

Adhesion-contractile balance in myocyte differentiation

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Accepted 17 August 2004
Journal of Cell Science 117, 5855-5863 Published by The Company of Biologists 2004
doi:10.1242/jcs.01496

Summary

Tissue cells generally pull on their matrix attachments and balance a quasi-static contractility against adequate adhesion, but any correlation with and/or influence on phenotype are not yet understood. Here, we begin to demonstrate how differentiation state couples to actomyosin-based contractility through adhesion and substrate compliance. Myotubes are differentiated from myoblasts on collagen-patterned coverslips that allow linear fusion but prevent classic myotube branching. Post-fusion, myotubes adhere to the micro-strips but lock into a stress fiber-rich state and do not differentiate significantly further. In contrast, myotubes grown on top of such cells do progress through differentiation, exhibiting actomyosin striations within one week. A compliant adhesion to these lower cells is suggested to couple to contractility and accommodate the reorganization needed for upper cell

striation. Contractility is assessed in these adherent cells by mechanically detaching one end of the myotubes. All myotubes, whether striated or not, shorten with an exponential decay. The cell-on-cell myotubes relax more, which implies a greater contractile stress. The non-muscle myosin II inhibitor blebbistatin inhibits relaxation for either case. Myotubes in culture are thus clearly prestressed by myosin II, and this contractility couples to substrate compliance and ultimately influences actomyosin striation.

Supplementary material available online at
<http://jcs.biologists.org/cgi/content/full/117/24/5855/DC1>

Key words: Myotube, Differentiation, Prestress, C2C12 cells, Substrate compliance

Introduction

On thin and flexible films, tissue cells visibly contract and induce wrinkles, doing so without a loss of attachment (Harris, 1984; Harris et al., 1980; Leader et al., 1983). Implications for cell phenotype are presently uncertain, but traction forces are clearly transmitted to the matrix, occurring primarily through specialized adhesion and cytoskeletal linkages. The cellular forces generate substrate displacements, but only if allowed by the softness of the substrate (Stamenovic et al., 2002). Cytochalasin disruption of F-actin moderates such cell tractions as does 2,3-butanedione monoxime (BDM), which inhibits myosin (Pelham and Wang, 1997). BDM also inhibits the development of striations or myofibrillogenesis in skeletal muscle differentiation, which suggests a need for actomyosin crossbridge cycling in differentiation (Ramachandran et al., 2003). However, calcium regulation is also affected by BDM (Turvey et al., 2003), and calcium has a key role in mechanosensory processes (Munevar et al., 2004). Additional pharmacological probes are therefore needed to test and distinguish myosin contributions and other mechanisms in contractility and differentiation.

Although tissue cells are generally found to spread and organize classical focal adhesions on stiff or rigid substrates (Pelham and Wang, 1997; Wong et al., 2003), once the cells are maximally spread and immobilized, their contractions are necessarily more isometric than on a softer substrate. On softer substrates, which could in principle include an underlayer of

soft cells, typical tissue cells such as smooth muscle cells are often more rounded and display more dynamic adhesions (Engler et al., 2004a). In principle, this should allow for finite contractions, and should foster differentiation of myocytes. Additionally, stress fibers that predominate in cells growing on rigid substrates are largely missing in cells growing on soft substrates (Engler et al., 2004a). A sustained balance between a compliant type of adhesion and contractility thus implies that cells 'feel' their substrate and feedback in response to it.

Because of the highly organized cytoskeletal structure of fully differentiated myotubes, skeletal muscle cells provide a relatively well-defined, even geometric system for evaluating contractile prestress and relating it to structure. In differentiating skeletal muscle, non-muscle myosin II is increasingly replaced by muscle-specific myosin II, which assembles with F-actin into sarcomeres that run the length of the multinucleated myotube and clearly define the contractile axis of these cells. However, classical cultures present a problem in distinguishing the effects of myotube adhesion versus contraction. This is because myotubes typically form complex, branched networks with uncontrolled myotube-myotube adhesion and substrate detachment occurring uncontrollably. In order to foster a more physiological and linear form to the myotube, we separated cells here from each other by using micropatterned substrates. Myoblasts can be constrained to line up and fuse into well-defined geometries on patterned substrates that provide limited or long-term cell-

shape definition (Griffin et al., 2004; Ra et al., 1999). In addition, as these cells differentiate further with striation in classic culture conditions, they stiffen, as measured using atomic force microscopy (AFM) (Collinsworth et al., 2002). Stiffness of smooth muscle cells (measured by twisting magnetic beads) also increases linearly with contractile stress (Wang et al., 2002). An increased stiffening of myotubes with differentiation thus leads to a prediction, demonstrated here, that contractile prestress (a basal tensile stress carried by the contractile apparatus) increases as muscle striates.

Here we identify the role of non-muscle myosin II in the contractility of skeletal myotubes, both striated and non-striated, by perturbing the adhesion and allowing partial relaxation of the cells. Prestress might be related to the tissue-level twitch tension typically measured in muscle cells (Carlson, 1974; Hack et al., 1999). This prestress probably has a significant earlier role during cell differentiation: maturation of the actomyosin cytoskeleton (myofibrillogenesis) appears to be fostered by a compliant substrate that accommodates, thereby signaling perhaps, reorganization within the cell. Although striation increases cellular prestress, the process of striation in a nascent myotube surprisingly requires that the cell is not too focally attached to an overly rigid substrate during post-fusion differentiation. Factoring substrate compliance into the nature of cell adhesion, the adhesion-contractile balance of skeletal muscle cells has wide implications. If adhesion is inadequate, as is likely in many muscular dystrophies, even basal levels of contractile stress can be damaging and impact differentiation. On the other hand, hyper-contractile muscle might detach itself from its matrix in the absence of adequate adhesion and thus influence apoptotic or other pathways. The study here is thus an initial investigation into contractile prestress and its effect(s) on adherent myotubes.

