong Jiang, Ling Zhang, and Feng Wang

Department of Ophthalmology, First Affiliated Hospital of Harbin Medical University, Harbin, China

Correspondence: Feng Wang, Department of Ophthalmology, First Affiliated Hospital of Harbin Medical University, 150001 China; wangfd@126.com.

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PURPOSE. The purpose of this study was to study the effectiveness of arsenic trioxide (ATO) in regulation of trabeculectomy in an animal model.

METHODS. This study was designed as random, comparative, and prospective study. Primary Tenon’s capsule fibroblasts (TFs) were cultured and their viability after arsenic trioxide (ATO) treatment was detected using 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) and bromodeoxyuridine incorporation (BrdU) assays. The dose of ATO was determined by 50% growth inhibition (IC50 values) and cell-cycle analysis. The expression of extracellular matrix (ECM) components and proliferating cell nuclear antigen (PCNA) was determined by Western blotting analysis. The status of filtration bleb was detected by haematoxylin and eosin (HE) stain.

RESULTS. Based on the MTT and BrdU assays, IC50 values was observed in rabbit TFs (rTFs) cells after treatment with ATO at a concentration of 6 μM arsenic trioxide for 72 hours. In addition, the ATO treatment caused not only a significant reduction in the expression of ECM proteins, fibronectin, collagen IV, and laminin in rTFs, but also reduced the expression of PCNA expression in a time dependent manner. The histologic observation showed high-level proliferation of rTFs in both vehicle and control groups, and proliferative ability of rTFs of experimental group was less than in vehicle and control group. Moreover, no inflammation was observed in the experimental group, and the filtering bleb was detected in the experimental group after 14 days of trabeculectomy.

CONCLUSIONS. Arsenic trioxide inhibited the proliferation of rTFs after trabeculectomy and may improve the success ratio of trabeculectomy in an animal model.

Keywords: trabeculectomy, arsenic trioxide, fibroblast

Glaucoma is a leading cause of blindness and is usually caused by increased IOP, which eventually leads to the optic nerve injury. Trabeculectomy is a filtering surgery that lowers the IOP and is usually used for treatment of moderate–advanced glaucoma. However, the proliferation of Tenon’s capsule fibroblasts (TFs) causes loss of filtering bleb function. The subconjunctival fibrosis has been shown to play a vital role in the failure of filtering surgery for scar-caused glaucoma, thus indicating that extracellular matrix (ECM) deposition is a major contributor to fibrosis-induced scar.

Antimetabolites like Mytomycin C and 5-fluorouracil have been used to reduce subconjunctival fibrosis and improves the success ratio of filtering surgery. However, they usually also induce severe complications, even sight threatening–like filtering bleb leak and infectious endophthalmitis.

Gene therapy appeared effective in suppressing fibroblast proliferation after glaucoma surgery. Our previous study has shown that Skp2 small interfering (si)RNA inhibited the proliferation and decreased the viability of rabbit TFs (rTFs) in vivo and in vitro by downregulating the expression of proliferating cell nuclear antigen (PCNA).

Arsenic trioxide, a broad-spectrum anticancer traditional Chinese medicine, induces the apoptosis of multiple cancer cells, including gastrointestinal and hepatocellular cancers. Recently, arsenic trioxide has been found to induce cancer cell apoptosis and inhibit cancer cell migration and invasion through suppressing matrix metalloproteinase (MMP)-2 and MMP-9 expression in solid tumors.

In the current study, we aimed to investigate if arsenic trioxide mediated suppression of rabbit TFs proliferation involves the downregulation of the expression of ECM proteins after trabeculectomy in an animal model.

