Biochemical and Biophysical Research Communications xxx (2016) 1–6

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Lysyl oxidase expression in cardiac fibroblasts is regulated by α2β1 integrin interactions with the cellular microenvironment

Albert E. Gao [a], Kelly E. Sullivan [a], Lauren D. Black III [a, b, *]

[a] Department of Biomedical Engineering, Tufts University, Medford, MA 02155, USA
[b] Cell, Development and Molecular Biology Program, Sackler School of Graduate Biomedical Sciences, Tufts University School of Medicine, Boston, MA 02215, USA

ARTICLE INFO

Article history:
Received 2 May 2016
Accepted 7 May 2016
Available online xxx

Keywords:
Lysyl oxidase
Cardiac fibroblast
Cardiac fibrosis
TGF-beta
Integrins
Cell-matrix interactions

ABSTRACT

Lysyl oxidase (LOX) catalyzes crosslink formation between fibrillar collagens and elastins and an increase in LOX activity has been associated with cardiac fibrosis following myocardial infarction (MI). It has been previously reported that LOX expression is regulated by growth factors and cytokines including transforming growth factor (TGF-β1); however, it is unclear how the biophysical and biochemical properties of the cellular microenvironment affect LOX expression. In this study, we isolated rat cardiac fibroblasts (CF) and infarct cardiac fibroblasts (ICF), from healthy and 1-week post-MI left ventricular tissue respectively, and cultured them under varied substrate conditions *in vitro* to assess their influence on LOX expression. Culture of ICF on collagen I-coated plates increased LOX expression versus uncoated plates with an additional increase observed with the presence of TGF-β1. To further investigate the effect of integrin interactions with collagen I on LOX expression, we inhibited the α2β1 integrin from binding to collagen I and found gene and protein expression of LOX to be downregulated. Together, this demonstrates that the interaction of α2β1 integrin to collagen I in the cellular microenvironment can regulate expression of LOX. Further studies investigating additional integrin interactions may identify therapeutic targets for treating cardiac fibrosis.

1. Introduction

Heart disease is the leading cause of death in the United States [1] and it is rapidly increasing in prevalence worldwide. The response of the cells within the heart to myocardial infarction (MI)-often induced via a blockage of one of the coronary arteries by an atherosclerotic plaque – can lead to cardiac fibrosis, which decreases tissue compliance, thereby significantly limiting the pumping capability of the heart [2,3]. Ultimately, continued progression of cardiac fibrosis can lead to diastolic dysfunction and subsequent heart failure. While efforts in attenuating fibrosis have been explored, the complex milieu of signals that promote the continued progression of cardiac fibrosis is still not fully characterized. It is known that there are significant alterations to both ECM composition and modulus in the remodeling infarct [4]; however, the manner by which these changes to the ECM feedback into continued ECM remodeling of cardiac fibroblasts (CF), are not fully understood. As such, there is a need to better understand how the changes to cardiac tissue feedback to affect the progression of fibrosis in order to develop strategies which may ultimately prevent fibrosis, diastolic dysfunction, and heart failure as a result of MI.

CF make up 70% of the cells in the adult heart and are the primary cell type responsible for maintenance and turnover of the extracellular matrix (ECM) [5]. Generally, in the adult heart, CF are quiescent; however, in the presence of stimuli brought on by cardiac injury, CF are activated to a myofibroblast phenotype (MF), which are classically characterized by increased proliferation, mobility, secretion of ECM proteins and expression of alpha-smooth muscle actin (αSMA) [6]. With this altered phenotype, MF actively remodel the ECM and increase the density of collagen in the infarcted tissue. While this is essential in stabilizing the necrotic tissue and preventing tissue rupture in the early stages following MI, over time, sustained secretion and accumulation of fibrillar collagens causes the formation of a dense, collagen-rich scar [5–7]. As the ECM becomes more collagen-based, this collagen is crosslinked by lysyl oxidase (LOX) to produce more mature collagen

* Corresponding author. Department of Biomedical Engineering, Tufts University, 4 Colby Street, Medford, MA 02155, USA.
E-mail address: lauren.black@tufts.edu (L.D. Black).

http://dx.doi.org/10.1016/j.bbrc.2016.05.037
0006-291X/

fibers which are more resistant to matrix metalloproteinase (MMP) degradation and also contribute to increases in local modulus [8]. Variations to local modulus, protein composition, and the degree of mechanical stretch have been shown to affect expression of proteins involved in fibrosis, such as MMPs and collagens in CF [9,10]. This behavior is likely regulated through integrin binding and associated downstream signaling [9,11,12].

LOX is a copper-dependent amine oxidase which catalyzes lysine residues to produce crosslinks in fibrillar collagen and elastin resulting in an increase of insoluble collagen fibers [13]. While additional LOX-like genes have been identified in the LOX family, LOX is the most prominent form within the heart [8]. LOX crosslinking can create mature collagen fibers to promote an increased tissue modulus [14] and its expression has been shown to be regulated by transforming growth factor-beta (TGF-β) [15], tumor necrosis factor-alpha (TNF-α) [16], and hypoxia inducible factor 1-alpha (HIF-1α) [17] which are all present in the infarct environment. While the implications of LOX activity have been of great interest in the development of fibrosis in numerous tissue types, there is still a lack of understanding in how LOX expression is regulated by cellular interactions with the ECM. This is especially important given that there has been extensive research showing that cells, including fibroblasts, respond to substrate modulus and composition and that these interactions can modulate the expression of collagens through integrin signaling.

