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Abstract

Cells and tissues are mechanical as well as biochemical machines, and cellular response to mechanical cues can have as large an influence on structure and function as chemical signals. The mechanical properties of cells are largely determined by networks of semiflexible polymers forming the cytoskeleton, which has viscoelastic properties that differ in important ways from the viscoelasticity of common synthetic materials. Two such features are the high resistance to deformation achieved by a remarkably low volume fraction of protein, and the increase in stiffness that occurs when the cytoskeletal network is deformed. The actin filaments, microtubules and intermediate filaments that comprise the cytoskeleton of most cell types are linear polymers with some important similarities but also some fundamental differences. The stiffness of the individual polymer types is vastly different, with persistence lengths ranging from 1 mm for the 24 nm diameter microtubules to a few 100 nm for the 10-14 nm diameter intermediate filaments. The material properties of these biopolymer networks are proposed to function as part of the mechanosensing mechanism in cells, and the stiffness of cytoskeletal networks is similar to that of common extracellular protein networks such as those formed by collagen and fibrin in which many cell types function. Examples of the morphologic differences in fibroblasts and astrocytes grown on chemically identical surfaces overlying gels with elastic moduli spanning the range from 50 to 12,000 Pa illustrate the large effect of stiffness differences on cell structure and function.

Comments

BIOPOLYMER NETWORKS AND CELLULAR MECHANOSENSING

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ABSTRACT

Cells and tissues are mechanical as well as biochemical machines, and cellular response to mechanical cues can have as large an influence on structure and function as chemical signals. The mechanical properties of cells are largely determined by networks of semiflexible polymers forming the cytoskeleton, which has viscoelastic properties that differ in important ways from the viscoelasticity of common synthetic materials. Two such features are the high resistance to deformation achieved by a remarkably low volume fraction of protein, and the increase in stiffness that occurs when the cytoskeletal network is deformed. The actin filaments, microtubules and intermediate filaments that comprise the cytoskeleton of most cell types are linear polymers with some important similarities but also some fundamental differences. The stiffness of the individual polymer types is vastly different, with persistence lengths ranging from 1 mm for the 24 nm diameter microtubules to a few 100 nm for the 10-14 nm diameter intermediate filaments. The material properties of these biopolymer networks are proposed to function as part of the mechanosensing mechanism in cells, and the stiffness of cytoskeletal networks is similar to that of common extracellular protein networks such as those formed by collagen and fibrin in which many cell types function. Examples of the morphologic differences in fibroblasts and astrocytes grown on chemically identical surfaces overlying gels with elastic moduli spanning the range from 50 to 12,000 Pa illustrate the large effect of stiffness differences on cell structure and function.

INTRODUCTION

Mechanical effects can have as great an influence on cell structure, function, and gene expression as signaling by chemical stimuli. The importance of gravity-dependent loads on such structures as bone, cartilage and muscle has been known in both clinical and research studies, and similar response to forces has been documented in soft tissues and single cells. Cellular response to mechanics has two aspects. Mechanical forces imposed from an external source to the outside of the cell membrane (Galbraith et al., 2002) may be transmitted directly to structures in the cell interior or trigger a variety of signals such as the opening of an ion channel (Glogauer et al., 1997), or local unfolding of a protein domain (Ohashi et al., 1999), to activate chemical signaling cascades that alter structure or function in a manner analogous to the way chemical signals such as growth or motility factors function. Alternatively, even in

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the absence of an external force, cells probe the mechanical properties of their environment by applying internal stresses to sites where they attach to surfaces or extracellular matrices. The generation of force on sites of cell-substrate attachment has been demonstrated by deformation of flexible substrates (Harris et al., 1980) and the significance of substrate stiffness on cell structure and motility was elegantly demonstrated in a recent study of fibroblasts moving on protein-laminated hydrogels (Pelham and Wang, 1997). These studies showed that the structure of the fibroblast cytoskeleton and the rate of movement were strongly dependent on the stiffness of the material to which the cell was bound. Related results have now been demonstrated in numerous cell types including smooth muscle, neurons, and epithelial cells. In these studies, the cytoskeleton was implicated as part of the mechanosensing or response mechanism.

