Live imaging of glioblastoma cells in brain tissue shows requirement of actin bundles for migration

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Live-cell imaging of glioblastoma U373 and U87 cells transfected with actin cytoskeleton markers has been used to study the re-arrangements that are associated with migration in two- and three-dimensional matrices and in brain tissue. In collagen gels and in brain slices, both cell types developed neuronal-like processes with ruffling membranes and filopodia. Blebbing cells were also observed, but these were mainly immobile. The retraction of trailing cell processes in a tissue environment was associated with the transient development and contraction of bundles of axial stress fibers. The inhibition of Rho-kinase caused glioblastoma cells in brain slices to become immobile and develop neurite-like processes at random, which indicates the requirement of Rho signaling and contractility for migration. Actin stress fibers were also observed in glioblastoma cells injected into the brains of living mice. Thus, invading glioblastoma cells use neurite-like extensions to penetrate between neuronal fibers and contractile actin bundles for traction of the cell body.

Keywords: Actin cytoskeleton, brain tissue, live imaging, motility, glioblastoma

Glioblastoma multiforme (GBM) is the most common of the malignant astrocytic brain tumors in humans. Glial tumors are classified according to the World Health Organization system, based on the aggressiveness of the tumor, with grade I the most benign and grade IV the most malignant (Kleihues and Sobin, 2000) and invasive (Palfi *et al*., 2004). In this classification, GMB is graded as 'IV'. Despite aggressive treatment, most patients die of the disease with a median survival of ~1 year. In addition, complete excision of tumorigenic tissue is difficult because of ill-defined boundaries. The invasion of healthy tissue by transformed cells, which leads to malignant tumor growth, requires the induction of the migration machinery of the invading cells. Although migration is a property of normal cells during embryonic growth, wound repair and immune defense, the process is deregulated during invasion. In the brain, dissemination of glioma cells follows myelinated fiber tracts and structures that contain extracellular matrix, such as the basement membranes of blood vessels (Giese *et al*., 1998). These patterns represent the two main routes of invasion that are observed in clinical disease. Different experimental approaches have already probed the molecular mechanisms that underlie the growth (Kilic *et al*., 2000; Uhl *et al*., 2004) and invasion (Gondi *et al*., 2004) of glioblastoma cells. However, little is known about the dynamics of their migration in the brain. In this context our study focuses on the characterization of the motility determinants of these cancer cells using different brain-tissue experimental systems and the brains of living mice. Our results, obtained using high-resolution, livecell imaging, reveal reorganization of the actin cytoskeleton in invading glioblastoma cells, with the presence of actin-like bundles during retraction of elongations and a reversible switch between ruffles, blebs and filopodial protrusions.

OBJECTIVE

Because little is known about the migration dynamics of glioblastoma cells in the brain, our study focuses on characterizing the re-organization of the cytoskeleton of these cells in three dimensional synthetic and tissue environments, including brain slices and the

brains of live mice. Cells transfected with green fluorescent protein (GFP)–actin were introduced into tissue models and their re-organization and motility monitored by high-resolution, live-cell imaging, using confocal microscopy.

METHODS

Cells culture, transient and stable transfections

U373 and U87 human glioblastoma cell lines (American Type Culture Collection) were maintained in a-MEM medium containing 10% FBS. Cell lines were then transiently transfected with the following DNA constructs: GFP–actin (Clontech), Dsred fluorescent protein (Dsred)–SM22 (kindly provided by Dominique Brandt, University of Hannover, Germany), Dsred–vasodilator-stimulated phosphoprotein (VASP), GFP–zyxin (kind gifts of J. Wehland and co-workers, GBF Braunschweig Germany) and yellow fluorescent protein (YFP)–phosphotyrosine-binding domain of the src proto-oncogene tyrosine-protein kinase (SH2) (kind gift of J. Kirchner, Weizmann Institute, Rehovot, Israel). U373 cells that stably express either GFP or GFP–actin were generated by a combination of G418 selection (1.4 μM) and several rounds of cell sorting with a Cytomation MoFlow to enrich for cell populations that express the respective proteins.

Flexible substrates and collagen gels

For cell-motility studies on soft substrates, glioblastoma cells were allowed to spread for ~15 hours on coverslips coated with soft polyacrylamide flexible substrates (Wang and Pelham, 1998) (Young's Modulus: 14 kN m^{-2}). Introduction of human glioblastoma cells into three-dimensional collagen gels was performed according to a method developed by P. Friedl (Friedl and Broker, 2003).

Brain slices in culture

Long-term culture of mouse brain slices

For organotypic, long-term culture of mouse brain slices, we followed the method introduced by Gahwiler (Gahwiler *et al.*, 1997). Briefly, after aseptic removal from seven-day-old (P7) mice (C57B/6), brains were sliced into sagittal sections (200– 250 μm thick) using a tissue chopper (McIlwain), still in aseptic conditions. Slices were put onto polycarbonate membrane filters (0.4 μm pore size) and placed into multi-wells fed from the bottom of the well with astrocytic medium consisting of Dulbecco's Modified Medium (DMEM, Sigma) with high glucose, supplemented with glutamine and 10% fetal calf serum. Alternatively, brain slices were put onto coverslips coated with 200 μg ml^{-1} collagen (Sigma) and leant on the bottom of multi-wells, only partially covered by medium. After 1–2 days in culture at 37°C in the presence of 5% CO2, slices were fed with N4 medium for neuronal cultures (Minimal Essential Medium, supplemented with 0.6% glucose and 0.5% mixed hormone solution). In a few days, brain tissue attached to either the membrane surface or the collagen-coated glass and started spreading. By exchanging the neuronal medium every 2–3 days, slices survived for a couple of weeks in these conditions. From a total of 480 brain slices from 26 mice, 55 were used for seeding glioblastoma cells.

Short-term culture of mouse brain slices

For short-term culture mouse brain slices, the brain was removed from P0 mice, plunged immediately into pre-warmed 4% agarose solution and left until it became completely solidified. Brains were then cut by vibratome into 350 μm thick slices. The slices were leant onto polycarbonate filters (8 μm pore size) inside wells refilled with neurobasal medium (Gibco) and left for 1 day at 37°C in the presence of 5% CO2 before injection of cells.

