Migrating glioma cells activate the PI3-K pathway and display decreased susceptibility to apoptosis

Anna M. Joy*, Christian E. Beaudry, Nhan L. Tran, Francisco A. Ponce, David R. Holz, Tim Demuth and Michael E. Berens

The Translational Genomics Research Institute, 400 North 5th Street, Suite 1600, Phoenix, AZ 85004, USA
*Author for correspondence (e-mail: ajoy@tgen.org)

Summary

Glioma cells that migrate out of the main tumor mass into normal brain tissue contribute to the failure of most gliomas to respond to treatment. Treatments that target migratory glioma cells may enhance the therapeutic response. Multiple lines of evidence suggest that suppression of apoptosis accompanies activation of the migratory phenotype. Here, we determine whether migration and apoptosis are consistently linked in glioma cells and whether manipulation of migration influences cytotoxic therapy-induced apoptosis. Camptothecin and Trail-induced apoptosis were decreased 2-5-fold in actively migrating glioma cells relative to migration-restricted cells. Consistent with a mechanistic link between migration and apoptosis, the dose-response for stimulation of migration on laminin was inversely proportional to apoptosis induction. Treatment of glioma cells with migration inhibitors alone had little effect on basal rates of apoptosis and had little effect on Trail-induced or camptothecin-induced apoptosis in migration-restricted cells. By contrast, migration inhibitors increased camptothecin and Trail-induced apoptosis in actively migrating glioma cells. Migrating glioma cells have increased amounts of phosphorylated Akt and its downstream substrate glycogen synthase kinase-3 relative to migration restricted cells. Treatment of migrating cells with a specific inhibitor of phosphoinositide 3-kinase (PI3-K), LY294002, blocked the phosphorylation of Akt and increased the sensitivity to apoptosis. LY294002 had no effect on the migration of restricted cells. This suggests that migrating glioma cells activate the PI3-K survival pathway, protecting migrating cells from apoptosis. Taken together, these data provide support for a link between migration and apoptosis in glioma cells. In addition, evidence indicates that treatment with migration inhibitors, while not affecting apoptosis-induction in migration-restricted cells, can sensitize migrating glioma cells to cytotoxic agents.

Movie available online

Key words: Glioma, Migration, Chemotherapy, Akt, PI3-K

Introduction

Current treatments for the most common form of brain tumor, glioblastoma multiforme (GBM), are disappointing in their effectiveness. Most patients diagnosed with a GBM survive less than a year despite intensive treatment, which may include surgical resection, radiation and chemotherapy. Treatment failure is due, in part, to the infiltration of glioma cells into normal brain. Because they reside within normal brain, migrating glioma cells are not typically removed by surgical resection or targeted by radiation and chemotherapy. Therapies that effectively target invasive glioma cells may significantly improve therapeutic outcome.

Multiple lines of evidence indicate that apoptosis is suppressed when cells adopt a migratory phenotype (Mariani et al., 2001). We have obtained the gene expression profile of human glioma cells that have invaded into normal brain tissue in vivo and compared it with the profile of the noninvasive glioma cells residing within the tumor core. Invasive glioma cells show a shift in the expression of several apoptosis regulatory genes consistent with a decreased ability to undergo apoptosis (Mariani et al., 2001).

Other groups have obtained molecular evidence of a link between apoptosis and migration. There appears to be a coordinate modulation of apoptosis and migration on overexpression of migration-promoting genes. For example, overexpression of CAS and Crk, proteins that bind to activated focal adhesion kinase, promotes both invasion and survival of COS-7 cells (Cho and Klemke, 2000a). Overexpression of the survival enhancer bcl-2 in glioma cells promotes both accelerated migration and invasion into brain aggregates (Wick et al., 1998). Rac activity is important for migration by regulating de novo actin polymerization at the cell periphery, resulting in membrane ruffling and lamellipodia extension (Ridley et al., 1999; Nobes and Hall, 1995). Suppression of Rac activity induces the apoptosis of human glioma cells (Senger et al., 2002). These studies indicate that modulation of the migratory phenotype may influence vulnerability to apoptosis.

