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The mechanical rigidity of the extracellular matrix regulates the structure, motility, and proliferation of glioma cells

Theresa A. Ulrich[1,2], **Elena M. de Juan Pardo**[1,†], and **Sanjay Kumar**[1,2,*]

[1] Department of Bioengineering, University of California, Berkeley

[2] University of California San Francisco-University of California Berkeley Joint Graduate Group in Bioengineering

Abstract

Glioblastoma multiforme (GBM) is a malignant astrocytoma of the central nervous system associated with a median survival time of 15 months, even with aggressive therapy. This rapid progression is due in part to the diffuse infiltration of single tumor cells into the brain parenchyma, which is thought to involve aberrant interactions between tumor cells and the extracellular matrix (ECM). Here we test the hypothesis that mechanical cues from the ECM contribute to key tumor cell properties relevant to invasion. We cultured a series of glioma cell lines (U373-MG, U87-MG, U251-MG, SNB19, C6) on fibronectin-coated polymeric ECM substrates of defined mechanical rigidity and investigated the role of ECM rigidity in regulating tumor cell structure, migration, and proliferation. On highly rigid ECMs, tumor cells spread extensively, form prominent stress fibers and mature focal adhesions, and migrate rapidly. As ECM rigidity is lowered to values comparable to normal brain tissue, tumor cells appear rounded and fail to productively migrate. Remarkably, cell proliferation is also strongly regulated by ECM rigidity, with cells dividing much more rapidly on rigid than compliant ECMs. Pharmacological inhibition of nonmuscle myosin II-based contractility blunts this rigidity-sensitivity and rescues cell motility on highly compliant substrates. Collectively, our results provide support for a novel model in which ECM rigidity provides a transformative, microenvironmental cue that acts through actomyosin contractility to regulate the invasive properties of GBM tumor cells.

Keywords

glioblastoma multiforme; cytoskeleton; cell adhesion; myosin; tumor invasion; Rho GTPase; mechanotransduction

Introduction

Glioblastoma multiforme (GBM) is a high-grade astrocytoma associated with a median survival time of 15 months, even with surgical care, chemotherapy, and radiotherapy (1). This uncommon aggressiveness is partly derived from diffuse infiltration of single tumor cells into the surrounding brain parenchyma prior to diagnosis, making complete tumor debulking virtually impossible. A central therapeutic goal has been to develop strategies to limit invasion,

*Address correspondence to: Sanjay Kumar, MD, PhD, Department of Bioengineering, University of California, Berkeley, Berkeley, CA 94720-1762, Phone: 510-643-0787, FAX: 510-642-5835, E-mail: skumar@berkeley.edu.
†Current Address: University of Navarra, San Sebastian and Pamplona, Spain.

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thereby rendering the tumor addressable by local therapies. This has led to an expansive effort to identify key molecular regulators of GBM tumor cell motility *in vitro* and *in vivo* (1–4).

Among the key regulators of cell motility are the extracellular matrix (ECM) and the cellular components needed to recognize and process ECM-derived cues, including adhesion proteins and molecular motors. Several *in vitro* studies have demonstrated the importance of fibronectin, laminin, collagen, and other ECM proteins in stimulating a migratory phenotype in both GBM cell lines and biopsy explants (5). Strong correlations between matrix metalloproteinase (MMP) activation, GBM invasion, and poor prognosis indicate that tumor cells can extensively remodel the surrounding matrix during invasion (1). This remodeling is frequently accompanied by secretion of ECM proteins such as tenascin-C, which has been associated with angiogenesis and enhanced cell motility (6). Because these behaviors are central to tumor progression, a growing body of work has begun addressing the functional contributions of key mediators of cell-ECM interactions, including integrins (7), focal adhesion proteins such as vinculin and focal adhesion kinase (FAK) (8,9), and molecular motors such as nonmuscle myosin II (NMMII) (2). These interactions have taken on new significance in light of the recent Cancer Genome Atlas (TCGA) sequencing effort, which has revealed a preponderance of genomic lesions across GBM tumors in the EGFR/Ras/PI3K pathway (10), which has been previously linked at multiple levels to ECM-based signaling in the context of glioma invasion (11).

However, while it is clear that biochemical signaling from the ECM is an important regulator of GBM invasion, the biophysical components of this crosstalk are comparatively poorly understood, particularly in light of the recent explosion of work demonstrating the powerful influence of biophysical inputs such as the density, rigidity, and geometry of the ECM on cell fate, migration, and morphogenesis (12,13). For example, recent studies have shown that manipulating substrate elasticity in a 2D cell culture system can strongly influence lineage specification of naïve human mesenchymal stem cells (14), the migration, adhesion, and cytoarchitecture of smooth muscle cells (15), and the outgrowth of neurons versus glia in mixed cortical cultures (16). A growing literature indicates that the biomechanical properties of cells and the surrounding ECM directly influence and are influenced by the progression of neoplastic disease (17,18). For example, a modest change in substrate elasticity during *in vitro* culture of mammary epithelial cells is enough to cause otherwise normal cells to develop early hallmarks of a growing tumor (19). With respect to invasion, ECM rigidity can control the motility of human prostate carcinoma cells in 3D ECMs (20) and the density and activity of tumor cell invadopodia, which spatially focus proteolytic secretion (21). Promising new chemotherapeutics have already begun targeting components of the contractility and adhesion machinery, including the integrin antagonist Cilengitide (7) and small-molecule inhibitors of FAK (8).