Living cells have shapes and mechanical properties that depend on the three-dimensional architecture of the underlying cytoskeleton. The three main cytoskeleton filament systems are actin microfilaments (F-actin), microtubules (MTs) and intermediate filaments (IFs). These systems interact to provide strength and allow the cell to have unique and complex viscoelastic properties. Studying the properties of these biopolymer systems individually has led to a more lucid understanding of the mechanics of the cell in its entirety.

Knowledge of the mechanical role of cytoskeletal elements can be deduced from in vitro rheology (the quantitative analysis of deformation and flow) of F-actin, MTs and IFs. Simultaneous measurements of deformation and applied force (quantified as stress = force/area) over a range of deformation rate and extent (or strain) is essential to define the mechanical properties of non-ideal fluids and soft solids (Hvidt and Heller, 1990). More simply and relevant to examining cytoskeletal polymers, rheology is important for the category of materials whose mechanical properties change dynamically and non-uniformly when a force is applied. F-actin, microtubules and intermediate filaments all fall under this category as do most of the filaments that form the extracellular matrix that surrounds most tissue cells. In this report we characterize some of the structural and mechanical properties of the cytoskeletal polymers that determine cellular mechanics and show how strongly the structure of fibroblasts and astrocytes depends on the stiffness of the materials to which these cells bind.

MATERIALS AND METHODS
Protein purification Actin was purified from rabbit skeletal muscle by methods previously described (Spudich and Watt, 1971) and was stored in monomeric form in a buffer containing 2 mM Tris, 0.2 mM $CaCl_2$, 0.2 mM dithiothreitol (DTT), 0.5 mm ATP, pH 8.0.

Polymerization was initiated by addition of 1/20 volume of concentrated salt solution containing 40 mM $MgCl_2$; 3 M KCl, 50 mM Tris, pH 7.4. Vimentin, purified from Ehrlich tumor cells as described elsewhere, was a generous gift from Prof. Peter Traub, and was dissolved in monomeric form in a buffer containing 10 mM Tris, 6 mM DTT, pH 7.6 and polymerized by addition of KCl to 150 mM (Janmey et al., 1991). Microtubules (MT) a generous gift of Jean-Francois Leterrier, were prepared in a semi purified form containing also microtubule associated proteins (MAPS) from two cycles of polymerization /depolymerization of extracts prepared from bovine brain (Leterrier et al., 1982). Depolymerized MT were stored on ice in solutions containing 100 mM MES, pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM GTP, 0.4 M sucrose and 0.1 mM PMSF. Polymerization was initiated by addition of 2 mM $MgCl_2$ and raising the temperature to 37°C.

Rheology

Measurements of shear modulus were made in a Rheometrics RFSII instrument in oscillatory mode using a deformation frequency of 10 rad/s and varying the maximal strain amplitude from 1 to 100%. All samples were polymerized under the conditions defined in the previous section within the plates of the rheometer to ensure an intact gel structure and good adhesion to the plate surfaces. Measurements were made at 23°C for vimentin, F-actin and polyacrylamide gels and at 37°C for microtubules. Under the conditions used to initiate assembly, the degree of polymerization of each protein is very high and the polymerization reaction goes nearly to completion. For the case of actin the polymeric form is in steady state with approximately 0.1 µM actin monomers, so that for 2 mg/ml (48 µM) actin, approximately 95% of the protein is assembled into polymers of typical average length 5 microns, corresponding to a degree of polymerization of 1850 (Janmey et al., 1994; 1988). Similar parameters characterize the other two polymer types. Therefore the materials formed by these proteins are well characterized as linear polymers with contour lengths many times greater than the apparent mesh size, which under these conditions is a few 100 nms.