It is possible that drugs that suppress the migratory phenotype may increase the susceptibility of migrating glioma cells to cytotoxic treatment. Here, we determine that migration and susceptibility to apoptosis are tightly linked in glioma cells and obtain evidence for survival signaling activated in migrating cells. We report that multiple migration-inhibiting drugs, while having little effect on basal rates of apoptosis, or apoptosis-induction in migration-restricted cells, increased the

susceptibility of migrating glioma cells to chemotherapy and Trail-induced apoptosis.

Materials and Methods

Cell culture and drug treatment

SF767 and T98G cell lines were isolated from a glioblastoma multiforme tumor and were obtained from the University of California at San Francisco and American Type Culture Collection (ATTC), respectively. Cells were maintained in MEM (Waymouth medium, Life Technologies) supplemented with 10% fetal calf serum at 37°C, 5% CO_2 in a humidified chamber. Cells are routinely tested for mycoplasma infection using Mycoplasma Plus (Stratagene, Cedar Creek, TX). For drug treatment, media was exchanged for serum-free media containing 1 μM camptothecin, 100 ng/ml Trail (plus enhancer as described by the manufacturer) or as prescribed in dose-response experiments, and cells incubated for 24 hours or 16 hours, respectively. Rapamycin (CalBiochem, La Jolla, CA) was used at 100 ng/ml and was added concurrently with Trail. For controls cells were incubated for the appropriate time period in serum-free media plus solvent used to deliver drug.

Reagents

Laminin was obtained from Gibco (Gaithersburg, MD), dazmegrel (3-(1*H*-imidazol-1-yl-methyl)-2-methyl-1*H*-indole-1-propanoic acid) was obtained from Pfizer (Sandwich, England); the phospholipase C inhibitor U73122 and the phosphoinositide 3-kinase (PI3-K) inhibitor LY294002 were obtained from Sigma (St Louis, MO); the anti-β1 integrin antibody, which can be used to block β1-containing integrins, was obtained from Chemicon (MAB1951, Temecula, CA). Camptothecin was from Calbiochem (La Jolla, CA) and TRAIL from Alexis (San Diego, CA). Antibodies against activated cleaved caspase-3 and Cy3 conjugated secondary antibody were from Promega (Madison, WI).

Cell-migration assay

Migration assays were performed using the microliter scale radial monolayer migration assay as previously described (Berens et al., 1994; Giese et al., 1994). Briefly, 10-well slides (Erie Scientific, Portsmouth, NH) were coated with 0.1% bovine serum albumin (BSA), 10 μg/ml laminin or as prescribed in experiments with varying laminin concentrations. Cells were seeded through a cell sedimentation manifold (CSM Inc., Phoenix, AZ) at 2500 cells/well to establish a circular 1 mm diameter confluent monolayer at the center of the substrate-coated well. One to two hours post-seeding, a circle circumscribing the cells was measured. The cells were allowed to migrate out over a 24 hour time period and another circle circumscribing the cells was drawn. Migration results are reported as the change in the diameter of the circle circumscribing the cell population over a 24 hour period (μm/day). Measurements were taken using an inverted microscope (Axiovert, Carl Zeiss, Thornwood, NY), digitalized using a Spot camera (Diagnostics Instrument, Sterling Heights, MI) and image analysis performed (Scion Image, Frederick, MD).

For experiments using migration inhibitors, cells were seeded in the migration assay format and allowed to adhere. Media was then exchanged for serum-free media containing the specified concentration of migration inhibitor or solvent control, and migration rate was evaluated as usual.

Assessment of apoptosis

Apoptotic cells were evaluated by nuclear morphology of 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI)-stained cells. Cells with condensed, fragmented chromatin were scored as apoptotic. Five replicates of at least 200 cells were evaluated and data reported as apoptotic cells/total cells × 100. Activated caspase 3 was detected by immunocytochemistry using an anti-activated caspase 3 antibody (Promega, Madison, WI) on cells stained with DAPI. At least 200 cells per treatment were evaluated for condensed chromatin and activated caspase 3.