Several lines of evidence indirectly suggest that ECM-derived biomechanical cues may play an important role in GBM specifically, although the pathophysiological significance of these cues remains poorly understood. Neurosurgeons anecdotally report that GBM tumors are stiffer than the surrounding parenchyma, consistent with the efficacy of ultrasound elastography in guiding surgical resection (22). Moreover, the tremendous anatomic variation in stiffness within the brain (23) may feature prominently in invasion; for example, the infiltrative path of GBM cells tends to favor interfaces between mechanically distinct structures, such as the basement membrane of blood vessels and white matter tracts (24). Recent work suggests that the mechanical properties of the cellular microenvironment may fundamentally alter the migration of glioblastoma cells *in vitro* and *in vivo* (2,25,26). For example, the migration rate of SNB19 cells cultured on elastomeric films correlates with substrate mechanical properties (controlled by altering the duration of heating and distance from a Bunsen burner) (25), and several 3D studies have shown strong correlations between matrix density and invasion from

tumor spheroids (26,27). Glioma cell migration depends on actomyosin-generated contractile forces and involves dynamic, spatially-regulated changes to the cytoskeleton and cell-matrix adhesion complexes. Many of these motility-mediating interactions are shaped by ECM mechanics, and the expression levels of several contractility-mediating signaling molecules, including RhoA and RhoB, are thought to correlate with tumor malignancy (28,29). Indeed, Rosenfeld and colleagues recently demonstrated that NMMII is needed to deform the nuclei of glioma cells to enable amoeboid motion through ECM pores, and invading tumor cells *in vivo* significantly upregulate NMMII expression relative to endogenous brain cells (2).

Motivated by the growing evidence that mechanobiological cues are present in human gliomas, by the limited existing information regarding the potential pathophysiological role of ECM elasticity, and by the significant implications that this knowledge could have for the creation of mechanobiologically-inspired therapeutics, we sought to test the hypothesis that micromechanical cues from the ECM influence fundamental properties of GBM tumor cells relevant to growth and invasion. We fabricated ECM substrates with independently-defined mechanical and biochemical properties, and with rigidities spanning the range between normal and tumor tissue. Our studies reveal for the first time that ECM elasticity strongly affects GBM cell structure, motility, and proliferation, and that this mechanosensing requires a competent actin cytoskeleton, Rho GTPase-based signaling, and NMMII.

Materials and Methods

Synthesis and characterization of ECM substrates

We followed a previously-established method for fabricating defined-rigidity polyacrylamide ECMs (15,30) with minor modifications (see Supplementary Materials and Methods). All substrates were functionalized with human plasma fibronectin (Millipore Corp., Temecula, CA) to achieve a nominal surface density of 2.6 $\mu g/cm^2$. The macroscopic elastic shear modulus of each gel formulation was measured at 37°C using an Anton Paar Physica MCR 301 rheometer with 25-mm parallel plate geometry. Amplitude sweeps over the range γ=0.1–10% were used to identify the linear regime; frequency sweeps at 5% strain over 0.1–10 Hz were then used to extract the storage, loss, and complex moduli of each sample. Three measurements were made on each sample, and at least three independent samples were measured per condition. Elastic moduli of 0.08 kPa, 0.25 kPa, 0.8 kPa, 19 kPa, and 119 kPa were measured for gels containing final acrylamide/bis-acrylamide (A/B) percentages of 3%A/0.05%B, 4% A/0.075%B, 5%A/0.1%B, 8%A/0.6%B, and 15%A/1.2%B, respectively, as described in detail elsewhere.*

Cell culture

U373-MG human glioma cells were obtained from the Tissue Culture Facility at the University of California, Berkeley, U87-MG and U251-MG human glioma cells were kindly provided by Dr. C. David James (University of California, San Francisco), and human SNB19 and rat C6 glioma cells were kindly provided by Dr. Andrew Wurmser (University of California, Berkeley). U373-MG and U87-MG cells were cultured in Dulbecco's Modified Eagle's medium (DMEM, Gibco) supplemented with 10% Calf Serum Advantage (J.R. Scientific, Woodland, CA) and 1% penicillin/streptomycin, MEM nonessential amino acids, and sodium pyruvate (Gibco). SNB19, U251-MG, and C6 cells were cultured in DMEM supplemented with 10% calf serum and 1% penicillin/streptomycin.

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Microscopy, fluorescence staining, and morphometric analysis

All live-cell and fluorescence imaging was performed using an inverted Nikon TE2000-E2 microscope equipped with a motorized, programmable stage (Prior Scientific, Inc, Rockland, MA), an incubator chamber to maintain constant temperature, humidity, and CO_2 levels (In Vivo Scientific, St. Louis, MO), a digital camera (Photometrics Coolsnap HQ II, Roper Scientific, Tucson, AZ), and SimplePCI software (Hamamatsu Corporation, Sewickley, PA). Cells were fixed and stained for F-actin and vinculin as described in detail in Supplementary Materials and Methods. Cell spreading measurements were obtained by quantifying the area of phalloidin-stained cells using Image J software (National Institutes of Health, Bethesda, MD). High-magnification epifluorescence images acquired through polyacrylamide gels in Figs. 1 and 5 were enhanced by applying a uniform background subtraction to the entire image; subsequent adjustments to brightness and contrast were applied as necessary.

Measurement and analysis of cell migration

Cells were plated at a subconfluent density of 1000 cells/cm^2 at least 10 hours prior to the start of imaging in at least three independent experiments. In each experiment, 10× phase contrast time-lapse images were acquired every 15 minutes over a 12 hour period for at least 10 representative fields of view per substrate and at least 2 substrates per condition. A representative subset of time-lapse videos was analyzed using SimplePCI software, where the periphery of each cell in each frame was used to define an object, and the Motion Tracking and Analysis module of SimplePCI was used to track the centroid of each object throughout the time sequence.