MATERIALS AND METHODS

Culturing of Rabbit Tenon’s Capsule Fibroblast Cells

Rabbits were anaesthetized with ketamine (60 mg/kg) combined with xylazine (80 mg/kg). Rabbit TFs were collected from subconjunctival Tenon’s capsule, and seeded onto the bottom of 6-well plates (Corning Costar Corporation, Cambridge, MA, USA) with sterile cover slips, which were
covered with Dulbecco’s modified Eagle’s medium/Ham’s nutrient mixture F12 medium (DMEM F12; Gibco BRL, Gaithersburg, MD, USA) supplemented with 50 ng streptomycin and 500,000 U/L penicillin (Gibco Ltd., Uxbridge, UK). To ensure fibroblast proliferation, the medium was added with 10% fetal calf serum (Invitrogen, Carlsbad, CA, USA). The rTFs of passages 3 to 5 were used for all experiments, and all the cells were cultured at 37°C in a ventilated incubator containing 5% CO2 (Heal Force, Hong Kong, China).14

The cell proliferation of rTFs treated with arsenic trioxide (Sigma-Aldrich Corp., St. Louis, MO, USA) at different doses of 0.5 to 11 μM for 24, 48, and 72 hours, was detected using 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazoliumbromide (MTT) assay. The rTFs cultured in DMEM F12 medium containing 10% fetal bovine serum served as control group and add 0.9% NaCl in the medium above as vehicle group. In fact, the cell proliferation inhibition, which appeared to be in a dose-dependent manner was assessed as described previously.15,16

Western Blotting Assay

Total protein from rTFs cultured in the serum-free medium containing 0.5 mg/mL BSA (HyClone Labs, Logan, UT, USA) was extracted after their treatment with or without arsenic trioxide. Next, the protein concentration was determined using Bradford chromometry and the total protein was separated by 10% SDS-PAGE. The proteins were then transferred to nitrocellulose (NC) membrane and the membrane was blocked with 5% nonfat milk. Subsequently, the membranes were incubated with primary antibodies against fibronectin, collagen IV, laminin, and PCNA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 4°C overnight, and later incubated with specific secondary antibodies. Finally, the nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) was assayed using the enhanced chemiluminescence (ECL) kit (Thermo Scientific, Rockford, IL, USA), and scanned with the ChemiDocDoc XRS+ system (Bio-Rad). The density of each band was acquired using the image analysis software, Quantity One version 4.6.2 (Bio-Rad), and was described as fold change relative to the control treatment. The expression level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology, Inc.) was used as a loading control.17

BrdU (Bromodeoxyuridine) Incorporation Assay to Assess Cell Proliferation

To allow BrdU incorporation, the rTFs were first grown on cover slips, in vitro, and then fixed with 4% paraformaldehyde at 4°C for 30 minutes. Next, they were rinsed with 0.1 M PBS (pH 7.4) containing 1% Triton, and later incubated with 1 N HCl on ice. Subsequently, rTFs were incubated with 2 N HCl at temperature for 10 minutes, and followed by incubation.
with the anti-BrdU or recombinant anti-BrdU primary antibody overnight. Finally, incubation with the secondary antibody, BrdU-positive cells were identified. The nuclei of rTFs were simultaneously stained with 10 g/mL 4',6-diamidino-2-phenylindole (DAPI). Cells with different BrdU-incorporation patterns were analyzed and counted with an optical microscope (Olympus, Tokyo, Japan) as described previously.18

**MTT Assay**

The viability of rTFs was measured using the MTT Cell Proliferation Assay Kit (ATCC, Manassas, VA, USA) following the manufacturer’s instructions. Quadruple samples of rTFs were grown on 96-well plates and were either transfected with different vectors or not transfected. After 2, 4, 6, 8, and 14 days, plates were incubated in a medium containing yellow tetrazolium for 20 hours. Cell viability was calculated using the following formula. Cell viability (%) = (1−A value of transfection group/A value of nontransfection group) × 100%.14