Mouse embryo neural-tube explants

For mouse embryo neural-tube explants, heads from 13.5-dayold embryos (E13.5) were cut at the level of rhombomeres with the otic vesicle as a caudal reference. Thereafter, the neural tube was opened along the dorsal midline and maintained like an 'open book'. Explanted tissue was placed on polycarbonate filters (8 μm pore size) with 10% fetal bovine serum in DMEM (Echevarria *et al*., 2001) and maintained for 24 hours at 37°C in the presence of 5% CO2 before injection of cells. All studies with mice were done in accordance with the relevant international laws and policies. A total of eight experiments were performed with embryo explants and eight with vibrotome-cut slices.

Cell seeding and injection onto brain slices

For seeding U87 and U373 cells onto long-term brain-slice cultures, either transiently or stably transfected cells were resuspended in 100–200 μl medium and placed inside a plastic ring around an individual tissue slice to prevent the cells swimming away. Cells were allowed to enter the slice for at least 15–20 hours at 37°C and 5% CO2 before taking time-lapse videos. A pellet of transiently transfected glioblastoma cells resuspended in 30–50 μl medium, together with fluorescein-Dextran were injected into vibratome-cut mouse brain slices and neural tube explants from mouse embryos using a programmable pressure injector (WPI). After ~15–20 hours at 37°C, slices and explants were fixed with 4% paraformaldehyde for microscopic observation.

Cell injection into freshly excised mouse brain

The brain of a P7 mouse was removed and stably transfected U373 cells injected immediately into the stem region using a 1-ml syringe (Braun Omnifix) with a needle of 0.45 μm internal diameter. Then, the brain was quickly put on a glass coverslip, placed in a heating chamber at 37°C, and refilled with glioblastoma and neuronal cell media (1:1), with the bottom face towards the microscope objective for video acquisition.

Cell injection into brains of living mice

P45 adult mice (C57/B6) were anesthetized by intraperitoneal injection of Ketamin + Rompun (10 mg g^{-1} mouse weight). Stably transfected glioblastoma cells were soaked with 50 μM CMTMR, (C2927; Molecular Probes) a rhodamine-derived cytoplasmic dye, to identify cells and to visualize the injection site (Uckermann *et al*., 2004). Cells were injected into the occipital visual cortex, close to the medial part of the brain and to the lamboid suture, 1 mm under the brain surface in the corpus callosum region. The injection was performed using a stereotaxic instrument (Stoelting) and, after the surgery, the brain bone box was reclosed and the head skin sewn. The mice were kept

alive for 4 days before being anesthetized using ether and perfused with 4% paraformaldehyde. The brain was removed and post-fixed overnight at 4°C. Thereafter, they were included in 4% agarose in PBS and cut frontally with a vibratome (Leica VT1000) into 80 µm-thick slices for fluorescence-microscopy observation.

Rho kinase inhibitor assays

For studies *in vitro*, 50 µM of the Rho kinase inhibitor Y-27632 (N-(4-pyridyl)-4-(1-aminoethyl)cyclohexanecarboxamide) (Tocris Cookson) was added to the medium in the microscope heating chamber ~20–30 minutes before video-imaging acquisition. For experiments in brain slices, 250 µM Y-27632 was added to the medium in the microscope heating chamber for diffusion into the tissue.

Fluorescence microscopy

Fluorescence images of fixed samples were recorded on a Zeiss Axioscope microscope equipped with an Axiocam using two Plan Neofluar objectives (5×, NA 0.15; and 10×, NA 0.30; Zeiss) and a Plan Apochromat oil-immersion objective (63×, NA 1.4; Zeiss). Fluorescence images of cells in three-dimensional collagen gels after 15-minute fixation with 4% paraformaldehyde, were obtained using a spinning-disk confocal microscope (Visitech-International) and a water-immersion objective (63×, NA 1.2: Zeiss) and collected using standard filters for GFP. High-resolution images of cells injected into the brains of live mice, after vibratome cutting, were acquired using laser-scanning confocal microscopes (Zeiss LSM510 on Axiovert 200M, Jena, Germany; Leica TCS SL, Leica Microsystems), with standard filters for GFP and an oil-immersion objective (63×, NA 1.4; Zeiss).

Time-lapse imaging

For experiments *in vitro*, cells were observed in an open chamber filled with cell medium at 37°C using an inverted microscope (Axiovert 135TV; Zeiss) equipped for epifluorescence and phase-contrast microscopy with an oil-immersion objective (63×, NA 1.4; Zeiss). Data were acquired using a back-illuminated, cooled CCD Micromax camera (Visitron System) driven by IPLab software (version 3.5.5, Scanalytics). For cells plated on flexible substrates, into three-dimensional collagen, brain slices and whole, fresh brain, observations were performed in an open heating chamber at 37°C, using a spinning-disk confocal microscope (Visitech-International) and a water-immersion objective (63×, NA 1.2: Zeiss). Images were collected using standard filters for GFP and Dsred. In all cases, time-lapse images of moving cells were taken every 60 seconds for up to 2 hours. Three-dimensional reconstructions were made using the IPLab software described previously. Quantification of the processes length and migration speed of cells was performed using Metamorph software (Metamorph Offline). Three-dimensional reconstructions of cells (cell images) were performed using IPLab and Image-J software.

RESULTS

Cells and environments

Glioblastoma cell lines were transfected, either permanently or transiently, with GFP alone and either GFP-tagged or DsRed-tagged constructs that mark actin filaments such as actin and SM22, and sites of matrix adhesion such as VASP (Rottner *et al*., 1999), zyxin (Rottner *et al*., 2001) and YFP–SH2 (Kirchner *et al*., 2003). SM22 localizes to actin filaments and a DsRed-tagged construct of SM22 (Gimona *et al*., 2003) labels actin in different cell types without forming aggregates of the fluorescent protein, which is a problem with other proteins. The transfected cells were either plated onto or injected into the different substrates and tissue environments and observed either live or after fixation. In this study we compared solid and flexible, two-dimensional substrates with collagen gels, brain slices and intact brains.