Immunocytochemistry

Cells were fixed in 2% paraformaldehyde, permeabilized with 0.1% triton X-100, and nonspecific binding sites were blocked with 1% BSA, 2% goat serum in PBS. Blocked cells were rinsed and incubated with primary antibody overnight at 4°C, rinsed then incubated with Cy3 conjugated reporter antibody (Jackson Laboratories, West Grove, PA) for 30 minutes at room temperature. Anti-Akt and phospho-Akt (Ser 473) were obtained from Cell Signaling Technology (Beverly, MA) and used at a concentration of 1:250. Cells were viewed with a LSM 5 Pascal laser scanning confocal microscope (Zeiss, Thornwood, NY) or a microscope equipped with a rhodamine filter for Cy3 fluorescence and a 450-490 band pass excitation filter and 515 long pass emission filter for DAPI fluorescence.

Cell lysis, SDS-PAGE and western analysis

Cells were routinely collected in 2× sample buffer (0.25 M Tris-HCl, pH 6.8, 2% SDS, 25% glycerol) containing protease and phosphatase inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotinin, 20 mM NaF, 1 mM PMSF, 2 mM sodium orthovanadate) and protein content determined with the BCA Pierce protein assay. Samples containing equivalent amounts of protein were subjected to reducing SDS-PAGE gel electrophoresis and transferred onto nitrocellulose. Immunoblotting was performed using anti-Akt, anti-phospho Akt Ser-473, anti-phospho Akt Thr-308 and anti-phospho glycogen synthase kinase-3 (GSK-3) α and β Ser-21/9 (Cell Signaling, Beverly, MA), all at 1:1000 dilution or GSK-3ß (clone #7, BD Transduction Laboratories, San Diego, CA) at 1:2500, or α-tubulin (clone DM1A, Oncogene Research, Boston, MA) at 1 μg/ml, all overnight at 4°C. Signals were visualized using enhanced chemiluminescence.

Results

Actively migrating glioma cells suppress apoptosis-induction

A microscale radial monolayer migration assay was used to assess the migration rate of cells plated onto the nonpermissive substrate BSA and the migration-stimulating substrate, laminin. A typical migration assay for cells on laminin is shown in Fig. 1A, 2 hours after plating (t=0) and after allowing cells to migrate for 24 hours (t=24). For cells on laminin, the migration assay after 24 hours presents a densely packed core of cells and actively migrating rim cells (Fig. 1A). Laminin effectively stimulated the migration of SF767 and T98G glioma cells approximately 8-12-fold relative to cells on BSA (Fig. 1B).

To investigate whether engagement of the migratory phenotype affects the cells' susceptibility to apoptosis, migration-restricted and migration-activated glioma cells were treated with an apoptosis inducer then the percentage of apoptotic cells present was determined. Migration-activated cells are defined as those cells on laminin actively migrating at the rim of the migration assay. Migration-restricted cells are operationally defined as those cells at the rim of the migration assay plated onto the nonpermissive substrate BSA, as well as
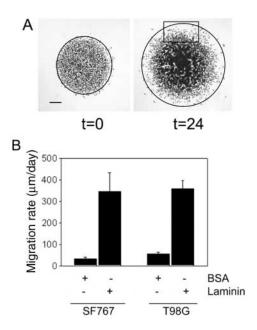
Fig. 1. Laminin stimulates glioma cell migration. (A) A typical migration assay for cells on laminin at the initial migration measurement (2 hours following plating, t=0) and after allowing cells to migrate out for 24 hours (t=24). The circle circumscribing cells used to calculate migration rate is shown. Also shown within the rectangle is a representative field of rim cells used to calculate apoptosis. Bar, 2 mm. (B) SF767 and T98G cells were plated onto 0.1% BSA- or 10 µg/ml laminin-coated slides and migration rate obtained after 24 hours. See movie online (http://jcs.biologists.org/supplemental).

cells in the densely packed core of the migration assay plated onto laminin, which were physically blocked from moving by their neighbors.

