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Microtubule regulation of corneal fibroblast morphology and mechanical activity in 3-D culture

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Abstract

The purpose of this study was to investigate the role of microtubules in regulating corneal fibroblast structure and mechanical behavior using static (3-D) and dynamic (4-D) imaging of both cells and their surrounding matrix. Human corneal fibroblasts transfected to express GFP-zyxin (to label focal adhesions) or GFP-tubulin (to label microtubules) were plated at low density inside 100 μ m thick type I collagen matrices. After 24 hours, the effects of nocodazole (to depolymerize microtubules), cytochalasin D (to disrupt f-actin), and/or Y-27632 (to block Rho-kinase) were evaluated using 3-D and 4-D imaging of both cells and ECM. After 24 hours of incubation, cells had well organized microtubules and prominent focal adhesions, and significant cell-induced matrix compaction was observed. Addition of nocodazole induced rapid microtubule disruption which resulted in Rho activation and additional cellular contraction. The matrix was pulled inward by retracting pseudopodial processes, and focal adhesions appeared to mediate this process, when present. Following 24 hour exposure to nocodazole, there was an even greater increase in both the number of stress fibers and the amount of matrix compaction and alignment at the ends of cells. When Rho-kinase was inhibited, disruption of microtubules resulted in retraction of dendritic cell processes, and rapid formation and extension of lamellipodial processes at random locations along the cell body, eventually leading to a convoluted, disorganized cell shape. These data suggest that microtubules modulate both cellular contractility and local collagen matrix reorganization via regulation of Rho/Rho kinase activity. In addition, microtubules appear to play a central role in dynamic regulation of cell spreading mechanics, morphology and polarity in 3-D culture.

Keywords

Corneal Fibroblast; Microtubules; Collagen Matrices; Cell Mechanics; Rho-Kinase

INTRODUCTION

Microtubules are dynamic polymers of α/β tubulin heterodimers, arranged head to tail to form hollow cylindrical tubes of 25nm diameter, up to several micrometers long. Microtubules regulate numerous aspects of cell behavior including cellular transport of vesicles, control of cell shape, cell division, spindle assembly and chromosome motion during mitosis (Honore et al., 2005). In addition, microtubules play a key role in various aspects of cell mechanical

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behavior. For example, microtubule behavior and organization is modulated by the small GTPases Rho, Rac and Cdc42 in order to regulate cell polarization and directed cell migration on 2-D substrates (Watanabe, 2005). Fibroblast spreading is also regulated by microtubules, however the role of microtubules in cell spreading is highly dependent on substrate geometry and stiffness (Rhee et al., 2007; Tomasek and Hay, 1984). Specifically, intact microtubules are required for dermal fibroblast spreading in unrestrained (floating) 3-D collagen matrices, but not on rigid 2-D substrates (Rhee et al., 2007).

Microtubules may also be involved in modulation of cell contractility. Kolodney et al. used an isometric force transducer to quantitatively monitor the tension exerted by a dense population of chick embryo fibroblasts within collagen matrices (Kolodney and Elson, 1995; Kolodney and Wysolmerski, 1992). Disrupting microtubules induced a 2–3 fold increase in force over several minutes and reached maximum at about 30 minutes. A similar pattern of increased phosphorylation of the myosin regulatory light chain was identified, suggesting that actomyosin contractile activity may underlay the increase in force. Consistent with these observations, more recent studies have demonstrated that microtubules sequester Rho-GEF, and that release of Rho-GEF following microtubule disruption induces Rho/Rho kinase activation and cellular contraction (Krendel, 2002; Kwan and Kirschner, 2005; Liu BP, 1998; Ren et al., 1999; Zhang et al., 2001).

While previous studies suggest that microtubules play a role in regulation of cell spreading and contractility within 3-D collagen matrices, these processes have not been assessed dynamically in individual cells. Furthermore, their influence on the subcellular pattern of force generation and local cell-induced matrix reorganization has not been established previously. In this study, we use high resolution imaging of isolated cells and their surrounding ECM to directly investigate the role of microtubules in regulating fibroblast structure and mechanical behavior at the subcellular level. We found that after allowing the cells to spread for 24 hours, treatment with nocodazole induced rapid microtubule disruption which resulted in Rho activation, cellular contraction and local matrix reorganization. Following 24 hour exposure to nocodazole, there was an even greater increase in both the number of stress fibers and the amount of matrix compaction and alignment at the ends of cells. In contrast, when Rho kinase was inhibited, cells were elongated and had dendritic processes, as previously described (Kim et al., 2006). Subsequent addition of nocodazole induced retraction of some dendritic processes without associated pulling in of the ECM. Interestingly, this process was immediately followed by formation and extension of broader “lamellipodial” processes from random locations along the cell body, which eventually lead to a convoluted, disorganized cell shape. Taken together, our data indicates that microtubules modulate both cellular contractility and local collagen matrix reorganization via regulation of Rho/Rho kinase activity. In addition, microtubules also appear to play a central role in dynamic regulation of spreading mechanics, morphology and polarity in 3-D culture.