Atomic Force Microscopy

AFM (atomic force microscopy) imaging was done with a Bioscope IIIa instrument (Digital Instruments, Santa Barbara, CA). Samples (300 µl) were transferred to freshly cleaved mica double taped on a plastic mini-dish. Imaging was performed in fluid tapping mode using DNP-S oxide-sharpened silicon nitride probes (Digital Instruments, Santa Barbara, CA) with a spring constant of 0.32 N/m at a scanning frequency of 0.8-1.2 Hz. Images were processed using Nanoscope (R) IIIa software (v. 5.12; Digital Instruments), and the WSxM freeware (v. 3.0; Nanotec, Madrid, Spain) was used for height measurements, flattening and inserting scale bars. The G-

scanner was calibrated using a standard grid. All buffers were filtered using a sterile 0.22 µm mesh-size filter.

Preparation of Polyacrylamide Substrates

Polyacrylamide substrates were synthesized by adapting two previously described methods (Schnaar et al., 1978, Wang and Pelham, 1998). All gels consisted of either 10% (for fibroblasts) or 7.5% acrylamide (for astrocytes) to maintain a consistent polymer mass. The bis-acrylamide concentration, however, varied between 0.03% and 0.3% to alter stiffness. A mixture comprised of an aqueous layer of acrylamide, bis-acrylamide, ammonium persulfate and N,N,N',N'-tetramethylethylenediamine (TEMED) under a layer of toluene containing 0.5% acrylic acid N-hydroxy succinimide ester (Sigma) was polymerized between two chemically modified coverslips. The N-succinimidyl acrylate copolymerized within the gel resulted in the top surface of the gel being chemically active. The reactive group was displaced by an amine-group containing polypeptide, either fibronectin or poly-L-lysine (Sigma). After extensive washing with HEPES buffer pH 8.2 to remove toluene and traces of unpolymerized acrylamide or N-succinimidyl acrylate which are cytotoxic, astrocytes or fibroblasts were plated, and their integrins engaged the bound polypeptide resulting in a surface coated with adherent cells.

Cell Culture and Microscopy

Rat primary astrocytes were obtained from prenatal rats and maintained for 14 days in culture before experimental use. Briefly, embryos (E17-E19) were removed by caesarean section from a timed-pregnant Sprague-Dawley rat and the hippocampi of the left and right hemispheres were surgically removed. The tissue was digested in trypsin/DNAse at 37°C, centrifuged (1000g x 5 min) and filtered to derive a cell suspension from each pup. Cells were grown in an incubator at 37°C and 5% CO_2 in DMEM (BioWhittaker) supplemented with Ham's F12 (Sigma) and 5% fetal bovine serum (Hyclone) for 7 days followed by an additional 5 days culture in Neurobasal medium (Gibco) also supplemented with 5% fetal bovine serum, 2 mM l-glutamine, 50 µg/mL streptomycin and 50 u/mL penicillin. Cultures used for experiments were >98% astrocytes based on this method. NIH 3T3 fibroblasts were maintained at 37°C and 5% CO_2 in DMEM supplemented with 5% normal calf serum, 50 µg/mL streptomycin and 50 u/mL penicillin.

Cultured astrocytes and fibroblasts were plated on gels at a density of 300,000 cells in 2 ml placed on a 35 mm well containing an 18 mm diameter gel. At 48 hours, the cells were examined using a Leica inverted microscope with a 40x objective, and phase images were acquired with a Hamamatsu digital camera.

Evaluation of Cell Morphology

Quantification of individual cell morphology was performed using NIH Image J software. For each gel stiffness, a minimum of 100 cells was traced, and cell area in square microns was determined. Only single cells were

measured to eliminate the effect of cell-cell interaction on morphology.