Solid and flexible two-dimensional substrata

Supplementary video 1 shows the typical morphology of U373 cells migrating on glass. Both U373 and U87 cells show stress fiber bundles of actin in the cell body with active protrusion of lamellipodia and filopodia at the cell edge. Prominent surface ruffling activity in the form of 'actin flowers', as described in other transformed cells (Ballestrem, 1998) is also observed, more frequently in U373 cells (Fig. 1). Double-labeling for actin and phosphotyrosine showed typical focal adhesions at stress fiber termini (data not shown). Pelham and Wang (1997) have developed flexible, two dimensional substrates based on polyacrylamide, the softness of which is regulated by varying the level of chemical crosslinker. We used a substrate stiffness in the lowest range obtainable by this method (~14 KN m^{-2}), which is in the region of pliability of tissue environments (Wang and Pelham, 1998). On these flexible substrates, glioblastoma cells adopted elongated shapes, with actin bundles only along the lateral edges of the cells (Fig. 2) and active ruffling at protruding fronts. Wang and co-workers described similar phenotypic changes with fibroblasts (Beningo *et al*., 2002). Of particular interest is the finding that motile cells switch surface morphologies reversibly between ruffles and blebs. In these cases, blebbing was associated with a temporary detachment of peripheral substrate adhesions, rounding-up of the cells and arrest of motility (Fig. 2). Cells transfected with both Dsred–SM22 and YFP– SH2 (to label adhesions) showed focal complex formation at protruding edges and the differentiation of focal complexes into focal adhesions. At retracting edges, large actin bundles were characteristically associated with prominent focal adhesions that slid during retraction (not shown).

Collagen gels

When plated in collagen gels (1.67 mg ml^{-1}) both U87 and U373 cells extended long, narrow processes with ruffling membranes composed of lamellipodia and filopodia at their tips (Fig. 3a). Individual processes were up to 60 μm long (20.3 ± 5 μm, $n = 8$ cells) and retracted at a maximum rate of 23 μm $minute^{-1}$. This morphology differed strikingly from that observed on two-dimensional surfaces. However, single actin filament bundles that resemble stress fibers were observed in these extended processes and became particularly pronounced during phases of retraction (Fig. 3b,c and Supplementary video 2). Immunofluorescent labeling of tubulin and actin showed that microtubules extended into the base of ruffling processes (data not shown).

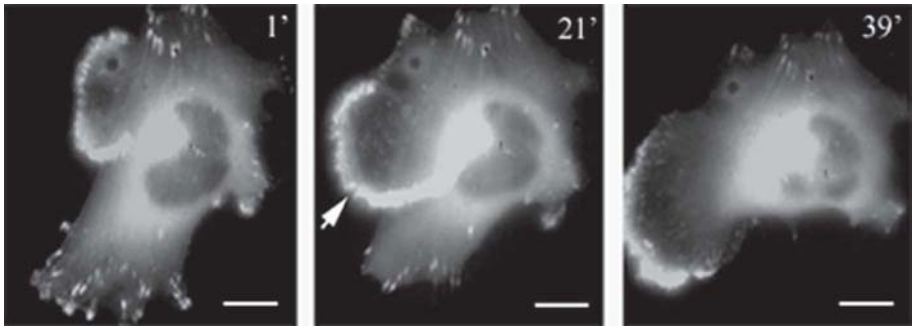


Fig. 1. Video frames of a U373 glioblastoma cells moving on a fibronectin-coated glass. U373 cells were transfected with GFP–zyxin. Note the ruffling activity in the form of 'actin flowers' (arrow). Scale bars, 3 μm. 1', 21' and 39' indicate time (in minutes).

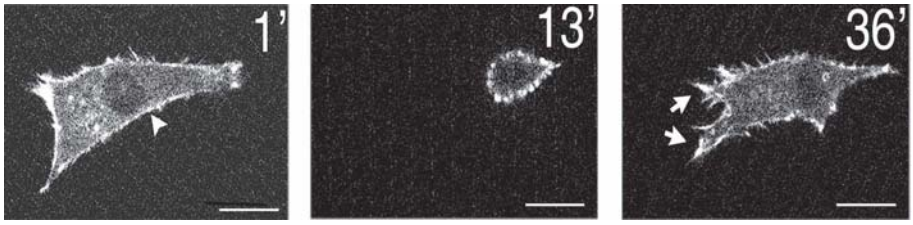


Fig. 2. Video frames of a U87 glioblastoma cell moving on a soft, flexible substrate. The cell was transfected with dsRed–SM22. 1', 13' and 36' indicate time (in minutes). Note the reversible switch from a spreading (outside panels) to a blebbed phenotype (middle panel). Actin filaments are concentrated in filopodia and ruffling protrusions (arrows) and in actin bundles immediately under the plasma membrane (arrowhead). Scale bars, 5 μm.

Preparations of mouse brain in culture

To investigate the behavior of glioblastoma cells in a brain-tissue environment ,we employed four types of preparations. The first was thin slices, cut nominally at 250 μm using a microtome)., from fresh P7 mouse brains, cultivated for 4–7 days on coverslips according to Gahwiler (Gahwiler, 1981;). Gahwiler *et al*., 1997). The second preparation was thicker (300 μm) vibratome-cut slices of P0 mouse brain, which were used within 24 hours. The third type was explants of spinal cord from E13.5 mice, according to Echeverria *et al*. (2001). Lastly, P7 mouse brain was excised *in toto* and cells injected into the brain stem. After several days in culture, microtome slices spread into a thin layer 100–200 μm thick. Glioblastoma cells plated onto such slices showed two different morphologies, one characterized by an elongated shape with long extensions bearing ruffles and filopodia, the other one round, with filopodia or bleb-like protrusions. From a total of 111 cells analyzed, 48% showed elongated protrusions, 39% were round and 12% had a mixed morphology. The motility of elongated cells was driven by protrusive lamellipodia and filopodia on neurite-like, leading extensions (Supplementary video 3), like that observed in collagen gels. Labeling for actin in the main cytoplasm was generally diffuse, however well defined actin bundles were evident in processes of elongated cells, especially during retraction (Fig. 3d,e and Supplementary video 4). The processes extended at 25 ± 3 μm $hour^{-1}$. As on flexible substrates, reversible transitions between cells with blebs, filopodial protrusions and ruffling activity were also observed (Fig. 3f,g and Supplementary video 5). In the latter case, the cells were transfected with DsRed–VASP, with the intention of highlighting adhesion foci. However, as yet we are unable to observe adhesion sites in three-

dimensional environments, probably because of the high level of transfection in the cells recorded.