MATERIALS AND METHODS

Cells

Studies were performed using a previously characterized telomerase-infected, extended lifespan human corneal fibroblast cell line, HTK (Jester et al., 2003). HTK cells were maintained in medium consisting of Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA), supplemented with 1% penicillin, 1% streptomycin, and 1% amphotericin B (Fungizone; BioWhittaker, Inc., Walkersville, MD) and 10% fetal bovine serum (FBS; Sigma-Aldrich).

Transfection

For expression of GFP-zyxin or GFP-tubulin, human zyxin or tubulin in a pEGFP-N1 vector (BD Biosciences-Clontech laboratories, Inc., Palo Alto, CA) were used. These probes have successfully been used previously to visualize focal contacts (zyxin) and microtubules (tubulin) in a variety of cell types (Kaverina et al., 1999; Petroll and Ma, 2003). Human corneal fibroblasts were plated on six-well plates (80,000 cells/well) using complete media without antibiotics 24 hours before transfection. Transfection was performed with a commercial reagent (Fugene Roche, Indianapolis, IN). For each well, 3 μ g of reagent was mixed with 97 μ L of DMEM for 5 mins and 1 μ g of DNA was added to the mixture for 20 mins at room temperature. Cells were washed twice with serum-containing media prior to transfection. Cells were then incubated with DNA-Fugene reagent complex mixed with additional 2ml of DMEM and 10% FBS.

Collagen Matrices

Hydrated collagen matrices were prepared by mixing neutralized bovine dermal collagen (Purecol; Inamed, Fremont, CA) with 10X DMEM to achieve a final collagen concentration of 2.48 mg/ml (Petroll and Ma, 2003). For plating cells inside of the matrix, a 50 μ L of suspension of HTK cells was mixed with 500 μ L of collagen solution. The pH of the collagen solution was adjusted to 7.2–7.4 by addition of 0.1 N NaOH or 0.1 N HCL. The cell/collagen mixture was preincubated at 37°C for 4 minutes, and 30- μ L aliquots (containing approximately 2000 cells) were then poured onto culture dishes (Delta T; Biopetechs, Inc., Butler, PA). Each aliquot was spread over a central 12 mm diameter circular region on the dish and was approximately 100 μ m thick. The dish was then placed in a humidified incubator (37°C, 5% CO₂) for 60 minutes for polymerization and overlaid with 1.5 ml of media, and incubated for 24 hours prior to imaging.

Time-Lapse Digital Imaging

Matrices were incubated for 24 hours before each time-lapse experiment. In each experiment, cells were allowed to acclimate to the microscope's microincubation system for 30 minutes before time-lapse imaging. To maintain cell viability during imaging, an objective heater (Biopetechs) was used and cells were continuously perfused with complete medium containing 50 mM HEPES buffer at rate of 6 ml/hour (Petroll and Ma, 2003). The cell density was sparse enough to focus on the mechanical activity of a single cell in each matrix, minimizing the potential interference caused by neighboring cells.

In most experiments, microscopy was performed with an inverted microscope equipped for time-lapse DIC imaging (Model TE300; Nikon Inc., Melville, NY) (Petroll and Ma, 2003; Petroll et al., 2003). The hardware was controlled using a computer running image-analysis software (Molecular Devices, Sunnyvale, CA). The activity of a single cell was imaged for up to 3 hours using either a 20X dry (0.75 numerical aperture [NA], 1 mm working distance) or 60X oil-immersion objective (1.3 NA, 220 μ m free working distance). Three dimensional data sets were obtained at each time point by repeating the acquisition at five to six sequential focal planes in z steps of 2–3 μ m. For DIC, 3-D image stacks were collected every 2 minutes.