Materials and Methods

Cell culture

C2C12 skeletal myocytes (CRL-1772, ATCC, Rockville, MD) were maintained in 75-cm² tissue culture flasks (Corning Glass Works, Corning, NY) at 37°C and 5% CO₂. Cells were grown in 10 ml DMEM (Gibco Laboratories, Grand Island, NY) supplemented with 20% fetal bovine serum (Gibco), 0.5% chick embryo extract (US Biological, Swampscott, MA) and 0.5% penicillin/streptomycin at 10,000 U/ml and 10,000 µg/ml, respectively (Gibco). Cells were passaged every 2-3 days by detaching with trypsin-EDTA (Gibco).

Substrate characterization

Glass slides or coverslips were patterned as previously described (Griffin et al., 2004). Briefly, glass slides were cleaned, spin-coated with photoresist (Shipley, Marlborough, MA), exposed to 2400 Joules of 300- to 500-nm UV light through a mask and the exposed photoresist was developed away. This process produced slides patterned with photoresist surrounding rectangles, or strips, of untreated glass 20 µm wide by 200-1000 µm long (Fig. 1A). The newly exposed glass strips were then coated with allyltrimethylsilane (Sigma, St Louis, MO) and covalently bound to an interpenetrating polymer network (IPN) of polyacrylamide and polyethylene glycol (Polysciences, Warrington, PA), resulting in p(AAm-co-EG) IPN-patterned slides. Next, the untreated glass rectangles were grafted with 3-aminopropyltriethoxysilane (Sigma) and coated with rat tail collagen-I (BD Biosciences, Palo Alto, CA) by overnight incubation at 4°C. The complete pattern consisted of glass strips coated with

collagen and surrounded by IPN (Fig. 1A). Cells plated on IPN-patterned slides remained patterned with high pattern recognition for up to 14 days.

The effectiveness of the patterning is due in part, we believe, to the compliance contrast between the IPN and the collagen-coated glass. Apparent elastic moduli of the IPN and the collagen-coated glass were measured by AFM (Engler et al., 2004a). Briefly, the apparent elastic modulus was obtained by fitting a thin-film Hertz model (Dimitriadis et al., 2002) to the first 5-50 nm of indentation profiles of the collagen-coated glass strips and the surrounding IPN. The measurements were performed with an Asylum Epi-Force AFM (Santa Barbara, CA). Although the materials were too thin (~10-50 nm) for accurate determination of the exact moduli, there was a significant difference in their relative stiffnesses and the collagen-coated glass appeared at least twice as stiff as the IPN.

The long-term quality of the patterning is also a result of the non-fouling nature of the IPN: proteins do not readily adsorb to the IPN. We tested the longevity of this resistance to protein deposition by adsorbing fluorescently labeled collagen (Molecular Probes, Eugene, OR) onto IPN patterns for 2-4 weeks and comparing the intensity of

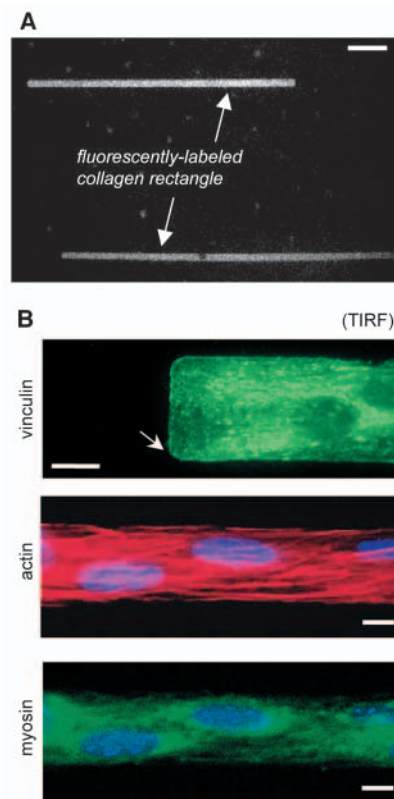


Fig. 1. Nascent C2C12 skeletal myotubes on collagen patterns. (A) Patterns of collagen-coated rectangles, 20 µm wide and 200-1000 µm long, surrounded by interpenetrating polymer network (IPN). Even after 14 days of incubation with fluorescent collagen, the IPN shows no significant adsorption based on intensity. In addition, when measured by AFM, the apparent elastic modulus of the IPN is approximately half that of the collagen-coated glass. Scale bar, 200 µm. (B) Myoblasts plated onto the patterned coverslips were either stained for vinculin (and imaged by TIRF for clarity) or triple-stained for actin (red) myosin (green) and nuclei (blue). The cells invariably fuse to form multinucleated myotubes. The arrow points to the vinculin clustered at the edges of the patterned myotube, indicative of classical focal adhesion structures. The actin and myosin images show no striations and are of the same nascent myotube at day 6. Bars: in A 200 µm; in B 10 µm.

the IPN (I_{IPN}) at day 14 to the baseline I_{IPN} at day 1. The intensity at day 1 was used as the baseline for comparison because Healy and co-workers have shown with XPS (X-ray photoelectron spectroscopy) that the IPN completely resists protein deposition after 24 hours incubation in protein solution (Bearinger et al., 1997). Fluorescently labeled collagen on patterned slides was observed with a Nikon TE300 inverted microscope (Optical Apparatus, Aardmore, PA) using a 40 \times objective (N.A. 0.45) and images were recorded with a liquid nitrogen-cooled CCD camera (Model CH360, Photometrics, Tucson, AZ). After 14-28 days of incubation in collagen solution, there was no significant increase in fluorescence compared to day 1, leading to the conclusion that no collagen adsorbed to the IPN even after incubation for 28 days.

Relaxation experiments

In preparation for relaxation experiments, $\sim 10^4$ cells were plated onto fully patterned IPN substrates. The media was changed to differentiation media [DM; DMEM supplemented with 10% horse serum (Gibco) and 0.5% penicillin/streptomycin] on the first day after plating and changed every other day. Relaxation experiments were performed after a total of 6 days in culture.

Before beginning the relaxation experiments, the medium was changed for one of three control (solutions 1-3 in Table 1) or three experimental solutions (solutions 4-6 in Table 1). The cells were then incubated for 20 minutes at 37°C and 5% CO₂ (this 20-minute incubation period was skipped for a subset of control cells in unaltered DM).