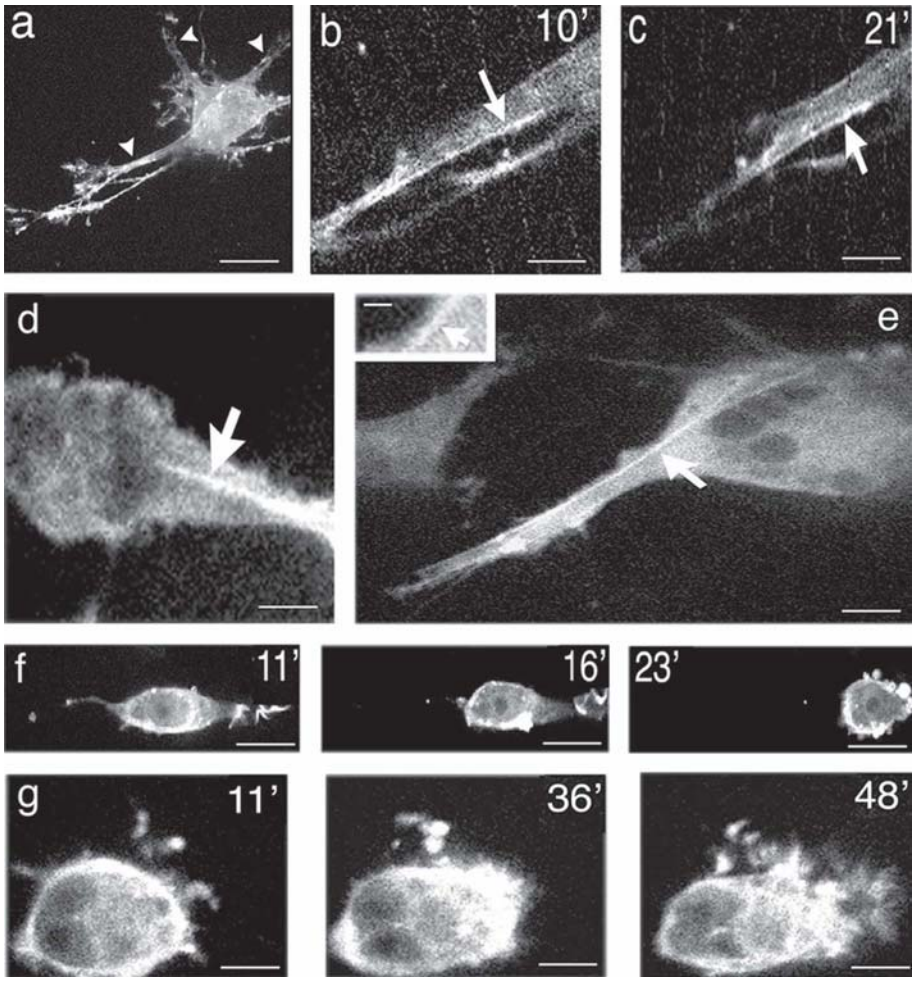


Fig. 3. Glioblastoma in collagen gels and microtome-cut brain slices. (a–c) GFP–actin-expressing U373 glioblastoma cells in a three-dimensional collagen gel. (a) Confocal microscope reconstruction of a living cell illustrates the long, dendrite-like extensions (arrowheads). (b,c) Video frames of the cell in (a) showing long, stress-fiber-like bundles in the retracting regions (arrows and Supplementary video 4). (d,e) GFP–actin-expressing U373 in a P7 mouse brain slice (200 μm). Selected video frames show the transient appearance of actin bundles during retraction phases (arrows and insert). (f,g) Video frames of Dsred–VASP-transfected U87 (f) and U373 (g) glioblastoma cells in microtome-cut brain slices show the blebbing phenotype. Note the switch between elongated and blebbing phenotype (f) and the transition from blebbing to ruffling (g). Scale bars: (a) 18 μm; (b,c) 8 μm; (d) 4 μm; (e) 6 μm, insert 1.2 μm; (f) 10 μm; (g) 12 μm. Time indicated as in Fig. 1.

With vibratome-cut slices, transfected cells were injected into the slice and the slices fixed 24 hours later and viewed by fluorescence microscopy. Fig. 4a shows a typical slice that contains cells that express Dsred–SM22 to label actin. Individual cells had a characteristic, elongated morphology with ruffling protrusions and long extensions (27 of 48 cells analyzed) (Fig. 4b) which is similar to cells in microtome slices. Examples of glioblastoma cells injected into explants of E13.5 embryos or into the stem of excised P7 mouse brain are shown in Fig. 4c,d. Again, long ruffling protrusions extend from the cell bodies. The protrusions were composed of lamellipodia mesh-works and filopodia; actin bundles are resolved in the extensions (Fig. 4d). As in collagen gels and brain-slice cultures, the average length of projections was 40 μm, with rates of extension of up to 20 μm $hour^{-1}$.