In order to obtain a more detailed visualization of cell-matrix interactions, additional time-lapse experiments were performed in which fluorescent (for GFP) and reflected light (for collagen fibrils) 3-D optical section images were acquired simultaneously using laser scanning confocal microscopy (LSCM, Leica SP2, Heidelberg, Germany). Transfection efficiency for the GFP-tagged proteins was approximately 30%, which was adequate since we only image one cell per matrix in our time-lapse experiments. A HeNe laser (633nm) was used for reflected light and Ar (488nm) laser was used for fluorescent imaging. Laser power settings were kept to a minimum to avoid phototoxicity. A stack of optical sections (z-series) was acquired for

each cell imaged by changing the position of the focal plane in 1 μm steps using a 63X water immersion objective (1.2 numeric apertures [NA], 220 μm free working distance). No chemical processing, physical sectioning, or staining of the matrices was required since reflection is an intrinsic optical property of biopolymers including collagen. For LSCM, image stacks were collected every 10–15 minutes.

For both DIC and LSCM, following 30 minutes of time-lapse imaging, nocodazole (10 μM) was added to the media to disrupt microtubules. After an additional 40 to 60 minutes, cytochalasin D (25 μM) was added to assess the effect of F-actin on the cell-matrix interactions (Petroll and Ma, 2003; Roy et al., 1999b). Image processing and creation of time-lapse movies were then performed using MetaMorph. The concentration of nocodazole was selected based on preliminary dose-response experiments that demonstrated similar efficacy of microtubule disruption and 10, 20 and 50 μM .

Finite Element Modeling

Finite element modeling (FEM) was used to better visualize and quantify the pattern of matrix deformation induced by nocodazole and/or cytochalasin D. FEMs were created using engineering analysis software (Ansys, Canonsburg, PA), as previously described. (Roy et al., 1999b; Vishwanath et al., 2003) Briefly, nodes were defined at coordinates coinciding with ECM landmarks from the DIC image. Boundary nodes were placed at the periphery of a 600- μm diameter circular field around this central set of nodes. A 2-D plane stress model was created from the nodes with linear elastic triangular elements. For simplicity, the matrix was assumed to be isotropic, with a Young's modulus of $3.89 \times 10^{-10} \text{ N}/\mu\text{m}^2$, an effective thickness of 15 μm , and a Poisson's ratio of 0.3. (Roeder et al., 2002; Roy et al., 1999b) To generate maps of ECM deformation, the displacements measured from time-lapse recording were applied to the corresponding nodes in the model. The resultant strains induced on the matrix were calculated and displayed.

Assessment of Cell Spreading and Morphology

In order to assess the role of microtubules in initial cell spreading within 3-D collagen matrices, experiments were performed in which reagents were added to the media at the time of cell plating. Matrices were incubated for 24 hours with basal media (control), the Rho-kinase inhibitor Y-27632, nocodazole, or both Y-27632 and nocodazole. A standard concentration of 10 μM of Y-27632 was used, which is optimal for specific inhibition of Rho-kinase (Anderson et al., 2004; Kim et al., 2006; Tamariz and Grinnell, 2002; Vishwanath et al., 2003). Cells were then fixed using 3% paraformaldehyde in phosphate buffer for 10 min, permeabilized with 0.5% Triton X-100 in phosphate buffer for 3 min, and blocked with 3% FBS (fetal bovine serum) containing buffer for 30 min. Cells were then incubated in Alexa Fluor 488 Phalloidin (1:50, Molecular Probes, Eugene, OR) for 60 min, and imaged using LSCM as described above. Changes in cell morphology were measured using MetaMorph. Projected cell length and breadth was calculated by outlining the maximum intensity projection image of a cell (generated from the f-actin z-series), thresholding, and applying the Integrated Morphometry Analysis (IMA) routine. The length is calculated by IMA as the span of the longest chord through the object; the breadth is the caliper width of the object, perpendicular to the longest chord. In each experiment, matrices were plated in triplicate for each of the four treatment groups. The entire experiment was repeated four times.

Biochemistry

To measure changes in Rho activation in response to microtubule disruption, cells were plated within collagen matrices (0.2 ml volume and 2×10^5 cells each), and cultured for 24 hours in serum containing media. Nocodazole (10 μM) was added for 0 (control), 10 or 30 minutes, and cell lysates were collected as previously described (Grinnell et al., 2003). GTP Rho was used

as a positive control. To measure Rho activation, the G-LISA™ activation kit (Kit #BK124, Cytoskeleton, Inc., Denver, CO) was used according to the manufacturer's instructions. This assay uses a Rho-GTP-binding protein linked to the wells of a 96 well plate. Active, GTP bound Rho in cell lysates binds to the wells while inactive, GDP-bound Rho is removed during wash steps. Bound GTP Rho is detected by incubation with a RhoA specific antibody followed by a secondary antibody conjugated to HRP and a detection reagent. The signal was read by measuring absorbance at 490 nm using a microplate reader (Beckman Coulter, Model DRX 880, Fullerton, CA). For each experiment, cell lysates were collected and pooled from four collagen matrices for each of the three treatment groups (control, 10 min of nocodazole treatment, and 30 min of nocodazole treatment). The entire experiment was repeated three times.

