

INTERNSHIP REPORT

Measurement of actin depolymerization induced by phospho HSP20 peptide in keloid fibroblasts

An internship report presented in partial fulfillment of the requirement of the Professional Science Master's in Computational Biosciences

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Abstract

The role of connective tissue growth factor (CTGF) and TGF β 1 in fibrosis and excessive scarring is well known. And since the expression of CTGF protein requires an intact actin cytoskeleton, disruption of the actin cytoskeleton decreases CTGF expression. This consequently decreases excessive scarring. Activation of cyclic nucleotide pathways leads to the phosphorylation of the small heat shock related protein (HSP20) which induces relaxation of smooth muscle and inhibits intimal hyperplasia by mainly affecting the actin cytoskeleton. Phospho HSP20 displaces phospho-cofilin from the 14-3-3 scaffolding protein complex leading to the activation of cofilin as an actin depolymerizing protein. AZX100 a phosphopeptide analog of HSP20 containing a protein transduction domain mimics the role of HSP20. In the present study we investigated the effect of AZX100 on actin depolymerization by measuring amount of G actin in keloid fibroblasts. Cultured keloid fibroblasts were treated with TGF β 1, TGF β 1+ AZX100, or AZX100 alone for 30 minutes. The amount of G actin was measured by separating F actin and G actin and analyzing by SDS-PAGE and western blotting. The cells treated with TGF β 1 alone showed a decrease in monomeric G actin suggesting actin polymerization, whereas the cells treated with AZX100 alone or AZX100 + TGF β 1 showed a significant increase (19%) in monomeric G actin. The increased monomeric G actin suggests depolymerization of actin and disruption of the actin cytoskeleton. Since AZX100 treatment disrupts actin cytoskeleton which further decreases CTGF expression it may be involved in reducing excessive scar formation. So, AZX100 may be used as a therapeutic agent for excessive scarring and fibrotic disorder.

Goals of the Project

1. The goal of the project is to measure Phospho-Hsp20 peptide induced actin depolymerization in keloid fibroblasts.
2. The amount of G actin (monomeric actin) was measured in the cells treated with AZX 100, TGF- β 1, and AZX 100 + TGF- β 1 to determine the amount of depolymerization induced by AZX100 in keloid fibroblasts.

Internship Details and Requirements

1. The internship mainly aims to measure the amount of g actin induced by AZX 100.
2. F/G actin assay kit (cytoskeleton) was used to measure the amount of G actin induced by AZX100.

Introduction

Wound healing is a natural process which involves various cell types, cytokines and signal transduction cascades. Natural wound healing process results in a scar formation, however sometimes excessive scarring occurs which leads to fibrosis resulting in conditions such as pulmonary fibrosis, liver cirrhosis (Kryger,Z.B et al.,2007).

The Transforming Growth Factor Beta (TGF- β 1) is a multifunctional protein which has three isoforms TGF- β 1, TGF- β 2 and TGF- β 3 respectively. TGF- β 1 plays an important role in processes like cell growth, differentiation, and extracellular matrix formation in wound healing process. TGF- β 1 plays an important role in the first week of wound healing. After the first week of wound healing the elevated levels of TGF- β 1 suggests a role in the formation of hypertrophic scars involving collagen synthesis and excess deposition of collagen (Kryger, Z.B et al., 2007). TGF- β 1 plays a vital role in excessive scarring and fibrosis (Leask, A et al., 2004). Its role in the pathway to fibrosis and scarring is clearly demonstrated in previous studies as shown in figure 1.

