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Fibroblast expression of α -smooth muscle actin, $\alpha 2\beta 1$ integrin and $\alpha v\beta 3$ integrin: Influence of surface rigidity

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Abstract

Open wound contraction necessitates cells and connective tissue interactions, that produce tension. Investigating fibroblasts responses to tension utilizes collagen coated polyacrylamide gels with differences in stiffness. Human foreskin fibroblasts were plated on native type I collagen-coated polyacrylamide gels coverslips with different rigidities, which was controlled by bis-acrylamide concentrations. Changes in α smooth muscle actin (α SMA), $\alpha 2\beta 1$ integrin (CD49B) and $\alpha v\beta 3$ integrin (CD-51) were documented by Immuno-histology and Western blot analysis. Cells plated on rigid gels were longer, expressed $\alpha v\beta 3$ integrin and α SMA within cytoplasmic stress fibers. In contrast, cells on flexible gels were shorter, expressed $\alpha 2\beta 1$ integrin and had fine cytoskeletal microfilaments without α SMA. Increased tension changed the actin makeup of the cytoskeleton and the integrin expressed on the cell's surface. These in vitro findings are in agreement with the tension buildup as an open wound closes by wound contraction. It supports the notion cells under minimal tension in early granulation tissue express $\alpha 2\beta 1$ integrin, required for organizing fine collagen fibrils into thick collagen fibers. Thicker fibers create a rigid matrix, generating more tension. With increased tension cytoskeletal stress fibers develop that contain α SMA and $\alpha v\beta 3$ integrin replaces $\alpha 2\beta 1$ integrin, consistent with cell switching from collagen to non-collagen proteins interactions.

Keywords

Myofibroblast; Tension; Polyacrylamide gel; Collagen; Integrin; α Smooth Muscle Actin

Introduction

Fibroblasts play a central role in restoring the integrity of the dermis in response to dermal loss. The wound fibroblast performs many activities: migration into the dermal defect,

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Conflict of Interest

The authors declare there is no conflict of interest.

proliferation, synthesis of macromolecules, organization of the newly synthesized macromolecules into a new connective tissue matrix, and then disappearance by apoptosis. In the initial stage of wound healing, the cavity caused by tissue loss is filled with a fibrin matrix that has two primary functions. First, a deposited fibrin clot controls bleeding. Secondly the fibrin matrix serves as the highway for the invasion of inflammatory cells and wound fibroblasts into the wound site (Desmouliere et al., 2003). Chemotactic cytokines are secreted in the local wound environment by platelets and leukocytes, activating quiescent fibroblasts to migrate into the wound (Hinz and Gabbiani, 2003). Activated fibroblasts contain fine actin cytoplasmic filaments associated with cell locomotion, which enhances fibroblast migration into the wound site. Young fibroblasts produce and release macromolecules, which includes collagen, the structural component the newly-generated granulation tissue matrix that replaces the fibrin matrix. The reorganization of collagen fibrils into thicker collagen fiber bundles by fibroblast compacts the newly deposited connective tissue matrix within granulation tissue, leading to wound contraction.

The manner in which wound fibroblasts respond to surrounding tension influences wound repair. The compaction of granulation tissue pulls on the surrounding dermis, generating tension. The wound fibroblast matures and transforms into a myofibroblast, characterized by prominent cytoplasmic stress fibers with the alpha smooth muscle actin (α SMA) isoform of actin. The myofibroblast is the icon of fibrosis, associated with granulation tissue and wound contraction, as well as chronic forms of fibrosis that include hypertrophic scar and cirrhosis (Desmouliere et al., 2005). Released soluble factors that direct cell-cell interactions and cell-matrix interactions are involved in initiating and controlling numerous fibroblast activities. One of these factors, transforming growth factor β_1 (TGF- β_1), is released from fibroblasts as well as inflammatory cells and is a major factor directing the transformation of fibroblasts into myofibroblasts. In this study, the focus is on changing the fibroblast phenotype to the myofibroblast through ECM-fibroblast interactions, rather than by the exogenous introduction of cytokines such as TGF- β_1 .