Statistics

Statistical analyses were performed using SigmaStat version 3.11 (Systat Software Inc., Point Richmond, CA). One way analysis of variance (ANOVA) was used to compare group means. Post-hoc multiple comparisons between groups were performed using the Holm-Sidak method. Differences were considered significant if $P < 0.05$.

RESULTS

Microtubule disruption induces cellular shortening and contractile force generation

To assess the role of microtubules in dynamic regulation of cell morphology and mechanical activity, corneal fibroblasts were plated inside collagen matrices with serum-free media for 24 hours, then transferred to the microscope for time-lapse imaging. After 30 minutes of microincubation to allow cells to adapt, nocodazole was added. DIC images allowed detailed visualization of the cells and collagen fibrils surrounding them, and matrix displacement could be assessed by tracking individual collagen fibrils. As seen in Figure 1A–C, retraction of cellular processes was observed following microtubule disruption. However, retraction did not directly correlate with movement of collagen fibrils. This is more clearly demonstrated in zoomed DIC images (Fig. 1D–F). Large retractions of the pseudopodial processes were observed, but much smaller displacements of the ECM were induced (red tracks). Retractions often occurred in a stepwise fashion (Movie 1), suggesting sequential rupturing of cell-matrix adhesions along the pseudopodial processes. Roy et al. previously demonstrated that stimulation of serum-starved cells by 1 μ M LPA induced stress fiber formation, focal adhesion assembly, and cellular contraction with corresponding collagen ECM displacement (Roy et al., 1999a). However, more rapid force generation induced by treating 10 μ M LPA caused contraction of cellular processes without matrix retraction, presumably because the cell-matrix adhesions were not strong enough to support the larger contractile force. Addition of nocodazole under serum-free conditions in the current study appears to have a similar effect.

In order to stimulate the formation of stronger adhesive structures (as are observed during corneal wound healing), cells were next incubated for 24 hours in serum-containing media prior to time-lapse imaging. Consistent with previous studies, collagen at the ends of these cells appeared to be aligned parallel to the long axis of pseudopodia (Fig. 1G), due to cellular force generation (Petroll and Ma, 2003; Petroll et al., 2003). Nocodazole induced cellular shortening and significant inward displacement of the ECM (red tracks, Fig. 1H). Addition of vehicle alone had no effect on cell behavior or morphology (not shown). Cytochalasin D reversed the process and induced cell elongation (Fig. 1I) and matrix relaxation.

FEM (finite element modeling) was used to better visualize and quantify the pattern of cell-induced force generation by analyzing matrix displacement. The FEM maps show the change in strain from one condition to the next. Cellular contraction after nocodazole treatment caused

and increase in the matrix tension at the ends of the cell and compression in the middle (Fig. 1K) as compared to baseline (Fig. 1J). Adding cytochalasin D reversed the process and resulted in decompression of the ECM under the cell, and reduction of tension at the ends (Fig. 1L). Taken together, these data demonstrate that disruption of microtubules induces cellular force generation which deforms the ECM, and that this force is actin-dependent.

Microtubule disruption induces matrix compaction and alignment

In order to obtain a more detailed visualization of cell-matrix mechanical interactions, time-lapse confocal imaging was used. Cells were cultured for 24 hours prior to transfer to the microscope stage. Fluorescent imaging was used to visualize GFP-tagged proteins, and reflected light imaging was used to visualize collagen. Figure 2A shows color overlay of GFP-zyxin (green) and collagen fibrils (red) for a cell cultured in basal (serum-containing) media. GFP-zyxin is organized into focal adhesions along the pseudopodia, which appear to be attached to the collagen fibrils (Fig. 2A, Movie 2). Collagen fibrils at the ends of cells generally appeared to be aligned parallel to the long axis of pseudopodia, consistent with previous studies (Fig. 2D) (Kim et al., 2006). Microtubules emanated from a microtubule organizing center, and were generally aligned with the long axis of the cells (Fig. 2B), consistent with previous observations of fibroblasts within restrained 3-D collagen matrices (Tomasek and Hay, 1984). Nocodazole disrupted microtubules and induced retraction of cell processes (Fig. 2C). Collagen fibrils surrounding the cell were pulled inward by retracting cellular process (Fig. 2E, F), which resulted in compaction of collagen fibrils surrounding the cell (white arrows in Fig. 2F). This process appeared to be mediated by focal adhesions (Movie 3). Cytochalasin D reversed the process and induced both cell and matrix relaxation (Movie 4), further demonstrating that the increase in cell contractility and matrix reorganization observed following microtubule disruption is mediated by f-actin. The interaction between focal adhesions and extracellular matrix is more clearly demonstrated in Figure 2G-I. Green arrows indicate the movement of focal adhesions and red arrows indicate the movement of collagen fibrils following nocodazole treatment. Note that the adhesion and collagen movement are generally correlated (Fig. 2I). It should be noted that the magnitude of tracked collagen displacement was always less than that of the focal adhesions, presumably due to realignment of individual fibrils, or slippage between fibrils (Petroll et al., 2003).

