AZX100 Modulates Actin Dynamics in Hypertrophic and Keloid Myofibroblasts

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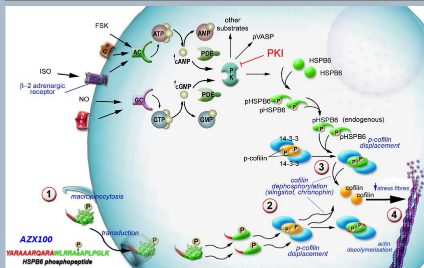
Abstract

Keloid and hypertrophic scar-derived myofibroblasts are characterized by increased stress fibers and contractile function as compared to normal dermal fibroblasts. Increased stress fiber formation is characterized by increases in filamentous (F) actin. The present study investigated the ability of AZX100 to reduce the F-actin content of dermal myofibroblasts. Experimentally, keloid and hypertrophic scar-derived fibroblasts were serum starved for 24 hours, followed by treatment with Transforming Growth Factor Beta 1 (TGFβ1) at 2.5ng/ml alone or with 25μM AZX100 for 24 hours. F-actin was then separated from G-actin by ultra centrifugation and the amounts of each were determined by Western blot analysis. AZX100 treatment of hypertrophic and keloid derived myofibroblasts decreased the pool of F-actin by 20% and 16%, respectively ($p < 0.001$). Moreover, AZX100 treatment of keloid and hypertrophic myofibroblasts visually reduced stress fiber formation and focal adhesions as observed by immunofluorescence. In addition, treatment of TGFβ1-differentiated fibroblasts with AZX100 reduced the amount of α-smooth muscle actin mRNA, a myofibroblast marker. The data suggest that AZX100 disrupts the cytoskeleton network in persistent myofibroblasts, potentially decreasing fibrotic scar formation.

Introduction

AZX100

- 24-amino acid synthetic peptide
- Represents amino acids of the human Heat Shock Protein Beta 6 (HSPB6 or HSP20)
- N-terminal protein transductions domain (PTD)
- Depolymerizes the actin cytoskeleton



-Modified from Dreiza et al Cell Stress and Chaperones, 2009 [1]

Previous research suggests that AZX100, a 24 amino acid phosphopeptide analogue of Heat Shock Protein Beta 6 (HSPB6 or HSP20) containing a protein transduction domain (1), binds to 14-3-3 displacing phospho-cofilin (2) and increasing cofilin-dependent depolymerization of actin (3), resulting in an decreased pool of F-actin (4).

Materials and Methods

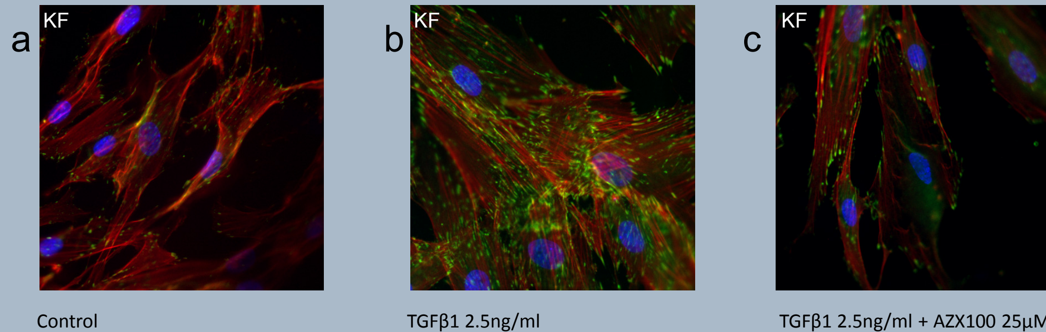
Keloid and hypertrophic scar-derived cells were plated on coverslips at 1x 10^6 cells/ml. Cells were treated at 85-90% confluency for 24hrs with TGFβ1 (2.5ng/ml) or TGFβ1 (2.5ng/ml) + AZX100 (25mM) in full serum. Coverslips were fiixed with 4% formaldehyde for 10 mins. Cells were permeabalized with 0.5% Triton X for 10mins. Cells were stained with anti-vinculin (Invitrogen) (1:250) and anti-mouse Alexa Fluor 488 (Invitrogen) (1:500), DAPI (Invitrogen) (1:1000), Phalloidin Alexa Fluor 568 (Invitrogen) (1:500). Images were taken with an Axiovert microscope at 40x magnification.

In order to quantitatively show increased cytoskeleton activity, an actin assay was run. Keloid and hypertrophic scar-derived cells were plated in 10mm dishes at 2.5 x 10^6 cells/ml. Cells were treated for 24hrs with the same treatments as those on coverslips. Lysates were ultracentrifugated at 100,000 g for 1hr at 37°C in order to separate the soluble fraction (G-actin) from the pellet (F-actin). Both fractions were depolymerized with Cytochalasin D at room temperature for 1hr, Westerns Blots were run using anti-actin antibody (Cytoskeleton). Blots were scanned with Licor Odyssey Imager (Licor) and intensities were measured.

The mRNA of α-smooth muscle actin was measured to ensure the fibroblast were indeed differentiatied into myofibroblasts. Keloid and hypertrophic scar-derived cells were serum starved and co-treated for 48 hours with 1.25ng/mL TGFβ1 and two concentrations of AZX100 (25mM 12.5mM). The mRNA expression was determined by real-time PCR and comparative C_T analysis, normalized to GAPDH.

