Acta Biomaterialia xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Myofibroblast persistence with real-time changes in boundary stiffness

Mehmet H. Kural, Kristen L. Billiar *

Department of Biomedical Engineering, Worcester Polytechnic Institute, Worcester, MA, USA

ARTICLE INFO

Article history:
Received 14 April 2015
Received in revised form 15 December 2015
Accepted 16 December 2015
Available online xxxx

Keywords:
Valvular interstitial cell
Myofibroblast
Stiffness
Tension
Fibrin
Three-dimensional
TGF-β1
Apoptosis
Mechanobiology

ABSTRACT

Myofibroblasts are critical for connective tissue remodeling and wound healing since they can close wound beds and shape tissues rapidly by generating high traction forces and secreting abundant extracellular matrix proteins and matrix metalloproteinases. However, their presence in excessive numbers is associated with fibrotic and calcific diseases and tissue thickening in engineered tissues. While activation of the myofibroblast phenotype has been studied extensively, whether myofibroblasts are "cleared" by phenotypic reversal or by apoptosis remains controversial. The goal of this work is to test the hypothesis that mechanical inhibition of myofibroblast force generation leads to de-differentiation or apoptosis depending upon the magnitude of the decrease in tension. To test this hypothesis, we cultured valvular interstitial cells (VICs) in fibrin micro-tissues suspended between flexible posts and dynamically altered the ability of the cells to generate tension by altering boundary stiffness via magnetic forces applied to posts. The flexible posts capped with magnetic beads enable the measurement and modulation of tension generated by the cells within the tissue. As expected, the cell-generated forces were elevated with dynamically increased boundary (post) stiffness, yet surprisingly, the forces continued to increase following dynamic reduction of boundary stiffness back to baseline levels. Increased apoptosis and reduced α-SMA staining were observed with complete freeing of the tissues from the posts but not upon removal of the magnet, resulting in a twofold decrease in post stiffness. Together, these data indicate that an increase in myofibroblast force generation, even if modest and temporary (1 day), can have lasting effects on myofibroblast persistence in tissues, and that a significant reduction in the ability of the cells to generate tension is required to trigger dedifferentiation and/or apoptosis. The ability to dedifferentiate myofibroblasts to a quiescent phenotype and to control the percentage of apoptosis would be of great benefit for therapeutic and tissue engineering applications.

Statement of Significance

Myofibroblasts play an important role in tissue remodeling and wound healing. However, excessive activation of this phenotype is associated with fibrotic diseases and scar formation. Being able to dedifferentiate these cells or controlling their clearance with apoptosis (programmed cell death) would be beneficial. It is known that releasing rigid tissue boundaries trigger apoptosis, while reducing the substrate stiffness can cause myofibroblast dedifferentiation. However, the mechanical tension was not quantified in any of the studies. Here we used micro-cantilever posts at tissue boundaries to measure tension and to regulate boundary stiffness in real time by pulling posts with magnets. We show that temporary stiffening of boundary causes irreversible myofibroblast activation and the magnitude of tension drop controls apoptosis.

1. Introduction

Myofibroblasts play a key role in tissue remodeling and wound healing [1,2]. Being able to generate high traction forces and secrete abundant extracellular matrix (ECM) proteins, myofibroblasts can reorganize their surrounding matrix, rapidly close wounds and repair matrix damage [3]. However, prolonged presence of myofibroblasts due to excessive activation of fibroblasts and/or insufficient myofibroblast apoptosis leads to fibrocontractive remodeling and scar tissue [3–5]. By compacting the surrounding matrix, digesting native ECM and secreting excessive amounts

* Corresponding author at: Department of Biomedical Engineering, Worcester Polytechnic Institute, 100 Institute Road, Worcester, MA 01609, USA.
E-mail address: kbilliar@wpi.edu (K.L. Billiar).

http://dx.doi.org/10.1016/j.actbio.2015.12.031
1742-7061/

of stiff collagen under high residual tension, myofibroblasts contribute to disease states of many different organs [6–8].

In heart valves, activation of a large proportion of valvular interstitial cells (VICs) to the myofibroblast phenotype is associated with valve fibrosis which may cause regurgitation, valve failure [9]. Moreover, in over 80% of calcified aortic valves, myofibroblasts are colocalized with the calcified regions and apoptotic cells [9]. In addition to being associated with disease of native heart valves, myofibroblasts are responsible for tissue thickening and retraction in engineered heart valves [10,11]. Thus, being able to reverse the myofibroblast phenotype (i.e., dedifferentiate VICs back to the quiescent state) and/or control the rate of clearing of myofibroblasts from the tissue via apoptosis would be of great benefit for therapeutic applications.

High substrate modulus and transforming growth factor-beta1 (TGF-β1) have been shown to be key activators of fibroblasts and VICs to the myofibroblast phenotype [12–14]. Specifically, on high modulus substrates in the presence of TGF-β1, fibroblastic cells form α-SMA-rich stress fibers – the defining visual hallmark of the myofibroblast phenotype – and generate high intracellular tension. We recently demonstrated quantitatively that this synergistic relationship between stiffness and TGF-β1 in modulating VIC-generated forces also applies to three-dimensional (3D) tissue models [15].