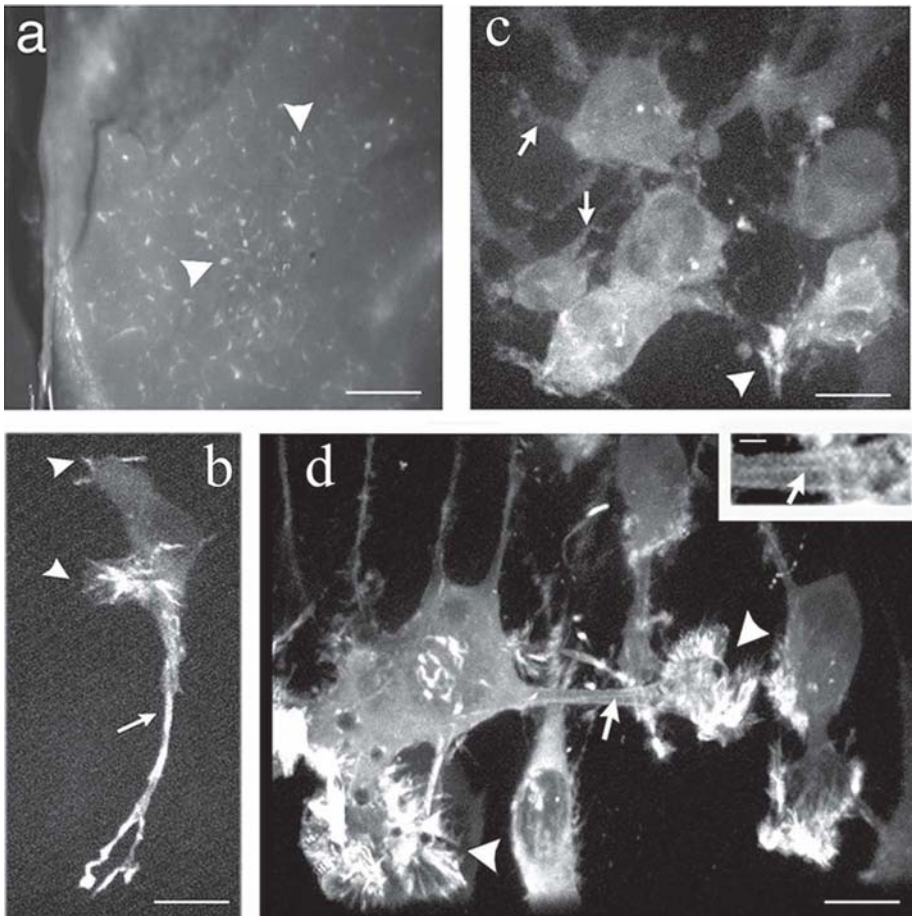


Fig. 4. Glioblastoma cells in different preparations of brain tissue. (a,b) U87 glioblastoma cells transfected with Dsred–SM22 were injected into a P0 mouse brain slice (vibratome type). (a) Fluorescence image acquired ~24 hours after injection shows the random spreading of cells (arrowheads) away from the injection site. (b) Confocal reconstruction of one of these cells at high resolution show a long projection that labels strongly for actin (arrow) and peripheral ruffles (arrowheads). (c) Confocal microscope reconstruction 15 hours after injection of U373 cells transfected with actin into a spinal cord explant from a mouse E13.5 embryo. Note expression of ruffling protrusions (arrowheads) and long projections (arrows). (d) U373 cells transfected stably with GFP–actin were injected into the brain stem-region of a freshly excised mouse brain (P7). The panel shows a confocal microscope reconstruction of a cell imaged 90 minutes after injection. Note the development of long extensions with tips bearing ruffles and filopodia (arrowheads). Stress-fiber-like bundles occur in the extensions (arrow and insert). Scale bars: (a) 0.28 mm; (b–d) 11 μm, insert 1.9 μm.

Injection into the brains of live mice

To investigate the migration characteristics of glioblastoma cells *in vivo*, we injected U373 cells that express GFP–actin into the brains of live, adult mice. The injection point was marked by co-injection of the rodamine-derived cytoplasmic dye CMTMR. Because tumor cells occur most commonly in white matter and often migrate from one hemisphere to the other across the corpus callosum (Duffau *et al*., 2004), we injected cells into the white matter and the corpus callosum in the region indicated in Fig. 5a (see Methods for details). Fig. 5b shows a vibratome frontal section from the cortical brain of a mouse sacrificed 4 days after injection. Cells that had already invaded the surrounding tissue had an extended shape (Fig. 5c) and migrated in rows either radially into the hippocampus (Fig. 5c), probably in the direction of neuronal afferent fibers, or along the border of the white matter and corpus callosum (data not shown). No cells

migrated outwards towards the external part of the cerebral cortex. High-resolution, confocal microscopy revealed actin filament bundles in long extensions that were close to the plasma membrane (Fig. 5d,e). Either round or mixed phenotypes were also visible, with diffuse actin label. Cells with bleb-like protrusions were observed also, but only close to the injection site (data not shown), which indicates that they did not migrate.

Rho kinase inhibition and motility

Tail retraction of cells migrating *in vitro* depends on the contraction of bundles of stress fibres by activation of myosin II via the Rho–ROCK (Rho kinase) pathway (Worthylake *et al*., 2001; Nakayama *et al.*, 2005). Inhibition of Rho kinase activity by Y-27632 induced gross transformations in U373 and U87 cells *in vitro* (Fig. 6a). The peripheral cytoplasm retracts and is replaced by actively motile, neurite-like processes that extend radially from the cell body (Supplementary video 6). Y-27632 induced a similar changes in cells in brain slices (27 of 48 cells analyzed), namely the formation of either short or long, thin, neurite-like protrusions (Fig. 6b). Time-lapse analysis showed that these cells are unable to undergo directional migration (Supplementary video 7). In the short term, this inhibitor of Rho kinase causes a loss of actin bundles in the cytoplasm (see Supplementary Fig.1).

