Myofibroblasts work best under stress

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Summary Myofibroblasts are reparative connective tissue cells that contribute to the reconstruction of injured tissue by secreting new extracellular matrix and by exerting high contractile force. Deregulation of these activities results in tissue contracture and development of fibrosis which makes the myofibroblast an important target for anti-fibrotic therapies. Two principle factors drive the development of myofibroblasts from different precursor cells and guarantee maintenance of the contractile phenotype: mechanical stress and transforming growth factor beta (TGFβ1). In this mini-review, we recapitulate the current understanding (1) of how myofibroblasts feel stress using specialized matrix adhesions, (2) of the level of stress that is required to induce their development and (3) of how myofibroblast mechanical activity can have a direct influence on the level of TGFβ1 activation. From these findings it emerges that the specific matrix adhesion structures of myofibroblasts are promising targets to modulate myofibroblast differentiation and activity.

Introduction

When tissues are injured, locally residing connective tissue fibroblasts and cells recruited from other sources acquire smooth muscle features by forming contractile stress fibers and by neo-expressing α-smooth muscle actin (α-SMA) (Hinz, 2007) (Figure 1). The high contractile activity generated by α-SMA in stress fibers plays a fundamental role in remodeling injured tissue such as during skin wound healing (Hinz et al., 2001a). Of high clinical relevance are the retractile phenomena, caused by excessive myofibroblast contractile and extracellular matrix (ECM) secreting activity, which characterize the vast majority of fibrocontractive diseases. This includes fibrosis affecting vital organs, such as heart (Virag and Murry, 2003), liver (Desmouliere et al., 2003), kidney (Lan, 2003)
and lung (Phan, 2002) and fibrosis reducing life quality in scleroderma (Rajkumar et al., 2005), hypertrophic scars (Desmouliere et al., 2003; Gabbiani, 2003), airway remodeling in asthma (Chiappara et al., 2001) and Dupuytren’s disease (Tomasek et al., 1999). Moreover, myofibroblasts at the tumor invasion front are activated by the transformed epithelium to stimulate tumor growth and invasion by promoting angiogenesis and tumor cell migration (Desmouliere et al., 2004). But don’t get fooled—the myofibroblast is not necessarily the ‘bad guy’ as it contributes to physiological wound healing and its absence has been associated with development of chronic wounds (Gabbiani, 2003). In addition, contractile fibroblasts have been suggested to generate a beneficial mechanical stress or ‘tonus’ in some normal tissues, such as in the alveolar septa in the lung and possibly the human dorsal fascia, as it has emerged from the first Fascia Research Congress in Boston 2007.

To develop novel strategies counteracting myofibroblast malfunction in fibrosis and controlling beneficial myofibroblast action in physiological tissue repair, it is important to understand the molecular mechanisms that regulate myofibroblast formation and activity. Over the past 15 years, the pro-fibrotic cytokine transforming growth factor beta1 (TGFβ1) has been established as the most potent inducer of myofibroblast differentiation (Desmouliere et al., 1993; Ronnov-Jessen and Petersen, 1993). However, during tissue repair, reparative fibroblasts are not only in contact with an altered chemical milieu but face a dramatically changed mechanical microenvironment. In fact, expression of α-SMA demands both, a mechanically restrained environment and the action of TGFβ1. In this mini-review, we will elaborate that mechanical stress and TGFβ1 are actually two sides of the myofibroblast differentiation coin, as recently reviewed in greater detail (Wipff and Hinz, 2008).

How stiff is the myofibroblast environment?

Having spent most of their lives shielded by a protective ECM, fibroblasts are exposed to considerable stress when the protective ECM structure is lost after tissue injury (Tomasek et al., 2002). In response to this mechanical challenge, fibroblasts develop tension on their own by building up a contractile stress fiber apparatus that is used to stiffen newly secreted ECM. What is a ‘stiff’ ECM in a physiological sense and how does ECM stiffness develop during tissue repair and remodeling? The stiffness of the provisional ECM of early wounds is comparable with the elastic modulus of ~10–100 Pa of newly polymerized collagen gels, frequently used in vitro models of tissue repair (Carlson and Longaker, 2004; Grinnell, 2003). Fibroblasts initiated in such gels organize actin filaments in a sub-membrane cortex (Tamariz and Grinnell, 2002). Gel and tissue stiffness at this stage have not been measured but neo-formation of stress fibers in fibroblasts grown on soft two-dimensional culture substrates occurs at an elastic modulus of ~3000–6000 Pa (Discher et al., 2005; Yeung et al., 2005).

Expression of α-SMA and incorporation into pre-existing stress fibers requires significantly higher ECM stiffness that begins to develop in anchored collagen gels after 2–3 days of remodeling (Hinz, 2007).
How do myofibroblasts feel the stress?