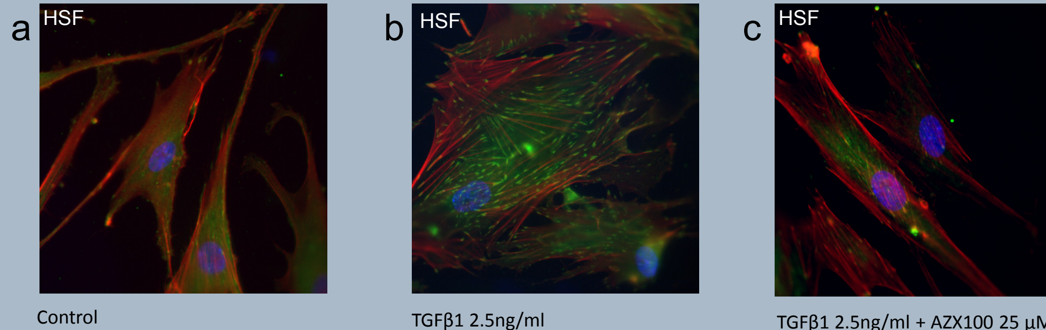
Results

AZX100 reduces vinculin associated with stress fibers and decreases total actin in Keloid Fibroblasts at 24hrs (Figure 1)



Keloid fibroblasts (Fig. 2a-c) were plated on fibronectin coated coverslips and treated for 24hrs with or without TGFβ1 (2.5ng/ml) and AZX100 (25μM) in 10% FBS. Cells were stained with DAPI (blue), anti-vinculin Alexa Fluor 488 (green), and Phalloidin Alexa Fluor 568 (red). Images were taken at 40x magnification with Axiovert microscope (Carl Zeiss).

AZX100 reduces vinculin associated with stress fibers and decreases total actin in hypertrophic scarring fibroblasts at 24hrs (Figure 2)



Hypertrophic scar fibroblasts (Fig. 2a-c) were plated on fibronectin coated coverslips and treated for 24hrs with or without TGFβ1 (2.5ng/ml) and AZX100 (25μM) in 10% FBS. Cells were stained with DAPI (blue), anti-vinculin Alexa Fluor 488 (green), and Phalloidin Alexa Fluor 568 (red). Images were taken at 40x magnification with Axiovert microscope (Carl Zeiss).

AZX100 reduces Filamentous actin (F-actin) in keloid and hypertrophic scar-derived fibroblasts (Figure 3)

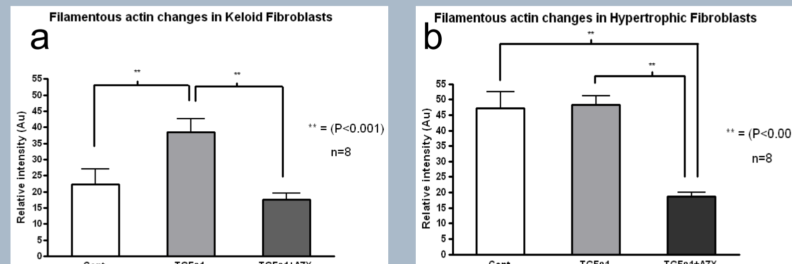


Figure 3a and b describe the AZX100 effect on filamentous actin in primary cell lines of keloid and hypertrophic scar derived fibroblasts. Treatment with TGFβ1 ensured full differentiation to myofibroblasts at 24hrs and shows an increase or maintenance of actin levels in keloid and hypertrophic scar cells, respectively. Upon co-treatment with TGFβ1 and AZX100, there was a significant decrease in filamentous actin, suggesting an AZX100-dependent reduction of the actin cytoskeleton in pathological dermal myofibroblasts.

n=8 for all cell types.

Results

AZX100 reduces alpha smooth muscle actin (ACTA 2) mRNA at 48 hrs in keloid and hypertrophic scar-derived fibroblasts (Figure 4)

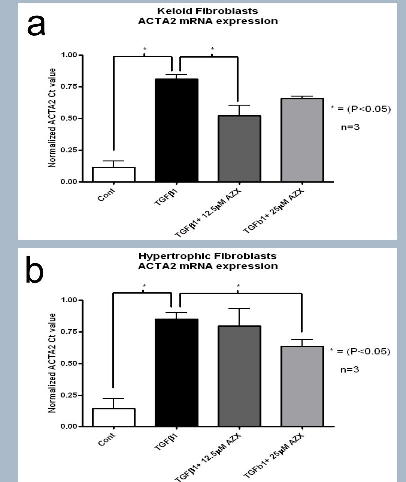


Figure 4a and b show a significant increase in the amount of α-smooth muscle actin with 48hrs of TGFβ1 treatment. Increased α-smooth muscle actin is a key marker of fibroblasts differentiation to myofibroblasts. Co-treatment of fibroblasts with TGFβ1 and AZX100 significantly reduced ACTA2 mRNA expression compared to TGFβ1 treated cells, suggesting an AZX100-dependent decrease in myofibroblast differentiation.

n=3 for all cell types.

Summary

- AZX100 (25 μM) significantly reduces F-actin in the presence of exogenous TGFβ1(2.5ng/ml) after 24 hrs of treatment.
- AZX100 (25 μM) treatment for 24 hours showed visual decreases in the number of focal adhesions (vinculin) of TGFβ1-differentiated myofibroblasts.
- AZX100 (12.5 μM, 25 μM in keloid and hypertrophic derived differentiated myofibroblasts respectively) treatment for 48 hours significantly decreases α-smooth muscle actin (ACTA2) mRNA in TGFβ1-differentiated myofibroblasts.

Thus, AZX100 affects cytoskeleton structure and α-smooth muscle actin expression, and may reduce keloid and hypertrophic fibrotic scarring during wound healing.

Bibliography

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