Measurement of cell proliferation

Cell proliferation was measured with a bromodeoxyuridine (BrdU) assay as described in Supplementary Materials and Methods.

Inhibition of cell contractility

Rho-associated kinase (ROCK) inhibitor Y-27632 (Calbiochem, La Jolla, CA), NMMII inhibitor blebbistatin (Sigma-Aldrich), and actin polymerization inhibitor cytochalasin D (Sigma-Aldrich) were added to the cell culture media in relevant timelapse and immunofluorescence experiments after the cells had been allowed to adhere for at least 10 hours.

Statistical analysis

Data are reported as mean ± standard error unless otherwise noted. Statistical comparisons between three or more sets of data were performed with a one-way analysis of variance (ANOVA) followed by a Tukey-Kramer HSD (honestly significant difference) test for pair-wise comparisons. A student's unpaired t-test was performed if statistical comparisons were made between two sets of data. p-values less than 0.01 denote statistical significance.

Results

To test the hypothesis that ECM rigidity influences the behavior of cultured glioma cells, we fabricated a series of fibronectin-coated polyacrylamide ECMs of variable stiffness as we and others have done previously (15,30,31). Here, ECM stiffness is dictated by the ratio of monomer (acrylamide) to crosslinker (bisacrylamide), and fibronectin is covalently grafted at fixed density to the gel surface. Because polyacrylamide does not support appreciable passive protein adsorption, this system enables independent control of ECM stiffness and ligand density. The rigidity of our ECMs ranged from one order of magnitude below normal brain tissue (0.08 kPa) to three orders of magnitude above (119 kPa).

ECM rigidity alters glioma cell morphology and cytoskeletal organization

We began by asking whether changes in ECM rigidity were sufficient to grossly and systematically alter glioma cell morphology and cytoskeletal organization. To answer this, we cultured cells on ECMs with varying rigidity and captured both cell-ECM adhesion area and cytoskeletal F-actin organization. The adhesive contact area of U373-MG cells decreased dramatically with decreasing substrate stiffness (Fig. 1), a finding that was qualitatively reproducible for U87-MG, U251-MG, SNB19, and C6 cells (Supplementary Fig. S1). U87-MG (Fig. 1B, C) and U373-MG (Supplementary Fig. S2) cells cultured on rigid substrates were typically well-spread with visible actin stress fibers and discrete, elongated vinculin-positive focal adhesions. Importantly, cells cultured on glass and the stiffest polyacrylamide substrates were similar in this respect, suggesting that the conjugation chemistry does not significantly interfere with adhesion-based cytoskeletal assembly. Cells cultured on progressively softer substrates showed decreasing spreading area, along with a rigidity-dependent dissipation of stress fibers and focal adhesions. Cells on the softest substrates were uniformly rounded with cortical rings of F-actin and small, punctate vinculin-positive focal complexes. Interestingly, cell rounding on the softest substrates did not reflect apoptosis, as evidenced by positive staining for the nuclear antigen Ki67 (Fig. 1A).

ECM rigidity regulates the random motility of glioma cells

Given that productive cell motility is critical to invasion, we next asked whether changes in ECM rigidity could alter migration speed in culture (Fig. 2). To address this question, we used time-lapse imaging to record the random motility of sparsely-cultured cells over 12 hours. Mean migration speeds fell dramatically with decreasing substrate rigidity for both U373-MG and U87-MG cells, with statistically indistinguishable speeds observed on the most rigid substrates and glass (Fig. 2A,B; Supplementary Movies S1–4). Cells on the glass surface adjacent to polyacrylamide substrates exhibited similar morphology and qualitatively similar motility to cells on fibronectin-coated glass substrates (not shown), effectively ruling out the possibility that rigidity-dependent changes in motility are related to altered paracrine signaling.

Concurrent with these reductions in cell speed, we observed a gradual transition in the mode of cell motility (Fig. 2C, Supplementary Movies S5–S8). Cells on glass moved in a smooth, gliding fashion with broad lamellipodia and continuous actin turnover at the leading edge. As ECM stiffness was reduced, this motility began transitioning to a “stick-slip” pattern in which cells would thin and extend as the leading edge advanced, with the trailing edge abruptly detaching and snapping forward to catch up to the cell body. While cells on glass and 119 kPa ECMs migrated with broad lamellipodia, the lamellipodia of cells on 0.8 kPa ECMs were smaller and less stable. Cells on the most compliant ECMs (0.08 kPa), actively extended small, thin filopodial processes over periods of 6–12 hours but failed to establish lamellipodia capable of supporting migration.

While the migration of all cell lines was highly sensitive to ECM rigidity, we observed variation between cell types. Specifically, on rigid substrates, U373-MG, SNB19, and U251-MG cells typically exhibited prominent broad, ruffled lamellipodia and a polygonal morphology, whereas the U87-MG and C6 cells exhibited a comparatively elongated spindle morphology (Supplementary Fig. S1), as reported by others (32, 33). However, all cell lines reduced to a mutually indistinguishable rounded morphology with largely non-productive filopodial extension when cultured on the most compliant substrates (0.08 kPa).