In this study, we assessed the impact of biophysical and biochemical environmental stimuli, such as local modulus and ECM protein composition, on LOX expression. We demonstrated that LOX expression is regulated by integrin interactions with the ECM and that inhibition of the α2β1 integrin reduced the integrin-mediated effects on LOX expression. Identifying the role of these integrin-mediated interactions within the specific context of the cellular microenvironment present following MI could provide new targets for development of potential therapeutics, which may promote healthier matrix remodeling and limit deleterious fibrosis following MI.

2. Methods

2.1. CF and ICF isolation

All animal experiments were performed in accordance with the US Animal Welfare Act and institutional guidelines and were approved by the Institutional Animal Care and Use Committee at Tufts University. MI was induced in male Sprague–Dawley rats (250–275 g) by permanently ligating the left coronary artery and left ventricle blanching of 40% or more was observed to confirm generation of a significant infarct as previously described [18]. Animals were allowed to recover for 1 week following infarction at which point the animals were sacrificed and the left ventricle (LV) was excised for isolation of infarct cardiac fibroblasts (ICF). The tissue was first minced into pieces less than 1 mm^3 in size and digested in collagenase solution [12]. The cell suspension was collected and passed through a 40 μm strainer and centrifuged to collect the cells. The pelleted cells were then suspended in DMEM with 15% FBS and 1% Penicillin-Streptomycin and plated for 4 h at which point media was exchanged and remaining suspended cells were discarded. Adherent cells were cultured until confluent and passaged. Additionally, LV of healthy non-infarcted male Sprague-Dawley rats of similar size were digested via the same method to obtain CF. All collected cell populations were further evaluated by immunohistochemistry and were found to stain positively for Vimentin. All further experiments were conducted with ICF and CF below passage 6.

2.2. Collagen and ECM coating plates and experimental cell culture conditions

To evaluate the influence of collagen I on CF and MF gene and protein expression, cells were cultured on coated and uncoated plates. Culture plates were incubated with 10 $\mu g/cm^2$ collagen I (ThermoFisher) in 0.1 M acetic acid for 4 h and rinsed with PBS prior to cell seeding. In all experiments, cells were cultured in serum-free media composed of a 50/50 DMEM/F12 blend with 0.2% (wt.vol.) bovine serum albumin (Sigma), 0.5% insulin-transferrin-selenium (ThermoFisher), and 1% penicillin-streptomycin (ThermoFisher). Additional dosing of 10 ng/mL TGF-β1 (Peprotech) was added 48 h following cell seeding and replenished at each subsequent feeding for the duration of the study.

2.3. RNA isolation and qPCR

RNA was isolated with RNeasy mini kit (Qiagen) followed by DNase treatment with TURBO DNA-free (Life Technologies). Reverse-transcription was performed with high capacity cDNA reverse transcription kit (Applied Biosystems) and quantitative real-time PCR with Taqman Universal PCR Master Mix (Life Technologies) on a qPCR machine (Stratagene, Mx3000P). Taqman primers for GAPDH, LOX, Col1α1, Integrin β1, and Tagln were used. (Life Technologies). Samples were normalized to Gapdh. Fold change was calculated by $2^{-\Delta\Delta ct}$ to Gapdh and experimental control values.

2.4. Protein isolation and westerns

Western blots were run as previously described [19]. Cells were trypsinized, collected via centrifugation and lysed with ice cold cell lysis buffer (NP40, sodium deoxycholate, sodium orthovanadate, aprotinin, pepstatin, leupeptin, and diH20). Lysates were run on 10% gels (Company) and transferred to a PVDF membrane (Company). Blots were blocked in 5% non-fat milk and probed for pro-LOX/LOX (NB100-2527, NOVUS, 1:100) and β-actin (A5441, Sigma-Aldrich, 1:1000). HRP-conjugated Secondary antibodies (Jackson ImmunoResearch) were incubated at 1:1000. Membranes were then imaged using enhanced chemiluminescence reagents and a G:BOX Chemi XR5 (Syngene, Frederick, MD) gel imager. All blots were imaged at the same exposure rate intervals and for each protein the same exposure time was used. Western blot analysis was carried out using the built-in gel analysis macro in the ImageJ software package (National Institutes of Health).

2.5. Immunohistochemistry

CF and ICF were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton-X100. Cells were probed for vimentin (Santa Cruz, sc-6260), α-smooth muscle actin (Santa Cruz, sc-32251), and LOX (NOVUS, NB100-2527) all at 1:200 concentration. Fluorescent labeling was carried out using Alexafluor 488 secondary antibodies (ThermoFisher) at 1:400. Hoescht stain (ThermoFisher) was used to label nuclei (1:10,000). Collected images were analyzed using a custom built pipeline in CellProfiler (Broad Institute) to calculate cell spread area.

2.6. Integrin inhibition

To further assess the role of integrin signaling in LOX expression of ICF, we specifically blocked the α2β1 integrin from binding to collagen I. The α2β1 integrin has been identified as a collagen binding receptor which can also impart mechanical contraction [20]. α2β1 integrin binding inhibitor BTT3033 (Cat #:4724, Tocris

Please cite this article in press as: A.E. Gao, et al., Lysyl oxidase expression in cardiac fibroblasts is regulated by α2β1 integrin interactions with the cellular microenvironment, Biochemical and Biophysical Research Communications (2016), http://dx.doi.org/10.1016/j.bbrc.2016.05.037