A total of 16 cells tagged with either GFP-tubulin or GFP-zyxin were studied by time-lapse confocal microscopy. Thirteen cells that did not undergo the transfection procedure were used as controls. The same pattern of cellular contraction and local matrix deformation was observed in both control cells (Figure 1) and transfected cells (Figures 2 and 3) in response to nocodazole, indicating that the transfection procedure did not significantly alter the mechanical behavior. To assess reversibility and further confirm viability, we reperfused with basal (serum-containing) media beginning after 1 hour of nocodazole treatment. As the nocodazole was washed out, microtubules reformed (not shown), cells regenerated their processes, and the matrix returned to a more relaxed state (Fig. 3, Movie 5).

It should be noted that not every cell process pulled the matrix inward during nocodazole-induced retraction. When present, thin dendritic cell processes often retracted without much impact on the surrounding matrix, similar to what was observed in serum-free conditions. Some pseudopodial processes also appeared to detach from the matrix and rapidly retract, resulting in a release of pre-existing tension on the matrix and outward displacement of adjacent collagen fibrils (Movie 3, bottom right process). It is likely that the cell-matrix adhesions along these processes are disrupted following addition of nocodazole.

Microtubule disruption activates Rho

Previous studies have demonstrated that microtubules sequester Rho-GEF, and that release of Rho-GEF following microtubule disruption induces Rho/Rho kinase activation and cellular contraction (Krendel, 2002; Kwan and Kirschner, 2005; Liu BP, 1998; Ren et al., 1999; Zhang et al., 2001). To assess whether similar changes occur in corneal fibroblasts, cells were plated in collagen matrices, cultured for 24 hours, and exposed to nocodazole for 0 minutes (control), 10 minutes or 30 minutes. The level of Rho activation was then measured using the G-LISA method. Figure 4 shows the mean and standard deviation of three independent experiments for each treatment group, as well as the GTP Rho positive control. In all three experiments, both 10 and 30 minutes exposure to nocodazole increased the level of Rho activation as compared to controls; this increase reached statistical significance at 30 minutes.

Microtubules regulate cell polarization and morphology in 3-D culture

Our results suggest that microtubules may regulate corneal fibroblast contractility by sequestering/releasing Rho GEF, thereby modulating Rho/Rho-kinase activation. However, microtubules are also known to regulate other aspects of cell behavior including cell polarization (Finkelstein, 2004; Sloboda, 1980; Watanabe, 2005). In order to assess these Rho kinase independent functions in corneal fibroblasts, we studied the effects of microtubule disruption in the presence of the specific Rho-kinase inhibitor Y-27632.

Figure 5 shows time-lapse DIC images before and after nocodazole treatment in the presence of Y-27632. Cells treated with Y-27632 for 24 hours were elongated and had dendritic processes (Fig. 5A, arrowheads), as previously described (Kim et al., 2006). Nocodazole induced retraction of some dendritic processes (Fig. 5B) without associated pulling in of the ECM (Movie 6). Interestingly, this process was immediately followed by formation and extension of broader “lamellipodial” processes from random locations along the cell body (Fig. 5B and C arrows, Movie 6). These extending lamellipodia were able to generate tractional forces, as indicated by inward displacement of collagen fibrils (Movie 6). Thus there was a switch from thin dendritic processes at the ends of cells to broader lamellipodial processes along the cell body when microtubules were disrupted.