To initiate relaxation, one end of a cell was mechanically detached with a glass micropipette (pre-incubated with BSA (Sigma) in order to minimize cell-pipette interaction). The cell was then allowed to relax for up to 6 minutes. The process was observed with a Nikon TE300 inverted microscope using a 10 \times (N.A. 0.17) phase objective and recorded onto video through a video-rate camera (model JE8242, Javelin Systems, Torrance, CA). When necessary, the stage was repositioned in order to keep the cell within the field of view.

Cell-on-cell experiments

For experiments where cells were grown on top of other cells, $\sim 10^4$ C2C12 myoblasts were plated onto IPN-patterned slides. On day 1 after plating, a second layer of $\sim 10^4$ myoblasts was plated on top of the cells. The media was changed to DM 2 days after plating of the bottom layer of cells and every other day thereafter. Control samples were also prepared by plating a single layer of $\sim 10^4$ cells both onto IPN patterns as well as onto Aclar sheets (Ted Pella, Redding, CA) pre-adsorbed with 0.2 mg/ml collagen I. Although the pattern recognition began to deteriorate after 14 days, we readily distinguished cells growing on other cells from cells growing directly on glass.

Fluorescent staining

Cells were stained for actin, myosin, vinculin and nuclei as previously described (Griffin et al., 2004). Briefly, cells grown on patterned coverslips were fixed with formaldehyde (Fisher Scientific, Atlanta, GA) permeabilized with 0.1% Triton-X 100 (Fisher) and blocked with 0.5% BSA in PBS for 1 hour at 37°C. Next, the cells were permeabilized and incubated overnight at 4°C in 1:50 mouse antibody to skeletal myosin heavy chain (Zymed, San Francisco, CA) or 1:200 in mouse anti-vinculin (Sigma) in PBS. Cells treated with 50 μ M blebbistatin in 0.5% DMSO were also stained in parallel for vinculin. The cells were then incubated with secondary antibody (1:100 FITC-conjugated anti-mouse IgG, Sigma) and cells stained for myosin were also incubated in 60 μ g/ml TRITC-phalloidin (Sigma). Before mounting, the nuclei were stained with 1:100 Hoechst 33342 in PBS (Molecular Probes). Fluorescently labeled cells were examined under

Table 1. Experimental setup

Controls	Experiments
1. Unaltered DM	4. 50 μ M blebbistatin in 0.1% DMSO
2. 0.1% DMSO in DM	5. 50 μ M blebbistatin in 0.5% DMSO
3. 0.5% DMSO in DM	6. 5 μ M latrunculin-A in 0.5% DMSO

epifluorescence using a Nikon TE300 inverted microscope with a 60 \times (1.4 N.A.) objective. Total internal reflectance microscopy (TIRF) images were obtained with an Olympus IX71 microscope (B&B Microscopes, Warrendale, PA). Images were recorded with a cascade CCD camera (Photometrics, Tucson, AZ). Confocal microscopy was performed on Nikon TE300 inverted microscope fitted with a Bio-Rad Radiance 2000 MP3 system (Bio-Rad Laboratories, Inc., Hercules, CA). Images were observed through a 60 \times objective (N.A. 1.45), and recorded with the software Lasersharp 2000 (Bio-Rad Laboratories, Inc.).

Results

Cell patterning and rigidity-inhibited differentiation

The patterned substrates here consist of cell-adhesive rectangular strips of glass-adsorbed collagen (Fig. 1A). The strips prove stable for the weeks of culture required for myotube differentiation. The thin film of polymer gel surrounding the collagen-coated strips contributes to the long-term efficiency of the cell patterning (Fig. 1B) not only by inhibiting protein deposition, but also perhaps by its soft and compliant nature, which tends to inhibit cell spreading (Engler et al., 2004a; Pelham and Wang, 1997; Wang et al., 2000; Wong et al., 2003). Within 1-2 days, motile myoblasts have crawled onto the micropatterned collagen strips and initiated differentiation.

After one week in culture, the C2C12 cells on the strips have invariably fused to form syncytial, multi-nucleated myotubes (Fig. 1B). These cells do not generate sarcomeres, however. Even after one month in culture, no actin-myosin striations are seen. Instead, F-actin maintains a network of stress-fibers, and the skeletal-muscle myosin remains diffusely distributed within the cell. The fluorescence intensity (indicative of differentiation) seen in nascent myotubes is higher than that typically seen in myoblasts.

Cell-on-cell and asymmetric differentiation

Full differentiation to striated myotubes is seen only with C2C12 cells growing on top of a lower layer of cells (Fig. 2A-C). This is achieved by adding myoblasts one day after the first plating. Within one week in culture, these upper cells not only fuse into a myotube separate from the lower cell, but the upper myotubes also show a very strong tendency to striate.

In the cells on top of cells, skeletal muscle myosin is clearly upregulated and often organized into sarcomeric striations by day 6. F-actin begins to striate as well. Multiple experiments confirm striation for a majority of cells-on-cells at this early time point (Fig. 2D). In comparison, none of the cells in direct contact with the patterned coverslips, including cells underneath other cells, show striations. By day 24, the percentage of cells-on-cells showing sarcomeres increases to 80-90%, and for these upper cells, the actin is also fully striated. No cells in direct contact with the coverslip at day 24 appear striated.

Substrate compliance may have a direct influence in the cell-on-cell differentiation. Although the upper cell sees only a soft

