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Original Article

Arsenic trioxide inhibits proliferation of retinal pigment epithelium by downregulating expression of extracellular matrix and p27

Ying Su[1], Feng Wang[1*], Qi Hu[1], Yixin Qu[1], Ying Han[2*]

[1]Department of Ophthalmology, The First Affiliated Hospital of Harbin Medical University, Harbin, China; [2]Department of Geriatrics, The First Affiliated Hospital of Harbin Medical University, Harbin, China. [*]Equal contributors.

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Abstract: The present study aimed to investigate the effect of arsenic trioxide (ATO) on the proliferation of retinal pigment epithelium (RPE) and its mechanism. RPE cells were cultivated with 0.5-11 μmol/L ATO for 24, 48, and 72 h and their survival and growth were measured by MTT assay. The expression of p27 and proliferating cell nuclear antigen (PCNA) in RPE cells was detected using cell immunofluorescence and western blotting. Dose-dependency was evident in both the experimental and control groups. The 50% inhibitory concentration was obtained at a concentration of 6 mol/L with cells treated for 3 days. The optimum concentration of ATO was 6 μmol/L based on the result of MTT. After the third day of ATO treatment, the number of cells was significantly lower in the experimental group compared with the control group. The expression of extracellular matrix (ECM) components decreased relative to the control group. The expression of p27 and PCNA declined gradually in cells treated for 72 h at 6 μmol/L ATO compared with the control group. The difference between the experimental and control groups was significant (*P=0.005*). ATO has the ability to inhibit the growth and proliferation of RPE cells by regulating the expression of the ECM components' p27 and PCNA, in a time- and dose-dependent manner. Thus, ATO may lead to an innovative method for the treatment of proliferative retinopathy.

Keywords: Retinal pigment cell, arsenic trioxide, proliferation, extracellular matrix, proliferating cell nuclear antigen

Introduction

Proliferative vitreoretinopathy (PVR) is the main cause of failure after rhegmatogenous retinal detachment surgery. PVR indicates a serious stage of diabetic retinopathy (DRP) and retinal vein occlusion (RVO), which are both ischemic diseases [1, 2]. Oxidative stress plays an important role in the mechanism of DRP and RVO. The contraction and traction of extensive fibrous proliferative membranes on the surfaces of retinal and vitreous bodies are key events in the pathogenesis of retinal detachment [3]. PVR is caused by contraction of epiretinal membranes, which contain extracellular matrix as well as various types of cells [4, 5]. PVR is a fibrotic complication as well as a form of abnormal repair caused by vitreous hemorrhage, retinal detachment, or posterior segment ocular trauma [6-8]. Experimental evidence has indicated that retinal pigment epithelia (RPE) and glial cells can differentiate and transform into myofibroblasts, the major type of contractile cells in epiretinal membranes [9, 10]. The complications of PVR include recurrence, endophthalmitis, and secondary glaucoma [11-13].

RPE cells form early in life and undergo minimal proliferation during life but can be activated in diseased states. RPE cells migrate and proliferate when stimulated by pathologic damage. In addition, they may transform into fibroblast-like cells and secrete collagen, eventually leading to membrane formation [14, 15]. The exploration of drugs that can inhibit the proliferation of RPE cells is therefore important to prevent PVR. Studies investigating the inhibition of RPE cell proliferation at the molecular level are also underway [16]. Proliferating cell nuclear antigen (PCNA) is highly expressed during the for-

mation of PVR, and PCNA antisense oligonucleotide can inhibit the expression of PCNA as well as cell proliferation in RPE cells [16-18]. Studies show that suramin can inhibit RPE cell proliferation *in vitro* and maintain a continuous rebound suppression effect even after the withdrawal of drugs [19-21].

Arsenic trioxide (ATO) is the main active ingredient in cancer treatment. It has been confirmed that ATO can inactivate some important intracellular enzymes such as catalase and superoxide dismutase, which interfere with cell metabolism and inhibit cellular DNA synthesis. In recent years, studies have shown that ATO can induce apoptosis in a variety of tumor cells and has a considerable therapeutic effect, particularly in acute promyelocytic leukemia, but also in gastric cancer, lung cancer, prostate cancer, and breast cancer [22, 23].

Our previous studies demonstrated that ATO can significantly inhibit Tenon's fibroblast proliferation after trabeculectomy by downregulating the expression of extracellular matrix (ECM) and PCNA [24]. In the present study, we investigated whether ATO can inhibit the proliferation of RPE cells and the resulting pathogenesis.

