·Basic Research·

Effects of endothelin-1 on the cytoskeleton protein F-actin of human trabecular meshwork cells *in vitro*

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Abstract

• AIM: To observe the effect of endothelin-1 (ET-1) on the cytoskeleton protein F-actin of cultured human trabecular meshwork (HTM) cells.

• METHODS: Cultured HTM cells were randomly divided into four groups: control group, low-dose ET-1 (10^{-9} mol/L) treatment group, middle-dose ET-1 (10^{-8} mol/L) treatment group, and high-dose ET-1(10^{-7} mol/L) treatment group. After treated with ET-1, the expression of cytoskeleton protein F-actin in trabecular meshwork was analyzed with Western-blot and the distribution of F-actin was detected with FITC-Phalloidin probe.

• RESULTS: ET-1 dose-dependently and significantly increased F-actin in trabecular meshwork cells ($P<0.05$). The F-actin stress fiber and periphery actin fiber highly increased and manifested mild reorganization after treated with ET-1; and there were much more cell-to-cell and cell-to-extracellular matrix attachments formation in ET-1 treated HTM cells than that in the untreated HTM cells.

• CONCLUSION: ET-1 promotes the expression of cytoskeleton protein F-actin and induced the trabecular meshwork actin cytoskeleton reorganization.

• KEYWORDS: trabecular meshwork;endothelin-1; cytoskeleton; F-actin

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INTRODUCTION

Glaucoma is one of the leading causes of irreversible blindness in the world. The abnormal restriction of aqueous humor drainage through the trabecular meshwork, which results in high intraocular pressure, is the major reason of open angle glaucoma (POAG). Recently, researchers have found that alteration of cytoskeleton of TM cells reorganization and cell-to-cell adhesion can reduce aqueous humor outflow and contribute to pathogenesis of glaucoma [1]. Endothelin-1 (ET-1) is a novel vasoactive peptide and inflammatory mediator. Many researches indicated that ET-1 is also an active regulator of intraocular pressure[2]. ET-1 level in aqueous humor and serum elevated obviously in POAG, normal intraocular tension glaucoma, and animal model of glaucoma [3-5]. There is now accumulating evidence for a role of ET-1 in the pathogenesis of glaucoma. However, it is not known that the effect of ET-1 on the reorganization of cytoskeleton in trabecular meshwork cells. In this study, we observed the effect of ET-1 on the cytoskeleton protein F-actin in cultured human trabecular meshwork (HTM) cells to explore the role of ET-1 in the pathogenesis of glaucoma.

MATERIALS AND METHODS

Materials Fluorescent probe of FITC-phalloidin (The Beyotime Institute of Biotechnology); recombined human ET-1 (Sigma), anti-F-actin monoclonal antibody (Abcam); fetal bovine serum (Hyclon); DMEM/F12 culture fluid (Gibco), clean bench and CO_2 incubator (Heraeus), inverted microscope (MO81, Olympus), Fluorescence microscope (NICON), HTM tissues obtained from donated healthy eyeball, and HTM cells were cultured according to reference 6[6]. The third generation subcultured cells were identified by immunohistochemistry stain using anti-FN, LN, NSE and F Ⅷ Rag antibody. Cultured HTM cells presented with various shapes: fusiform, triangulum, irregular shape and so on and have many cell processes, abundant cytoplasm and littler pigment granules in cytoplasm. The cell nucleuses present round or ellipse. The identification by immunohistochemistry stain displayed that anti-FN, LN, NSE were positive (cytoplasm present yellow brown granulo) and anti-

FⅧRag was negative. This is a list, this needs to be written into a paragraph of the manuscript.

Mehthods The third generation HTM cells were divided into four groups randomly as follows: control group (0 mol/L ET-1), low-dose ET-1 (10^{-9}mol/L) treatment group, middle-dose ET-1(10^{-8}mol/L) treatment group and high-dose ET-1 (10^{-7}mol/L) treatment group. HTM cells were treated with ET-1 for 72 hours and harvested and cell total protein was extracted. Protein levels were determined using the Bio-Rad DC protein assay. Protein samples (30μg total protein) were separated on SDS-PAGE gels and transferred to nitrocellulose membranes. After blocking, the membranes were incubated with primary antibody against F-actin (1:1 000), and then horseradish peroxidase (HRP)-conjugated secondary antibody. Signals were detected using enhanced chemiluminescence (ECL) reagents. The data was analyzed by software Quantity One 4.62.

