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TGF-beta1 increases cell rigidity by enhancing expression of smooth muscle actin: Keloid-derived fibroblasts as a model for cellular mechanics

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ABSTRACT

Background: Mechanical transduction contributes to appropriate cell functions. Clinically, keloid, an uncontrolled fibrous overgrowth and scarring, preferentially affects skin areas subject to higher mechanical tension than others. Keloid-derived fibroblasts have exaggerated TGF-beta1-mediated responses, including smooth muscle actin (SMA) expression, cellular contraction, and tissue remodeling, to mechanical strain compared to normal fibroblasts.
Objective: This study asked if SMA contributes to cellular intrinsic rigidity using keloid -derived fibroblasts as a model.
Method: Using atomic force microscopy and confocal microscopy, we measured cellular rigidity and the expression of SMA in keloid fibroblasts treated with exogenous TGF-beta1.
Result: There was an increase of SMA expression in keloid tissue as well as keloid-derived fibroblasts. The cell rigidity increased by TGF-beta1 in keloid fibroblasts occurred concomitantly with increases in SMA expression. TGF-beta1 receptor 1 kinase inhibitors reduced TGF-beta1-induced cellular rigidity and SMA expression. Knocking down SMA with interference RNA resulted in a reduction of TGF-beta1-enhanced rigidity, suggesting that TGF-beta1 increases cell rigidity via SMA expression.
Conclusion: We conclude that TGF-beta1 increases cell rigidity through TGF-beta1 receptor-SMA axis. This study reports that SMA, at least in part, contributes to cell rigidity in fibroblasts. SMA might be an appealing pharmaceutical target in keloids.

1. Introduction

Keloids are common fibrotic lesions resulting from aberrant and uncontrolled wound repair, an overgrowth of tissue starting at the site of a healed skin injury. Keloids often affect anatomic sites with increased tension, such as the anterior chest, arm, and back. Because they usually progressively expand over time and frequently recur following surgical excision, keloids are challenging for both patients and physicians. Current treatments are inadequate and recurrences are common [1].

The pathogenesis of keloids is complicated, with several hypothesized causes, including altered mechanical tensions [1]. In addition to their preference in tensed skin, at the tissue level, tissue viscoelasticity or rigidity has been found to be increased in keloids [2]. At the cellular level, keloid fibroblasts have a more exaggerated response to mechanical strain than normal fibroblasts, leading to increased production of pro-fibrotic growth factors, including TGF-beta1 [3]. Moreover, dermal fibroblasts from different body sites differ in their responses to both tension and TGF-beta1 [4]. Functional assay and microarray studies have identified TGF-beta1 as a major candidate mediator in the pathogenesis of keloids [5,6]. TGF-beta1 has diverse effects on cell function, regulates collagen production and tissue remodeling in keloids [7]. However, how TGF-beta1 affects the intrinsic mechanical property in keloids remains unknown.

The mechanical properties of cells, which are critical in such cellular processes as locomotion, division and differentiation, are determined by both extracellular substrate and intracellular cytoskeleton. In several cancers, aberrant increase in substrate stiffness activates integrins, which in turn not only promote proliferation but also augment cell contractility, ultimately increasing matrix stiffness [8,9]. In addition, fibroblasts can sense

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changes in physical properties in their extracellular matrix environment, transduce mechanical information into chemical information, and incorporate these signals with growth factor derived stimuli to regulate specific changes in gene expression [10]. In keloids-derived fibroblasts, they have an increased transcriptional response to extrinsic mechanical strains due to increased focal adhesion complex formation [3]. However, how the intrinsic cellular rigidity is altered in the keloid fibroblasts is not known.

In the cell, the intrinsic cellular rigidity is largely determined by the interior cytoskeleton, which is made of several polymeric networks, including actin, microtubuli, and intermediate filaments [11,12]. In static conditions, actin filaments are less rigid than microtubules [11]. In dynamic processes, organized actin fibers, or stress fibers, contribute to cell contractility in fibroblasts sensing substrate stiffness or other activation factors. Smooth muscle actin (SMA), a form of actin well-known for its role in myofibroblast contractility, is expressed in keloids and hypertrophic scars [13,14]. It remains unknown if SMA contributes to intrinsic cell rigidity, however. Stiffness tomography in the attached cells is, to date, best determined via the indentation measurements by atomic force microscope (AFM) [15]. The displacement of the AFM probe reflects the regional cell rigidity in single cells. Although the location of the AFM probe on the cells may affect the measurements of cell rigidity, the nuclear location is known to accurately present the cell rigidity [16]. Coupled with confocal microscopy to measure SMA expression, we studied the molecular mechanisms underlying the mechanical properties and cellular rigidity, using keloid fibroblasts as a model.