Materials and methods

RPE cell culture

This experiment was approved by the First Affiliated Hospital of Harbin Medical University Ethics Committee and conformed to the requirements of the American Committee of Visual Science Research.

ARPE-19 cells were purchased from the American Type Culture Collection (ATCC). Cells ($4*10^5$) were cultured in 60-mm dishes with alpha-modified Eagle's medium (α-MEM) containing 10 mL/L N1 supplements (Sigma-Aldrich Corp., St. Louis, MO), 10 mL/L MEM nonessential amino acid, 1 mM sodium pyruvate, and 2 mM glutamine with 20% fetal bovine serum in addition to 1% penicillin and streptomycin at 37°C in an incubator with 5% CO_2. Cell culture medium was replaced 2-3 times a week. Cells from generations 8 to 12 near confluence were used for all experiments.

Drug interference; preparation for detecting cell viability by MTT assay

Cells were transferred to a 96-well plate with 200 μL in each well and adjusted to 10000 cells/well (the edge holes were filled with sterile PBS). The holes were divided into zero holes, experimental groups, and control groups. Once the cells had adhered to the bottom of the plate, they were treated with ATO at 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 10, and 11 μmol/L and cultured for 72 h. MTT solution (20 μL; 5 mg/mL, 0.5% MTT) was added to each well. Cultivation was stopped after 4 h and the culture medium was carefully removed. Then, 150 μL DMSO was added to each well and the well plate was oscillated on a shaker at low speed for 10 min to sufficiently dissolve the crystals. The absorbance of each well was measured at OD=490 nm for the enzyme-linked immunosorbent assay [25].

Bromodeoxyuridine (BrdU) detection

Cell viability was detected with BrdU immunological staining. To perform BrdU, RPE cells grown on a cover slip were fixed with 4% paraformaldehyde at 4°C for 30 min, then rinsed with 0.1 M phosphate buffered saline (PBS) containing 1% Triton (pH 7.4). The cells were then incubated using HCl (1N) on ice with HCl (2N) for 10 min, followed by the same method at room temperature. After culture with anti-BrdU antibody (Santa Cruz Biotechnology, Inc., Dallas, TX) overnight, anti-BrdU-positive cells were detected with the secondary antibody (Santa Cruz Biotechnology, Inc). The nuclei were stained with 10 g/mL 4,6-diamidino-2-phenylindole simultaneously. BrdU-embedded cells were analyzed and counted using an optical microscope (Olympus, Tokyo, Japan) [18].

Western blot

Pre-cooled PBS (4°C; 3 mL; 0.01 M, pH 7.2 to 7.3) and 400 μL lysate containing PMSF were added to each bottle of cells (blank control, group A; 6 μmol/L, group B). Cells were transferred to 1.5 mL EP tubes after sitting on ice for 30 min, and the tubes were centrifuged at 6037×g for 5 min at 4°C. After the protein content of each tube was measured, the sample was boiled for 5 min to denature the protein. Electrophoresis was performed for 4-5 h at 40 V. The membrane was wetted with TBS from the bottom to the top, oscillated, and blocked on the decoloration table for 1 h at room temperature. The primary antibody was diluted with 5% nonfat dry milk to appropriate concentrations (in a 1.5 mL centrifuge tube, 1:1000 fibro-

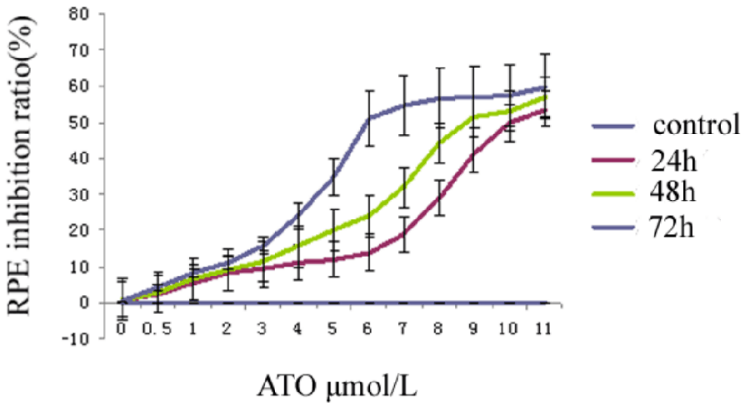

Figure 1. Inhibition of RPE cell growth with different concentrations of arsenic trioxide (ATO) according to MTT assay. After treating RPE cells using 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11 μmol/L ATO, respectively, MTT assay revealed that RPE cell activity decreased significantly in a dose-dependent manner compared with the control group.