An in vitro model for studying fibroblast interaction with the surrounding connective tissue matrix is the fibroblast populated collagen lattice (PCL), which was first introduced by Bell and his co-workers (Bell et al., 1979). The Bell fibroblast PCL is a three-dimensional, free-floating, cell-populated collagen matrix, which undergoes lattice contraction, containing exclusively fibroblasts, as evidenced by cells that retain an elongated morphology as the PCL are reduced in size and that fail to express α SMA (Arora et al., 1999; Eckes et al., 2006; Ehrlich, 1988). An alternate model of contracting fibroblast PCL is the attached-delayed-released PCL. Fibroblast PCL are anchored to the underlying surface of a tissue culture dish for 4 days. At day 4 the entire cell population consists of myofibroblasts, all cells expressing α SMA in cytoskeletal stress fibers. The release of these attached lattices produces rapid lattice contraction through the contraction of the resident myofibroblasts (Tomasek et al., 1992).

The expression of α SMA in myofibroblast stress fibers can be induced by TGF- β_1 and by placing cells under tension. Including TGF- β_1 with dermal fibroblasts in monolayer culture increases the proportion of cells expressing α SMA in stress fibers (Desmouliere et al., 1993). Mechanical tension also enhances α SMA expression within fibroblasts maintained on a stiff collagen-coated surface. Increased mechanical tension on cells increases the expression of α SMA (Arora et al., 1999). The $\alpha_2\beta_1$ integrin on the surface of fibroblasts fixes fibroblasts to collagen and is required for free-floating fibroblast PCL contraction (Gullberg et al., 1989). Increased $\alpha_2\beta_1$ expression is a common feature of fibroblasts suspended in free-floating PCL (Eckes et al., 2006). Initially, expression of $\alpha_2\beta_1$ integrin is increased in fibroblasts within contracting PCL, but the level of expression returns to baseline following the completion of compaction activities (Klein et al., 1991). Compared to

cells growing on plastic culture dishes, fibroblasts on collagen-coated dishes express greater amounts of $\alpha 2\beta 1$ integrin. In contrast, those same cells maintained on a rigid plastic surface express α SMA and express little $\alpha 2\beta 1$ integrin, which suggests that myofibroblasts attachment to collagen differs from fibroblasts (Ehrlich et al., 1998).

Thin, collagen-coated polyacrylamide gels adherent to glass cover slips is a technique for the study of substrate rigidity on fibroblast physiology. Because of the inert nature of the polyacrylamide surface, cells interact with the substrate solely through the collagen-coated surface. For the study of mechanical tension on cell physiology, these gels have advantages over the PCL model (Beningo et al., 2002; Wang and Pelham, 1998). Through the thin gel surface, cells are observed and imaged at high resolution. The polyacrylamide substrate displays nearly ideal elastic properties over a wide range of stiffness. Substrate rigidity can be varied in a controlled, sequential and consistent manner by altering the concentration of the cross-linking element of the gel, bis-acrylamide. Such stiffness-controlled properties facilitate a more precise measurement of fibroblast response to substrate tension.

In this study, collagen-coated polyacrylamide gels of varying flexibility or stiffness examine the effects of substrate rigidity on fibroblast morphology and the expression of α SMA, $\alpha 2\beta 1$ integrin, and $\alpha \nu \beta 3$ integrin. We compare changes in human dermal fibroblasts phenotype, growing on relatively flexible surfaces and on rigid substrates. Fibroblasts responses to tension are to increase the expression of α SMA and $\alpha \nu \beta 3$ integrin, while limiting the expression of $\alpha 2\beta 1$ integrin.

