Journal of Biosciences and Medicines, 2016, 4, 24-29
Published Online August 2016 in SciRes. http://www.scirp.org/journal/jbm
http://dx.doi.org/10.4236/jbm.2016.48004

HSP47 Deletion Inhibits TGF-β1 Stimulation on Proliferation and Collagen Synthesis of Human Tenon Fibroblasts

Bin Gao[1], Ping Chen[1], Qifeng Jiang[2*]

[1]Department of Medical Information, Chongqing Medical University, Chongqing, China
[2]Department of Biochemistry and Molecular Biology, Chongqing Medical University, Chongqing, China
Email: *jqf1008@aliyun.com

Received 7 July 2016; accepted 7 August 2016; published 10 August 2016

Abstract

Heat shock protein 47 (HSP47) is a collagen-specific molecular chaperone that is required for molecular maturation of various types of collagens. Many studies have shown a close association between increased expression of HSP47 and excessive accumulation of collagens in scar tissues of various human fibrotic diseases. However, the role of HSP47 in formation of scar after glaucoma filtration surgery is still unclear. In this study, we deleted the expression of HSP47 in human tent on fibroblasts (HTFs) by virus infection, and then the proliferation and collagen synthesis were compared between HSP47 deletion cells and control upon TGF-β1 stimulation. Our data showed that HSP47 deletion could significantly inhibit the proliferation and collagen synthesis of HTFs upon TGF-β1 stimulation, HSP47 gene suppression might be a novel method to against the formation of scar after glaucoma surgery.

Keywords

Heat Shock Protein 47, Proliferation, Collagen Synthesis, Fibroblasts

1. Introduction

Filtration surgery remains the most effective therapy for patients with glaucoma [1] [2]. However, conjunctival scarring at the wound site greatly affects the surgical treatment of glaucoma. Especially, excessive postoperative scarring of the Tenon at the sclerostomy site is associated with filtration failure and the key players in ocular wound healing are the fibroblasts in the Tenon's capsule [3] [4]. Although anti-mitotic agents such as mito-my-

*Corresponding author.

How to cite this paper: Gao, B., Chen, P. and Jiang, Q.F. (2016) HSP47 Deletion Inhibits TGF-β1 Stimulation on Proliferation and Collagen Synthesis of Human Tenon Fibroblasts. *Journal of Biosciences and Medicines*, **4**, 24-29.
http://dx.doi.org/10.4236/jbm.2016.48004

cin-C and 5-fluorouracil help to prevent post-surgical scarring and improve the surgical outcome, their use is associated with severe and potentially blinding complications [5]. Alternative and safer agents are therefore necessary, especially those with more physiological actions. However, identification of such agents requires further elucidation of the mechanisms involved in the cellular processes of wound healing in the eye.

Heat shock protein 47 (HSP47) is a collagen-specific molecular chaperone that is required for molecular maturation of various types of collagens [6]. Collagen biosynthesis is a multi-step complex process that includes both intracellular and extracellular events. HSP47 assists in the correct folding and stabilization of triple-helical procollagen molecules [7], which are eventually transported to the extracellular space across the Golgi complex, where N- and C-propeptides are cleaved by procollagen N- and Cproteinases to assemble into collagen fibrils [8]. Recent studies have shown a close association between increased expression of HSP47 and excessive accumulation of collagens in scar tissues of various human and experimental fibrotic diseases [9]-[14]. The profibrotic effects of HSP47 make it a potential target for developing anti-fibrotic therapy. Because collagen is the only substrate for HSP47, it provides a selective target to manipulate collagen production, a phenomenon that might have huge impact on controlling fibrotic diseases [15]. It is presumed that the increased levels of HSP47 in fibrotic diseases assist in excessive assembly and intracellular processing of pro-collagen molecules and, thereby, contribute to the formation of fibrotic lesions. Because HSP47 is a specific chaperone for collagen synthesis, it provides a selective target to manipulate collagen production, a phenomenon that might have enormous clinical impact in controlling a wide range of fibrotic diseases. Recently, a renal fibrosis model using human proximal tubular epithelial cells stimulated with TGF-β1, demonstrated that ERK 1/2, JNK and MAPK inhibitors were able to block HSP47 expression induced by TGF-β1 [16]. In their model, the inhibition of those pathways suppressed the over-expression of ECM proteins and PAI-1, thus limiting fibrogenesis. The authors suggested that during renal fibrosis TGF-β1 enhance HSP47 expression through the ERK 1/2 and JNKMAPK signaling pathways, thereby enhancing collagen synthesis.