Another aliquot of HTM cells were seeded on a cover slip and was fixed by 40g/L paraformaldehyde after treated with ET-1 for 72 hours. HTM cells were wash three times with 1X phosphate-buffered saline (PBS) containing 1g/L Triton X-100 (cell permeabilization) and incubated with fluorescent probe FITC-Phallodin in dilution buffer (PBS containing 10-50g/LBSA and 1g/L Triton X-100,dillution:1:200) for 1 hour at 37℃, then washed with PBS containing 1g/L Triton X-100 three times. F-actin distribution in HTM cells were screened under fluorescence microscope after mounted in 30% glycerol.

Statistical Analysis Statistical comparisons were performed using ANONA (analysis of variance) with SPSS software. $P< 0.05$ is considered as statistical significance.

RESULTS

F−actin Expression in HTM Cells Western-blot analysis revealed that the expression of F-actin increased obviously in a dose-dependent manner at range of 10^{-9}mol/L-10^{-7}mol/L of ET-1(Figure 1).

F −actin in HTM Cells In control group, the actin microfilament distribute uniformly in parallel lines along with the longitudinal axis of HTM cells detected by fluorescent probe FITC-Phallodin. There are fewer cell-to-cell and cell-to--extracellular matrix (ECM) actin attachments. However, in ET-1 treated group, actin microfilament became thickened, tense and distributed with radial or parallel manner. Periphery actin microfilament increased obviously and the borderline of HTM cells became sharper. And cell-to-cell and cell-to-extracellular matrix (ECM) actin microfilament attachments increased obviously in numbers compared with control group, especially in high-dose group (Figure 2).

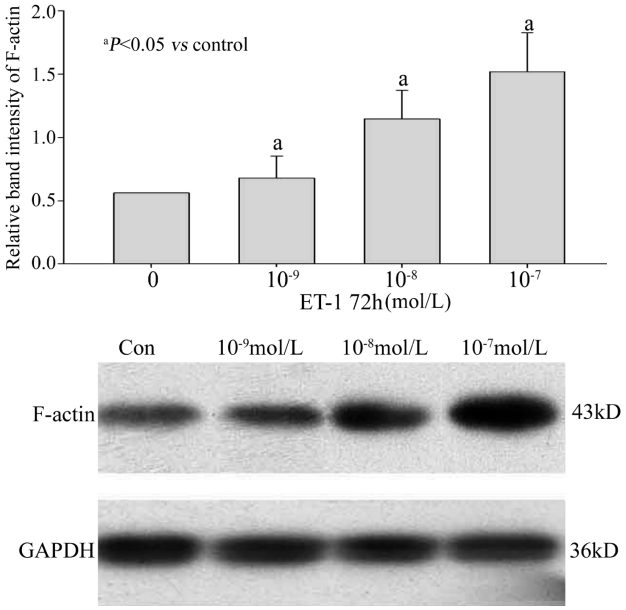

Figure 1 Western −blot analysis of F −actin expression A: SDS-PAGE; B: Quantitative analysis

DISCUSSION

The high resistance of aqueous humor outflow via the trabecular meshwork is the major reason of POAG. The cell shape, constractile properties, cell- to-cell/ECM attachments of the TM cells are to the key factors to influence the resistance of aquour houmor outflow [1]. It is reported that reorganization of cytoskeleton can change cell structure and strengthen cell-to-cell or cell-to-ECM attachments, which induce abnormal high resistance to aqueous humor outflow and promote the advance of POAG [7]. F-actin is the major component of cytoskeleton and highly organized as stress fibers and peripheral actin within cells. The stress fibers distribute along with the longitudinal axis of cells and contribute mainly to maintain the rigidity, tenacity and morphology of cell. The peripheral actin distribute along with cell plasma membrane and mainly contribute to stabilization of plasma membrane and form cell-to-cell attachment or cell-to-ECM focal adhesion. There is abundant F-actin in those cells located in aqeous humor outflow pathway, especially in TM cells. So if alteration of the F-actin cystoskeleton of TM cells could change the morphology, cell attachments, and structure, which will modulate obstacle of aqueous fluid outflow. Research proved that dexamethasone induced cell-to-cell attachment formation between TM cells [8], which increase the stability of actin, hence restrict contraction and dilatation of TM cell and decrease aqueous fluid outflow. On the contrary, Y-27632 [9]. a inhibitor of rho enzyme, and Caldesmon [10], multifunctional regulator of the cytoskeleton, can destroy the actin fiber, cell-to-cell or cell-to-ECM attachments, , which