ECM rigidity regulates glioma cell proliferation

The above results demonstrate that ECM rigidity can substantially regulate the structure and motility of cultured glioma cells. Given that alterations in shape and motility have been previously correlated with alterations in tumor growth (34), we reasoned that ECM rigidity

might concurrently alter cell proliferation. We first observed that in long-term cultures, cells on stiff substrates reached confluency more rapidly than cells on soft substrates and at a level that could not be solely attributed to differences in cell spreading area (not shown). To test this connection more rigorously, we used BrdU incorporation to measure percentages of dividing cells as a function of ECM rigidity (Fig. 3). These studies revealed a remarkable correlation between the proliferation rate of U373-MG and U87-MG cells and ECM stiffness, with an approximately 5-fold increase in the percentage of BrdU-positive cells on the stiffest substrates (119 kPa) compared to those on the softest substrates (0.08 kPa). Consistent with our previous measurements of cell structure and motility, BrdU incorporation on the stiffest substrates was comparable to glass.

Pharmacologic disruption of intracellular tension tempers mechanical regulation of glioma cell morphology, cytoskeletal architecture, and migration

Increasing ECM stiffness is associated with increased cell spreading and formation of actomyosin stress fiber bundles (Fig. 1), suggesting that ECM rigidity controls NMMII-mediated intracellular contractility. This relationship has been directly observed in other systems (14,35) and led to the hypothesis that NMMII and its upstream regulators are critical to processing rigidity-encoded cues. To test this hypothesis in our system, we asked whether glioma cells remained sensitive to ECM rigidity when actin cytoskeletal assembly and contractility are disrupted. Direct pharmacological inhibition of NMMII or ROCK blunted sensitivity of U87-MG (Fig. 4A) and U373-MG (Supplementary Fig. S3; Supplementary Movies 9–12) cell morphology to ECM rigidity, with cells exhibiting a stellate morphology in all cases. Remarkably, inhibition of either NMMII or ROCK on the softest ECMs not only enhanced adhesion but rescued cell motility, with cells spreading and resuming migration within minutes of drug addition (Fig. 4B). For all ECMs, ROCK- and NMMII-inhibited cells lacked prominent stress fibers and vinculin-positive focal adhesions (Fig. 5). To confirm that this rescue of cell motility requires competent actin polymerization, we treated cells with cytochalasin D, which disrupts F-actin and inhibits new polymerization. Indeed, treatment with cytochalasin D caused cytoskeletal collapse and loss of motility on stiff substrates but failed to induce spreading or rescue migration on the softest substrates (Figs. 4, 5).

Discussion

Although mechanistic studies of glioma growth and invasion have historically focused on biochemical and genetic factors, studies with other cell types have revealed that biomechanical cues can also powerfully regulate cell behavior. Here, we have begun to explore the role of ECM-based mechanical cues in controlling cell behaviors central to GBM pathophysiology. Our studies reveal for the first time striking stiffness-dependent differences in glioma cell structure, migration, and proliferation. This mechanoregulation is especially significant in light of the contrasting mechanical microenvironments associated with normal and tumor brain tissue. Importantly, we were able to temper stiffness-dependent differences in cell structure and migration by inhibiting NMMII-dependent contractility, suggesting that mechanical features of the tumor microenvironment, and the molecular systems that sense and process these features, may serve as handles for understanding and manipulating glioma cell physiology.

The phenomenon of durotaxis (or mechanotaxis) was first defined by Wang and colleagues after they observed directed migration of fibroblasts *in vitro* from soft to stiff regions of ECM (36); other investigators have observed that changes in ECM rigidity can either increase or decrease cell migration speed, with the relationship depending strongly on cell type, degree of adhesion, and other factors (15,25). High ECM stiffness enhances the expression and activity of contractility-mediating proteins such as Rho and ROCK (19), which intuitively correlates

with the enhanced expression of contractile proteins in many solid tumors (37). In the case of GBM, the role of Rho GTPases in mediating tumor growth and spread *in vivo* is complex and remains incompletely understood (28,29). However, lysophosphatidic acid-mediated NMMII activation can strongly stimulate astrocytoma motility *in vitro* (3), and Rho/ROCK inhibition sensitizes glioma cells to apoptosis induced by radiation (38) and chemotherapy (39). We observe stiffness-dependent enhancement in the robustness of cytoskeletal and focal adhesion structures, cell spreading, and migration (Fig. 1,2), consistent with the predominant model of dynamic mechanical reciprocity in which cells respond to rigidity-encoded cues through "inside-out" signaling that includes reinforcement of contractile and adhesive structures (13). How expression of contractility-mediating proteins in glioma cells varies with substrate rigidity is an intriguing issue that has not yet been addressed.

Pharmacological inhibition of NMMII or its upstream regulator ROCK blunts the sensitivity of glioma cells to ECM rigidity, with cells adopting a stellate morphology and becoming highly motile, even on compliant ECMs (Figs. 4–5; Supplementary Movies 9–12). Together, these results suggest that NMMII and its activators form a critical component of the ECM rigidity-sensing pathway in glioma cells, consistent with past observations in other cell types (14,15, 19,36). These results are also consistent with recently-discovered contributions of NMMII isoforms (NMMIIA, NMMIIB, and NMMIIC) to cell motility, traction generation and rigidity sensing. Specifically, NMMIIB-null fibroblasts rapidly alter cell shape by extending and retracting protrusions; analogous to our observations of glioma cells under NMMII inhibition, these fibroblasts migrate faster and are less morphologically sensitive to ECM rigidity than wild-type fibroblasts (40). Moreover, acute depletion of NMMIIA in mouse embryonic fibroblasts speeds cell spreading and slows retrograde flow of actin, suggesting that NMMIIA acts as a brake on cell spreading by globally retarding actin cytoskeletal remodeling (41). Additional mechanistic insights come from recent observations that glioma cell motility can be stimulated by inhibition of either NMMII (42) or ROCK (4); the latter effect may be blocked by concomitant inhibition of Rac GTPase, implying that the enhanced motility is due to Rac pathway disinhibition. Importantly, this balance between Rho and Rac activation may also be indirectly disturbed in glioma cells by pharmacologically inhibiting Ras (43), which offers an unexpected but potentially important mechanistic connection between Rho GTPase-based mechanosensing and the EGFR/Ras/PI3K pathway. Thus, in our studies, ROCK inhibition may have the dual effect of both releasing NMMII-based restrictions on cell spreading and enhancing Rac-mediated cell motility, explaining why ROCK inhibition rescues motility on compliant substrates more potently than direct inhibition of NMMII.