nectin sc-18825, 1:500 laminin sc-74531, 1:500 of P27 sc-1641, 1:1000 of PCNA sc-56, and 1:3000 of GAPDH sc-365062) (Santa Cruz Biotechnology, Inc.) and 1:1000 collagen IV (ab6586, Abcam). After 1-2 h of incubation at room temperature, the primary antibody was washed twice with TBST for 10 min each at room temperature and once with TBS for 10 min. The secondary antibody conjugated with HRP (1:1000) (Santa Cruz Biotechnology, Inc.) was incubated at room temperature for 1-2 h, then subsequently washed with TBST twice for 10 min each at room temperature and with TBS once for 10 min. The chemiluminescence reaction was executed and the expression level of GAPDH served as the internal reference of the optical density analysis. Quantity One version (Bio-Rad Laboratories Inc., Hercules, CA) was used to obtain the band density and analyze the value compared with the control group. The experiment was repeated three times [26].

Statistical methods

SPSS14.0 statistical software (SPSS, Inc., Chicago, IL) was used for the analysis of each observation by time point and group. Data are displayed as means ± standard deviation. Comparisons among different groups were analyzed using one-way ANOVA. The parallels between the two groups were anlyzed by the Dunnett's *t*-test. P<0.05 was considered significant.

Results

The influence of ATO on RPE cell proliferation

RPE cells in the control group grew well. The numbers of RPE cells in each experimental group were not significantly different between the first day and second day compared with the control (*P*>0.05). However, the numbers decreased over time and with an increased ATO concentration after the third day. Thus, the inhibition of RPE proliferation was time- and dose-dependent. To determine the effect of ATO on cell proliferation, RPE cells were incubated with ATO concentrations of 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 11 mol/L for 24, 48, and 72 h. ATO inhibited the growth of RPE cells in a dose-dependent manner because their growth was inhibited by the 50% inhibitory concentration, 6 μmol/L, after 72 h as measured by BrdU (**Figure 1**) and MTT (**Figure 2**). The results suggest that ATO inhibits RPE cell growth because the results of the MTT assay showed that the survivability of RPE cells treated with ATO was reduced compared with the control group in a dose-dependent manner. The 8 mol/L and 10 mol/L groups were not remarkably different from the 6 mol/L ATO group. Therefore, an ATO concentration of 6 mol/L was used for the subsequent experiments.

Effect of ATO treatment on the expression of fibronectin, collagen IV, and laminin in RPE cells

Fibronectin, collagen IV, and laminin are the major ECM components, hence we investigated their expression by western blotting of RPE cells treated with ATO. Our results suggested a significant reduction in the expression of ECM components in ATO-treated RPE cells compared with vehicle and control groups (**Figure 3**, *P*<0.01). These data suggest that ATO may downregulate the expression of ECM components.

Decreased expression of p27 in ATO-treated RPE cells detected by western blot

p27 expression declined in all experimental groups but not in the blank control group. The

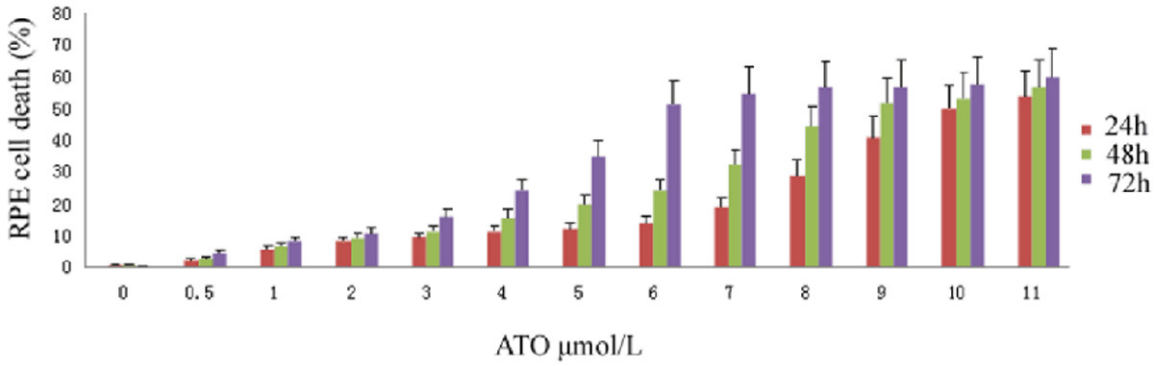

Figure 2. ATO inhibited the proliferation of RPE cells according to BrdU assay. RPE cells were treated with different concentrations of ATO for different time periods and BrdU expression was detected, quantified, and plotted. BrdU-positive RPE cells in the experimental group decreased drastically compared with the control and vehicle groups.