TGF-β1 production in the eye after surgery is locally derived from tissues and inflammatory cells. It has been elucidated that TGF-β1 induced overproduction and reduced degradation of matrix proteins to promote fibrogenesis. So, TGF-β1 has been identified as important in the pathogenesis of several ocular scarring diseases [17]-[19]. In this study, the primary HTFs were used as *in vitro* model, and then we used TGF-β1 to stimulate HTFs to mimic *in vitro* glaucoma condition. After deletion of HSP47 expression in HTFs by virus infection, the proliferation and collagen synthesis were compared between HSP47 deletion cells and control upon TGF-β1 stimulation. Our data showed that HSP47 deletion could significantly inhibit the proliferation and collagen synthesis of HTFs upon TGF-β1 stimulation, HSP47 gene suppression might be a novel method to against the formation of scar after glaucoma surgery.

2. Material and Methods

2.1. Cell Culture

Tenon fibroblasts were isolated from tissue surrounding the bleb at 10 days after surgery (total 50 patients with glaucoma had received comprehensive information and provided written consent for the procedure). The tissue was digested for 3 h at 37˚C with collagenase (2 mg/ml) in order to yield a suspension of Tenon fibroblasts. The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum. Identity of the cultured cells was assessed on the basis of both the distinctive morphology of Tenon fibroblasts and their reactivity with antibodies to vimentin in immunofluorescence analysis. Solutions of TGF-β1 were prepared in DMEM with 1% bovine serum albumin (BSA; Sigma, Dorset, UK). The concentration of TGF-β1 in the study was 10^{-9} M.

2.2. Cell Infection

Human tenon fibroblasts were seeded in 30-mm dishes and cultured to 50% to 60% confluence. HSP47 shRNA lentiviral particles were purchased from Sigma. All experimental procedures were followed with manufacturer instruction. Briefly, cells were seeded in 12-well plates till 50% confluent. Then, the medium was replaced with 1 mL complete medium containing 5 μg/mL polybrene (Sigma) and 15 μL HSP47 shRNA lentiviral particles. The infection control was the lentiviral particles packed with scrambled shRNA sequence (Sigma). The infection medium was replaced with 1 mL complete medium and incubated at 37˚C and 5% CO_2 overnight. To select the stable clones expressing shRNA, the Puromycin dihydrochloride (Sigma) was added to each well with the 5

μg/mL concentration. The complete medium containing fresh puromycin was changed every 3 - 4 days till resistant colonies can be identified. Western-blotting was followed to check the knock-down efficiency of HSP47 in HTFs.

2.3. Western-Blotting

The expression level of endogenous HSP47 reduced by infection was evaluated by Western-blotting analysis using an ECL system (Amersham Biosciences). Briefly, after 24 h incubation, culture medium was removed, and cells were rinsed twice with ice-cold PBS. Proteins were extracted by adding to each well 100 μl of lysing buffer (Santa Cruz, CA). The plates were incubated on ice for 30 min and scraped. Total cell extracts were loaded onto sodium dodecyl sulfate (SDS)-polyacrylamide gels and the separated proteins were transferred to nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ). Blots were incubated with HSP47 monoclonal antibody (Abcam, Cambridge, MA) diluted in TBST (containing 0.1% Tween 20% and 2% BSA) overnight at 4˚C. Then, blots were washed and incubated with monoclonal anti-rabbit secondary antibody and detected.

2.4. Immunofluorescence Analysis

HTFs were plated on glass coverslips, cultured overnight, and then serum starved for 24 h. Then, the cells were fixed in 2% paraformaldehyde-PBS for 15 minutes and then blocked in 2% BSA for 30 minutes. Coverslips were incubated with anti-vinculin antibody (Sigma-Aldrich) or anti-collagen antibody for 30 minutes. Samples were washed with PBS and incubated with secondary antibody (Chemicon, Temecula, CA) for 30 minutes. After they were washed, the cells were mounted in anti-fade medium and observed via the fluorescence.

2.5. Cell Proliferation Assay

Proliferation of cultured HTFs was measured by use of the commercially available MTT (3-(4, 5-dimethyl-2-thiazyl)-2, 5-diphenyl-2H-tetrazolium bromide) cell proliferation kit (Sigma), according to the manufacturer's instructions. Cells were plated at a density of 10^4 cells per well in 96-well plates and were allowed to adhere for 24 hours. After cultures were washed with phosphate buffered saline (PBS), then treated with 5 mg/mL MTT for 4 hours at 37˚C. The relative active number of cells was determined by an automated plate reader (Bio-Rad, Hercules, CA).

2.6. Statistical Analysis

Quantitative data are presented as means ± SE and were analysed by Student's t test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Primary Culture of HTFs and HSP47 Stable Deletion

Human tenon fibroblasts were isolated from tissue surrounding the bleb. Identity of the cultured cells was assessed on the basis of both the distinctive morphology of Tenon fibroblasts and their reactivity with antibodies to vimentin in immunofluorescence analysis, nearly all of the cells presented positively staining of vimentin (**Figure 1**). Western-blotting results showed that the expression of HSP47 in HSP47 knock-down (KD) cell was significantly decreased (**Figure 2(a)** and **Figure 2(b)**).