Camptothecin-induced apoptosis was approximately 4-5-fold lower in actively migrating rim SF767 cells plated on laminin relative to the migration-restricted cells at the core of the assay and migration-restricted cells plated on BSA (Fig. 2B). SF767 cells were not sensitive to Trail-induced apoptosis. Apoptosis suppression in the migrating cells cannot be attributed exclusively to laminin exposure because the SF767 cells at the core and rim were both residing on laminin, but only the actively migrating rim cells showed a significant reduction in apoptosis.

Apoptotic cells were also quantitated by immunofluorescent detection of activated caspase 3 (Fig. 2D) and loss of mitochondrial membrane potential (data not shown). Similar trends were obtained when apoptotic cells were detected using these methods (data not shown). All cells staining positive for caspase 3 had condensed fragmented chromatin, although not all cells with condensed chromatin contained activated caspase 3. This indicates that camptothecin induces a conventional, intrinsic apoptosis cascade involving the loss of mitochondrial membrane potential, activation of caspase 3 and chromatin condensation.

Most anticancer agents activate the intrinsic or mitochondrial cell death pathway (Strasser et al., 1995). Experiments were performed to determine whether migrating glioma cells also suppress the death receptor pathway. Trail, a

ligand for death receptors 4 and 5, is of interest clinically in the treatment of brain tumors because it preferentially induces apoptosis in malignant glioma cells but not in normal human astrocytes (Hao et al., 2001). T98G but not SF767 cells were sensitive to Trail (Fig. 2B). Migrating T98G cells also suppressed apoptosis induction by Trail. Actively migrating rim cells plated on laminin were approximately twofold less sensitive to Trail-induced apoptosis than the migration-restricted rim cells plated on BSA and 1.6 times less sensitive than the migration-restricted cells at the core of the assay (Fig. 2B).

To determine whether decreased apoptosis in migrating cells can be overcome with increasing concentrations of cytotoxic agent, a dose-response profile was obtained. Differential sensitivity to apoptosis was maintained in both cells lines at higher concentrations of both camptothecin and Trail (Fig. 2C). Therefore, the link between migration activation and apoptosis suppression is evident in two glioma cell lines and manifests using a death receptor pathway and a DNA damaging agent; it cannot be overcome with increasing concentrations of cytotoxic agent.

Migration rate is inversely proportional to apoptosis induction

If a functional link exists between activation of the migratory phenotype and apoptosis suppression, then migration rate should be inversely proportional to apoptosis induction. The relationship between migration rate and camptothecin- or Trail-induced apoptosis of SF767 and T98G cells on increasing concentrations of laminin is shown in Fig. 3. Both SF767 and T98G cells increase the migration rate with increased concentration of laminin. Camptothecin-induced and Trail-induced apoptosis of SF767 and T98G cells, respectively, decrease with increased migration rate. The inverse relationship between migration rate and apoptosis induction supports a link between migration and susceptibility to apoptosis.

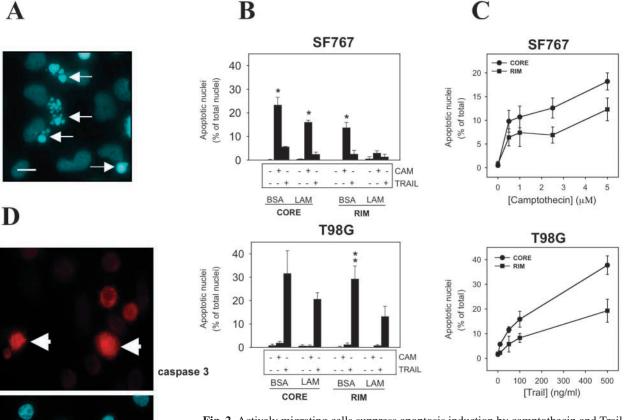## Suppression of migration increases apoptosis induction