Finally, we observe that ECM rigidity strongly regulates glioma cell proliferation, with the stiffest ECMs supporting fivefold more proliferation than the softest ones (Fig. 3). While the magnitude of this effect is somewhat surprising, ECM rigidity has been observed to modulate cell growth in other systems, including fibroblasts (44), hepatocytes (45), and neural stem cells (46). One potential explanation is that changes in ECM rigidity might alter the speed of progression through the cell cycle by altering mechanochemical feedback during mitosis. Indeed, direct application of mechanical force can slow cytokinesis and induce shape asymmetries, which cells can actively correct by mobilizing NMMII to produce a restoring force (47). Second, ECM rigidity might regulate mitosis by synergistically triggering mechanotransductive and mitogenic signaling pathways. As described earlier, ECM rigidity can transform cultured breast epithelial cells from a benign, highly-differentiated phenotype into a dysplastic and proliferative one (19). This matrix-driven transformation is accompanied by activation of ERK, Rho GTPase, and NMMII-based contractility, is recreated by overexpressing constitutively active Rho or spontaneously-clustering integrins, and is reversed by inhibition of ROCK or ERK. Importantly, many of these pathways have also been implicated in epithelial-mesenchymal transition (48). All of this is consistent with a paradigm in which tumor cells and their pre-malignant progenitors sense matrix rigidification through enhanced

integrin clustering, which in turn activates ERK and mechanosensory signaling, thereby stiffening the cell and inducing proliferation. Suppression of these mitogenic pathways on compliant matrices may also explain why we fail to observe high proliferation in the face of low motility, as would be predicted by the “go or grow” hypothesis (34). The relationship between ECM rigidity, cellular mechanics, and EGFR/Ras/ERK signaling remains largely unexplored in GBM and other non-epithelial tumors, and given that 88% of clinical GBM tumors in the TCGA group bore mutations in the EGFR/Ras/PI3K pathway (10), it would be intriguing to ask if rigidity-dependent proliferation is accompanied by alterations in EGFR-based signaling or could be indirectly modulated by EGFR pathway manipulation.