RESULTS AND DISCUSSION
Atomic force microscope imaging of cytoskeletal polymers
Both similarities and differences among the three classes of cytoskeletal polymers are evident from the images shown in Figure 1. All three types of protein form linear, unbranched polymers that can be several microns long. The diameters of the filaments, most accurately measured from the heights derived from AFM shown in the lower panels, range from 24 nm and 11 nm for the approximately cylindrical microtubules and intermediate filaments, to 6 nm for the thinner and more ribbon-like F-actin. The most striking structural difference, however, is in filament flexibility. MTs are nearly inflexible on the length scale of a micron and other studies have shown that the persistence length of these polymers is on the order of mm (Gittes et al., 1993), in contrast to values of 10 microns for F-actin (Gittes et al., 1993) and <1 micron for IFs (Hohenadl et al., 1999). F-actin (Figure 1C) is still sufficiently stiff that its contour is approximately straight on the length scale of a few hundred nm, or the mesh size of the cytoskeletal network, but vimentin IFs (Figure 1B) like other IFs are much more flexible and form loops within a single polymer chain and tangles between polymers. The flexibility of IF is not understood structurally since these filaments are larger in diameter than F-actin but at least an order of magnitude more flexible (Janmey et al., 2003). The increased flexibility is the basis for the unusual elastic properties of IF networks shown in Figure 2.

Viscoelastic properties of cytoskeletal polymer gels
The stiffness of different cytoskeletal polymer gels, as quantified by the shear storage modulus (G') over a range of deformations, quantified as shear strain, is shown in Figure 2. Despite the fact that MTs have by far the greatest stiffness of all cytoskeletal filaments, the networks they form have very low elastic moduli, even when MTs are polymerized in the presence of microtubule-associated proteins (MAPS) that stabilize their polymeric state and promote associations between polymers. At small strains, F-actin forms the stiffest gels, but this network structure is fragile and fails at moderate strains on the order of 10 %, as also reported elsewhere (Janmey et al., 1994; 1988). Vimentin, a type of intermediate filament forms networks that have low moduli at small strain, but their stiffness increases at larger strains and can become larger than the stiffness of F-actin at strains above 50% that occur in tissues and single cells (Simon and Schmidt-Schonbein, 1990). Reproducibility of measurements of the absolute values of elastic moduli for these proteins gels is no greater than 50% in most cases, but the general trends shown in this figure are highly reproducible: microtubule networks never strain stiffen at the deformations shown in this figure and tend to flow, whereas F-actin gels stiffen and then fail at much smaller strains compared to vimentin gels which generally show the largest degree of strain stiffening.
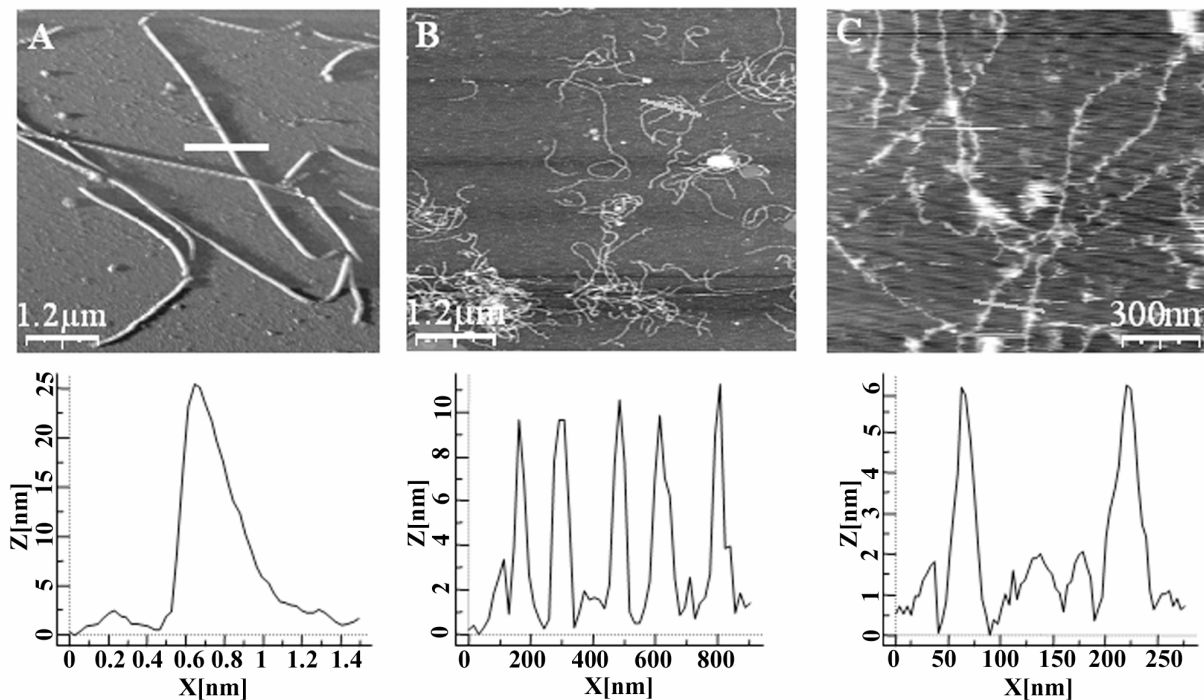