It is widely accepted that fibroblastic cells perceive mechanical signals from the ECM by utilizing integrins, i.e. the same transmembrane proteins that anchor stress fibers to the substrate through a complex of cytoplasmic proteins (Bershadsky et al., 2006; Chen et al., 2004; Giannone and Sheetz, 2006). Integrins are αβ heterodimers that combine to 24 currently identified cell receptors in mammals binding to various ECM proteins (Hynes, 2002; Luo et al., 2007; Sheppard, 2000). The signaling role of integrin-based ECM adhesions becomes clear from their maturation in response to mechanical challenge. Reinforcement of adhesion sites under stress starts with integrin clustering into nascent adhesions that further develop into focal complexes when associating with actin filaments. Increasing intracellular stress that needs to be balanced by a mechanically resistant substrate then lead to the enlargement of ~1 μm² focal complexes into classical focal adhesions (Bershadsky et al., 2006).

Ultrastructural analysis of myofibroblasts in fibrotic and wound granulation tissue has revealed massive cell–ECM contacts that are not formed by normal connective tissue fibroblasts (Eyden, 2005). Analogous to this so-called fibronexus, cultured myofibroblasts develop specialized ‘supermature FAs’, a designation that accounts for their significantly longer appearance (8–30 μm) (Dugina et al., 2001) compared with the FAs (2–6 μm) of α-SMA-negative fibroblasts (Geiger et al., 2001) (Figure 1). Supermature FAs exhibit a specific molecular composition which is subject of a more comprehensive recent review (Hinz, 2006). Establishment of supermature FAs depends both on the high intracellular contractile activity generated by α-SMA and on a stress-resistant ECM (Hinz, 2006). Myofibroblasts reduce supermature FAs to small classical FAs after inhibition of α-SMA-mediated contraction (Hinz et al., 2003) and when cultured on soft substrates and in newly polymerized collagen gels (Goffin et al., 2006; Hinz, 2006) (Figure 1). These studies suggest that fibroblasts gain information about the mechanical state of the ECM by assessing the level of tension in stress fibers, which is limited by the size of their ECM anchors. Myofibroblasts grown on arrays of FA adhesion islets (≤6 μm long) rapidly lose α-SMA from stress fibers (Figure 2), which however is preserved on supermature FA islets (>8 μm long).

It is believed that specific proteins in the cytoplasmic adhesion plaque serve as mechanosensors and act as molecular switches that change their conformation/activation state when force is applied (Giannone and Sheetz, 2006). However, adhesion-mediated mechanical switches and signal generators are not necessarily localized within the cell. The first ECM protein to be identified as a mechanosensitive protein was fibronectin, which reveals cryptic sites for auto-fibrillogenesis when unfolded upon cell traction (Zhong et al., 1998). ECM protein unfolding may similarly reveal specific integrin-binding sites and thus change cell adhesion-dependent responses such as cell migration, proliferation, survival, and differentiation as well as ECM organization and remodeling (Vogel and Sheetz, 2006).
TGFβ1 activation—when the insoluble becomes soluble

A novel mechanism to translate cell forces into biochemical signals is the liberation of growth factors from stores in the ECM. Such a mechanism was recently demonstrated for the activation of the pro-fibrotic cytokine TGFβ1 by epithelial cells (Jenkins et al., 2006) and by myofibroblasts (Wipff et al., 2007). TGFβ1 is synthesized as a homodimeric protein together with the latency-associated protein (LAP); activation of TGFβ1 requires its dissociation from LAP (Annes et al., 2003). The majority of cell types secrete TGFβ1 as part of the large latent complex (LLC), consisting of TGFβ1, LAP and the latent TGFβ1 binding protein (LTBP-1) (Annes et al., 2003; Todorovic et al., 2005) (Figure 3). LTBP-1 is an ECM protein that binds to several other ECM components, including fibrillin-1, fibronectin and vitronectin, thereby providing a reservoir of latent TGFβ1 in the ECM (Annes et al., 2003; Todorovic et al., 2005).

Activation of TGFβ1 by its dissociation from LAP and/or the ECM-bound LLC is promoted by various mechanisms, which differ according to the cell type and the physiological context. Latent TGFβ1 activation occurs upon proteolytic cleavage, by interaction with thrombospondin 1 and with the mannose-6-phosphate receptor (Annes et al., 2003; Jenkins, 2007). More recently, integrins αvβ5, αvβ6, and αvβ8, a yet unidentified β1 integrin and possibly αvβ3 integrin have been reported to participate in activating TGFβ1 (Sheppard, 2005; Wipff and Hinz, 2008). The knockout of integrin subunits which participate in TGFβ1 activation produces phenotypes that closely resemble that of a TGFβ1 knockout (Bader et al., 1998; Huang et al., 1996; Shull et al., 1992; Zhu et al., 2002).

Importantly, the lungs of β1 knockout mice are protected from bleomycin-induced fibrosis (Munger et al., 1999) and inhibition of αvβ5 integrin in vitro reduces the fibrogenic character of fibroblastic cells (Asano et al., 2006). Hence, interfering with the integrins that are implicated in TGFβ1 activation is a possible therapeutic strategy to counteract the harmful activity of active TGFβ1 in a cell-specific manner, without impairing its beneficial effects on other cell types.