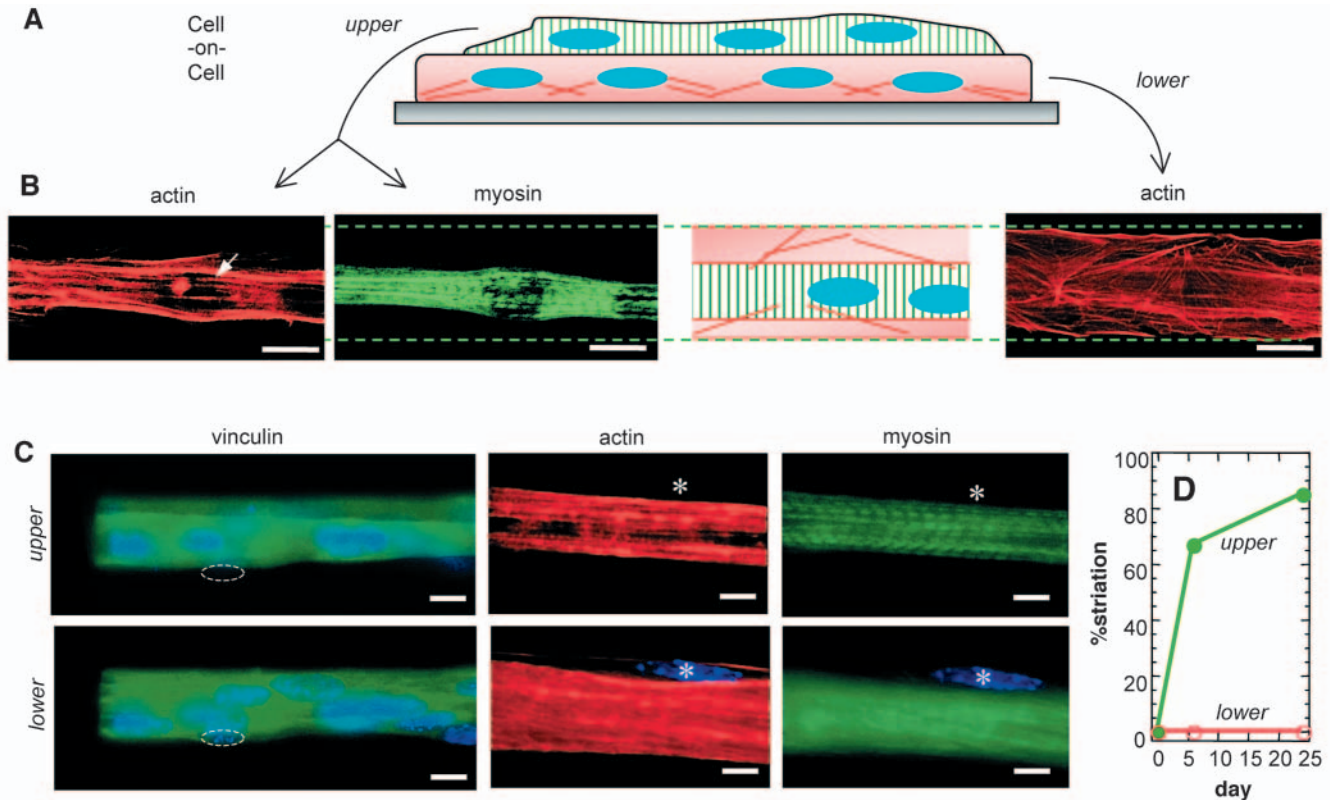


Fig. 2. Myotube striation occurs only on top of nascent myotubes. (A) Schematic showing that myotubes striate on top of other cells but not directly on IPN-patterned coverslips. Myoblasts plated on top of day 1-3 cells generate distinct cell-on-cell arrangements: in the upper myotube, actin and myosin will striate, whereas the lower myocyte bound to glass remains unstriated. (B) Confocal micrographs of the upper cell in a 2-week cell-on-cell system stained for actin and myosin. The actin shows a hint of striation, particularly near the arrow. The lower cell is wider, as in the middle sketch, and shows more isotropically oriented stress fibers. There is a 1.2 μm difference in height between the two actin images. The dashed lines represent the edges of the bottom myotube. Although the bottom cell is a fused myotube, the actin and myosin are striated in the top cell only. Bars, 10 μm . (C) Vinculin staining appears diffuse in both upper ($n=15$) and lower cells ($n=15$) in conventional fluorescence microscopy. A nucleus in the lower cell is not visible in the upper cell (dashed oval). The actin and myosin images show striation only in the upper cell ($\sim 6 \mu\text{m}$ higher focal plane) and again a nucleus (asterisk) is visible in one cell but not the other. Bars, 10 μm . (D) After 1 week in culture, 68% of cells ($n=34$) growing on top of other cells exhibit striated myosin compared to 0% ($n=25$) of cells growing directly on collagen-coated glass. By 4 weeks, more upper cells have striated with no evidence of lower cell striation.

cell below it, the lower cell always sees rigid substrate below it in spite of the soft cell above. The elastic modulus of early-stage myotubes has been determined previously by AFM indentation (Collinsworth et al., 2002) and proves remarkably similar to elastic moduli reported for the passive elasticity of skeletal muscle tissue. Separate studies of our own with C2C12 cells grown on collagen-coated polyacrylamide gels confirm that a tissue-like substrate modulus is indeed optimal for myotube differentiation (see Fig. S1 in supplementary material) (Engler et al., 2004b). These comparisons therefore suggest that the collagen-coated glass is too stiff and locks stress-fiber-rich nascent myotubes into tightly adherent, early differentiation states. An important role for substrate compliance in the differentiation of contractile cells such as C2C12s (Portier et al., 1999) is understandable.

Fully differentiated myotubes *in vivo* show vinculin organized into striated structures known as costameres (Pardo et al., 1983). Although costameres have been observed in C2C12 cells in culture by day 11 (Belkin et al., 1996), costameric vinculin has not been reported *in vitro*. We found that by day 6, myotubes growing either directly on the

collagen-coated glass or on top of other cells, show vinculin either in classical focal adhesion structures (Burrige and Connell, 1983; Katoh et al., 1995) or diffusely distributed (Fig. 1B, Fig. 2C). The same is true for C2C12 cells grown on collagen-coated, unpatterned plastic Aclar even after 2 weeks in culture (data not shown). TIRF microscopy of vinculin-stained cells grown on patterned glass reveals vinculin clusters at the edges of the cells (arrow in Fig. 1B), a phenomenon we have reported previously (Griffin et al., 2004). Vinculin organization and associated adhesion mechanisms in C2C12 cells up to two weeks thus seem relatively similar across the various cultures here. For the cell length relaxation studies presented below, these adhesions are probably a source of drag as the bonds are broken during myotube relaxation.

Lastly, although it is difficult to clearly focus even in confocal microscopy on the interface between the upper myotube and the lower myotube (Fig. 2B), by raising the focal plane only 1.2 μm , we can nevertheless see in cell-on-cell arrangements that what appears to be a bright but out-of-focus actin filament running down the center of the bottom cell, is in fact the actin-rich cell membrane cortex of the top cell.

