Hyaluronan Controls the Deposition of Fibronectin and Collagen and Modulates TGF-β1 Induction of Lung Myofibroblasts

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Abstract

The contribution of hyaluronan-dependent pericellular matrix to TGF-β1-driven induction and maintenance of myofibroblasts is not understood. Hyaluronan is an extracellular matrix (ECM) glycosaminoglycan important in cell adhesion, proliferation and migration, and is implicated in myofibroblast formation and maintenance. Reduced turnover of hyaluronan has been linked to differentiation of myofibroblasts and potentiation of lung fibrosis. Fibronectin is a fibril forming adhesive glycoprotein that is also upregulated following induction with TGF-β1. Although they are known to bind each other, the interplay between hyaluronan and fibronectin in the pericellular matrix during myofibroblast induction and matrix assembly is not clear. This study addresses the role of hyaluronan and its interaction with fibrillar matrix components during myofibroblast formation. Hyaluronan and fibronectin were increased and co-localized in the ECM following myofibroblast induction by TGF-β1. Inhibition of hyaluronan synthesis in TGF-β1-induced lung myofibroblasts over a 4 day period with 4-methyl umbelliferone (4-MU) further enhanced myofibroblast morphology, caused increased deposition of fibronectin and type I collagen in the ECM, and increased expression of alpha-smooth muscle actin and hyaluronan synthase 2 (HAS2) mRNA. Hyaluronan oligosaccharides or hyaluronidase treatment, which more effectively disrupted the pericellular matrix, had similar effects. CD44 and β1 integrins co-localized in the cell membrane and along some stress fibers. However, CD44 and hyaluronan were specifically excluded from focal adhesions, and associated primarily with cortical actin. Time-lapse imaging of the immediate effects of hyaluronidase digestion showed that hyaluronan matrix primarily mediates attachment of membrane and cortical actin between focal contacts, suggesting that surface adhesion through hyaluronan and CD44 is distinct from focal adhesion through β1 integrins and fibronectin. Fluorescein-labeled hyaluronan bound regularly along fibronectin fibers and co-localized more with β1 integrin and less with CD44. Therefore, the hyaluronan matrix can interfere with the assembly of fibrillar ECM components, and this interplay regulates the degree of myofibroblast formation. These data also suggest that adhesion through hyaluronan matrix impacts cytoskeletal organization, and is potentially part of a clutch mechanism that regulates stick and slip of myofibroblasts by affecting the adhesion to and organization of fibronectin and collagen.

Introduction

Differentiation of fibroblasts and other cells into the myofibroblast functional state is controlled by transforming growth factor-beta 1 (TGF-β1), a well-known driver of fibrosis in lungs and other tissues [1]. Expression of alpha smooth muscle actin (αSMA), which contributes to cellular contractility, is a characteristic feature of myofibroblasts, as is increased mechanical coupling [2], and production of extracellular matrix (ECM) components. Tissue or substrate stiffness and tensile mechanical loading also drive the formation of myofibroblasts as part of a detrimental feed forward loop in which cellular contraction serves to mechanically activate latent TGF-β1 via integrins that are implicated in

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myofibroblast mechanoperception and transduction [1]. However, how and whether specific ECM components control the differentiation and stability of the myofibroblasts is not clear. Matrix adhesion structures may be promising targets to modulate myofibroblast differentiation and activity and diminish fibrosis, and clarifying the interactions among matrix components and their cell surface receptors will be vital for developing the most effective strategies.

Hyaluronan is an extracellular glycosaminoglycan important in cell proliferation, migration, wound healing, and inflammation, but its role in fibrosis is only beginning to be studied. Reduced turnover of hyaluronan via knockout of the hyaluronan receptor, CD44, has been linked to potentiation of inflammation and lung fibrosis [3]. Hyaluronan appears to play a direct role in the differentiation of myofibroblasts [4], and previous studies have also suggested a role for pericellular hyaluronan in generating and maintaining the myofibroblast phenotype by modulating TGF-β1 signaling pathways [5,6]. As a surface coating, hyaluronan is primarily anti-adhesive for cells that already have it on their surface [7] and its effects on collagen gel contraction by fibroblasts in vitro are variable [8,9]. High hyaluronan production has also been linked to detachment of cells [10,11]. Therefore, the question of how hyaluronan controls myofibroblast adhesion, differentiation, and matrix assembly remains unclear.