**Cell Cycle Analysis**

Rabbit TFs were seeded in a 60-mm dish (5 × 10^5 cells) and incubated for 24 hours. For synchronization purpose, the cells were starved for 16 hours. Later, they were treated with various concentrations of ATO (2−10 μM). After the treatment, rTFs were treated with 0.25% trypsin for 5 minutes, followed by washing with 0.1 M PBS twice, before being fixed in a precooled 70% ethanol at −20°C for 24 hours. Before analysis, the cells were washed and then added in PBS (pH 7.4). The 120 μL of propidium iodide (50 μg/mL) was added to it and incubated for 10 minutes avoid of light. Then 150 μL RNase (80 μg/mL) solution was added to the cells and then incubated for 30 minutes at 4°C. The cells were analyzed on flow cytometer system (FACS Vantage, Becton Dickinson, San Jose, CA, USA) to determine the DNA histograms.18

**Pathological and Immunohistochemical Detection**

The rabbits were anesthetized with ketamine and xylazine (60, 80 mg/kg). The flap of the conjunctiva and Tenon’s capsule were dissected and then limbus-based scleral flap was outlined and dissected further, carefully. The tissue blocking the entrance into the anterior chamber was excised, and a peripheral iridectomy was performed. The flaps of sclera and conjunctiva were closed with a 10–0 and 8-0 nylon suture, respectively.14 Three days after trabeculectomy, 1 mL of arsenic trioxide (6 μM) was injected into the filtrating blebs of the left eye, while the right eye was injected with 1 mL of 0.9% NaCl, which served as control.

After 14 days of ATO injection, the hearts of all rabbits were perfused with 4% paraformaldehyde (Shanghai Biotechnology Company, Shanghai, China), followed by heparin infusion. The eyeballs were collected, placed overnight, and then transferred to 30% sucrose solution at 4°C overnight. Eyeball samples were then frozen in a cryostat, and cut into 5-μm sections. Subsequently, sections were thawed, transferred

**Figure 4.** Effect of arsenic trioxide on morphology of rTFs. The morphology of rTFs treated with 6 μM of ATO was assessed to be the optimal dose for arsenic trioxide inhibition on rTFs. Following 24-hour treatment with 6 μM arsenic trioxide, rTFs appeared whirlpool-like adherent growth, and no apparent changes were seen in the overall cell morphology (b) compared with control group (a). After 6 μM arsenic trioxide treatment for 72 hours, rTFs became round, most of which remained adherent to plate wall, and granule-like substances were observed (c). In addition, rTFs became ageing and appeared maldevelopment, some of which exfoliated from the flask wall. Scale bar: 40 μm.
FIGURE 5. Flow cytometry analysis of cell cycle events in ATO treated rTFs. The rTFs were treated with different doses of ATO ([A] the blank control group; [B] 2 μM; [C] 4 μM; [D] 6 μM; [E] 8 μM; [F] 10 μM) and the cell-cycle progression was investigated by flow cytometry.
to film-coated glass slides, and stained with hematoxylin and eosins (HE) stain as described previously.14

**Statistical Analysis**

All data are presented as the mean ± SD and Shapiro-Wilk test was used to evaluate for normality of distribution. The statistical analyses were performed using the statistical software SPSS version 10.0 (SPSS, Inc., Chicago, IL, USA). Difference between mean values was tested for statistical significance with Student's t-test, with a P value less than 0.05 indicative of statistical significance.

**RESULTS**

**Characterization of rTFs**

The isolated rTFs were identified by analyzing the expression of vimentin protein. Our data showed that vimentin is expressed in the cytoplasm of the fibroblasts isolated from the subconjunctival Tenon’s capsule (Fig. 1).

**Effect of Arsenic Trioxide on the Growth of rTFs**

In order to determine the effect of ATO on the cell proliferation of rTFs, the cultured rTF cells were treated with ATO at different concentrations ranging from 0.5 to 11 µM for 24, 48, and 72 hours. Our data in Figure 2, based on BrdU incorporation assay, showed that ATO inhibited the growth of rTF cells in a dose- and time-dependent manner. As seen here, the inhibitory rate of rTF proliferation after 72 hours of ATO treatment was significantly greater than 24- and 48-hours treatment (P < 0.01). Similarly, in MTT assay as seen in Figure 3, an approximately 50% inhibition was achieved following treatment with 6 µM ATO at 72 hours, and the inhibition curve appeared S-shaped. So, IC50 at 72 hours of ATO treatment was 6 µM. The BrdU assay in Figure 2 also suggested the IC50 value of ATO at 72 hours to be 6 µM, while the IC50 at 24 and 48 hours was seen at 10 µM concentration by both BrdU and MTT assays in Figures 2 and 3. This data suggested that ATO inhibits the proliferation of rTF cells at relatively low concentration of 6 µM, and thus this dose was selected for the subsequent experiments.