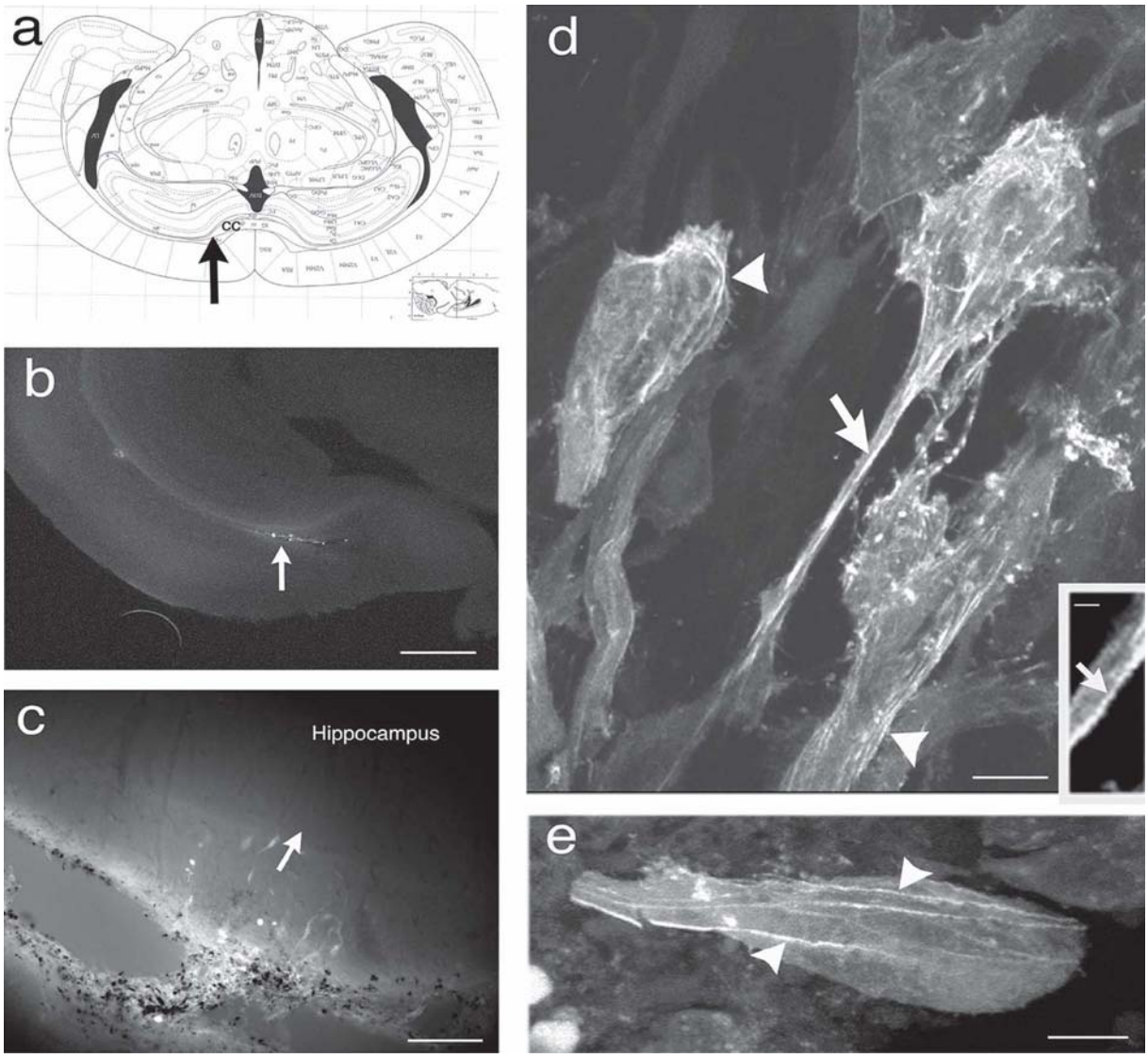


Fig. 5. Injection of U373 glioblastoma cells that express GFP–actin into the brains of adult, live mice. (a) The arrow illustrates the anatomical site of injection (white matter of the corpus callosum (CC). (b) Vibratome-cut section of a brain of a mouse 4 days after injection. Arrow indicates GFP-tagged cells. (c) Higher magnification of a section from the same brain shows cells migrating from the injection site. The labeled cells cross the hippocampus towards the center of the brain, typically in queues. (d,e) Confocal microscope images of the slice in (c) shows that glioblastoma cells close to the injection point have actin bundles in the long protrusions (arrow and insert) and under the cell membrane (arrowheads). Scale bars: (b) 0.29 mm; (c) 139 μm; (d) 5.9 μm, insert 0.5 μm; (e) 5.7 μm.