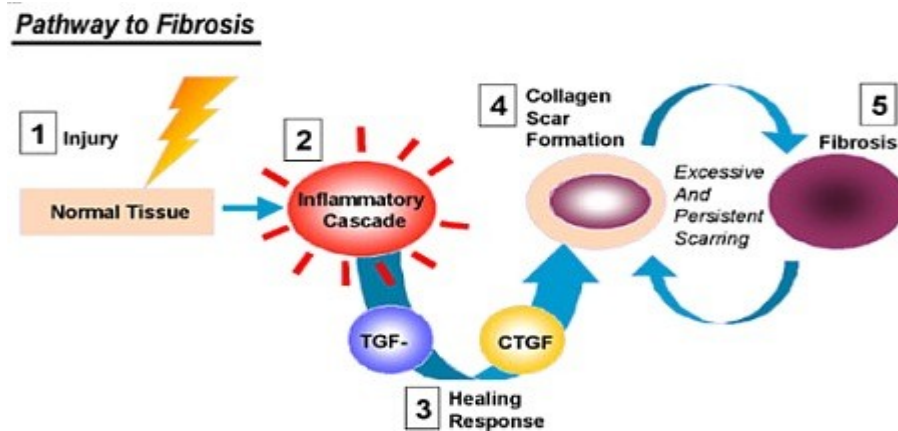


Figure 1: Pathway to Fibrosis

Source: http://www.fibrogen.com/i/fibrosis_pathway.jpg

Connective tissue growth factor (CTGF) is a protein which plays an important role in tissue repair, and fibrosis. A substantial body of evidence suggests that TGF- β 1 induced CTGF plays an important role in fibrosis and excess scarring. Injection of either CTGF or TGF- β 1 induced a transient fibrotic response in a mouse model whereas injection of CTGF and TGF- β 1 together induced sustained fibrotic response (Mori et al., 1999). It was also demonstrated that by inhibiting the CTGF expression the fibrotic response of TGF- β 1 can be prevented.

Previous studies have demonstrated that TGF- β 1 up regulates CTGF in keloid fibroblasts and that by blocking the CTGF expression, the excessive scarring and fibrosis can be reduced (Shi-Wen, X et al., 2008). Reorganization of cytoskeleton occurs when cells are exposed to mechanical force or stress. It has been shown that CTGF expression depends on an intact cytoskeleton. Cell cytoskeleton is a protein network with associated proteins which helps in

maintaining the cell shape, locomotion and other cellular functions. Actin filaments, intermediary filaments and microtubules constitute cytoskeleton and their organization is shown in the figure below.

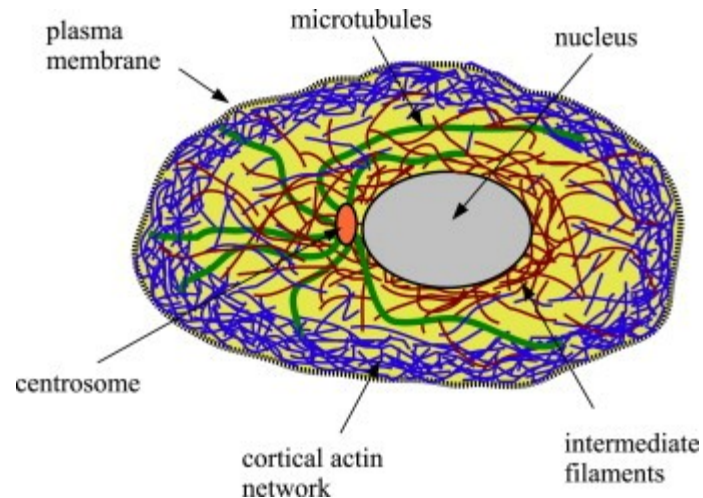


Figure 2: Cytoskeleton

Source: [doi:10.1016/j.physrep.2007.03.002](https://doi.org/10.1016/j.physrep.2007.03.002)

Actin is an important constituent of cytoskeleton which can undergo rapid depolymerization and repolymerization assisting cellular motion. Polymerized actin fibers can be visualized as stress fibers in cultured fibroblasts. Stress fibers help in the process of wound healing by exerting tension on the matrix of collagen surrounding the fibroblasts. CTGF expression increases due to the stress fiber formation because of the intact polymerized actin. Stabilization of actin cytoskeleton increases CTGF expression whereas actin depolymerization or increased monomeric actin decreases CTGF expression (Muehlich, S et al., 2007).

Increased cAMP levels have been demonstrated to abolish the actin cytoskeleton reorganization and CTGF expression (Kothapalli, D et al., 1998). Actin cytoskeleton reorganization is important for various cellular functions like muscular contraction and relaxation, cell adhesion and motility etc. Reorganization of actin is induced by Rho which results in formation of stress fibers and focal adhesions. Phosphorylation of cofilin by LIM kinase results in the actin polymerization. Previous studies have demonstrated that phosphorylation of HSP20 in response to increased cAMP leads to disruption of actin stress fibers (Dreiza, C.M et al., 2005).