Next, we observed the cell morphology of cultured rTF cells following 24 and 72 hours, ATO treatment with 6 µM arsenic trioxide. It was seen that after 24-hours of treatment, rTFs appeared whirlpool-like and adhered to the bottom of the plate and showed no apparent changes in the overall cell morphology. In contrast, 6 µM of ATO treatment for 72 hours, made rTFs looks like round morphology, and most of them remained adherent to the plate, and in addition, we observed granule-like substances. Moreover, rTFs looked aged and some of them even exfoliated from the flask wall as seen in Figure 4. Thus, overall results suggested that 6 µM was the optimal dose for ATO inhibition on rTF proliferation and it displayed minimum toxicity.

**Effect of ATO on Cell-Cycle Progression of rTFs**

To figure out the mechanism of inhibition of proliferation of rTFs by ATO, we performed the cell-cycle analysis by flow cytometry. In the control group, percentage of cells in G1 phase was 48.29%, S phase was 46.31%, and G2 phase was 5.4% (Fig. 5A). But, treatment with ATO at 2 µM for 72 hours, resulted in G1 phase cells to be 56.75%, S phase to be 54.04%, and G2 phase to be 9.2% (Fig. 5B). The ATO treatment at 4 µM, also showed the percentage of cells in G1 phase as 15.3%, S phase as 73.37%, and G2 phase had no cells (0%; Fig. 5C). Similarly, the ATO concentration of 6 µM, which was determined t be the optimum dose to inhibit proliferation of rTFs also revealed the percentage of cells in G1 phase as 24.57%, S phase as 75.43%, and G2 phase had no cells (0%; Fig. 5D). In addition we also analyzed the two concentrations
of ATO, which were higher than the optimal dose, which was 8 μM, which suggested the percentage of cells in G1 phase as 41.99%, S phase 45.50%, and G2 phase having 12.51% (Fig. 5E); while ATO at 10 μM, resulted in G1 phase cells to be 45.97%, S phase as 49.70%, and G2 phase as 4.33% (Fig. 5F). So, in conclusion, all the above experimental results demonstrated that rTF cells underwent G2/M arrest at 6 μM concentration of ATO, which is also the optimum dose that revealed the inhibition of rTF proliferation.

**Effect of ATO Treatment on the Expression of Fibronectin, Collagen IV, and Laminin in rTFs**

Because ECM deposition is a main contributor to fibrosis and fibronectin, collagen IV, and laminin are the major components of ECM, we tested their expression by Western blotting assay in rTFs treated with ATO. The data in Figure 6, revealed a significant reduction in the expression of fibronectin, collagen IV, and laminin in arsenic trioxide-treated rTFs as compared with vehicle and control group ($P < 0.01$). This data suggested that arsenic trioxide downregulated the expression of ECM components.

**Effect of Arsenic Trioxide on PCNA Expression**

In addition, we also analyzed the expression of PCNA protein, which is a marker of cell proliferation. The Western blotting analysis showed reduced expression of PCNA in rTF cells treated with ATO for 24, 48, and 72 hours in comparison to the control ($P < 0.05$) and PCNA expression appeared to be reduced with the increase in duration of the of the treatment ($P < 0.05$; Fig. 7).

**Effect of ATO on Filtrating Bleb Formation**

High-level rTF proliferation was observed in both vehicle and control groups. We observed a significant reduction in the number of rTFs of experimental group as compared with the control group, and furthermore, there was no observation of any inflammation in the filtering blebs of the experimental group even after 14 days of trabeculectomy (Fig. 8).