These data suggest that microtubules may be required for both the maintenance of dendritic cell processes and cell polarization. This is consistent with studies by Grinnell and coworkers showing that interfering with microtubules blocked the initial formation of dendritic extensions by dermal fibroblasts in relaxed (floating) 3-D matrices (Rhee et al., 2007). To further assess the role of microtubules on initial cell spreading and polarization in our model, experiments were performed in which reagents were added to the media at the time of cell plating. Fibroblasts were incubated for 24 hours in Y-27632, nocodazole, or both Y-27632 and nocodazole, stained for f-actin, and imaged using 3-D LSCM. Following culture in basal (serum-containing) media, corneal fibroblasts in 3-D collagen matrices had a bipolar morphology, and stress fibers were sometimes observed along the cell body (Fig. 6A). In contrast, cells treated with Y-27632 were more elongated, had a dendritic morphology (Fig. 6B). Collagen fibrils were compacted and aligned nearly parallel to the pseudopodial tips at the end of cells in basal media (Fig. 6A). However, little compaction and alignment of extracellular matrix is observed in the presence of Y-27632, consistent with previous findings (Fig. 6B) (Kim et al., 2006). Incubation with nocodazole reduced the amount of cell spreading and induced larger and more numerous stress fibers (Fig. 6C). Collagen fibrils were also more compacted and aligned along the cell body as compared to basal media. When treated with both nocodazole and Y-27632, cells lost their polarity and developed numerous convoluted branching processes (Fig. 6D). These processes were generally broader than the dendritic processes observed with Y-27632 alone (compare Figs. 6D and 6B). A more random collagen

organization was also observed, with no preferred matrix alignment at the ends of the cell and isolated areas of matrix compaction around the cell body.

Quantitative analysis demonstrated that cell length was significantly increased when Rho-kinase was inhibited, and decreased when microtubules were disrupted (Fig. 7A). To further assess the differences in cell shape, we compared the breadth/length ratio following nocodazole treatment. This ratio was higher when microtubules were disrupted, confirming a less polarized morphology (Fig. 7B). Taken together, these data demonstrate that microtubules play a role in cell spreading, polarization and morphology in 3-D collagen matrices.

DISCUSSION

Numerous studies using 2-D culture models have established an important role for microtubules in regulation of cell mechanical activities such as spreading, migration and contraction. However, cells reside within 3-D extracellular matrices *in vivo*, and ECM geometry has also been shown to effect cell morphology, mechanical activity, and adhesion organization and composition (Abbott, 2003; Bard and Hay, 1975; Cukierman et al., 2001; Cukierman et al., 2002; Doane and Birk, 1991; Friedl and Brocker, 2000; Grinnell et al., 2003; Tomasek et al., 1982). While it is known that microtubules play a role in cell spreading and contractility within 3-D collagen matrices, these processes have not been assessed dynamically in individual cells. Furthermore, their influence on the subcellular pattern of force generation and local cell-induced matrix reorganization has not been reported previously. In this study, we used both static (3-D) and time-lapse (4-D) imaging of cells and their surrounding ECM to directly assess the effects of microtubule disruption on the mechanical activity of human corneal fibroblasts. Our data indicates that microtubules modulate both fibroblast contractility and local collagen matrix reorganization via regulation of Rho/Rho kinase activity. In addition, under conditions where Rho-kinase is inhibited, disruption of microtubules leads to retraction of dendritic cell processes, and rapid formation and extension of lamellipodial processes at random locations along the cell body, eventually leading to a convoluted cell shape. Thus microtubules also appear to play a central role in dynamic regulation of corneal fibroblast spreading mechanics, morphology and polarity in 3-D culture.

Previous studies have demonstrated that as microtubules polymerize during cell spreading on 2-D substrates they sequester Rho-GEF (guanine nucleotide exchange factor), and that release of Rho-GEF following microtubule disruption induces Rho activation (Krendel, 2002; Kwan and Kirschner, 2005; Liu BP, 1998; Ren et al., 1999; Zhang et al., 2001). Consistent with these studies, our results also show that the level of Rho activation was significantly increased in corneal fibroblasts after nocodazole treatment. Activated Rho binds to and activates Rho kinase, which inhibits myosin light chain (MLC) phosphatase, resulting in elevated MLC phosphorylation and increased contractility (Amano et al., 1998; Amano et al., 1996; Chrzanowska-Wodnicka and Burridge, 1994; Kimura et al., 1996; Parizi et al., 2000). When corneal fibroblasts were plated inside 3-D collagen matrices and allowed to spread for 24 hours, subsequent addition of nocodazole induced rapid retraction of cellular processes. However, the impact of these retractions on the ECM were dependent on the culture conditions. Under serum-free conditions, retractions did not directly correlate with movement of collagen fibrils, and there appeared to be rupturing of cell-matrix adhesions along the retracting pseudopodial processes. In contrast, under serum conditions in which stronger focal adhesions develop, retraction of cell processes was accompanied by a corresponding centripetal displacement of the ECM surrounding the cell, indicating more effective transmission of cellular forces to the ECM.

