

Inhibition of latrunculin –A on dexamethasone – induced fibronectin production in cultured human trabecular meshwork cells

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Foundation items: National Natural Science Foundation of China (No.30772379); Education Department Project of Hunan Province, China(No.04A049)

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Received:2011-03-08 Accepted:2011-04-25

Abstract

• **AIM:** To determine the effects of a low dose latrunculin (LAT)-A on dexamethasone (Dex)-induced upregulation of extracellular matrix proteins fibronectin (FN) in cultured human trabecular meshwork (HTM) cells.

• **METHODS:** HTM cells were cultured to confluent and incubated with 0.4 μ mol/L Dex and/or 0.05 μ mol/L LAT-A. FN expression in HTM cells was evaluated by Western blot and immunofluorescence microscopy.

• **RESULTS:** Dex up-regulated FN production in HTM cells, failed to do so when co-incubated with LAT-A. LAT-A decreased production of FN in cultured HTM cells.

• **CONCLUSION:** This study indicated that LAT-A may modulate the expression of fibronectin in trabecular meshwork to achieve treatment for steroids and other types of glaucoma. It has an important prospect as an intraocular pressure-lowering drug.

• **KEYWORDS:** latrunculin-A;dexamethasone; human trabecular meshwork cells; fibronectin

DOI:10.3980/j.issn.2222-3959.2011.03.04

Wang Y, Tan J, Zhuo YH, Wang CL, Liu XY, Cai SP. Inhibition of latrunculin-A on dexamethasone-induced fibronectin production in cultured human trabecular meshwork cells. *Int J Ophthalmol* 2011;4(3):239-242

INTRODUCTION

In the human eye, the main aqueous humor outflow route is the conventional or trabecular outflow pathway. This pathway comprises the trabecular meshwork (TM) and the Schlemm's canal. TM is a tissue located in the anterior chamber angle of the eye, as a crucial determinant of intraocular pressure (IOP) because of its resistance to the evacuation of aqueous humor from the eye [1]. TM cells provide an endothelial-like lining for the entire pathway of aqueous humor drainage and are responsible for the secretion and metabolism of the complex extracellular matrix (ECM). Primary open-angle glaucoma is associated with structural changes in TM, which likely affect tissue rigidity and biomechanics, in particular, an increase in ECM material in the cribriform meshwork adjacent to Schlemm's canal and a loss of cells in TM were observed [2,3]. Therefore, modulation of ECM protein expression may be an important strategy in regulating outflow facility. Latrunculin-A (LAT-A), a macrolide derived from the Red Sea sponge *Latrunculia magnified*, is a specific actin disrupting agent capable of disrupting the actin filaments by sequestering monomeric actin. It is believed that the basis for IOP-lowering effect of LAT-A appears to be related to its effects on actin filaments and ECM of HTM cells. Fibronectin (FN) is a high molecular weight ECM glycoprotein that binds to membrane-spanning receptor proteins called integrins [4]. Altered FN, and organization were known to be associated with a number of pathologies, including and fibrosis [5]. Glucocorticosteroids (GC) such as Dex induced ocular hypertension and open angle glaucoma is a result of increased resistance to aqueous humor flow in TM [6,7]. Dex-induced IOP increases mimic primary open angle glaucoma in many ways [8]. Zhang *et al* [9] demonstrated that Dexamethasone can inhibit TM cell phagocytosis by

LAT-A on Dex-induced fibronectin production in cultured HTM cells

glucocorticoid receptor beta. GC induced increased expression of ECM molecules and decreased expression of several extracellular proteinases. We therefore hypothesize that low dose LAT-A might prevent or reverse Dex-induced IOP elevation through modulation of extracellular proteins. Previous study showed that TSP1 may be an important target of LAT-A in HTM cells^[10]. Here, we reported the effects of LAT-A at low dose on expression of another important extracellular protein FN in cultured HTM cells treated without, or with Dex.

MATERIALS AND METHODS

Materials HTM cells were a gift from State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou. These cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 50mL/L FCS, antibiotics (100mg/L penicillin and 100µg/mL streptomycin) at 37°C, 50mL/L CO₂ with 90% humidity. Passage eight cells were used after reaching confluence. 1mmol/L stock solutions of Dex were prepared by dissolving Dex in absolute ethanol. LAT-A, a solution in ethanol at 237µmol/L was purchased from Cayman chemical (Catalog number 10010630). Dex or LAT-A was added to growth medium to a final concentration of 0.4µmol/L and 0.05µmol/L. Control cells received equivalent volumes of absolute ethanol. The experiments were done three times with similar results.