**DISCUSSION**

Despite different antimetabolites (like 5-fluorouracil and mitomycin C) have been used to control subconjunctival
fibrotic scar formation following glaucoma filtering surgery, the efficacy of these antimetabolites has shown side effects, even inducing severe blinding complications, such as filtering bleb leak, conjunctival button-hole formation and blebitis, which results in late endophthalmitis and scleral necrosis.\textsuperscript{19,20} Therefore, it is necessary to find new drug to inhibit the proliferation of rTFs after trabeculectomy.

Arsenic trioxide has been proved to a safe and effective therapeutic drug. Preclinical in vivo and in vitro studies have demonstrated that arsenic trioxide alone or in combination with other antimielyoma agents showed inhibitory activity against myelomas.\textsuperscript{21–24}

In the present study, we identified that proliferation of cultured rTFs was inhibited with the ATO treatment of 6 μM. The ECM and PCNA proteins were also significantly reduced in ATO-treated rTFs. Even the ATO treatment weakened the viability of rTFs both in vivo and in vitro as analyzed by BrdU and MTT assays. The histologic result showed that good filtering bleb can be achieved after treatment with 6 μM of ATO.

The 6 μM of ATO concentration appeared to be the optimal dose based on BrdU and MTT assays. This concentration of ATO in our study was lower than the systemic concentration used during the treatment of retinoblastoma cells, leukemia, and hepatocarcinoma.\textsuperscript{25–27} Little congestion in the ATO-treated conjunctiva, in our study indicated its low cytotoxicity. We didn't even find blebitis after injection of ATO into the Tenon's capsule for 2 weeks.

In addition, the ECM components have been found to play an important role in the pathogenesis of scar formation.\textsuperscript{7} It has suggested that TFs can release TGF-β1, which regulates the ECM components expression.\textsuperscript{7} Our findings also showed a significant reduction in the expression of fibronectin, collagen IV, and laminin in arsenic trioxide-treated rTFs in comparison to the control treatment. This reduction in the expression of ECM proteins by ATO, may reduce the scar development. We hypothesize that ATO might also affect the TGF-β1 signaling pathway, and thus consequently decrease the ECM expression.\textsuperscript{28–29} The 5-FU and mitomycin C (MMC) have been confirmed to induce the apoptosis of TFs.\textsuperscript{30,31} However, there is little data that showed 5-FU and MMC affecting the ECM expression, which is important for the pathogenesis of wound healing. In our study, ATO can improve the filtering bleb formation similar to 5-FU and MMC. Therefore, we presume that ATO might be more effective in improving the filtering bleb formation than 5-FU and MMC and it can also affect both ECM expression and cell viability.

Proliferating cell nuclear antigen is another protein that is associated with cell proliferation and repair ability, and PCNA expression is usually detected in the G1 phase of the cell cycle, indicating its greater role in fibroblast proliferation. In addition, PCNA is also found to accompany DNA replication and repair.\textsuperscript{32} It has been demonstrated that 5-FU can reduce the PCNA expression in TFs.\textsuperscript{30} The MMC can also affect the PCNA expression in TFs of canine model of glaucoma surgery.\textsuperscript{31} However, there is no any report about ATO on regulation of PCNA expression at different time point. In this study, our result showed that ATO treatment significantly reduced PCNA expression, and the expression appeared to be decreased in a time-dependent manner. The results revealed a positive correlation between PCNA expression and fibroblast proliferation.

In conclusion, the results of this study demonstrated that arsenic trioxide effectively inhibited the rTF proliferation both in vivo and in vitro through downregulation of not only ECM protein expression but also PCNA expression. It thus suggested that arsenic trioxide may be used as a novel approach for suppressing rTF proliferation. Further studies will be required to evaluate the potential of ATO for reducing the subconjunctival fibrosis and increasing success ratio of filtering surgery in glaucoma patients.

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