To further assess the affects of microtubule disruption on collagen displacement and reorganization, confocal microscopy was performed. Time-lapse confocal imaging of GFP-

zyxin labeled cells provided direct visualization of the correlation between centripetal movement of collagen fibrils and focal adhesions. As the ECM was pulled inward by retracting cell processes, the collagen fibrils at the ends of the cells were compacted and aligned parallel with the direction of displacement. This pattern of reorganization is consistent with FEM analysis which showed increased tension in the ECM parallel to the cell axis at the ends of pseudopodia. Cytochalasin D reversed the process and induced cell elongation and matrix relaxation, demonstrating that the effects of nocodazole were actin-dependent. Taken together, the data suggests that actomyosin contractile activity underlies the increase in cellular force generation and matrix reorganization induced by treatment with nocodazole.

Following 24 hour exposure to nocodazole beginning at the time of plating, cells remained somewhat bipolar, but their length was significantly decreased, consistent with previous observations of corneal fibroblasts within attached (stressed) collagen matrices (Tomasek and Hay, 1984). In addition, a dramatic increase in both the number of stress fibers and the amount of matrix compaction and alignment surrounding the cells was also observed. This increase in local matrix reorganization is distinctly different from what occurs in floating (unstressed) collagen matrices, in which nocodazole inhibits global matrix contraction when added at the time of cell plating (Lee et al., 2003). Intact microtubules are required for cell spreading in unrestrained (floating) 3-D collagen matrices, but not on more rigid substrates (Rhee et al., 2007). Our attached matrices apparently provide enough mechanical resistance to allow microtubule-independent cell spreading and formation of the actomyosin contractile machinery which underlies local matrix remodeling.

An important finding in this study was that the effects of microtubule disruption were reversible. As nocodazole was washed out, microtubules reformed, cells regenerated their processes, and the matrix returned to a more relaxed state. Kolodney et al. previously used an isometric force transducer to measure the tension exerted by chick embryo fibroblasts within collagen matrices (Kolodney and Elson, 1995; Kolodney and Wysolmerski, 1992). Disrupting microtubules induced a 2–3 fold increase in force which correlated temporally with increased phosphorylation of the myosin regulatory light chain. The increase in both force and phosphorylation could be reversed by treating with paclitaxol to allow reformation of microtubules. Taken together with our other results, the data suggest that microtubules may be involved in dynamic regulation corneal fibroblast contractility by sequestering Rho GEF as cells lengthen, and releasing Rho GEF as they shorten, thereby modulating Rho/Rho kinase activation.

To investigate potential Rho kinase independent functions of microtubules in corneal fibroblasts, we further studied the effects of microtubule disruption in the presence of Y-27632. Cells treated with Y-27632 for 24 hours were elongated and had dendritic processes, as previously described (Kim et al., 2006). Nocodazole induced retraction of some dendritic processes without associated pulling in of the ECM. However, this process was immediately followed by formation and extension of broader “lamellipodial” processes from random locations along the cell body. Thus there was a dynamic switch from thin dendritic processes at the ends of cells to broader lamellipodial processes along the cell body when microtubules were disrupted. Grinnell and coworkers recently demonstrated that interfering with microtubules prior to cell spreading blocked the initial formation of dendritic extensions by dermal fibroblasts in relaxed (floating) 3-D matrices. In contrast, cells on 2-D substrates (which normally form lamellipodia) maintained the ability to spread in the absence of microtubules (Rhee et al., 2007). When corneal fibroblasts were treated with both nocodazole and Y-27632 for 24 hours (beginning at the time of cell plating), cells developed numerous convoluted branching processes. These processes were generally broader than the dendritic processes observed with Y-27632 alone. Quantitative analysis of cell shape demonstrated that cells did not achieve a normal bipolar morphology. A similar loss of polarity is observed when dermal