Phosphorylation of the small heat-shock related protein, HSP20, on serine 16, is one of the downstream events in the cAMP signaling pathway (Woodrum, D et al., 2003). Phosphorylation of HSP20 in response to increased cAMP levels leads to dephosphorylation of actin depolymerizing protein cofilin. Phospho HSP20 competes with p-cofilin for binding to scaffolding protein 14-3-3. Dephosphorylated cofilin then activates depolymerization of actin leading to disruption of the actin cytoskeleton. Phosphopeptide analog of HSP20 can control the binding of proteins to intracellular scaffolding protein 14-3-3 (Dreiza, C.M et al., 2005). The p-cofilin that is not bound to 14-3-3 protein is susceptible to phosphatases like slingshot. The mechanism of actin depolymerization induced by HSP20 phosphopeptide is shown in the figure3.

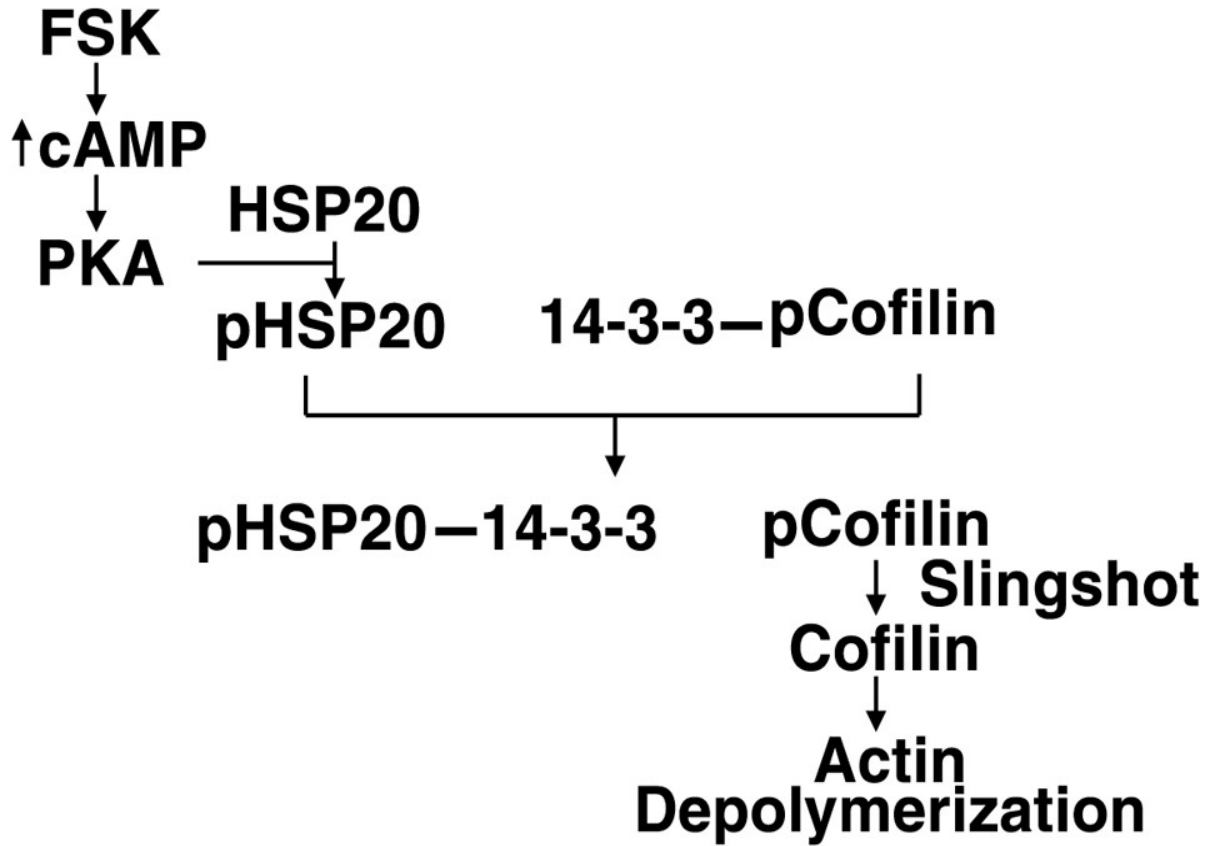


Figure3: Mechanism of actin depolymerization induced by HSP20 phosphopeptide.