FN Production Detection HTM cells were incubated with Dex and/or LAT-A for one week. Cells were gently washed twice with cold PBS, and then cell lysis buffer with protease inhibitor cocktail (P8340, Sigma, USA) was added into each well, cell lysate was sonicated and then centrifuged to remove cell debris, and analyzed by SDS-PAGE under reducing conditions. Following electrophoresis, proteins were transferred to PVDF membrane, blots were incubated with monoclonal anti-human FN antibody (sc-18825, Santa Cruz Biotechnology, Inc., USA) and developed using appropriate secondary antibodies and ECL reagent (P0018-2, Beyotime, Shanghai, China). These experiments were repeated twice with similar results.

Extracellular FN Level Detection Cells were washed twice with 1x PBS, and fixed with 40g/L paraformaldehyde without Triton X-100, then incubated with anti-human FN (P1H11) antibody at a dilution of 1:100 (sc-18825, Santa Cruz) followed by FITC conjugated goat anti-mouse secondary antibody for fluorescent. The immunostaining was done twice with similar results.

RESULTS

Cell Morphology The cell morphology of HTM cells in

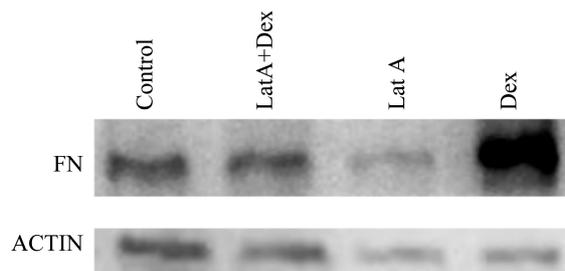


Figure 1 Effects of LAT-A and Dex on FN production by HTM cells

culture had the same characteristics as reported previously. Untreated cells were similar in morphology and size. However, Dex-treated cells appeared different in morphology and were arrayed in a more random orientation and these cells had increased in size and intercellular space. Cells treated with Dex and LAT-A appeared morphologically similar to untreated cells. LAT-A treated cells slightly rounded and the degree of rounding of LAT-A treated cells are dose-dependant. Cells became rounded and gradually detached from culture dish when these cells were treated with higher doses of LAT-A, such as 0.2µmol/L and above.

FN Production LAT-A dramatically decreased the level of FN production detected in HTM cell culture (Figure 1). Dex treatment alone had an apparent effect on FN production in HTM cells compared to LAT-A and control treated cells. In cells incubated with both LAT-A and Dex, reduced expression of FN compared to only Dex treatment was observed.

Extracellular FN Level Previous immunofluorescence studies showed that HTM cells treated with LAT-A exhibited a time- and dose-dependent disruption of the actin stress fibre network and intercellular adherens junctions. In Dex treated cells, actin fibre bundles were highly concentrated at the periphery of the cell with few actin filaments left in the central area^[10]. No study has done on the extracellular FN expression in HTM cells treated with LAT-A and Dex by fluorescent assay. In our study, we found that Dex increased extracellular FN expression at modest level (Figure 2A,D), LAT-A and Dex co-treatment decreased Dex induced extracellular FN expression (Figure 2C). We also found that LAT-A treatment dramatically decreased extracellular FN expression and extracellular FN staining pattern seen in medium treated cells was almost gone (Figure 2B). This result further indicated that LAT-A dramatically decreased extracellular FN expression and also decreased Dex induced extracellular FN production.