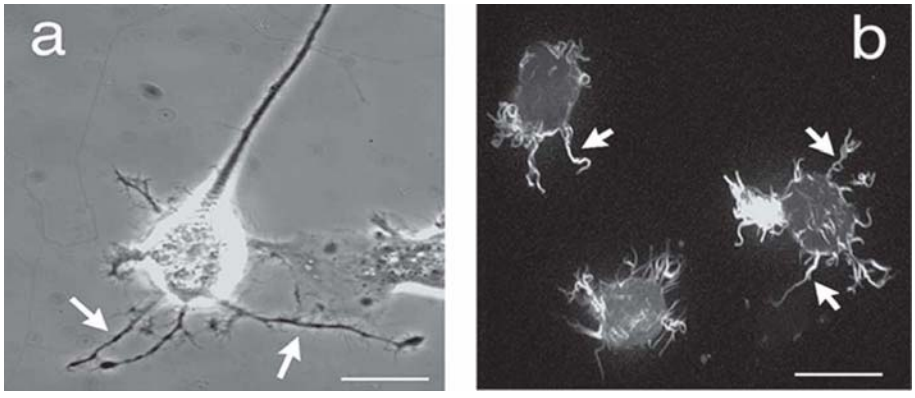


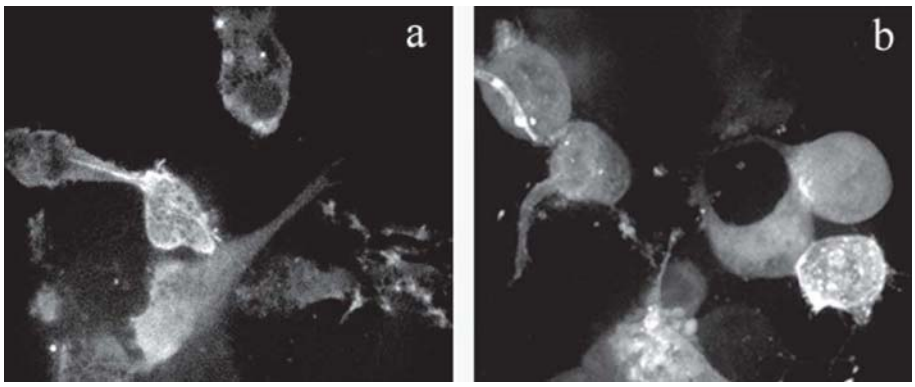
Fig. 6. Effect of inhibiting Rho kinase on the migration of glioblastoma cells. (a) Phase-contrast, time-lapse video frame of a U87 glioblastoma cell *in vitro*, 20 minutes after treatment with 50 μM Y-27632 shows the induction of neurite-like elongations (arrows) that result from retraction of the cell body. (b) U87 cells transfected with Dsred–SM22 (to mark actin) in brain slices in culture. Random protrusions are induced after treatment with Y-27632. These cells are immobile (see Supplementary video 7). Scale bars: (a) 14 μm; (b) 16 μm.

CONCLUSIONS

- The migration of glioblastoma cells in brain tissue involves the extension of long protrusions, which is driven by ruffling membranes and filopodia at their tips, and the subsequent retraction of the cell body, which is driven by the transient formation of contractile bundles of actin filaments.
- Blebs form on the surface of glioblastoma cells both *in vitro* and *in vivo*, but they do not contribute to cell migration.
- Rho kinase, which is upstream of contractility and actin bundle formation, is required for the directional migration of glioblastoma cells in brain tissue.

DISCUSSION

Cell migration in living organisms is a central theme in development (Trinkaus, 1984) and it has received increasing attention with the development of technologies to express fluorescent probes in defined tissues for *in vivo* imaging (Jacinto *et al.*, 2002). Different types of cells have also been visualized in three-dimensional matrices, based largely on collagen (Friedl, 2004) and such matrices serve as standard models to observe collective cell migration *in vitro*. Organotypic brain slices have been used previously to characterize the invasion of glioblastoma cells (Giese *et al*., 1998), and recent studies have employed glioma cells that express GFP both in brain slices (Jung *et al*., 2002) and live brain (Kilic *et al*., 2000). In the present study, we show for the first time the incorporation of glioma cells that express probes tagged for actin in models of brain tissue and intact brain, in conjunction with live-cell imaging. This allows us to visualize the cytoskeleton of these tumor cells in an environment in which they exhibit an invasive mode of migration. There is a major change in glioblastoma cell morphology following the switch from a solid, two-dimensional substrate to a non-rigid, three-dimensional substrate (either synthetic or



Supplementary Fig. 1. U373 glioblastoma cells, stably transfected with GFP–actin in an organotypic brain slice. The panels show corresponding fields of cells before (a) and after (b) inhibition of Rho kinase. Before inhibition actin bundles are detected in retracting protrusions, whereas they are absent after inhibition.

tissue-based). This change is typified by the extension of long, dendritic-like processes bearing protrusive regions of ruffling activity and filopodia, and by the down regulation of stress-fibre bundles. The same type of transition has been described previously for fibroblastic cells in collagen and matrigel (Cukierman *et al.*, 2001; Grinnell, 2003). Generally, stress fibers are considered an *in vitro* phenomenon, and they are reported to be expressed only in some situations in tissue environments using fixed samples, such as in endothelial cells under mechanical stress (Kano *et al.*, 2000). We show here that contractile actin bundles also occur in glioblastoma cells that are migrating in three dimensions and in living brain, but that they are transient and their numbers are limited. Live imaging shows that these bundles form specifically during retraction phases, which is consistent with the recruitment of filaments that pre-exist in the cell body through the activation of myosin II. Activation of myosin II is, in turn, signaled through Rho via the suppression of myosin light chain phosphatase by Rho kinase (ROCK), and the general requirement of ROCK for cell motility is established both *in vitro* and *in vivo* (Riento and Ridley, 2003). In the present study, the depolarization of glioblastoma cells observed in brain slices after ROCK inhibition further supports the requirement of the Rho–ROCK pathway for directional migration. It is difficult to determine exactly how much inhibitor reaches the cells and whether this is sufficient to block directional migration because it diffuses freely in the tissue from the medium. In neuronal cells *in vitro*, activation of Rac and Cdc42 promote neurite and growth-cone extension, whereas activation of Rho promotes neurite retraction (Aoki *et al*., 2000; Miyashita *et al*., 2004). Our results link retraction in a tissue environment with the transient formation of contractile bundles of actin filaments. Initially, membrane blebs were described in migrating cells of fish embryos (Trinkaus, 1984) and they are a common feature of cells when they are rounded up in culture. Their formation is linked to an increase in intracellular contractility, for which matrix anchorage is reduced (Fay and Delise, 1973; Torgerson and McNiven, 1998) and they also occur on cells undergoing mitosis (Trinkaus, 1984). Their formation is also linked to apoptosis, whereby contractility results from caspase 3-mediated cleavage of ROCK to form a constitutively active kinase (Sebbagh *et al*., 2001; Coleman *et al*., 2001). More recently, Sahai and Marshall (2003) describe two phenotypes of migrating tumour cells in three-dimensional matrices: conventional, extended cells and blebbing cells. Our analysis of live cells, also in tissue, shows that glioblastoma cells switch reversibly between extended and blebbing phenotypes, which indicates that this blebbing activity is not associated with apoptosis. Rather, blebbing appears to be associated with diminishing matrix adhesion and, generally, blebbing cells are less mobile. We found few examples of blebbing cells in tissue and brain preparations and indeed rounded cells often showed filopodial projections. Therefore, we conclude that a blebbing mode of motility is the exception rather than the rule in a brain-tissue environment. In the scheme outlined in Fig. 7 we summarize the potential signaling pathway for glioblastoma cell phenotypes. Human malignant glioma cells, in particular U87 and U373 cell lines, express semaphorins, the semaphorin receptors neuropilins and plexins (Rieger *et al*., 2003) and ephrin receptor tyrosin kinases (Nakada *et al*., 2004), all of which are implicated in axonal growth and guidance. U87 cells also aberrantly expresses the axonal cell-adhesion molecule TAX-1, which occurs transiently on the surface of a subset of developmental neurons, and whose inactivation profoundly reduces the migration of glioma tumor cells *in vitro* (Rickman *et al.*, 2001). Together, these observations indicate that human glioblastoma cells share similarities with neuronal progenitors and can mimic their motility in a brain-like environment. Our own findings support this: in brain tissue glioblastoma cells develop neurite-like protrusions and in intact brain they migrate along tracks, similar to