(Dreiza, C.M et al., 2005)

AZX100, a 24 amino acid containing phospho peptide analog of HSP20, (WLRRAS*APLPGLK) when attached to a protein transduction domain (YGRKKKRRQRR) mimics the effects of activation of cyclic nucleotide signaling pathways (Komalavilas, P et al., 2008).

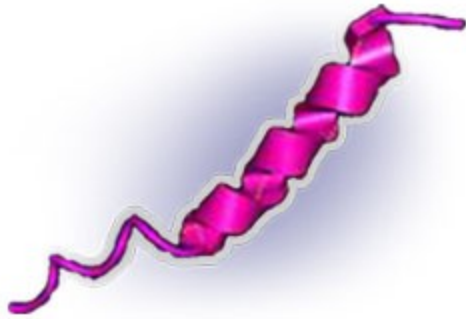


Figure 4: Ribbon representation of the AZX 100 peptide.

Source: www.orthologic.com

Treatment of cultured cells with the AZX 100 leads to changes in cellular morphology (loss of actin stress fibers and increases in monomeric G-actin) similar to the changes observed with activation of the cAMP signaling pathway (Dreiza, C.M et al., 2005). In this project, we study the effect of AZX100 on depolymerization of actin by quantitating ‘G’ actin. This may help to understand the mechanism of AZX100 induced cytoskeletal changes in keloid fibroblasts and it’s potential as a therapeutic agent in the treatment of fibrosis and excessive scarring.

Methods

Cell culture: Human Keloid Fibroblasts (HKF) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) FBS, penicillin (100 U/ml), and 100µg/ml streptomycin (Gibco/BRL), L-glutamine (4 mM) in a CO₂ incubator (10% CO₂) at 37 °C. Cells were seeded on 60mm dishes (70-90% confluent) and serum starved in medium supplemented with 0.5% fetal bovine serum for 24 hours prior to treatment.

Cell Treatment: Cells were either non-stimulated (control) or stimulated for 30 minutes with TGF- β 1(2.5ng) in the presence or absence of AZX100 (100µM). Following treatment, the media was aspirated off and cells were scraped off in lysis buffer prewarmed to 37°C. The G actin was separated from the F actin and quantitated using the kit from cytoskeleton. A bent 26 gauge syringe was used to pipette cells in lysis buffer to disrupt the cellular membrane. Cells were centrifuged at 800g for 10 min at 37 °C to pellet cellular debris. Equal amounts of supernatants were pipetted into ultra centrifuge tubes warmed to 37 °C and centrifuged for 1hr at 100,000g to separate globular actin from filamentous actin. After the ultracentrifugation the supernatants of each sample were pipetted into labeled tubes. Pellets of each sample were suspended in Milli-Q water and 10µM cytochalasin-D and left on ice for 1 hour to dissociate F actin. Supernatants and the pellets are analyzed by SDS-PAGE and western blot.

Western blot analysis Equal volumes of supernatants and pellets (8µl) of all samples were loaded onto 4-20% SDS-PAGE gel; proteins were electrophoretically separated and transferred onto an Immobilon membrane (Millipore, Billerica, MA).

Membrane was probed overnight at 4 C with the primary antibody: rabbit anti-actin antibody (Cytoskeleton, Denver, CO). After washing, the membrane was incubated with an IR Dye 800CW labeled Goat Anti-Rabbit Secondary Antibody (Li-Cor) for 1h at room temperature. The protein-antibody complexes were visualized using the Odyssey direct infrared fluorescence imaging system. The intensity of the bands was quantitated by densitometry and the amount of G actin was calculated by comparing to actin standard loaded on the same gel.