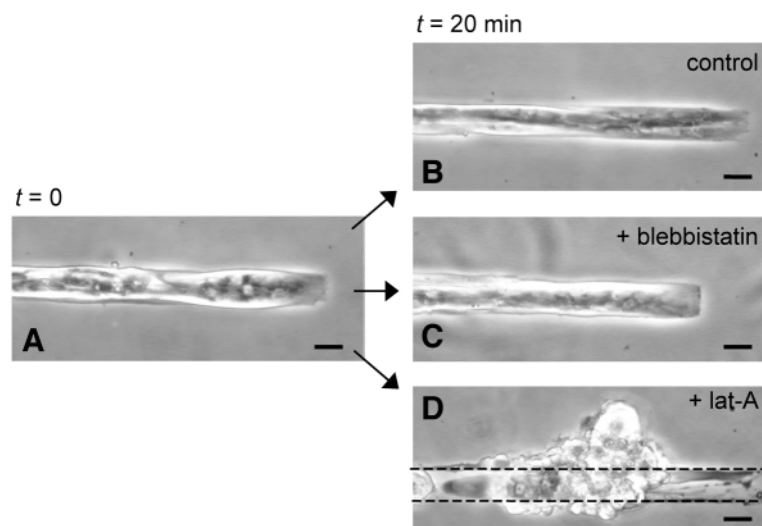


Fig. 3. Cell attachment response to actin depolymerization and myosin II inhibition. Cell-substrate attachment is influenced by lat-A but not by blebbistatin. (A) Before treatment, myotubes are fused and firmly attached to the substrate. (B,C) Control cells and cells incubated in 50 μM blebbistatin for 20 minutes exhibit no visually observable change. (D) After a 20 minute incubation in 5 μM lat-A, however, cells bleb and self-peel off the substrate. The dotted lines indicate the location of the collagen-patterned strip in the IPN. Bars, 20 μm .

Examination of confocal images indeed confirms that striations occur only in the top cell. In addition, the relaxation experiments discussed below show that the upper cell relaxes rapidly off the lower cell, consistent with the conclusion that the upper and lower myotubes are distinct: membrane fusion between the two layers has not occurred. The lack of ‘vertical’ fusion might reflect mechanistic limits to lateral fusion or more probably reflects the time delay between platings, with the lower myotube, plated first, perhaps being past the stage of ready fusion.

Relaxation of contractile stress

Cells treated for 20 minutes with the myosin II-specific inhibitor blebbistatin appear visually unaffected (Fig. 3B,C). Latrunculin-A (lat-A) exposure for 20 minutes shows dramatic effects, however: myotubes treated with lat-A detach almost completely from the substrate. As the lat-A-treated cells detach, they also bleb extensively (Fig. 3D). Because of these severe effects, cells treated with lat-A could not be studied further in the relaxation measurements below.

Quantitative studies of myotube relaxation were carried out with various control cells to compare to blebbistatin-treated cells. When mechanically detached slightly at one end, a myotube tends to shorten with a relative length change that decays exponentially (Fig. 4). We have previously shown that by using a simple viscoelastic model for relaxation, the decrease in myotube length generally fits well to the equation:

$$L/L_{\text{init}} = 1 - A(1 - e^{-t/\tau}),$$

where L is the cell length, L_{init} is the initial length, A is the shortening amplitude, and τ is the characteristic time (Griffin et al., 2004). Together with typical relaxation curves, snapshots of the relaxation processes at two different time points are shown in Fig. 4. The cells generally slow and stop relaxing upon reaching what must be a strong adhesion, which seems likely to be a collection or cluster of focal adhesions (see Fig. 1B). The location of this strong adhesion relative to the forces driving relaxation is indicated by the amplitude, A , of the fit (see equation). This decay amplitude varies with both the type of perturbation applied and the nature of the cell.

Compared to control cells (Fig. 4A), cells treated with blebbistatin relax to a lesser extent (Fig. 4B). Myotubes relaxing off a lower layer of cells also show slower relaxation dynamics but reach equilibrium at a much smaller L/L_{init} than control cells (Fig. 4C). Note that in the image of the cell-from-cell relaxation (Fig. 4C'), an unperturbed bottom layer of cells is exposed once the upper myotube has peeled back. From this we conclude that, as described earlier, cells growing on top of other cells have not fused with the bottom layer to form a single thick myotube. The two cell layers are mechanically as well as morphologically distinct.

In order to determine the effect of the carrier solvent DMSO, the relaxation dynamics of the different control cells were also compared (solutions 1-3 in Table 1). DMSO and several other organics have been noted by Sheetz and co-workers to reduce membrane surface tension (Dai and Sheetz, 1995; Hochmuth et al., 1996), and so we tested whether DMSO has an effect on the relaxation dynamics. By binning and averaging the relaxation data sets, we found that cells treated with 0.1% and 0.5% DMSO (solutions 2 and 3 in Table 1) showed no significant difference in relaxation dynamics. We then compared the data from DMSO-treated cells with the data from cells exposed only to unaltered media (solution 1 in Table 1). The L/L_{init} data sets from two separate experiments were each binned by time, averaged (Fig. 5A), and compared. Again, no difference was seen between the DMSO-treated cells and the untreated cells, and experiments in the three control solutions (1-3 in Table 1) are therefore grouped as ‘Controls’ in Fig. 5B. Relaxation dynamics for blebbistatin in 0.1% and 0.5% DMSO (solutions 4 and 5 in Table 1) also do not differ significantly, and so these cells are grouped together as ‘Blebbistatin’ in Fig. 5B. Thus, DMSO in relevant concentrations here does not affect myotube relaxation dynamics. Based on this and other observations (Dai and Sheetz, 1995; Hochmuth et al., 1996), membrane tension seems not to be a major contributor to the prestress seen here.