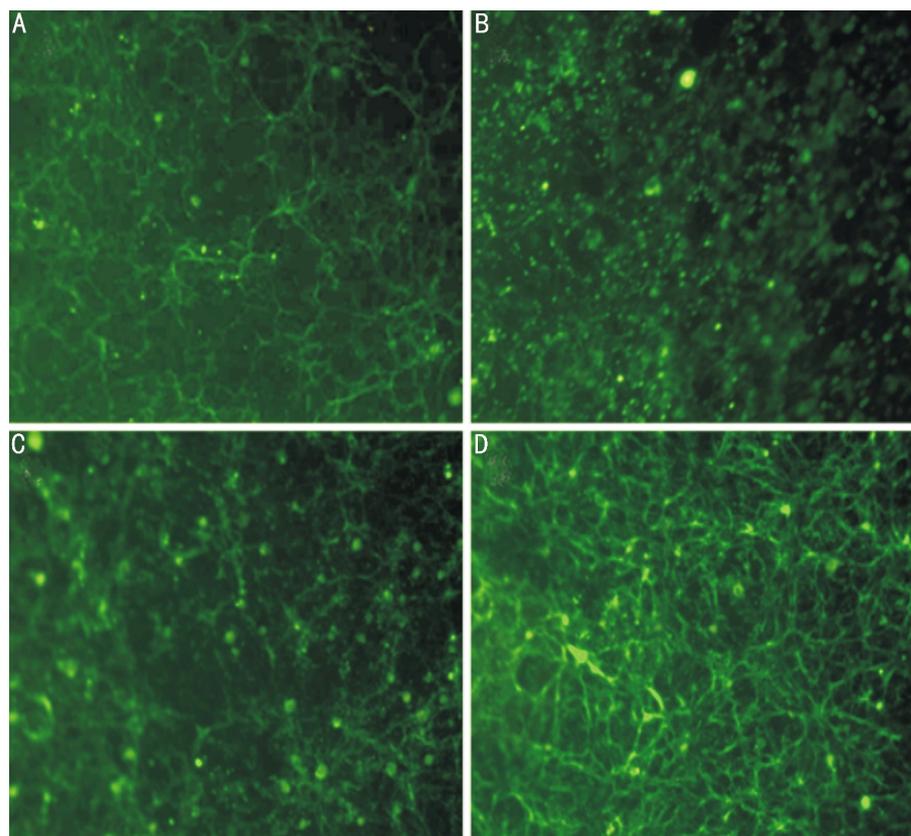


Figure 2 Immunofluorescence assay of extracellular FN in HTM cells A: Normal medium; B: LAT-A; C: LAT-A plus Dex; D: Dex

DISCUSSION

The TM can be considered a filter of the aqueous humor whose pore diameter changes due to the combined action of an extrinsic factor, the tone of the ciliary muscle, and several intrinsic factors, such as contractility, cellular volume, and extracellular matrix status^[11]. FN is a very rich component of extracellular matrix. The dimeric fibronectin protein exists in two forms: a soluble protomer in body fluids and an insoluble multimer in the extracellular matrix. The latter is the primary functional form and creates a substrate for cell migration, a role which makes fibronectin vital to embryogenesis and wound response. Fibronectin mediates cytoskeletal organization, cell attachment, and cellular signaling through interactions with cellular receptors. Further polymerization steps are regulated by fibronectin/integrin interactions and result in generation of the complex fibrils that constitute the fibronectin matrix. Previous studies also indicated that fibronectin and actin showed coincident staining in a large proportion of cells during spreading or when fully spread. The distributions of actin and fibronectin staining during the course of cell spreading progressed through a series of patterns. Certain actin patterns correlated with certain fibronectin patterns. When fibrillar patterns developed, there was correspondence between the two fibrillar arrays in 80-100% of the cells. These results suggest

a transmembrane relationship between microfilament bundles and fibronectin. This close relationship represent a point at which pharmacological control of IOP using actin disrupt agent which then change ECM expression pattern may be possible.

LAT-A, a Red Sea sponge product, depolymerizes actin filaments by sequestering monomeric actin in cells. It may also affect other actin-binding proteins by a feedback mechanism that may sense the cellular concentration of actin monomers, resulting in more complicated outcomes than that predicted by monomer sequestration alone. Organization of the actin-based cytoskeleton has multiple effects on adhesion-modulating matrix glycoproteins. Previous study showed that extracellular protein thrombospondin-1 (TSP1) was decreased in LAT-A treated HTM cells^[10], it suggested that IOP-lowering effect of LAT-A in animal model may be related to its effect on TSP1. We hypothesized that extracellular proteins FN may also play a very important role in LAT-A reduced IOP in animal model due to its close relationship with actin filaments. Dex-induced changes include reorganization of the actin cytoskeleton, ECM accumulation and the formation of cross-linked actin networks (CLAN) in TM tissues^[12-15]. Low dose LAT-A may be effective in counteracting the GC-induced cellular and extracellular changes in TM through its action on actin

filament. Our results showed that Dex failed to induce changes in the morphology of HTM cells when administered concurrently with LAT-A, and that LAT-A partly reversed Dex-induced morphology changes. When cells were treated with higher dose of LAT-A, cells became rounded and detached, indicating that both cell adhesion, mainly ECM proteins, and actin were affected. By Western blot assay using whole cell lysate, we found Dex stimulated FN production in HTM cells. LAT-A significantly inhibited FN production, decreased Dex-induced over-expression of FN. Our study indicated that the effects of LAT-A on IOP might be associated with its regulatory effect on FN expression through its action on actin filament. In conclusion, our study suggests that FN may be another important target for IOP modulation in TM cells in addition to TSP1. LAT-A at low doses may be useful in reducing IOP in primary glaucoma and GC caused glaucoma.

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