Although, the mechanical environment is clearly critical for triggering myofibroblast activation, little is known about the mechanical reversibility of this phenotype. Early *in vivo* data indicated that myofibroblasts are cleared following wound closure by apoptosis (i.e., programmed cell death) rather than by dedifferentiation [16]. Externally reducing the resistance to cell contraction has also been shown to trigger apoptosis; for example, releasing a stiff splint holding the edges of an excisional wound caused apoptosis in 8% of the total cell population [17]. In an analogous *in vitro* 3D system in which collagen gels seeded with dermal myofibroblasts were detached from rigid boundaries, apoptosis was triggered in 15% of the cells [18]. A similar study utilizing fibroblasts isolated from scar tissue [19] reported 40% apoptosis following the release of anchored collagen gels.

More recently, however, there is evidence that myofibroblasts may dedifferentiate without apoptosis in response to changes in the mechanical environment. Hinz and colleagues showed that α-SMA expression in dermal fibroblasts decreases without apoptosis when a splinted wound bed is released [20]. Anseth and her group seeded VICs on light-responsive gel substrates for three days, then dynamically decreased the Young's modulus of the gel [21,22]. They observed a significant decrease in the number of cells expressing α-SMA without inducing apoptosis above the level of stiffness-matched controls, thus indicating phenotypic reversion after two additional days of culture at the lower modulus. However, when they cultured VICs on stiff gels for a longer time period (seven days), reduction of substrate modulus did not result in dedifferentiation [22]. In the aforementioned 2D and 3D studies, the experimental manipulations reduce the mechanical restraints to cell-generated tension, but in none of the studies is the tension quantified; thus it is unclear if the magnitude of tension is a key parameter for differentially regulating dedifferentiation and/or apoptosis.

The goal of this work is to test the hypothesis that mechanical inhibition of myofibroblast force generation leads to either dedifferentiation or apoptosis, depending upon the magnitude of the decrease in intracellular tension. To test this hypothesis, we cultured VICs in 3D fibrin micro-tissues suspended between flexible posts, the deflection of which can be used to quantify the tissue tension. To determine the effect of mechanical tension drop on the myofibroblast phenotype, we stimulated cell-generated forces by doubling the boundary stiffness in real time by holding one of the micro-posts rigid via magnetic force applied to a nickel bead glued onto the post; the post was then released by removing the permanent magnet. To create a more severe tension drop, we released tissues completely from one of the micro-posts and cultured the tissues without constraining contraction towards the remaining micro-post. The findings of the present study provide insight for future studies aimed at directing myofibroblast fate in both diseased and engineered tissues.

2. Methods

2.1. Cell culture

Pig hearts were obtained from a local slaughterhouse (Blood Farm, Groton, MA). Aortic valves were excised within two hours after animals were killed. VICs were isolated according to a previously published protocol [23]. Aortic valve leaflets were rinsed with cold 1 × Dulbecco's phosphate buffered saline (DPBS, Cellgro, Manassas, VA), and submerged in a 600 U/mL solution of type II collagenase (Worthington Biochemical, Lakewood, NJ) in 1 × Dulbecco's Modified Eagle's Medium (DMEM, ThermoFisher, Grand Island, New York) with 1% penicillin/streptomycin/amphotericin B (PSA, Life Technologies, Grand Island, New York) and 10% fetal bovine serum (FBS, Life Technologies, Grand Island, New York). Both surfaces of the leaflets were rubbed by a sterile cotton swab to remove the valvular endothelial cells in a collagenase solution. Next, the leaflets were rinsed and incubated in 12 ml of collagenase solution at 37 °C for 12 h for enzymatic digestion. Finally, VICs were plated on tissue culture plastic flasks in DMEM with 1% PSA and 10% FBS. Cell passage numbers 3–8 were used. Two types of pre-treatments were utilized in the current study as follows: the first group was pre-treated with low serum media containing 1% FBS without TGF-β1 for four days to have a more quiescent population with lower tissue tension (low tension group), and the second group was pre-treated with 10% FBS and 5 ng/ml TGF-β1 for four days to activate all cells to a myofibroblast phenotype (high tension group) as per [18].

2.2. Force Calculations with micro-tissue gauges (μ-TUGs)

We utilized fibrin gels as our 3D scaffold (rather than collagen) due to the high efficiency of creating micro-tissues that could be cultured for long duration. In addition to its technical advantage, fibrin is abundant in damaged heart valve tissue, and it is extensively used in tissue engineered heart valve studies since fibrin gels, unlike reconstituted collagen gels, do not inhibit collagen production by the resident cells [10,24]. As we described previously [15], to measure and regulate cell-generated forces, fibrin-based micro-tissues were cultured in micro-wells containing two flexible posts made of poly(dimethylsiloxane) (PDMS, Dow Corning, Midland, MI). As cells compact the fibrin gel and form dog-bone shaped tissues around the posts, the forces applied to the posts can be calculated by measuring the posts' deflection and using the following beam bending equations: $= \frac{wt^3}{12}$ and $F = \frac{\delta * 6EI}{a^2 * (3L-a)}$, where δ is the post deflection and all the other parameters are dimensional parameters shown in Fig. 1.

Post deflections were measured at multiple time points and under various boundary conditions; in general, they were measured immediately before and after applying the magnet, ~10 h following applying the magnet, immediately before removing magnet, immediately after removing the magnet, 12 h after removing the magnet, and two days after removing the magnet. The post held rigidly by the applied magnetic force acts as an infinitely stiff boundary which causes a doubling of the effective boundary stiffness as explained in the next sections. The average force per cell

Please cite this article in press as: M.H. Kural, K.L. Billiar, Myofibroblast persistence with real-time changes in boundary stiffness, Acta Biomater. (2015), http://dx.doi.org/10.1016/j.actbio.2015.12.031