Binned relaxation lengths for control cells (\pm DMSO) were fitted to the equation (Fig. 5A), and then contrasted with cells relaxing off other cells and blebbistatin-treated cells grown on glass or on other cells (Fig. 5B). Based on the amplitude, A , for each fit, the blebbistatin-treated cells reach an equilibrium that is 4% smaller than their initial length, L_{init} . Control cells relax by 9%. Cell-from-cell relaxation is greatest after initial detachment, reaching equilibrium at a length 27% smaller than L_{init} , but cells-on-cells pre-treated with blebbistatin reach equilibrium after relaxing 8%. The extremely small error bars shown in the plots for the control and blebbistatin-treated cells reinforce the significance of the different relaxation

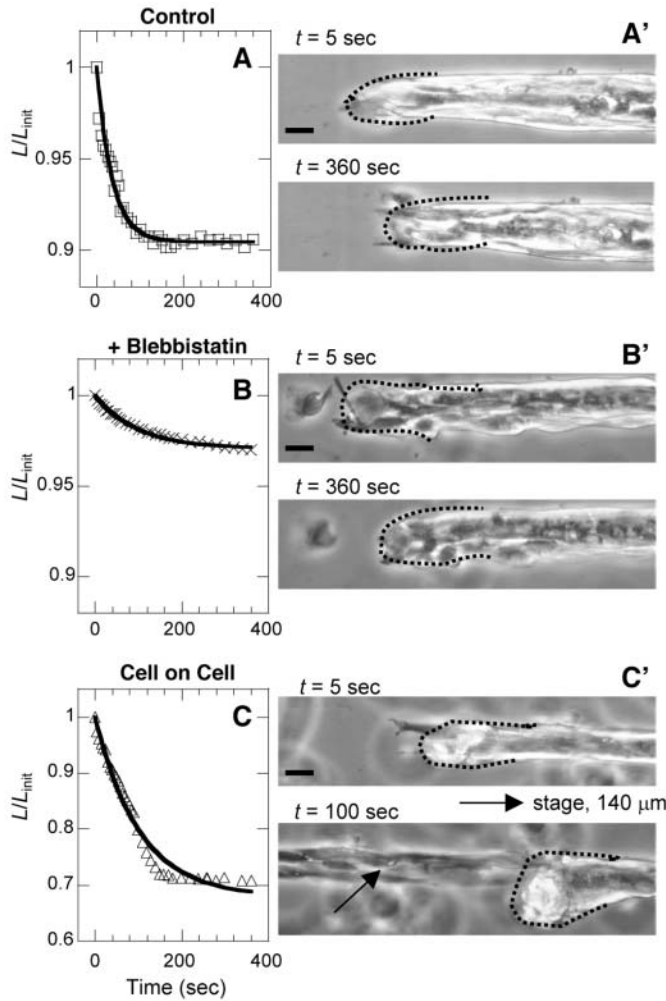


Fig. 4. Myotube relaxation after initial detachment. Representative length-relaxation of control cells (A), cells treated with 50 μ M blebbistatin (B) and cells relaxing off other cells (C). The data are fitted to the equation $L/L_{init} = 1 - A(1 - e^{-t/\tau})$, where A is the amplitude and τ is the time constant. The respective images in A', B' and C' are snapshots of the relaxation with cells outlined by dotted lines. In the case of untreated cells relaxing off other cells (C, C'), the relaxation is so large that the stage is moved to the right 140 μ m. The arrow points to the lower cell that is left unperturbed after relaxation of the upper cell. Bars, 10 μ m.

amplitudes. The untreated cell-from-cell results are also clearly below controls but show more variability.

Fitted values for both A and τ are compared in Fig. 5C,D. Typical of damped elastic systems, low initial loading or prestress, and/or more drag is expected to give a larger value for τ and a smaller value for A . Relative to control myotubes, blebbistatin decreases A (by 56%) and dramatically slows the relaxation (τ increases 190%). Blebbistatin therefore either decreases the intracellular prestress or it increases the drag on the cells, or both. However, as this drag in all likelihood represents the disruption of receptor-ligand bonds along the length of the cell, and as blebbistatin is known to target myosin II motor activity (Straight et al., 2003), we examined the effects of blebbistatin on focal adhesions. In thresholded TIRF images of vinculin-stained cells, we found that blebbistatin affected