2. Materials and methods

2.1. Cell culture and treatment

Tissue samples were collected from patients over age 20 years who were visiting the Department of Dermatology at Kaohsiung Medical University between 2006 and 2007. All the subjects, including those with keloids (n = 6, age 25–49 years; 4 males and 2 females) and normal controls (n = 6, age 21–49 years; 3 males and 3 females), were Taiwanese of Han Chinese origin (Table 1). Subjects included those with keloids exhibiting continuous growth beyond the margin 6 months after surgery and excluded those with invasive cancers or those treated with liquid nitrogen spraying or intralesional corticosteroid injection within 3 months, and those treated at any time with systemic immunomodulatory agents. Tissues were sampled from each subject by a punched biopsy in the center of the keloid with a 3 mm punch. Control skin was obtained using perilesional normal skin after surgical excision of melanocytic nevi. Dermis tissues were cut to 1–2 mm^3 and incubated in a culture dish with Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Grand Island, NY) supplemented with 10% FBS (Invitrogen), 2 mM L-glutamine (Invitrogen) and 0.1 mM 2-mercaptoethanol (Sigma, cat. no. M7522), along with 50 units/ml of penicillin and 50 g/ml of streptomycin (Invitrogen). The 3rd passage of human fibroblasts were used for the experiments. All participants signed written informed consent forms. The protocol for this study was approved by the Institutional Review Board (IRB) of Kaohsiung Medical University (KMU-IRB-950275). Inhibitors for TGF-beta1 receptor 1 kinase (LY-364947 and SB-431542), AKT (LY294002), and p38 (SB-202190) were all acquired from Sigma–Aldrich (St Louis, MO)

Table 1
Clinical demographic data of patients with keloids and normal controls.

	Gender	Age	Location	Duration (years)	Size (cm)
Case 1	Male	25	Shoulder	4	2.1
Case 2	Male	37	Chest	3	3.5
Case 3	Male	28	Knee	5	2.3
Case 4	Female	49	Back	7	4.1
Case 5	Female	35	Arm	8	3.2
Case 6	Male	42	Back	12	1.7
Control A	Male	32	Back	N/A	N/A
Control B	Female	27	Face	N/A	N/A
Control C	Male	21	Chest	N/A	N/A
Control D	Female	33	Shoulder	N/A	N/A
Control E	Male	45	Back	N/A	N/A
Control F	Female	49	Arm	N/A	N/A

2.2. Immunofluorescence staining

Sections (5 μm) of formalin-fixed, paraffin-embedded tissues were subjected to immunofluorescence staining according to our previous protocol [17]. Briefly, following deparaffinization and rehydration, slides were autoclaved in 10 mM citrate buffer for 20 min for antigen retrieval. All the sections were blocked by 10% goat sera or 3% BSA depending on the host species of the secondary antibody at room temperature for 2 h before they were incubated with primary antibodies. For double staining of actin and SMA, sections were stained with goat anti-human SMA antibody and stored at 4 °C overnight (1:500, R&D, MN). The next day we incubated the sections with rabbit anti-goat-IgG-FITC (1:1000, Sigma, St Louis, MO) for 1 h at room temperature. They were also incubated with rabbit anti-human actin (1:200, Millipore, Billerica, MA) followed by goat anti-rabbit-Alexa568 (1:1000, Invitrogen, Carlsbad, CA). Cells in the culture plates in vitro were stained in a similar manner. The stained specimens were mounted and observed under a confocal laser scanning microscope (LSM Fluoview 500, Olympus). Image analysis was performed by NIH imageJ, an open image-processing software, with a native plugin of "ROI manager". The intensity value in each region of interest (ROI) was generated by pseudocolor with gray-scale transformation (0–255). Mean fluorescence intensity index (0–255) was averaged from 5 random high power fields (or ROIs) in the dermis.

2.3. Western blotting

For SDS-PAGE and Western blotting of SMA, total cellular protein extract from fibroblasts was obtained by lysing the cells in ice-cold lysis buffer (50 mM tris, 5 mM EDTA, 0.1% triton X-100, 150 mM NaCl and mixed cocktail protease inhibitors). After 4000 × g centrifugation, supernatants were collected, and protein concentrations measured using a protein quantification kit (Bio-Rad, Hercules, CA). Samples containing 40 μg protein were subjected to SDS-PAGE on 12.5% acrylamide gels then transferred onto a nitrocellous membrane (Bio-Rad, Hercules, CA). The membrane was blocked in 2% skim milk/PBS with 0.5% Tween-20 for 1 h at room temperature. Antibodies against human SMA and actin (both from Abcam, San Francisco, CA) were used at a dilution of 1:500. Specific proteins in the nitrocellulous membranes were visualized using a chemiluminescence substraction kit (Pierce, Rockford, IL).

2.4. AFM indentation experiments

Atomic force microscope (AFM) experiments were performed using a commercial instrument (JPK NanoWizard II, Germany) mounted on an inverted optical microscope (Axiovert 200, Carl Zeiss, Heidelberg, Germany). In order to prevent cells from physical damage during experiments, the indentation tests were performed with a tipless silicon cantilever with an attached micro-size glass