3.2. Effect of HSP47 Deletion on HTFs Proliferation upon TGF-β1 Stimulation

Cell proliferation was measured by the MTT assay. The assay is based on measuring the intracellular formazan spectrophotometrically which is facilitated by active cells. Upon TGF-β1 stimulation, the optical absorbance of HSP47 deletion cells were 0.28 ± 0.21, while that of control cells was 0.69 ± 0.11 (**Figure 3**). The MTT assay revealed deletion of HSP47 could significantly suppress the proliferation of HTFs upon TGF-β1 stimulation.

3.3. Effect of HSP Deletion on Collagen Synthesis of HTFs upon TGF-β1 Stimulation

Compared with controls, HSP47 deletion caused significant loss of HTF-mediated collagen synthesis in the presence of TGF-β1 stimulation (**Figure 4(a)** and **Figure 4(b)**). At 24 hours after plating and incubation, the relative

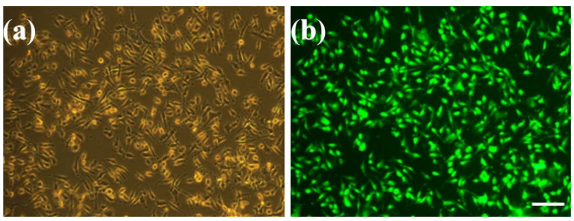

Figure 1. Immunofluorescence identification of HTFs with vimentin: (a) Phase-contrast image of HTFs; (b) Immunofluorescence image of HTFs with vimentin. Scale bar: 100 um.

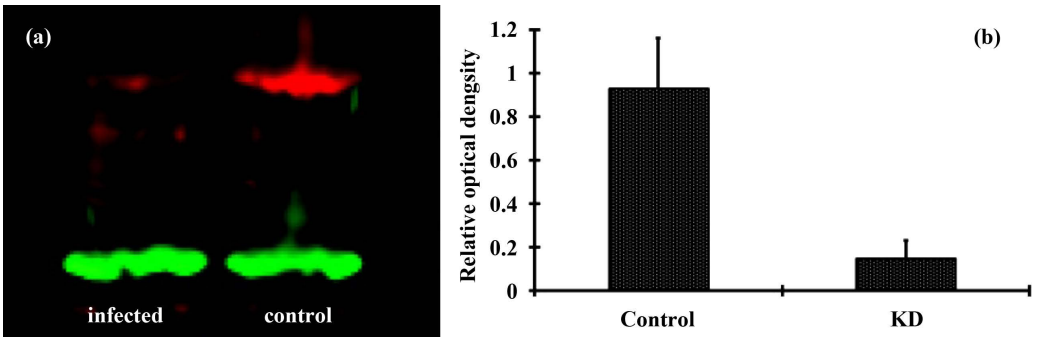

Figure 2. HSP47 deletion in HTFs with virus infection: (a) A blot representative for HSP47 deletion in HTFs; (b) The relative expression levels of HSP47 in deletion and control cells are shown in bar diagram. Data represents the mean of three independent experiments. $p < 0.05$.

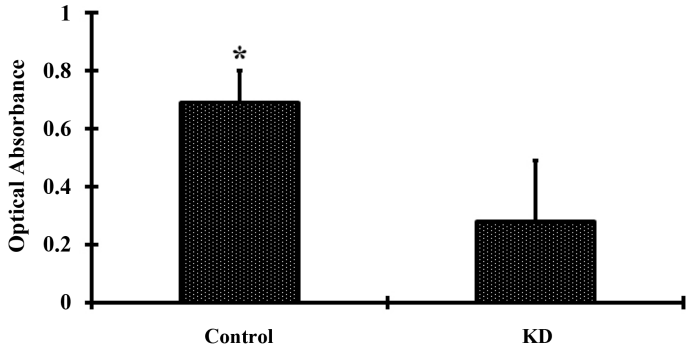

Figure 3. The optical absorbance of HTFs upon TGF-β1 stimulation between HSP deletion and control. The optical absorbance of HSP47 deletion cells were 0.28 ± 0.21, while that of control cells was 0.69 ± 0.11. Deletion of HSP47 could significantly suppress the proliferation of HTFs upon TGF-β1 stimulation.

optical density of the collagen in HSP47 deletion cells was 0.25 ± 0.18, while that of control cells was 0.87 ± 0.08 (**Figure 4(b)**). The results showed that deletion of HSP47 could significantly decrease the HTF-mediated collagen synthesis in the presence of TGF-β1 stimulation.