Methods

Preparation of polyacrylamide gel sheets

Thin polyacrylamide gel film was deposited on activated glass surfaces by the technique of Wang and Pelham (1998). Briefly, glass cover slips were activated by cleaning with ethanol, treating with sodium hydroxide, followed with 3-aminopropyltrimethoxysilane and fixed with glutaraldehyde. A 10% polyacrylamide solution with either 0.4% bis-acrylamide to create a rigid substrate or 0.025% bis-acrylamide for a flexible substrate was pipetted on the activated glass surface. The polyacrylamide was cross linked and coated with rat tail tendon acid extracted type I collagen (Ehrlich and Rittenberg, 2000). The stiff 0.4% and flexible 0.025% bis-acrylamide collagen coated glass surfaces were available for the plating human dermal fibroblasts. Primary derived human foreskin fibroblasts between their 4th and 9th passage maintained in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 10 μ g/ml gentamicin, referred to as complete DMEM, were released from tissue culture dishes by trypsinization, transferred to coverslips with collagen coated polyacrylamide surfaces and maintained in complete DMEM. Fibroblasts were observed by phase-contrast microscopy. Cells were photographed on day 1, 2 and 3 and cell lengths measured with ImageJ software (National Institutes of Health, 2007, Version 1.38x).

Immuno-histology

On days 1 and 3, fibroblasts maintained on either 0.4% or 0.025% bis-acrylamide substrates were fixed with 4% paraformaldehyde in cytoskeletal buffer (137 mM NaCl; 5 mM KCl; 4 mM NaHCO₂; 2 mM MgCl₂; 3.5 mM glucose; 2 mM EGTA, 1.5 mM phosphate buffer, and 5 mM PIPES buffer pH 6.1). Fixed cells were washed and then permeablized with 0.1% Triton X-100 in cytoskeletal buffer. Some fixed, permeablized cell preparations were incubated with rabbit monoclonal antibody directed to vinculin (Cat. # V4139 Sigma) and others were incubated with mouse monoclonal antibody directed to α SMA (Cat. # A2547 Sigma). Unbound primary antibody was washed away and the cell preparations incubated

with a secondary antibody: an Oregon-green-tagged goat anti-rabbit IgG antibody or a rhodamine-tagged donkey anti-mouse IgG antibody. All cell preparations received AlexaFluor phalloidin to label filamentous actin and DAPI to stain nuclei fluorescent blue (Invitrogen). Stained preparations were mounted on glass microscope slides and viewed with an inverted Zeiss fluorescent microscope. Photographs were taken, using a 40x water-immersion objective with a CoolSnap video camera.

Western blot analysis

On day 2, media was replaced with complete DMEM supplemented with 50 µg/ml ascorbic acid 1-phosphate sesquimagnesium salt (Sigma Aldrich, St. Louis, MO). On day 3, the fibroblasts were prepared for Western blot analysis. The fibroblast monolayer on the collagen-polyacrylamide surface was covered with 100 µl of lysis buffer [2% SDS in Tris buffer pH 6.8], glycerol and proteinase inhibitors (Roche Diagnostics, Indianapolis, IN) for 5 min. While keeping the collagen-polyacrylamide surface intact, cell contents were collected by scrapping the cells in lysis buffer onto a paraffin sheet. The harvested cell lysate was pipette into a micro centrifuge tube, sonicated, then boiled for 5 minutes and centrifuged to remove particulate matter. Equal volumes of lysate-supernatant from cultured fibroblast lysates were electrophoresed on a 4–20% Tris-HCl gradient gel. The proteins were transferred to nitrocellulose membranes by electrophoresis and the protein bands prepared for immune-precipitation detection. The membrane was probed with mouse monoclonal antibodies directed to α SMA (Cat. # A2547 Sigma Aldrich) and α -tubulin (Cat. # T5168 Sigma Aldrich), followed by a horseradish-peroxidase-conjugated secondary antibody (Jackson Immuno Research, West Grove, PA). Detection of antibody-protein bands used chemluminescence with SuperSignal West Dura Extended Duration Substrate (Thermo Scientific, Rockford, IL), and X-ray film. The antibody-protein complex bands were eluted from the membrane and the membrane was reprobed with mouse monoclonal antibodies directed to α 2 β 1 integrin/CD49b (Upstate Biotechnology, Lake Placid, NY) and the vitronectin receptor/CD51/ α v integrin (Zymed Laboratories, San Francisco, CA) and the protein bands detected as described above.