fibroblasts plated on top of collagen matrices are treated with nocodazole (Rhee et al., 2007). Taken together, our data suggest that microtubules play a central role in the mechanics of both the *initial formation* and the *dynamic maintenance* of corneal fibroblast structure and morphology within 3-D collagen matrices.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Dynamic assessment of the effects of nocodazole and/or cytochalasin D on cell mechanical activity. Following 24 hours of incubation in serum-free media (A–F) or serum-containing media (G–L), matrices were transferred to the microscope stage, and time-lapse DIC imaging was performed. **A–C:** Large retractions of the pseudopodial processes were observed following treatment with nocodazole, but much smaller displacements of the ECM were induced (cyan tracks). **D–F:** Zoomed DIC images from square region in A–C. **G–L:** Following culture in serum-containing media, nocodazole treatment induced cellular contraction, and collagen was simultaneously pulled inward (H, cyan tracks). Cytochalasin D reversed the process and induced cell elongation, and corresponding matrix relaxation (I, cyan tracks). **J–L:** FEM strain maps along the cell axis generated from matrix displacements (cyan tracks) in G–I. Color legend is in dimensionless units $\Delta L/L$ (change in length/initial length). Nocodazole treatment induced compression along the cell body, and tension at the end of the cells. In contrast, subsequent treatment with CytoD resulted in decompression of the ECM under the cell, and reduction of tension at the ends.

Figure 2.

Dynamic assessment of the effects of nocodazole and/or cytochalasin D on corneal fibroblast mechanical activity. Following 24 hours of incubation in serum-containing media, matrices were transferred to the microscope stage, and time-lapse confocal imaging was performed. Color overlays of GFP-tubulin (green, B–C) or GFP-zyxin (green, A, D–H) and collagen fibrils (red) allow interaction between cells and the extracellular matrix to be directly visualized.

A: Corneal fibroblast cultured for 24 hours in serum-containing media. GFP-zyxin is organized into focal adhesions along the pseudopodia, which appear to be attached to the collagen fibrils.

B: Microtubules emanated from a microtubule organizing center, and were generally aligned with the long axis of the cells. **C:** Same cell as in B. Nocodazole induced microtubule disruption and retraction of cell processes.

D–F: GFP-zyxin labeling revealed centripetal movement of focal adhesions and matrix reorganization (compaction and alignment) surrounding the cell following treatment with nocodazole.

G–I: A subset of focal adhesions (G, white arrows) was tracked following addition of nocodazole. The relationship between movement of focal adhesions (I, green arrows) and individual collagen fibrils (I, red arrows) is demonstrated.

Figure 3.

Dynamic assessment of nocodazole reversibility using time-lapse confocal imaging. Same cell as shown in Fig. 2D–F. After 50 minutes of nocodazole treatment (**A**), reperfusion with basal media (i.e. without nocodazole) induced re-extension of cellular processes and matrix relaxation (**B** and **C**).

Figure 4.

Rho activation data. Graph shows the mean and standard deviation of three independent experiments. Data shown is absorbance over background signal (wells incubated with lysis buffer alone instead of cell lysates). Rho activation was significantly increased following 30 minutes of nocodazole treatment ($P < 0.05$, ANOVA with Holm-Sidak method for multiple comparisons) as compared to untreated control cells.

Figure 5. Time-lapse DIC images before and after nocodazole treatment in the presence of Y-27632. **A:** Cells treated with Y-27632 for 24 hours were elongated and had dendritic processes (arrowheads). **B,C:** Nocodazole induced retraction of these dendritic processes without force generation (i.e. no associated pulling in of the ECM), followed by formation and extension of lamellipodial processes from random locations along the cell body (B and C, arrows).

Figure 6.

Assessment of the role of microtubules on initial cell spreading and polarization in 3-D culture. Fibroblasts were incubated for 24 hours in basal (serum-containing media), Y-27632, nocodazole, or both Y-27632 and nocodazole, stained for f-actin, and imaged using 3-D LSCM. **A:** Following culture in basal media, corneal fibroblasts had a bipolar morphology, and stress fibers were sometimes observed along the cell body. Collagen fibrils were compacted and aligned nearly parallel to the pseudopodial tips at the end of cells. **B.** Cells treated with Y-27632 were more elongated, had a dendritic morphology, and did not induce matrix reorganization. **C.** 24 hours of incubation with nocodazole reduced the amount of cell spreading and induced larger and more numerous stress fibers. Collagen fibrils were also more compacted and aligned along the cell body as compared to basal media. **D.** When treated with both nocodazole and Y-27632, cells lost their polarity and developed numerous convoluted branching processes. A more random collagen organization was also observed, with no preferred matrix alignment at the ends of the cell and isolated areas of matrix compaction around the cell body.

Figure 7.

Quantitative analysis of cell morphology following 24 hours of culture in serum (control), Y-27632, nocodazole (Noc) or Noc + Y-27632. Each bar shows mean and standard deviation from four independent experiments. **A.** Cell length was significantly increased when Rho-kinase was inhibited, and decreased when microtubules were disrupted. **B.** The breadth/length ratio was also higher when microtubules were disrupted, confirming a less bipolar morphology.