the routes taken by neuronal fibers (see Results). In this respect it is notable that glioma cells invade preferentially white matter where neuronal fibers dominate (Giese and Westphal, 1996).

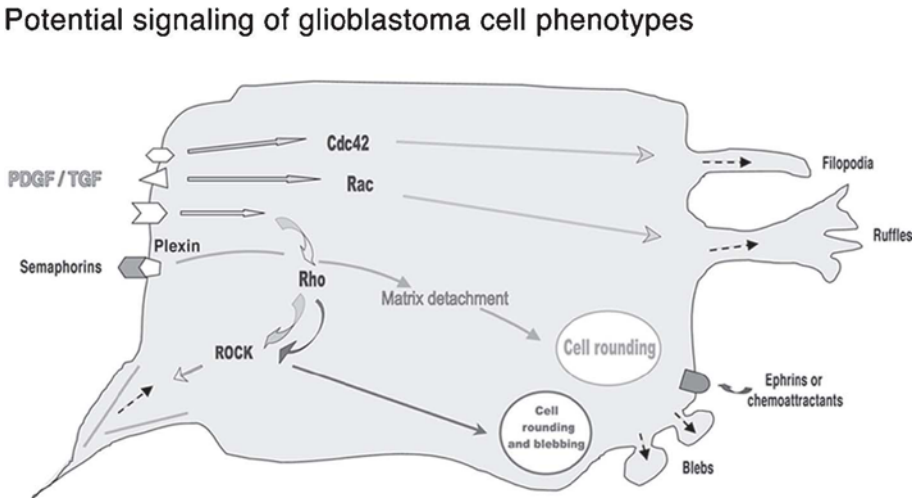


Fig. 7. Potential signaling in phenotypes of glioblastoma cells. Signals such as growth factors might activate different cellular pathways that are mediated by small GTPases such as Cdc42 and Rac (Laurent and Smithgall, 2004) and Rho–Rho kinase (Clements *et al*., 2005), leading to filopodia, ruffling protrusions and actinbundle formation. In addition, the Rho-Rho kinase pathway might lead to cell rounding and blebbing, as proposed by Sahai and Marshall (2003) for other tumour cell lines in three-dimensional matrices. Based on more recent findings (Barberis *et al*., 2004), signals that operate through receptors such as plexins, which are expressed by glioblastoma cells (Rieger *et al*., 2003), might activate a pathway that is independent of Rho kinase and leads to matrix detachment and cell rounding.

In vibratome-cut slices of fresh brain, in which the anatomy is preserved, live neurons can be visualized by vital dyes and shown to extend processes that are characteristic of neurons (Nadarajah *et al.*, 2002). We show that glioblastoma cells behave similarly in organotypic brain slices and in intact brain, and demonstrate the feasibility of high-resolution imaging of cytoskeletal structures in live cells in brain tissue. The use of specifically tagged cells in slice preparations and in intact brain, together with confocal imaging, offers the possibility of assessing the specificity of compounds that are designed to inhibit the invasion of glioblastoma cells.

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Supplementary video 1. U373 human glioblastoma cells transfected with GFP–actin migrating on fibronectin-coated glass. Prominent stress-fiber bundles cross the cell body, and lamellipodia and filopodia protrusion actively at the cell edge. Time between selected frames, 1 minute; total time 51 minutes.

Supplementary video 2. U373 human glioblastoma cells that express GFP–actin in a three-dimensional collagen gel. The presence of long, stress-fibre-like bundles in the retracting regions (arrows) is apparent. Time between selected frames, 1 minute; total time 57 minutes.

Supplementary video 3. GFP-transfected U373 human glioblastoma cells in a mouse brain slice (microtome type) in culture. The motility of elongated cells is driven by protrusive lamellipodia and filopodia on neurite-like, leading extensions. Note the migration of cells in three-dimensions (e.g. arrow). Time between selected frames, 75 seconds; total time 85 minutes.

Supplementary video 4. U373 human glioblastoma cells that express GFP– actin in a mouse brain slice (microtome type) in culture. Prominent actin bundles are evident during retraction. Also notable, in the last part of the video, is the switch between ruffles and blebs on the cell body. Time between selected frames, 1 minute; total time 52 minutes.

Supplementary video 5. U373 human glioblastoma cells transfected with Dsred–VASP in a mouse brain slice (microtome type) in culture. The video shows the switch of a round cell, which bears blebs and filopodia, to a spread cell with ruffling activity. Time between selected frames, 1 minute; total time 60 minutes.

Supplementary video 6. Phase contrast video of a U87 human glioblastoma cell on fibronectin-coated glass, recorded 20 minutes after addition of the Rho kinase inhibitor Y-27632. Y-27632 induces neurite-like elongations, which result from cell-body retraction. Time between selected frames, 90 seconds; total time 60 minutes.

Supplementary video 7. Dsred–SM22 transfected U87 cells in a tissueslice (microtome-cut) from mouse brain in culture. Treatment with the Rhokinase inhibitor Y-27632 results in cell depolarization, and induces randomprotrusions and loss of directional locomotion. Time between selected frames,1 minute; total time 30 minutes.Supplementary content can be viewed by going to journals.cambridge.org/jid_NGB and on to the specific article.

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