Results

Morphological features of fibroblasts grown on the more rigid substrate, 0.4% bisacrylamide, were different from fibroblasts grown on the more flexible substrate, 0.025% bisacrylamide. When cell lengths were measured from phase-contrast images, the cells on the rigid substrate were more elongated (Fig. 1A and 1C), as compared to cells maintained on the flexible substrate (Fig. 1B and 1D). The measured mean cell length of fibroblasts plated on the rigid substrate was 260 microns at day 3, compared to a mean cell length on more flexible substrate of 171 microns, an increase of 34%. These differences were highly significant with a p value < 0.0001 (Table 1). The increased differences in length of cells on stiff substrate versus flexible substrates was shown as early as day 1, where fibroblasts on stiff surfaces had an average a length of 245 microns as compared to 193 microns for cells on the flexible surface, a difference of 21%. That significant difference was found on day 2, 18% difference, reaching the maximum difference on day 3.

Images taken on days 1 and 3 with a fluorescent microscope showed differences in the organization of the fibroblast's cytoskeleton and α SMA expression. Fibroblasts grown on the more flexible substrate failed to express α SMA and had fine microfilaments arranged in stress fibers (Fig. 2B). At 1 day, fibroblasts plated and maintained on the rigid substrate had more prominent, thick, actin-rich microfilaments arranged in stress fibers, but expressed minimal levels of α SMA (Fig. 2A). At 3 days, thick actin-rich filaments were a common feature on the plasma membrane of fibroblasts grown on either firm or flexible collagen coated substrates. However, actin filaments within the cytoplasm of these cells showed

distinct variations. At 3 days, fibroblasts maintained on the rigid substrate had maintained their actin-rich cytoplasmic stress fibers that were now enriched in α SMA (Fig. 2C). Fibroblasts on the flexible surface had developed actin rich stress fibers, but they were devoid of α SMA (Fig. 2D). Based upon α SMA expression, the fibroblasts maintained on firmer 0.4% bis-acrylamide substrate for 3 days had transformed into myofibroblasts. Fibroblasts growing on the more flexible substrate at 3 days had retained their fibroblast phenotype and had not transformed into myofibroblasts. Western blot analysis presented in Fig. 3 confirms that fibroblasts maintained on the more flexible substrate at 3 days failed to express α SMA within cytoplasmic stress fibers.

As shown in Fig. 3, differences in protein levels for α SMA, $\alpha 2\beta 1$ integrin of the collagen-binding integrin CD-49b receptor, and αv integrin from the CD-51 receptor between fibroblasts maintained on a firm substrate compared to maintenance on a flexible substrate. At 2 days, there were clear differences in the density of the protein bands of these three proteins depending upon whether fibroblasts were growing on rigid collagen-coated surfaces or more flexible collagen-coated surfaces. The increased expression of α SMA in fibroblasts maintained on the 0.4% bis-acrylamide identified by immuno-histology was confirmed by Western blot analysis (Fig. 3A). In addition, fibroblast expression of the collagen receptor, $\alpha 2\beta 1$ integrin, was greater in fibroblasts plated on flexible substrate as compared to cells residing on rigid substrates (Fig. 3B). The $\alpha 2$ integrin was characteristically expressed in fibroblasts and minimally expressed in myofibroblasts. An integrin associated with myofibroblasts is the CD-51 receptor or the $\alpha v\beta 3$ integrin, which showed greater expression in myofibroblasts that developed on the firmer collagen-coated substrate (Fig. 3C).

Discussion

Changes in fibroblast morphology, where α SMA expression in cytoskeletal stress fibers are implicated in numerous disease processes (Desmouliere et al., 2003). In vitro a proportion of fibroblasts growing in monolayer culture on plastic with serum supplemented culture medium develop cytoskeletal stress fibers expressing α SMA (Desmouliere et al., 1992). The inclusion of TGF $\beta 1$ with fibroblasts in monolayer culture enhances the expression of α SMA within cytoskeletal stress (Desmouliere et al., 1993). Plating human dermal fibroblasts at low density in monolayer, generates greater than 95% of the cells expressing α SMA in stress fibers, while plating fibroblasts at high density generates few cells expressing α SMA in stress fibers (Masur et al., 1996). Fibroblasts growing on collagen coated dishes express little α SMA as compared to fibroblasts growing on naked plastic surfaces, which express more α SMA (Ehrlich et al., 1998). The mechanism for cell adhesion is influenced not only by the composition of the surface on which they adhere, but also to the rigidity of the substrate beneath that surface on which those cells adhere (Arora et al., 1999; Eckes et al., 2006). Here, fibroblasts on collagen coated surfaces of 0.4% bis-acrylamide (a ridged surface) express α SMA and $\alpha v\beta 3$ integrin compared to fibroblasts residing on collagen coated surfaces of 0.025% bis-acrylamide, a flexible surface (Yeung et al., 2005).