In summary, we have shown that increasing ECM rigidity can induce a suite of phenotypic changes in human glioma cells that includes increased cell spreading, faster motility, and enhanced proliferation. As described earlier, bulk brain tissue has an elastic compliance of 0.5–1 kPa, similar to the most compliant matrices considered in this study. While we are unaware of systematic and definitive measurements of the mechanical rigidity of GBM tumor tissue, intraoperative ultrasound clearly demonstrates that tumors and their surrounding stroma are stiffer than normal brain parenchyma. Placed in context of the notion that invasive glioma cells actively remodel their microenvironment from brain-like to tumor-like (1), this raises the intriguing hypothesis that GBM tumor cells stiffen their surroundings as they invade. We envision that this remodeling could occur through a combination of proteolytic degradation of existing matrix components, secretion of matrix components *de novo*, induction of strain-stiffening, and contractility-dependent bundling and alignment of ECM fibrils, as was recently observed for invading breast cancer cells (49,50). The resulting microenvironmental stiffening may deliver reciprocal mechanobiological signals to tumor cells that act through integrins, focal adhesion proteins, Rho GTPases, and the cytoskeleton to promote shape plasticity, motility, and proliferation. If this is the case, then therapeutic interventions that either interfere with mechanotransductive signaling or mechanical remodeling may hold value in slowing or arresting GBM invasion, analogous to the use of integrin and FAK inhibitors (7,8). Though challenging, revisiting these ideas in the setting of 3D ECMs or *in vivo* platforms that allow precise tracking of cell-mediated mechanical remodeling during invasion should permit more direct evaluation of this hypothesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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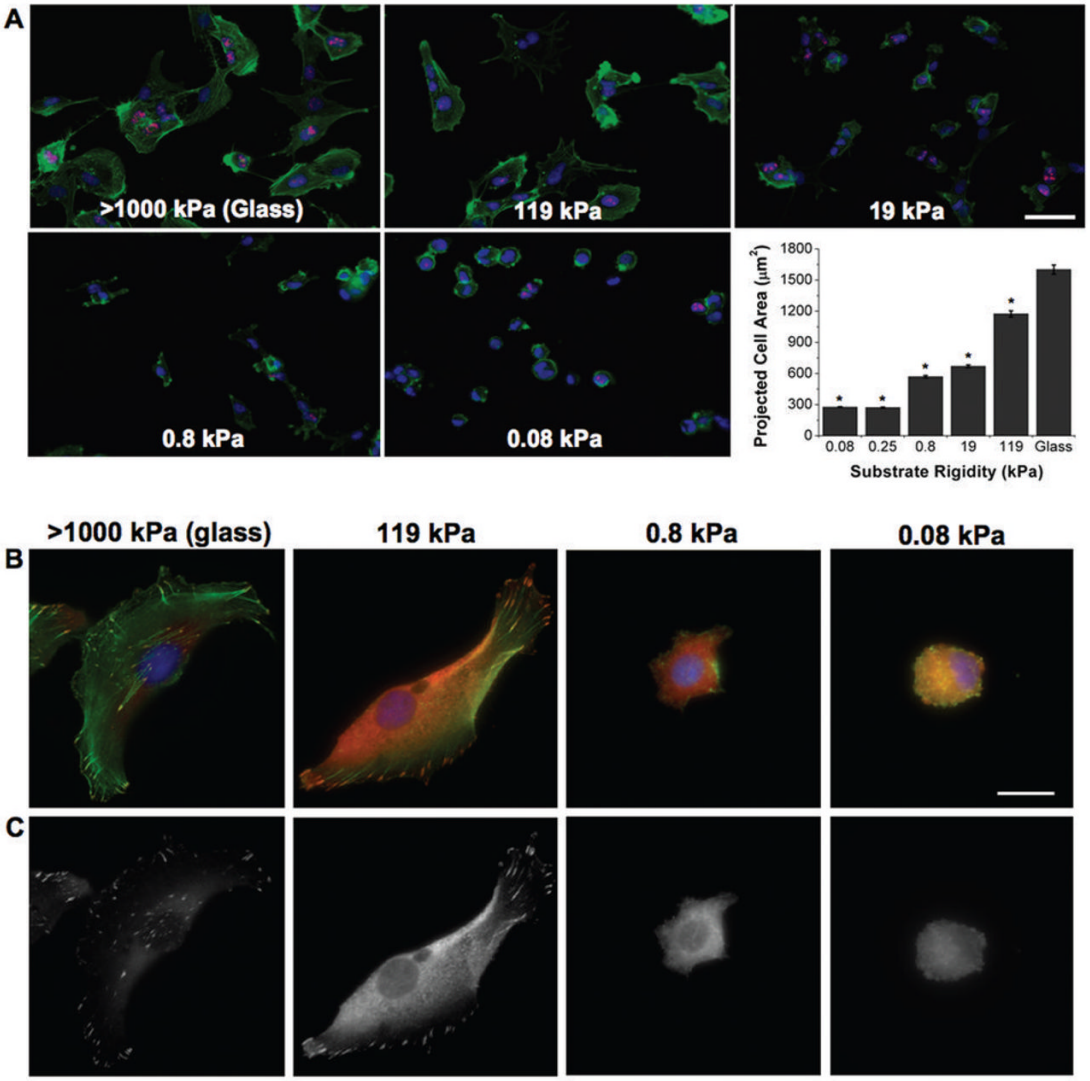