Statistical analysis: Statistical analysis was performed with Graph pad Prism software (version 4, Graph pad Prism software, Inc., San Diego, California, USA) and the results are shown as means ± SEM. The Western blot bands were quantified by densitometry. One-way ANOVA followed by Tukey test was used to compare treated samples with the control, with a significance level of $p < 0.05$. Standard error values were calculated for the Control, TGF- β 1, TGF- β 1+AZX100 and AZX100.

Results

Measurement of F/G actin

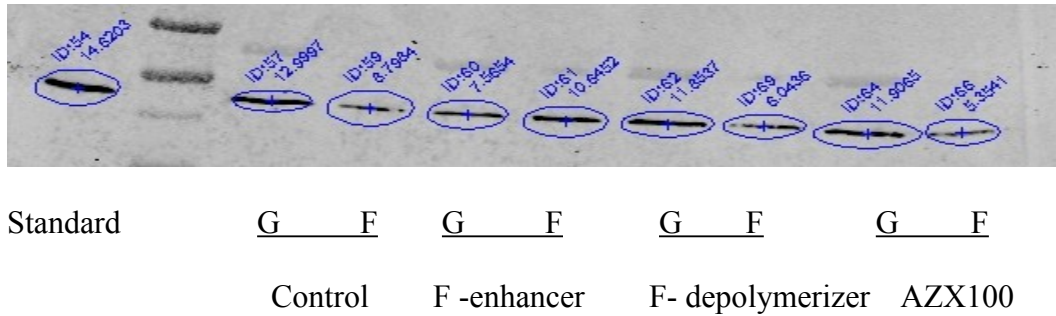


Figure 5: **Measurement of F/G actin**

The figure 5 shows a representative immunoblot of actin in soluble and insoluble fractions of the keloid fibroblast cells from one experiment. Treatment of AZX100 resulted in an increase in G - actin in keloid fibroblasts. The sample 2 and 3 were treated in vitro with F-actin enhancer and F-actin depolymerizer solutions. Sample 4 was treated in vivo with AZX100. As expected the amount of G actin was lower and F actin was higher in the sample treated with F- actin enhancer. Similarly the amount of G-actin was higher and F-actin was lower in the samples treated with F –actin depolymerizer suggesting that kit was working adequately to measure changes in the G- actin. Cells treated with AZX100 increased the G actin.

Measurement of F/G actin

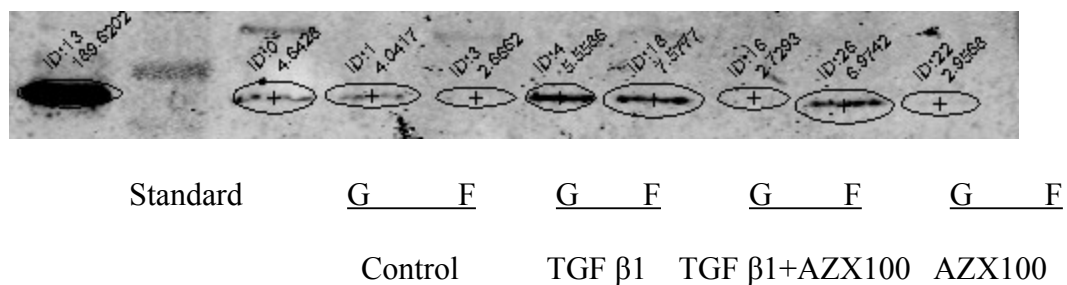


Figure 6: **Measurement of F/G actin**

Figure 6 shows a representative immunoblot of actin in soluble and insoluble fractions of the keloid fibroblast cells treated with TGF-β1, TGF-β1+AZX100 and AZX100 alone. The above blot shows the effect of TGF-β1 in the presence or absence of AZX100 on actin depolymerization in keloid fibroblasts. The amount of G actin is less and F actin is more in the sample treated with TGF-β1. The amount of G-actin is more and F-actin is less in the samples treated with TGF-β1+AZX 100 and AZX100.