Figure 1. Atomic force micrographs of microtubules (A) vimentin intermediate filaments (B) and filamentous actin (F-actin) adsorbed to mica and imaged by tapping mode in aqueous solution, as described in Materials and Methods. The lower panels show height measurements of the filaments made over the bar superimposed on the images in the corresponding upper panels.

The stability of intermediate filaments at large strains is thought to be due to the significant excess contour length between network branch points that can be extended as the network deforms leading to a non-linear increase in the force-extension relation in the range just before the filament becomes fully extended at which point either longitudinal elongation or filament brakeage occurs (MacKintosh et al., 1995). F-actin is much stiffer and more fragile to longitudinal elongation than are IFs and so actin networks rupture at much smaller strains. MTs are so stiff that they are already fully extended between junction points in the resting state, and so stresses are concentrated at junctions rather than being taken up by filament extension. Microtubule networks actually show a reduction in shear modulus when under strain, an effect that may be due to the orientation of the network and slipping of filaments in the direction of shear. Except in specialized settings such as cilia where bundles of MTs are tightly linked by motor proteins, cellular MTs may act not to resist shear or elongational stresses, but rather are proposed to resist compression, and therefore to counteract contractile forces imposed by actin filaments pulled by myosin motors (Ingber, 1997).

The magnitudes of moduli in these networks also depend on the nature and extent of crosslinking between filaments, and part of the softness of MT networks may also arise from the lack of stable crosslinking proteins to link them to each other as actin crosslinking proteins can function to stabilize actin networks. Quantitative measurements of elasticity for purified cytoskeletal networks provide estimates, when extrapolated to the higher concentrations of actin and vimentin found in cells, suggesting that the cytoskeleton may have elastic moduli on the order of several thousand Pa, a value consistent with some estimates of whole cell stiffness (Radmacher et al., 1996). However, cell stiffness can be rapidly altered by activation of factors that cause gel-sol transformations or other reorganization of the cytoplasmic matrix. Such estimates do however, suggest that stiffness ranges from 10 to 10,000 Pa are likely to span the range where the cytoskeleton may respond elastically, and help define conditions for designing synthetic materials with stiffness relevant to cellular response.
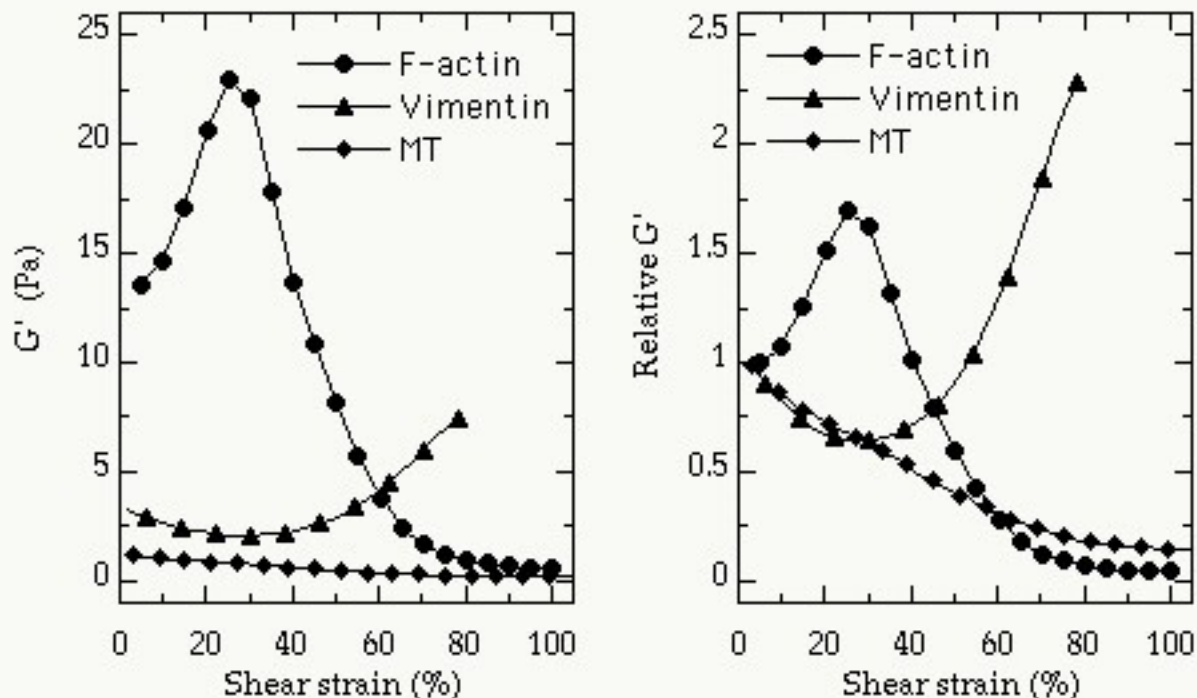
Figure 2. Shear storage moduli (G') measured from oscillatory deformation of samples at 10 rad/sec over a range of deformation magnitudes (strain) of networks formed by 8 mg/ml microtubules (diamonds) 3 mg/ml vimentin intermediate filaments (triangles) and 2 mg/ml F-actin (circles). Absolute values of shear modulus in Pa are shown in panel A and relative values normalized to the value in the limit of low strain are shown in panel (B).