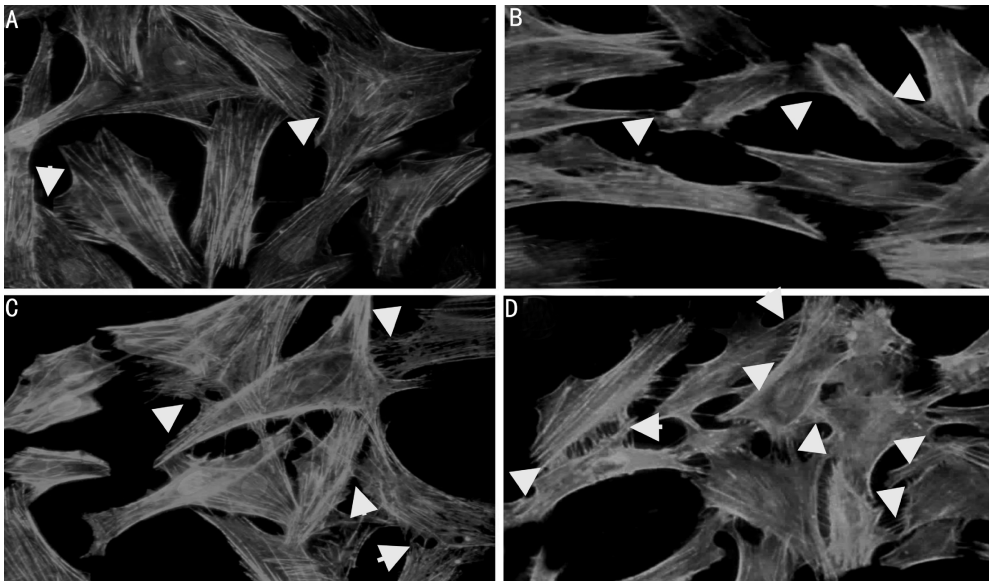

Figure 2 The expression of F-actin in HTM cells (FITC-phalloidin) A: Control group; B: ET-1 10^{-9}mol/L; C: ET-1 10^{-8}mol/L; D: ET-1 10^{-7}mol/L (arrow: The cell-to-cell and cell-to-ECM attachments)

facilitate the aqueous humor outflow and decrease intraocular pressure.

ET-1 is one of the most potent vasoactive peptides. It has been found to cause contraction of TM cells and play a role in the regulation of outflow pathway of the bovine and primate eye. In this study, we found ET-1 can increase the lever of F-actin protein expression in a dose-dependent (10^{-9}-10^{-7}mol/L) manner. Moreover, immunofluorescence studies have shown that HTM cells distributed with thicken and tense stress fiber, peripheral actin and formatted numbers of cell-to-cell/ECM attachments following ET-1 treatment. These results suggested that ET-1 induced the reorganization of F-actin cytoskeleton and altered the cell adhesive properties of HTM cells. Therefore, we propose that ET-1 elevation in aqueous humor could promote the expression of F-actin, alter the distribution of actin stress fiber, and induce cell-to-cell or cell-to-ECM attachment in TM cells. Then the tenacity and compliance of TM cell decreased and the overall tissue architecture and stability of the TM changed. Therefore, the outflow resistance of aqueous humor drainage from trabecular meshwork increased during the pathogenesis of glaucoma.

ET-1 is a major upstream factor of Rho kinase, a serine/threonine-specific protein kinase, and it reported that ET-1 induced the phosphorylation of myosin phosphatase targeting subunit (MYPT-1), a Rho-kinase substrate, and mediated the F-actin reorgnization in airway smooth muscle cells [11]. However, the detailed mechanism of ET-1 on the F-actin cytoskeleton expression and reorganization in HTM cells is not completely known and need further investigation.

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