How can integrins activate a growth factor? Two distinct mechanisms are currently discussed which appear to co-exist. In the first mechanism that is sensitive to protease inhibitors, integrins are proposed to serve as a common docking point for latent TGFβ1 and its activating proteases (Jenkins, 2007; Sheppard, 2005; Wipff and Hinz, 2008). The second mechanism is independent from any proteolytic action and involves cell traction forces which are directly transmitted to the LLC via integrins. The resulting conformational change in the latent complex is suggested to liberate TGFβ1 and/or to present it to its receptor (Figure 3). It appears that at least four conditions need to be satisfied for this conformational change: (1) specific integrin binding to the LLC, (2) the presence of the contractile actin cytoskeleton to generate force and/or to provide mechanical resistance, (3) incorporation of latent TGFβ1 into the ECM as LLC, and (4) an ECM that mechanically resists the cellular traction forces exerted to the LLC (Figure 3).

First evidence that integrins can directly activate TGFβ1 independently from any proteolytic activity came from studies on the epithelial integrin αvβ6 (Annes et al., 2004; Jenkins et al., 2006; Munger et al., 1999). Very recently, we elucidated that integrin αvβ5 and possibly αvβ3 integrin can...
directly activate TGFβ1 in myofibroblasts (Wipff et al., 2007). All integrins that contribute to the activation of TGFβ1, regardless of the underlying mechanism, physically interact with the LAP portion of the LLC (Sheppard, 2005). Interfering with the respective binding sequence abolishes TGFβ1 activation in cultured epithelial cells (Munger et al., 1999) and in myofibroblasts (Wipff et al., 2007). Knockout of this binding site in mice results in defects in vasculogenesis, a deficient immune system and enhanced inflammation, similar to a TGFβ1 knockout (Yang et al., 2007).

Incubating purified integrins with latent TGFβ1 alone is not sufficient to activate the LLC and association of integrins with the cytoskeleton appears mandatory for this action. Both disruption of actin filaments with cytochalasin D and truncation of the αvβ6 integrin cytoplasmic tail prevent TGFβ1 activation (Munger et al., 1999; Sheppard, 2005). Subsequent studies carried out with β6 integrin-transfected fibroblasts, overexpressing either constitutively active or dominant negative forms of the small GTPase RhoA, consolidated the role of the cytoskeleton in the activation process (Jenkins et al., 2006). Activation of RhoA is a central element in promoting actin-myosin contraction in these cells and the level of RhoA activity correlates with their level of TGFβ1 activation (Jenkins et al., 2006). First direct evidence for a contribution of mechanical stress in TGFβ1 activation was recently provided by experiments with myofibroblasts that activate TGFβ1 as a function of their contractile activity (Wipff et al., 2007). Inducing myofibroblast contraction with thrombin, angiotensin-II and endothelin-1 increases TGFβ1 activation; this effect depends on integrin binding to LAP (Wipff et al., 2007).

The third mandatory element for traction-mediated activation of TGFβ1 by integrins is incorporation of the LLC into the ECM. The connection between LLC–ECM binding and integrin-mediated release of active TGFβ1 was recently elucidated by identifying the pivotal ECM binding motif in the LTBP-1 protein (Annes et al., 2004). An engineered construct comprising only the LAP-binding domain and the N-terminal ECM-binding hinge region of LTBP-1 is sufficient to substitute for this function of the full-length LTBP-1 (Annes et al., 2004). Deletion of this ECM-binding hinge region from LTBP-1 abolishes αvβ6 integrin-mediated activation of TGFβ1 (Annes et al., 2004).

The physiological relevance of the relationship between binding of the LLC to the ECM and TGFβ1 activation is understandable in the context of connective tissue repair and development progressive fibrosis. In these situations, ECM stiffness gradually increases with ongoing fibroblast remodeling activity (Hinz and Gabbiani, 2003). To induce a conformational change in the latent TGFβ1 complex by integrin-mediated pulling on the LAP, the ECM must provide mechanical resistance.

![Figure 3](image-url)
(Figure 3). Indeed, myofibroblasts only activate TGF-β1 by integrin-mediated contraction when cultured on silicone substrates with a stiffness that corresponds to that of contracting fibrotic and granulation tissue but not when grown on substrates exhibiting the compliance of normal connective tissue (Wipff et al., 2007). We propose that linking the level of active TGF/β1 release with the degree of ECM stiffness restricts generation and autocrine maintenance of the myofibroblast phenotype to the appropriate (contractile) mechanical microenvironment (Figure 3).

Conclusion

Myofibroblasts adapt their often harmful contractile activity to the level of stress in their surrounding tissue. To detect mechanical changes in the microenvironment, they utilize the same proteins that ultimately transmit stress fiber contraction to the ECM, namely integrins. To prevent formation of the myofibroblast, we propose to target these stress-sensors, in other words to render them blind for mechanical inputs. In addition, it becomes increasingly clear that activation of TGF/β1 is confined to cells expressing the appropriate integrin in the appropriate physiological context. Clearly, this renders local targeting of specific integrins a possible therapy to reduce the profibrotic effects of TGF/β1.

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