Effects of Substrate Stiffness on Cell Morphology

To examine the effect of substrate stiffness on cell morphology, primary astrocytes were plated on a substrate with shear modulus (G') of either 50 Pa or 9500 Pa and NIH3T3 fibroblasts on gels with a G' of 500 Pa or 12000 Pa. Acrylamide was cross-linked with varying amounts of bis-acrylamide to create gels that varied in rigidity due to the number of bis cross-links while maintaining polymer mass. Rheological measurements performed verified that a range of stiffness from 50 Pa to ~12 kPa can be produced by 7.5 % polyacrylamide (data not shown). Polyacrylamide gels alone are unable to

trigger cell adhesion. The gel surface, therefore, was adapted by co-polymerizing an acrylic acid N-hydroxy succinimide which bound to the gel and from its N-terminus was able to bind an amine. The gel surface was incubated with the amine-containing protein fibronectin at 0.05 mg/mL for NIH3T3 fibroblast cultures and poly-L-lysine at 0.1 mg/mL for primary astrocyte cultures. An observable difference in morphology was evident in cells in contact with one another or in close proximity to other cells. Therefore, cells were plated at a low density to minimize cell-cell interaction and only single cells not in contact or near other cells were focused on for morphology analysis.

After two days in culture, a qualitative evaluation of cells on different substrates demonstrated that matrix rigidity had an obvious effect on cell shape (Figure 3A-3D). Astrocytes and fibroblasts on soft substrates were attached to the substrate but were rounder, smaller and extended fewer processes compared with cells on stiff substrates. Adhesion also appeared weaker on soft gels as there were visible phase bright rings surrounding many cells, suggesting reduced adhesion, and when equal densities of cell were plated there were significantly fewer cells bound after 24 hours on the soft gels. Quantification of the amount of bound fibronectin or polylysine showed no significant difference on the surfaces of different gel, so the difference in adhesion appears to have a physical and not a chemical origin. Although the cells on soft gels appeared rounded and less adherent, they were still metabolically active and capable of dividing as evidenced by examination of DNA synthesis levels (data not shown). On stiff substrates both cell types appeared polygonal with a larger cell body and processes extending from the cell body, a morphology similar to typical cells grown on tissue culture plastic.