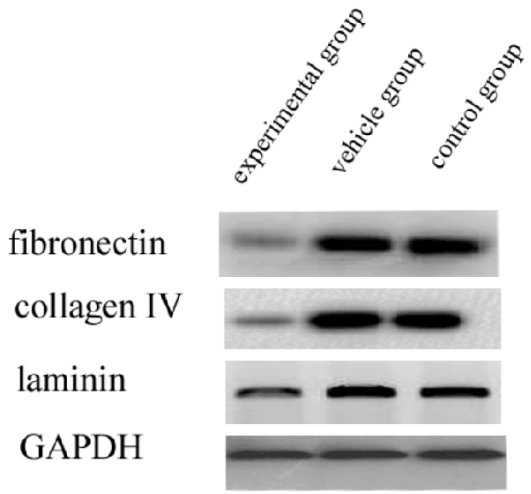

Figure 3. Effect of ATO treatment on the expression of ECM components in RPE cells. We found that there was a major reduction in the expression of ECM components in ATO-treated RPE cells in contrast with the vehicle and control groups ($P<0.01$). Our results indicated that ATO can downregulate the expression of ECM components.

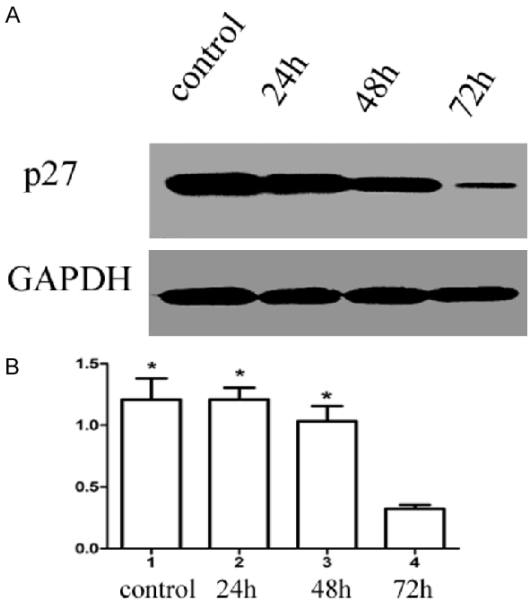

Figure 4. Western blot showed that ATO decreased the expression of p27. RPE cells were treated with 6 μmol/L ATO for 24, 48, and 72 h. The expression of p27 decreased over time.

expression of p27 protein was downregulated at 24, 48, and 72 h of treatment. The expression levels were significantly different (F= 25.646, P<0.005, **Figure 4**).

Decreased expression of PCNA protein in ATO-treated RPE cells by western blot

Our results showed that the expression of PCNA in cells treated for 24 h decreased compared with the control group. Expression levels showed a downward trend with prolonged treatment times. Compared with the blank control group, the expression of PCNA protein in the experimental group decreased gradually with time and the difference was statistically significant (F=58.141, P<0.001) (**Figure 5**).

Discussion

Proliferative vitreoretinopathy is a serious scarring process that strongly impairs visual function. In addition to RPE cells, TGF-β1/β2 is an important factor in PVR [27]. It was confirmed that there is increased expression of TGF, which has an important role in epithelial-mesenchy-

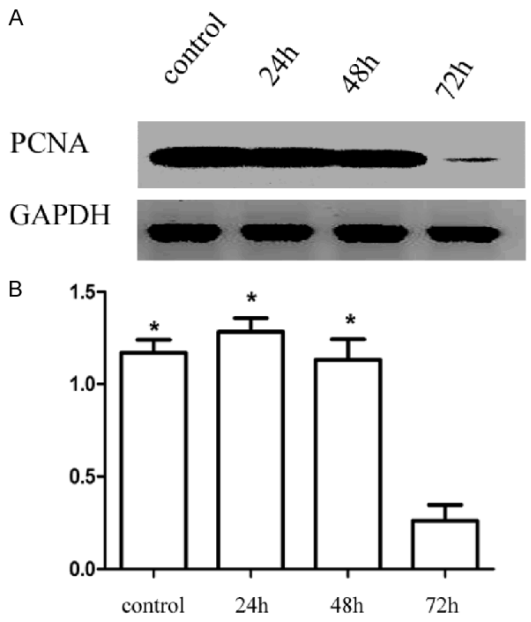

Figure 5. ATO decreased the expression of PCNA revealed by western blot. RPE cells were treated at 6 μmol/L ATO for 24, 48, and 72 h. Western blot analysis showed that PCNA expression lessened over time.

mal transition (EMT) in proliferative membranes of PVR patients. It was reported that ATO can inhibit TGF-β1 induced fibroblast to myofibroblast cell differentiation by downregulation of α-smooth muscle actin (α-SMA) and collagen I [28]. It was also confirmed that ATO can inhibit breast cancer cells through p21 and p27 [29].