4. Discussion

Scarring is a major reason for failure of filtration surgery. Several studies showed that sub conjunctival scarring of the scar tissue formation in a rabbit model of glaucoma filtration surgery [1] [2]. HTF perform an essential role in conjunctival scarring and provide a useful *in vitro* model of cellular activity during the scarring response [3] [4]. Our data showed that HSP47 played critical role in HTFs proliferation and collagen synthesis. HSP47 deletion cells presented significantly decrease of proliferation and collagen synthesis upon TGF-β1 stimulation.

In this study, the primary HTFs were used as *in vitro* model, and then we used TGF-β1 to stimulate HTFs to mimic *in vitro* condition after glaucoma surgery. The results showed that the primary cells in our study were positively stained for marker of HTFs (**Figure 1**). After infection with HSP47 specific siRNA, which packaged in virus, the expression of HSP47 was significantly decreased (**Figure 2**).

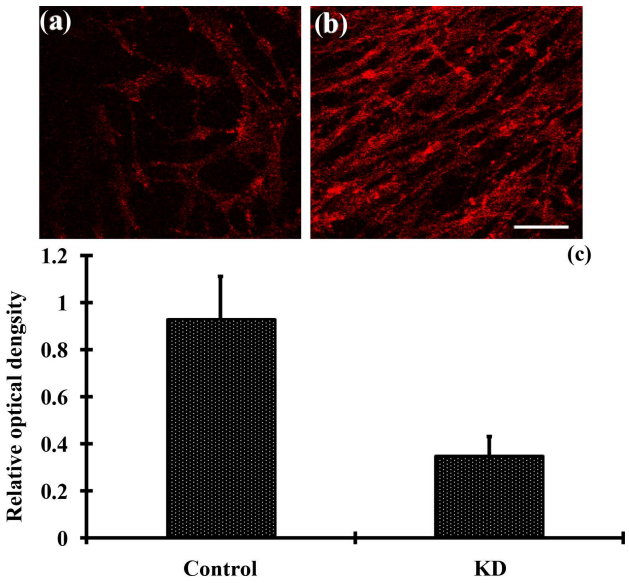

Figure 4. Collagen staining in HTFs. (a) (b) An Immunofluorescence representative for HSP staining in HSP47 deletion and control HTFs, respectively; (c) The relative optical densitys of HSP in deletion and control cells are shown in bar diagram. Data represents the mean of three independent experiments. Scale bar: 50 um. $p < 0.05$.

HTFs proliferation induced by TGF-β1 may occur via its modulation of c-fos, c-myc, and c-sis expression [19] or its induction of cyclin D and strong downregulation of p 27 expression, leading to passage from G1 to S phases of the cell cycle [18]. Kay *et al*. [20] have suggested that TGF-b may have an indirect mitogenic effect on HTFs, via its induction of fibroblast growth factor-2 (FGF-2). Our results showed that HSP47 could be a key factor in TGF-β1 signaling cascades to promote HTFs proliferation (**Figure 3**). The absence of HSP47 expression would significantly decrease the proliferation of HTFs upon TGF-β1 stimulation.

Increased expression of HSP47 with excessive accumulation of collagens is consistently observed in various human and experimental fibrotic diseases [12]. Whether over-expression of HSP47 in fibrotic diseases is an epiphenomenon has not been obvious in earlier studies. Subsequent *in vivo* studies, however, showed that blocking the bioactivities of HSP47 did not only alter collagen production, but also reduced the progression of fibrotic lesions, directly implicating HSP47 in fibrogenesis [15]. Increased glomerular expression of HSP47 correlated with increased deposition of collagens in sclera proliferative glomeruli in anti-thymocyte serum-induced experimental model of glomerulosclerosis [17]. Besides, previous studies have shown that the mechanism by which TGF-β1 may stimulate collagen contraction may via its effect in altering the expression of the integrin family of cell adhesion receptors [20]. Our data presented that the cross-talking between HSP47 and TGF-β1 might be another critical factor in collagen synthesis of HTFs. Once deleting the expression of HSP47 in HTFs, the promoting effect of TGF-β1 in collagen synthesis of HTFs was prompt blocked (**Figure 4**).

5. Conclusion

Our study indicates that, HSP47, which is a collagen-specific molecular chaperone that is required for molecular maturation of various types of collagens, plays an essential role in proliferation and collagen synthesis of HTFs. The deletion of HSP47 expression would significantly decrease the proliferation and collagen synthesis of HTFs upon TGF-β1 stimulation. HSP47 gene suppression might be a novel method to against the formation of scar after glaucoma surgery.

Acknowledgements

This study was supported by NNSFC (National Natural Science Foundation of China, No. 31201047 to Jiang), by SRFDP (Research Fund for the Doctoral Program of Higher Education, No. 20125503120018 to Jiang), by the open fund of Key Laboratory of Ministry of Education (No. CQKLBST-2012-005 to Jiang), by the Chongqing Yuzhong District Science and technology project (No. 20150115).

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