Figure 1. ECM rigidity alters glioma cell morphology and cytoskeletal organization

(A) Rigidity-dependent changes in cell structure. U373-MG cells cultured on fibronectin-conjugated glass and polyacrylamide gels over a range of stiffnesses were stained for F-actin (green), nuclear DNA (blue) and the nuclear antigen Ki67 (red). Note that a subset of cells on all substrates stained positive for Ki67. Bar is 50 μm. (*$p < 0.01$ with respect to glass; $n > 450$ cells for each condition) (B) High-magnification imaging of cytoskeletal and adhesive structures. U87-MG cells were stained for F-actin (green), nuclear DNA (blue), and the focal adhesion protein vinculin (red). Bar is 25 μm. (C) Isolated view of vinculin signal only, showing structure and distributions of cell-ECM adhesions.

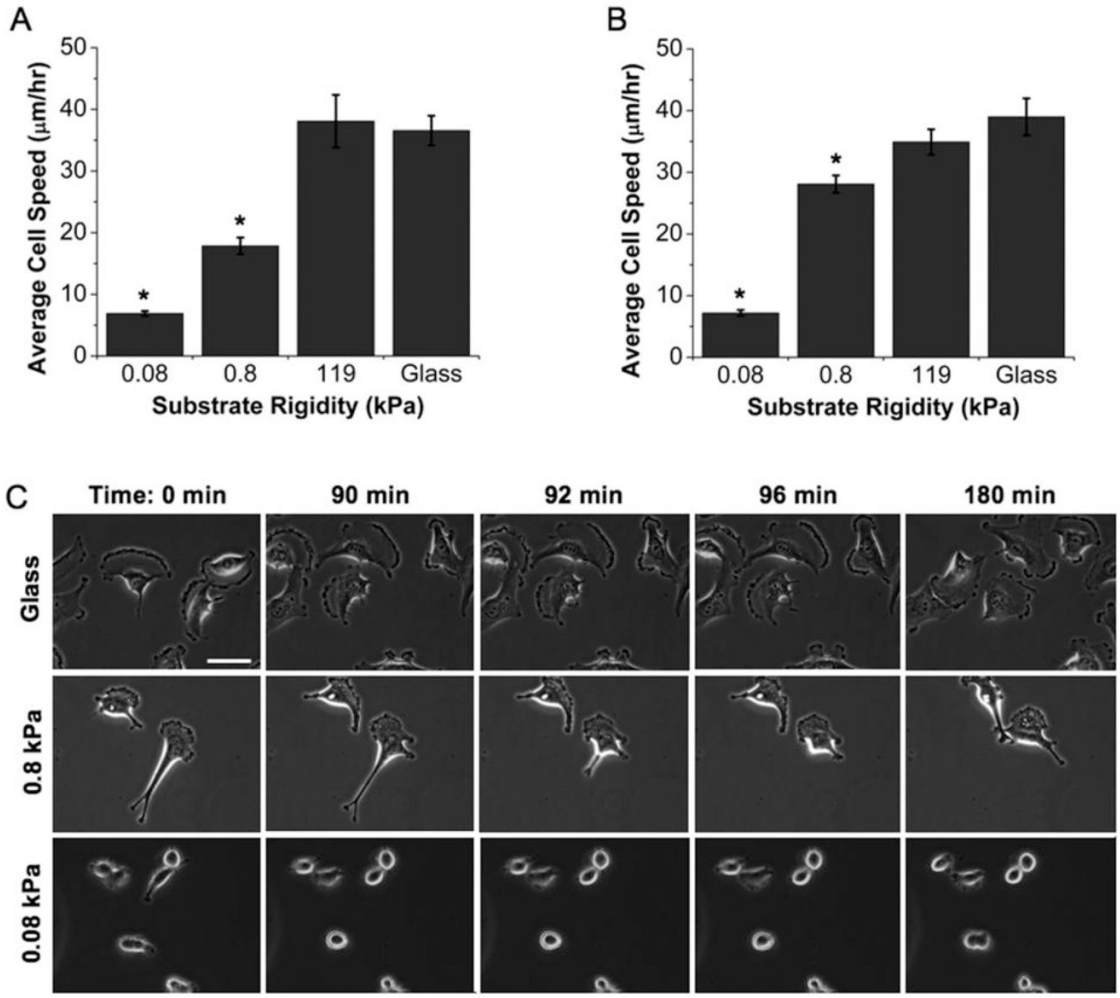

Figure 2. ECM rigidity regulates glioma cell motility

Effect of ECM rigidity on the random migration speed of (A) U373-MG and (B) U87-MG cells. Results represent the average migration rate from at least 15 cells per condition over 6–12 hours. Qualitatively similar dependences of migration speed on substrate stiffness were observed for SNB19, U251-MG, and C6 cells. (*$p < 0.01$ with respect to glass) (C) High-magnification imaging of U373-MG cell migration on ECMs of varying rigidity over both long time scales (columns 1–2 and 4–5) and short time scales (columns 2–4). Cells on glass (top row) migrate quickly, smoothly, and with broad, stable lamellipodia. Cells on 0.8 kPa ECMs (middle row) form smaller, less stable lamellipodia, and migrate in a "stick-slip" fashion, in which the cell thins and extends as it advances and then abruptly contracts as adhesions at the trailing edge rupture. Cells on 0.08 kPa ECMs (bottom row) continuously extend thin filopodia and fail to productively migrate. Bar is 50 μm.

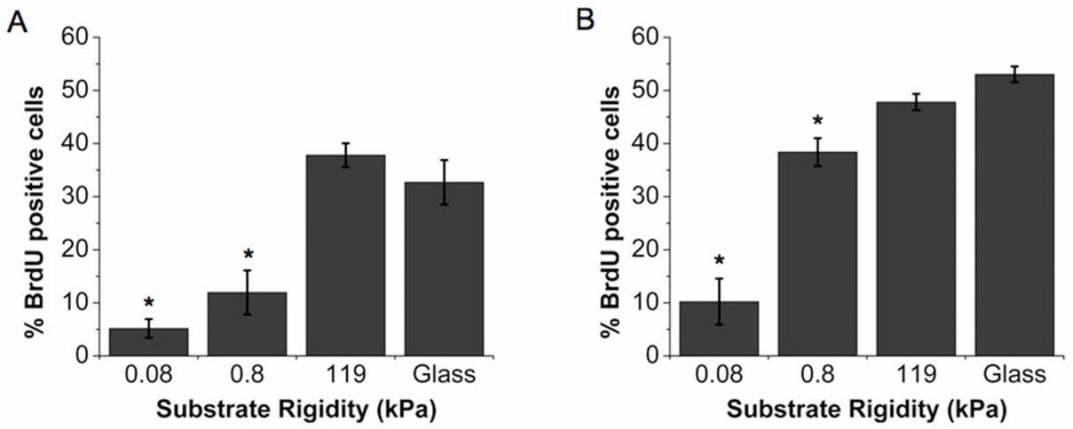

Figure 3. ECM rigidity regulates glioma cell proliferation

Effect of ECM rigidity on proliferation of (A) U373-MG and (B) U87-MG cells. Results represent quantification of n > 325 cells in at least 8 fields of view per substrate for at least five substrates per condition, where the percentage of dividing cells was determined as the average percentage of cells staining positive for BrdU incorporation. (*$p < 0.01$ with respect to glass).