Increased production of fibrillar ECM, particularly collagen and fibronectin, is a hallmark of myofibroblasts and the resulting fibrosis ultimately interferes with tissue function. However, the questions of how these fibrillar ECM components interact with hyaluronan, what controls their interactions, and how important these interactions are to myofibroblast formation and maintenance need to be addressed.

Hyaluronan, in part, plays a space filling role and was shown to affect collagen fibril spacing in synovial tissue [12]. Fibronectin is also deposited by fibroblasts during wound healing and requires β1 integrins to be organized into fibrils [13], but the effects of hyaluronan on fibronectin fiber formation are not known. Earlier studies suggested that hyaluronan binds to cellular extra domain A (EDA)-containing fibronectin [14,15]. Other data suggests there is cross talk between CD44 and β1 integrin receptors and cooperative binding of these receptors to fibronectin [16,17]. However, the physical relationship between these two matrix components and their receptors in myofibroblasts is not clear.

In this study, we test whether hyaluronan affects the assembly of fibrillar matrix components during myofibroblast induction by TGF-β1, as well as determine the relationships between hyaluronan, fibronectin, CD44, β1 integrins, and the cytoskeleton by immunocytochemistry. We wanted to know if inhibition of hyaluronan synthesis or disruption of pericellular matrix integrity during induction by TGF-β1 would affect deposition of fibrillar matrix components in human lung fibroblasts (HLFs) and influence myofibroblast differentiation.

Results

Hyaluronan and fibronectin are closely interwoven in the ECM

The spatial relationship of hyaluronan to fibrillar matrix components has not been extensively studied in myofibroblasts. Therefore, immunocytochemistry was used to compare the distribution of hyaluronan with fibronectin in the ECM of control fibroblasts and TGF-β1-induced myofibroblasts. Compared to non-induced fibroblasts (Fig. 1A), stronger staining for both hyaluronan and fibronectin was seen in the myofibroblasts (Fig. 1B), where the hyaluronan was present in the form of cable-like structures. The molecules co-localized on the substrate and in the matrix above the myofibroblasts. Control fibroblasts tended to have less pericellular hyaluronan, less fibronectin, and consequently less colocalization. However, higher magnification revealed that in both control fibroblasts and TGF-β1-treated cells, the processes of fibronectin fibril formation and hyaluronan pericellular matrix formation are closely juxtaposed along the membrane, controlled via the same microspikes and filopodia, indicating that these matrix components are spatially positioned to interact with each other directly upon secretion (Fig. 1C and D). As was previously described [13], cells use tractional forces to pull globular fibronectin that was deposited on the substrate into thick mature fibrils, and our results showed that the same filopodia and finer protrusions that participated in this process were also hyaluronan-positive. Other images clearly indicated that cells closely interweave the hyaluronan cables and fibronectin fibers as the matrix is laid down (Fig. 1E). This suggests that hyaluronan may coat the fibronectin fibrils to varying extents. Very little collagen was detected in control cells and only occasional colocalization of collagen with endogenous hyaluronan was seen in myofibroblasts (data not shown).

Exogenous hyaluronan binds fibronectin fibers

Previous studies have shown that hyaluronan binds cellular fibronectin [14,15], and others have shown a relationship between myofibroblast differentiation and reduced hyaluronan turnover [4]. We tested whether the interaction of hyaluronan and fibronectin would influence hyaluronan binding and uptake in myofibroblasts. Initial studies determined the degree of binding of exogenous fluorescein-labeled hyaluronan to control fibroblasts and TGF-β1-induced myofibroblasts during

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