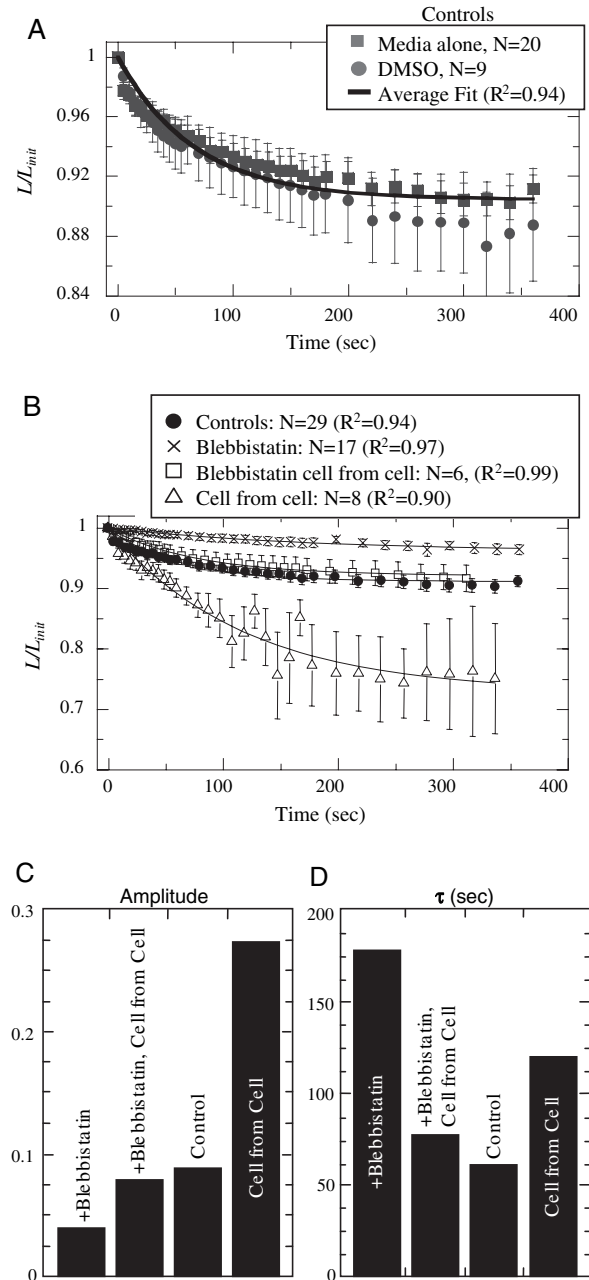


Fig. 5. Cumulative relaxation dynamics of the various myotubes. The results show that cells relax more quickly and to a greater extent when grown on top of other cells; and less so when treated with blebbistatin. (A) No significant difference is seen in the relaxation dynamics of cells exposed to DMSO (three cultures) compared to cells exposed only to media (five cultures). Data are time-binned averages all cells, with error bars representing the standard error of the mean. The bold line is the exponential fit for all 29 cells, $R^2=0.94$. (B) Control cells relax to an average equilibrium length that is $A=90\%$ of their initial length. Blebbistatin-treated cells (three cultures) relax significantly less, whereas cells relaxing off other cells (three cultures) relax more. Blebbistatin-treated cells relaxing off other cells (two cultures) also relax more than blebbistatin-treated cells on glass. (C) The amplitudes, A , of each fit, indicate clear differences in the fraction of cell relaxation at equilibrium. (D) In all cases, the time constant for relaxation is greater when the cell is perturbed; but the slowest relaxation occurs in blebbistatin cells-on-glass.

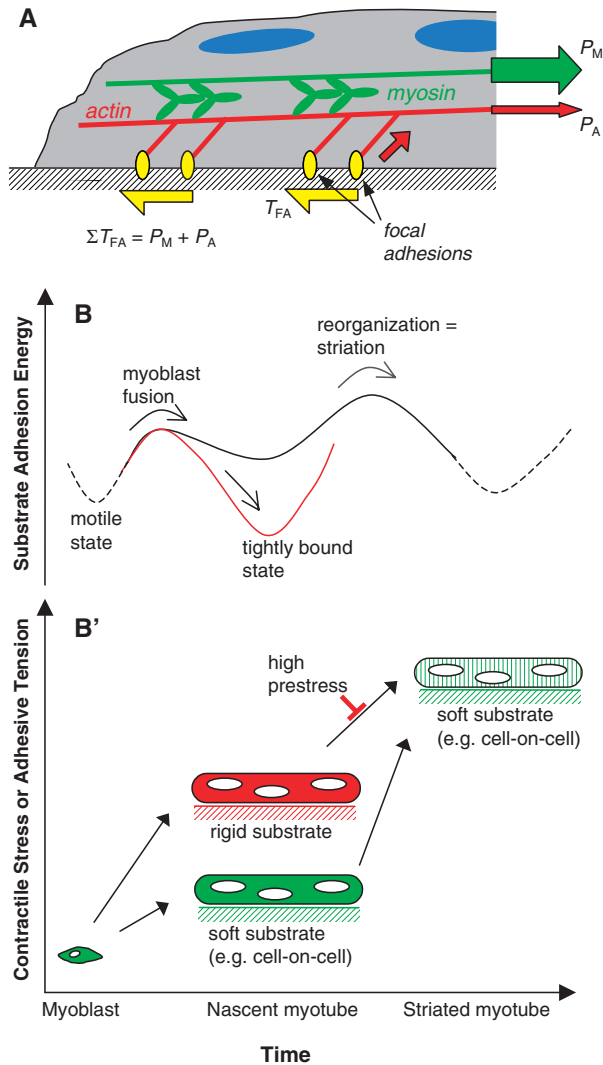


Fig. 6. Myotube adhesion versus prestress. (A) Schematic of a sectioned myotube illustrates the balance between the prestress imposed by the actomyosin cytoskeleton (P_A , P_M), and the adhesive tension of the focal adhesions (T_{FA}). (B,B') Schematic of the adhesive energetics, contractile stress, and adhesive tension during differentiation. After fusion, myotubes growing on rigid substrates progress to a tightly bound state with high adhesive tension. Cells growing on soft substrates, including other cells, exhibit a reduced adhesive tension and a shallower adhesion energy at this nascent myotube phase. This allows the latter to progress to a fully striated state of high prestress.

neither the fraction of cell area covered by focal adhesions nor the edge clustering of the focal adhesions. In contrast, the adhesions of lat-A-treated cells (Fig. 3D), were clearly undermined, probably because F-actin depolymerization disrupts focal adhesions and stress fibers. Therefore, the reciprocal changes in τ and A caused by blebbistatin probably result from a decrease in cellular prestress.

Relaxation parameters for cells-on-cells also seem to support the conclusions above. Compared to blebbistatin-treated cells-on-glass, A is higher (by 100%) for blebbistatin-treated cells-on-cells whereas τ is lower (by 60%). Untreated cells growing on other cells, however, have both a larger

average relaxation amplitude (by 200%) and a larger time constant (by 95%) than those of control cells. The majority of cells growing on other cells were shown to be striated (Fig. 2) and therefore had more of a contractile phenotype. Contractile prestress is therefore reasonably assumed to have increased in these cells and thereby drives cell relaxation. In comparing blebbistatin-treated and untreated cells-on-cells, we find that blebbistatin significantly decreases the relaxation amplitude, consistent with decreased prestress in the treated cells.

Discussion

Cells on matrix-like gels reveal contractile stresses that are visible in gel displacements, which increase with activation and decrease with disruption of the actomyosin cytoskeleton (Stamenovic et al., 2002). Likewise, experiments here with blebbistatin and lat-A reveal that the cytoskeleton also plays a key role in cellular prestress. In mechanical relaxation studies of a related type by Wang and coworkers (Pourati et al., 1998), adherent endothelial cells were cut or partially detached with a microneedle and were then seen to retract. Retraction occurred unless the actin cytoskeletal network was first disrupted with cytochalasin D. Recent studies of our own with GFP- (unpublished) and GFP-paxillin- (Griffin et al., 2004) transfected cells show that cells maintain their membrane integrity during cell peeling. Based on such findings and the results here, it can be concluded that the cytoskeleton imposes a contractile prestress on the cell and its bounding membrane.

Prestress is considerably relieved here in cells treated with blebbistatin (for both cell-on-cell and cell-on-glass systems). However, cells treated with lat-A collapse in upon themselves without the need for forced detachment from the substrate. The difference is undoubtedly due to the contribution of F-actin to adhesion complexes: disrupting the actin-myosin network inhibits focal adhesion assembly (Engler et al., 2004a).