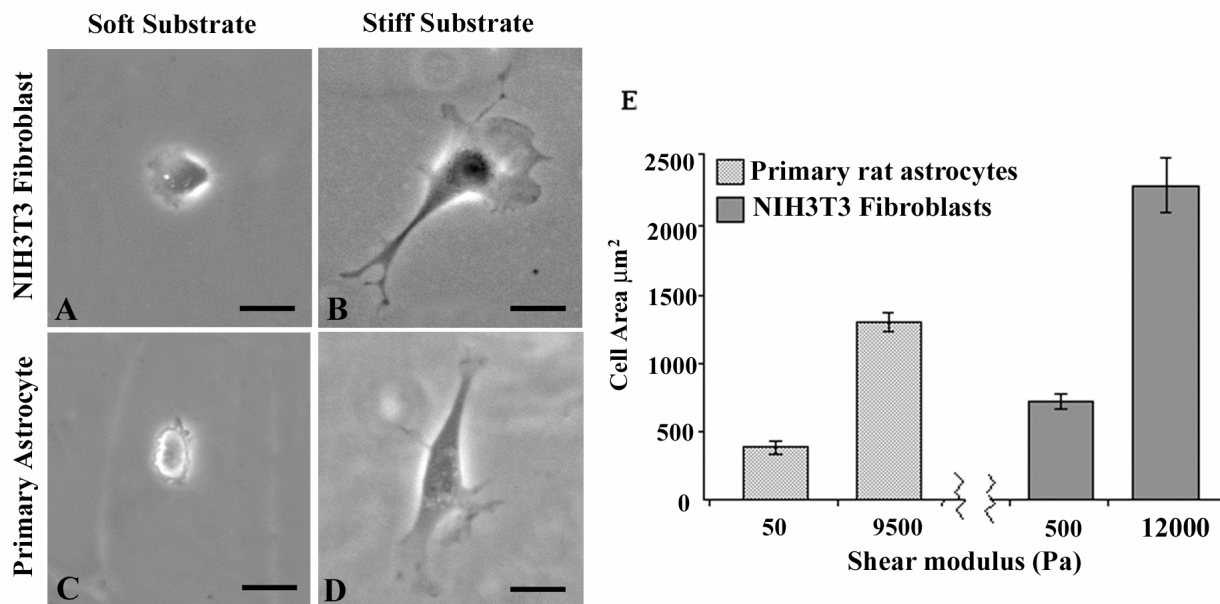
Figure 3. Cell morphology of astrocytes and fibroblasts plated on polyacrylamide gels of varying rigidities. *A,B,* Morphology of NIH3T3 fibroblast plated on soft gels (G'~500Pa) [A] and stiff gels (G'~12 kPA) [B]. *C,D,* Morphology of primary rat astrocyte on soft gel with G'~50 Pa [C] and stiff gel with G'~9.5 kPa [D]. *E,* Quantification of average cell area reveals an increase in area of both fibroblasts and astrocytes when plated on stiffer substrates. Scale bar = 14 μm.

To quantify this difference, individual cells were traced and their adherent areas were calculated. Under each condition, the variance of spread area was rather small, usually less than 10% of the average value. However, the spread areas of both astrocytes and fibroblasts cultured on surfaces with stiffness around 10 kPa were over 300% greater than the areas of the same cell types bound to softer materials (Figure 3E).

In most models of cell spreading, a cell must generate traction forces at adhesion sites that stretch or otherwise perturb the membrane of the cell, initiating actin polymerization (Pelham and Wang, 1997). Traction forces may not be able to form without adequate external resistance from a soft extracellular matrix. If the shear modulus of the matrix is very small, the cell will pull on the substrate and not meet any resistance, therefore, not initiating the counter-forces necessary for cell spreading (Glogauer et al., 1997, Lo et al., 2000). Without these forces, the cell membrane remains slack and the attached actin and intermediate filament cytoskeletons experience low strain where their shear moduli are relatively low. In this scenario cells on soft gels cannot send or receive the mechanical signals that cells on stiffer flexible substrates or on tissue culture plastic use to trigger morphological and molecular responses.

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