In the present study, MTT assay was used to detect the influence of different concentrations of ATO at different time points on RPE cells. Our previous experiment confirmed that the dose of ATO (>11 μmol/L) and longer time (>72 h) was almost the same as 11 μmol/L for 72 h (data not shown). Our results showed that RPE cells grew well in the blank control group. After 24 and 48 h of ATO treatment, the number of RPE cells in each group was not substantially different from that of the control group. Nonetheless, after 72 h, the number of cells in each experimental group was lower than that in the control group over time and with increasing concentrations. This result confirmed that ATO inhibited the proliferation of RPE cells in a time-dependent and dose-dependent manner. Moreover, the effective concentration was 6 μmol/L which is tenfold lower than achieved in humans. The different status of animal and human cells may cause the different concentrations of ATO.

The pathologic mechanisms of PVR include the proliferation of RPE cells and shrinkage of the ECM, while exposure of RPE cells to the vitreous body is a crucial event [30-32]. It was reported that RPE cells have complex and important physiologic and biochemical functions. Under stimulation from inflammatory factors, they can dissociate, migrate, and transform phenotypically, such as changing into fibroblast-like cells, which causes traction in retinal detachment through the secretion of collagen [33, 34]. Our results also showed that the expression of ECM proteins such as fibronectin, collagen IV, and laminin in RPE cells decreased after treatment with 6 μmol/L ATO for 3 days. The above results suggest that treatment with ATO may be a novel treatment for PVR.

PCNA is a suitable indicator for detecting cell proliferation [24]. p27 protein is one of the most important members of the kinase inhibitory protein family and inhibits the activities of various cyclin/cyclin kinase complexes. It is by far the most direct negative regulator of the cell cycle. The fluorescence intensity expressed by PCNA and p27 protein was detected using immunofluorescence staining. The results demonstrated that the fluorescence intensity of p27 and PCNA in RPE decreased significantly when treated at 6 μmol/L. In this study, we further detected the expression of p27 and PCNA proteins through western blot. According to the results, and comparing RPE cells treated with 6 μmol/L ATO for 24, 48, and 72 h with the blank control group, we found that the expression of PCNA was downregulated significantly in the group treated for 72 h, which is roughly the same as findings related to the relationship between PCNA and neoplastic disease. The decreased expression of PCNA observed in this study indicated that RPE cells were arrested in the G2/M phase after treatment with ATO and their proliferation was inhibited as well. These findings suggest that the expression level of PCNA is indicative of the proliferative ability of RPE cells. A study examining the inhibition of glioblastoma showed that the expression of p27 protein increased where interference was present [35]. We found that p27 expression in RPE cells decreased according to the duration of time after ATO treatment. Accordingly, we speculated that p27 may have an influence on RPE cell proliferation through the mechanisms

of transcription and post-transcriptional regulation. p27 promoted the proliferation of RPE cells by regulating the activity of proliferation proteins in RPE cells. After acting upon RPE cells, ATO inhibited the expression of p27 protein and the proliferation of RPE cells. The mechanism may also relate to the cell line and pattern of cell growth. Our result confirmed that ATO can downregulate the expression of p27 and PCNA which are important for regulation of cell proliferation. Further study will be required to investigate whether a mutual promotion or inhibitory relationship exists between PCNA and p27.

Conclusion

This study revealed that ATO inhibited the proliferation of RPE cells at both the cellular and molecular level by inhibiting the expression of p27 and PCNA proteins. The limitation of our study is that the molecular mechanisms of how ATO inhibits proliferation of RPE by downregulating expression of extracellular matrix, p27, and PCNA will require future research. ATO shows promise as a novel therapeutic approach for the treatment of proliferative vitreous lesions and consequently the prevention of visual damage.

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Disclosure of conflict of interest

None.

Address correspondence to: Feng Wang, Department of Ophthalmology, The First Affiliated Hospital, Harbin Medical University, Harbin 150001, China. Tel: +86-13359995660; E-mail: wangfd@126.com

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