An in vitro model for studying fibroblasts interactions with their surrounding connective tissue matrix is the fibroblast populated collagen lattice (PCL), which was first introduced by Bell and his co-workers (Bell et al., 1979). The Bell fibroblast PCL is a free-floating PCL, which undergoes a slow steady reduction in area, exclusively contains fibroblasts, cells that fail to express α SMA and retain an elongated shape as PCL contract (Arora et al., 1999; Eckes et al., 2006; Ehrlich, 1988). In a free floating PCL, a major degree of lattice contraction is achieved by 2 days in the absence of tension. Fibroblasts lack cytoskeletal stress fibers and fail to express α SMA (Ehrlich et al., 2006). By polarized light microscopy, contracting free floating PCL develop thick collagen fibers exhibiting birefringence. The collagen fibers birefringence runs parallel to the long axis of elongated fibroblasts (Ehrlich,

1988). Those same fibroblasts cast in an attached PCL for 4 days, where tension develops; all the cells express α SMA in stress fibers, having transformed into myofibroblasts (Tomasek et al., 1992). Upon release at 4 days, in minutes these once attached PCL undergo rapid lattice contraction via the contraction of the resident myofibroblast populations. However, unlike free floating PCL, where there is minimal tension, no collagen fibers birefringence develops (Ehrlich et al., 2006). The compaction of a collagen lattice under tension through rapid myofibroblast contraction fails to organize fine collagen fibrils into longer, thicker parallel oriented collagen fibers. The $\alpha 2\beta 1$ integrin, recognized as the fibroblast-collagen binding integrin (Klein et al., 1991) is required for free floating fibroblast PCL contraction (Gullberg et al., 1989). In unpublished studies there is enhanced $\alpha \nu \beta 3$ integrin expression in attached PCL.

By Western blot analysis human dermal fibroblasts growing on collagen coated rigid surfaces, experiencing mechanical tension, express increased levels of α SMA and $\alpha \nu \beta 3$ integrin, and decreased levels of $\alpha 2\beta 1$ integrin. CD-51 receptor, $\alpha \nu \beta 3$ integrin, contributes to the generation of α SMA expression. Treating cells with antibodies directed to the $\alpha \nu$ integrin, down regulates the expression α SMA (Lygoe et al., 2007). The $\alpha \nu \beta 3$ integrin binds to a variety of matrix proteins that include vitronectin and fibronectin. Blocking the expression of $\alpha \nu$ integrin reduces fibroblast migration (Lygoe et al., 2007). In the absence of $\alpha \nu$ integrin expression, TGF $\beta 1$ induced expression of α SMA is impaired (Lygoe et al., 2004). Mechanical stress renders fibroblasts more susceptible to growth factors. As an example ED-A fibronectin in combination with TGF $\beta 1$ together transform fibroblasts into myofibroblasts (Hinz et al., 2001). A proposed mechanism for controlling cell phenotype is through mechanical tension (Jiang et al., 2006). The sensing of mechanical tension utilizes intracellular signaling through Receptor-like Protein Tyrosine Phosphatase (RPTP α) and $\alpha \nu \beta 3$ integrin, both localized at the leading edge within cells experiencing tension (von Wichert et al., 2003). In the absence of tension, RPTP α cannot interact with the $\alpha \nu \beta 3$ integrin at the cell's periphery. Increasing $\alpha \nu \beta 1$ expression reduces the rate and degree of free floating PCL contraction (Boemi et al., 1999; Lygoe et al., 2004). The $\alpha \nu \beta 3$ integrin RPTP α complex senses mechanical tension at the leading edge of cells, passing on properties of the extracellular matrix to intracellular signal transduction pathways (Jiang et al., 2006).