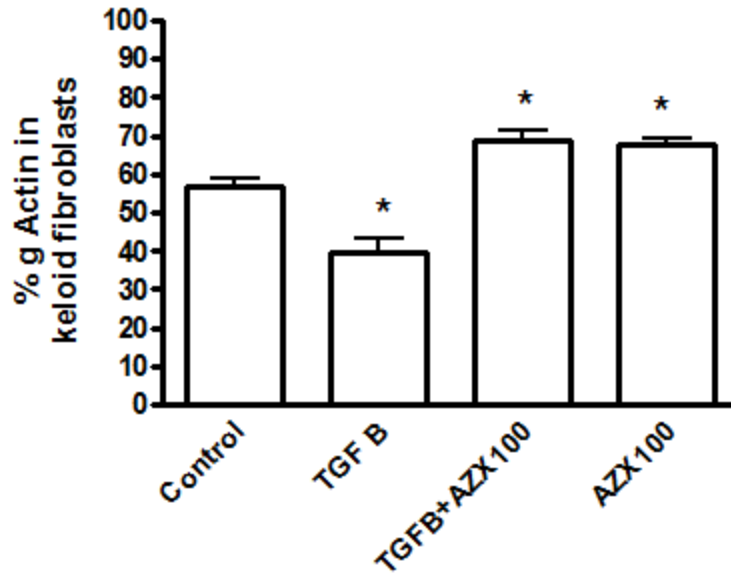


Figure 7: **Quantitation of G-actin**

The percentage of G-actin to the total actin in keloid fibroblasts treated with TGF- β 1, TGF- β 1+ AZX100 and AZX100 are shown in figure 7. The data are presented as means \pm SEM; n=3 experiments; *P < 0.05 compared to control. In untreated keloid fibroblasts the amount of G actin content was 56.85 \pm 1.99 % of total actin. In keloid fibroblasts that were treated with TGF- β 1 for 30 minutes, the amount of G actin content was 39.48 \pm 3.69% of total actin. In the keloid fibroblasts treated with TGF- β 1+ AZX100, the amount of G actin content was 68.62 \pm 2.52 % of total actin. In the keloid fibroblasts treated with AZX100 the amount of G actin content was 67.90 \pm 1.38% of the total actin. Compared to control there was a significant increase (19%) of G actin when cells were treated with AZX100. The cells treated with TGF- β 1 alone also showed a significant decrease in G actin compared to control. The cells treated with TGF- β 1 + AZX100 has shown a significant increase in G actin compared to control.

Discussion

In the present study, we investigated the effect of AZX100 on actin depolymerization by measuring G actin in keloid fibroblasts. The cells treated with TGF- β 1 showed significant decrease in G actin compared to control. The cells treated with AZX100 showed significant increase (19%) in G actin compared to control suggesting depolymerization of actin.

AZX100 displaces the actin accessory protein cofilin from 14-3-3 complex. Such displacement leads to dephosphorylation and activation of cofilin, which in turn mediates actin depolymerization and stress fiber disruption (Dreiza, C.M et al., 2005). According to previous research, AZX100 was also able to reduce CTGF expression induced by LPA suggesting that AZX100 is not specific for a certain pathway, but instead affects CTGF expression induced by the activation of several pathways, presumably because its mechanism of action is downstream and related to the cytoskeleton (Lopes et al.,2008).

One of the problems associated with protein-based therapeutics is that large molecules such as proteins and peptides do not cross cell membranes. By using a protein transduction domain (PTD) as a “carrier”, the attached active peptide can be transported across cell membranes (Flynn, C.R et al., 2003) . Without the PTD, the free HSP20 phospho peptide had no effect on TGF- 1-stimulated expression of CTGF and collagen, demonstrating that the protein transduction domain is necessary for intracellular delivery of this active moiety of HSP20.

AZX100 is novel in that a relevant phospho protein motif is directly delivered inside the cell and modulates downstream events that regulate scar formation. Our results indicate that AZX 100 increases monomeric G actin by depolymerizing actin which disrupts cytoskeleton. Since disruption of cytoskeleton decreases CTGF expression and prevent excessive scarring, AZX100 may be used as a therapeutic agent for excessive scarring and fibrotic disorders.

Future Studies

1. Future studies would be to develop a mathematical model describing the behavior of actin depolymerization at different time points induced by AZX100 will be useful in understanding the level of depolymerization and effect of AZX100 in the cytoskeleton.
2. A same conceptual process can be repeated in a tissue scarring condition.

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