The dramatic increase in shortening amplitude for cells relaxing off other cells is indicative of the prestress in myofibrillogenesis. In normal muscle tissue, myotubes bind through integrins and dystroglycans to a basement membrane sheath of ECM molecules such as laminin and collagen (Clark et al., 1997; Lyles et al., 1993) and myoblasts bind to other cells through cell adhesion molecules such as NCAM (Dickson et al., 1990). Actomyosin striations generally begin to appear 4-10 days after the introduction of differentiation medium (van der Ven et al., 1992). The immunofluorescence studies here indeed revealed that by one week, the skeletal muscle myosin in cells-on-cells has begun organizing into sarcomeres. Because the actin in these cells does not appear as organized as the myosin, and because spontaneous contraction was not observed in these cells (rare even in classical overgrown cultures of C2C12s), we cannot say that the upper cells are functionally differentiated. Nevertheless, it is clear that the upper cells have progressed beyond the differentiation state of the lower cells, and there are probably muscle-like contraction forces contributing to the prestress that cause these upper cells to relax. However, relaxation amplitudes (A), of cells-on-cells, as well as qualitative observations, suggest that the upper cells have adhesive properties that are different from cells growing directly on the substrate. Unlike substrate-bound cells, cells-on-cells seem to act like tensed strings 'pinned down' at a few

discrete points along their lengths, with particularly strong attachment at the ends.

Although the unstriated cells will have predominantly (or exclusively) non-muscle myosin II, differentiated cells (on cells) are expected to express muscle myosin II in their assembled sarcomeres. Consistent with this, the relaxation of blebbistatin-treated cells-on-glass is minimal compared to the significant, blebbistatin-resistant relaxation of treated cells-on-cells. Nonetheless, given the significant decrease in A (70%) for blebbistatin-treated cells-on-cells versus a 50% decrease for cells-on-glass, and the specificity of blebbistatin for non-muscle myosin II (Straight et al., 2003), there is evidently considerable non-muscle myosin II in the upper, striated cells. This is consistent with continued myofibrillogenesis and incomplete differentiation, despite clear striation.

It is not a trivial matter to fully separate the effects of adhesive drag and contractile prestress on the upper layer of cells-on-cells, but the present approaches are applicable to cells grown on substrates of cell-like compliance with better controlled ligand concentration. Preliminary data indeed shows that C2C12 cells will form striations when grown directly on suitable synthetic gels (see Fig. S1 in supplementary material) (Engler et al., 2004b). Such a system removes the possibility of complex signaling between the cells in the two cell layers. It also creates a more controllable system where prestress and adhesion can be probed independently. Finally, although the C2C12 cells used here are well recognized for displaying much of the differentiation of primary cells, we have also studied primary cells from mice, including cells derived from γ -sarcoglycan-deficient mice (unpublished results). Similar results to the C2C12 cells have been observed, including a definitive increase in the degree of myofibrillogenesis achieved in cells-on-cells versus cells-on-glass. Exploiting some of the adhesion defects and perhaps contractility defects, possibly in combination with inhibitors such as blebbistatin, should also add insight into the adhesion-contractile balance and its relationship to differentiation. Indeed, preliminary results show that γ -sarcoglycan-deficient myotubes striate more than normal, are more contractile when allowed to relax and have the same adhesion strength (when forcibly peeled), but clearly tend to apoptose in culture, which is consistent with reported observations *in vivo* (Hack et al., 1999).

In Fig. 6, we present a schematic diagram to help conceptualize time courses of the prestress and energetics of a differentiating myotube. The combined data resulting from the lat-A and blebbistatin studies led to the conclusion that the contractile prestress generated within the cell by actin and myosin (P_A and P_M , respectively) are balanced by the cumulated adhesive tension (T_A) for substrate resistance to compression. Given the dynamic balance of the stresses that must reflect myosin as well as adhesion kinetics, compliant substrates would tend to contract with similar dynamics as Harris and coworkers visualized many years ago (Harris, 1984). In contrast, cells growing on rigid substrates become highly prestressed, seemingly in order to match the compliance of the substrate. Because of the high adhesive tension (T_A), firmer adhesion is needed to prevent the cell from detaching. Cells on rigid or stiff substrates thus become very tightly bound upon becoming fused, nascent myotubes.

Fig. 6B attempts to convey the energetics of the summed adhesive interactions. Motile myoblasts seem likely to weaken

their substrate interactions as they fuse into myotubes that, on glass and other rigid substrates, leads to firmly adherent myotubes, but on top of other cells leads to more superficial adhesion. The latter, importantly, allows the sort of cytoskeleton reorganization evident in striation as more complete differentiation. Fig. 6B' depicts the related or conjugate pathway in the context of prestress.

In summary, when grown on rigid substrates with non-ideal compliance, skeletal muscle cells lock into a fused, non-striated, early differentiation state. 'Anchorage dependence' thus seems likely to mean far more than a simple distinction of fluid versus solid in describing the effects on a cell in adhesion. This is most likely associated with a shift in the balance between adhesion and prestress. In isolated myotubes, this prestress is quantifiable through mechanically induced relaxation. Also, if the actin is depolymerized, isolated myotubes will bleb and detach from the substrate because of the disruption in the prestress and the adhesive network, rendering relaxation studies impossible. When only myosin II is inhibited, however, the relative length change of relaxation is significantly decreased compared to that of untreated cells. Because the myosin of nearly all cells-on-cells is striated, we conclude that it plays a critical role in establishing the prestress of a myocyte cell.

We thank Alina Popescu-Hategan and Adam Engler of the University of Pennsylvania for their help with AFM measurements and for Adam Engler's work with flexible substrates for cell culture. We are also grateful to Andrew Griffin (Bell Laboratories) for his help with substrate patterning and to Gladys Gray-Board (University of Pennsylvania) for assistance with confocal microscopy. Finally, we thank Timothy Mitchison (MIT) and Aaron Straight (Stanford University) for providing us with blebbistatin, and Aaron Straight, Bhagavathi Ramamurthy (University of Pennsylvania) and Chris Yengo (University of Pennsylvania) for their help with the blebbistatin protocols. This work was funded by the NIH, MDA, AHA and NSF-PECASE.

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