Besides tension there are other reports of influencing the expression of α SMA in cultured cells in monolayer. Cells plated at low density, upon reaching confluence, most express α SMA in cytoskeletal stress fibers (Masur et al., 1996). Casting a collagen lattice over a confluent layer of low density plated cells, all expressing α SMA within stress fibers, eliminates the expression of α SMA (Ehrlich et al., 2006). In monolayer culture about 20% of fibroblasts plated on plastic express α SMA as well as fail to express $\alpha 2\beta 1$ integrin. Plating those same fibroblasts on a polymerized type I collagen surface, up regulates the expression of $\alpha 2\beta 1$ integrin, while knocking down the expression of α SMA (Ehrlich et al., 1998).

Wound fibroblasts are subjected to changes in tension as the repair process proceeds (Tomasek et al., 2002). After 1 week, fibroblasts within wound granulation tissue express α SMA in cytoskeletal stress fibers, becoming myofibroblasts. Tension within granulation tissue increases as granulation tissue is compacted, which pulls the surrounding skin into the defect. The musculature of surrounding skin response to these forces is pulling out, which contributes to generating tension. The myofibroblasts are the wound fibroblast phenotype that undergoes apoptosis in the remodeling phase of repair (Desmouliere et al., 2005). Tension can interfere with myofibroblasts undergoing programmed cell death. It is proposed myofibroblast populations identified within hypertrophic scars results from mechanical stress as well as inhibiting their entrance into apoptosis (Aarabi et al., 2007; Desmouliere et

al., 2005). In a mouse model a brief period of applied stress, during the proliferative phase of repair, generates excess fibrosis and retards myofibroblasts entrance into apoptosis by activating the pro-survival Akt pathway (Aarabi et al., 2007). Tension also increases the transcription of types I and III collagens as well as the synthesis of tissue inhibitors of metalloproteinases (MMP), which favors connective tissue accumulation (Derderian et al., 2005). Further study in the area of fibroblast response to mechanical stress may elucidate molecular pathways that will provide targets for intervening in the control of fibrosis and scar contracture.

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Fig. 1. Morphology of fibroblasts by phase-contrast microscopy. (A and B) are cultured cells as they appear on day 1. (C and D) are cultured cells as they appear on day 3. (A and C) are cells growing on 0.025% bis-acrylamide, the flexible substrate, (B and D) are cells growing on 0.4% bis-acrylamide, the rigid substrate. The bar represents 100 μ m

Fig. 2. (A) Morphology of fibroblasts grown on 0.025% bis-acrylamide or (B) 0.4% bis-acrylamide and viewed on day 3. Phalloidin stained filamentous actin is green, α -SMA red, and nuclei blue. The bar represents 100 μ m, (C) Fibroblast morphology on day 1 of culture on 0.025% bis-acrylamide and (D) 0.4% bis-acrylamide. Vinculin within focal adhesions is immuno-stained red; phalloidin stained filamentous actin in microfilaments is green, and nuclei are blue. **The bar** represents 100 μ m.

Fig. 3.

Western blots of cell lysates were performed on fibroblasts maintained on 0.4% and 0.025% bis-acrylamide collagen coated substrates for 2 days: (A) αSMA, (B) α2β1 integrin, and (C) αvβ3 integrin. Equivalent protein loading on gels was confirmed by equal band densities in both samples using α-tubulin (data not shown).

Table 1

Comparison of fibroblasts cell lengths grown on stiff compared to flexible substrate.

Day	0.025% Bis Mean Length $\pm$ SD (μ m)	0.4% Bis Mean Length $\pm$ SD (μ m)	Difference P value	% Increase in length
1	193 (54)	245 (90)	<0.003	21%
2	207 (74)	253 (111)	<0.002	18%
3	171 (77)	260 (103)	<0.0001	34%