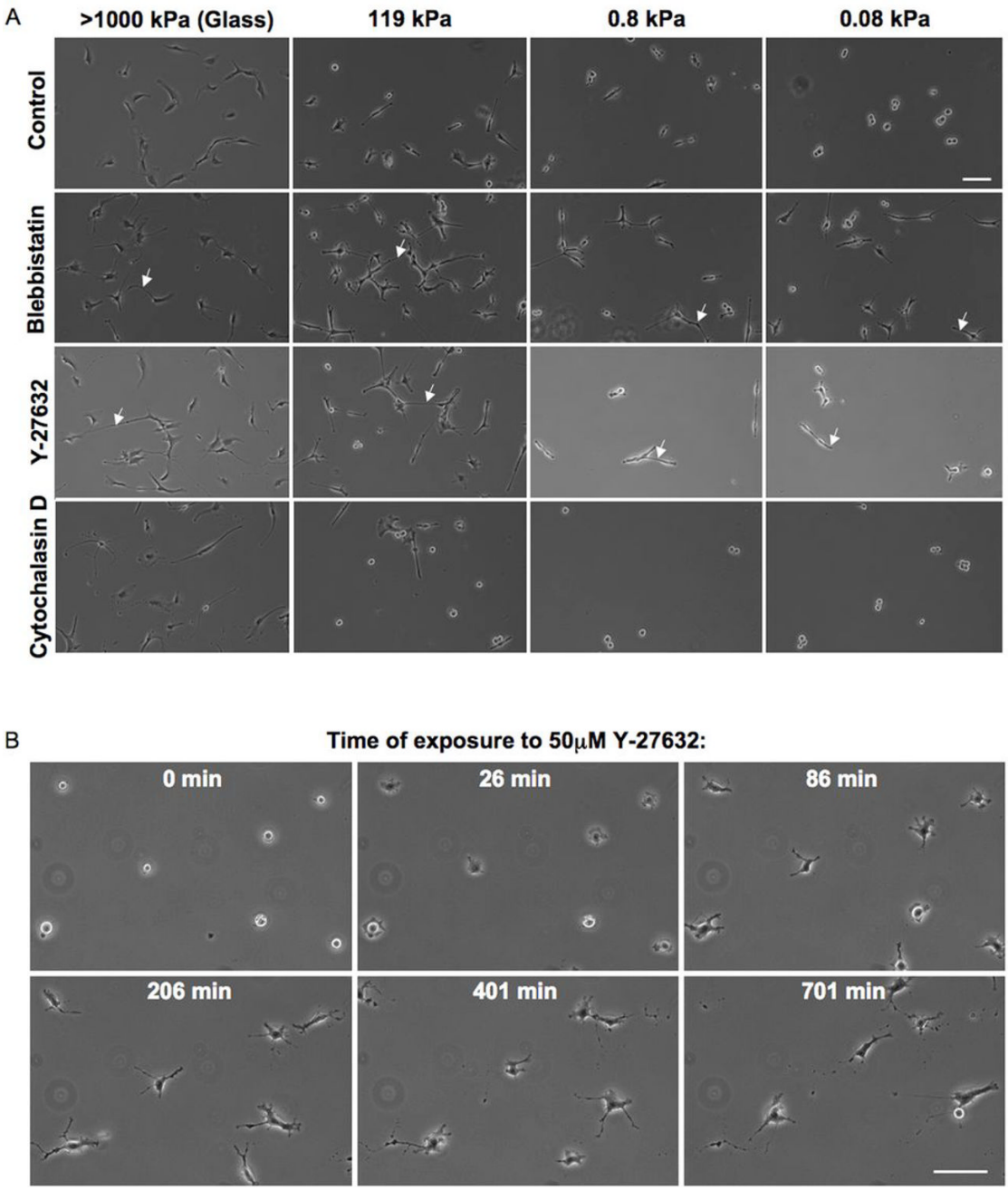

Figure 4. Pharmacologic inhibition of cytoskeletal contractility reduces stiffness-dependent differences in cell morphology

(A) U87-MG cells cultured on fibronectin-conjugated glass and polyacrylamide substrates in either the absence of drug (control) or 24 hours after addition of 25 μM blebbistatin, 10 μM Y-27632, or 1 μM cytochalasin D. Cells began extending actin-rich processes (arrows) within an hour after addition of Y-27632 or blebbistatin. Cytochalasin D induced stellation and rounding of cells on stiff substrates but had no appreciable effect on the morphology or migration of cells on the softest substrates. Bar is 100 μm. (B) U373-MG cells cultured on 0.08 kPa fibronectin-conjugated polyacrylamide substrates showed enhanced spreading and migration with addition of 50 μM Y-27632. Bar is 100 μm.

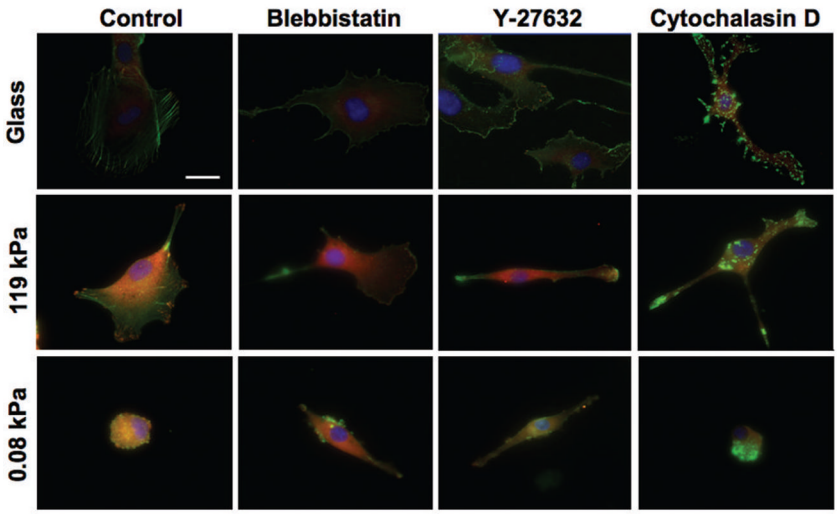

Figure 5. Pharmacologic inhibition of cell tension reduces stiffness-dependent differences in cytoskeletal and adhesive architecture

U87-MG cells cultured on fibronectin-coated glass and polyacrylamide substrates were treated with 25 μM blebbistatin, 10 μM Y-27632, or 1 μM cytochalasin D for 12–24 hours before fixation and staining for nuclear DNA (blue), F-actin (green), and vinculin (red). In all cases, the number and size of vinculin-positive focal adhesions was reduced with inhibition of cell tension. Blebbistatin and Y-27632 both induced cell spreading on the softest substrates, whereas cytochalasin D induced collapse of the actin cytoskeleton. Bar is 25 μm.