A set of experiments was performed to determine whether suppression of migration may potentially be exploited to enhance the response of glioma cells to cytotoxic therapy. Migration of both glioma cell lines was effectively suppressed by a wide spectrum of migration inhibitors including the phospholipase C-γ1 inhibitor U73122 (Khoshyomn et al., 1999), by the thromboxane synthase inhibitor dazmagrel (Yoshizato et al., 2002) and by an antibody against β1 integrin (Giese et al., 1996) (Fig. 4A).
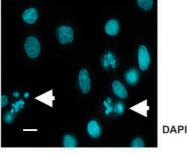
Treatment with migration inhibitors had little effect on the basal levels of apoptosis or on camptothecin-induced apoptosis of migration-restricted SF767 cells at the core of the assay (Fig. 4B; gray bar is cytotoxic agent alone, black bars are migration inhibitors plus cytotoxic agent). By contrast, both the PLC-γ and thromboxane synthase inhibitor sensitized actively migrating rim SF767 cells to camptothecin-induced apoptosis. All the migration inhibitors sensitized T98G rim cells to Trail-induced apoptosis (Fig. 4B). Because migration inhibitors sensitized apoptosis only in the actively migrating rim cells and had little effect on apoptosis induction of migration-restricted

core cells, this data further supports a link between migration and apoptosis. In addition, it suggests that migration inhibitors, while not appreciably affecting basal rates of apoptosis by themselves and having little effect on nonmigrating cells, may

increase the response of migrating cells towards cytotoxic therapy.
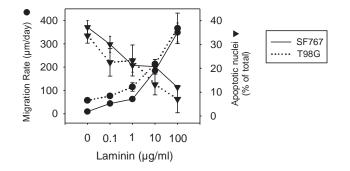
Data from the pharmacological inhibition of migration and from the laminin dose-response for both cell lines were plotted
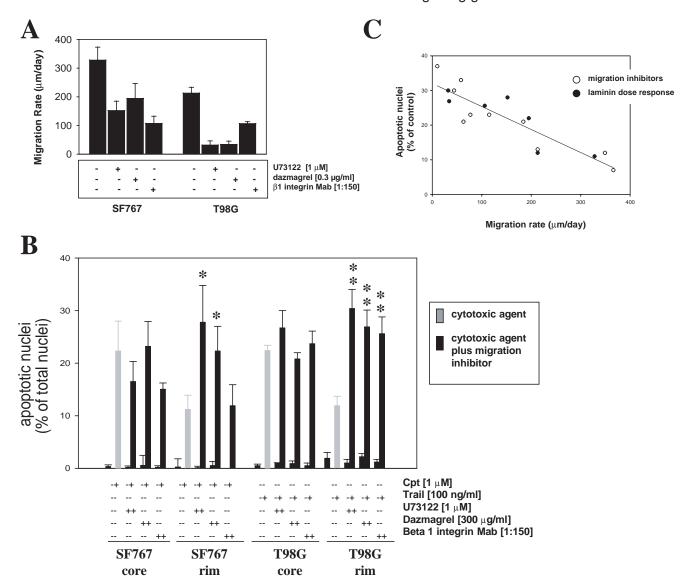

Fig. 2. Actively migrating cells suppress apoptosis induction by camptothecin and Trail. (A) Representative micrograph of Trail-treated, fixed and DAPI stained T98G cells. Arrows identify cells scored as apoptotic. (B) SF767 and T98G cells were plated onto laminin- (LAM, 10 µg/ml) or 0.1% BSA-coated slides in the migration assay format and allowed to migrate for 16 hours. Cells were treated with 1 µM camptothecin for 24 hours or 100 ng/ml Trail for 16 hours, fixed and stained with DAPI. Cells in rim and core were scored for total and apoptotic cells. Values represent the mean and standard deviation of five replicate measurements. Significance with unpaired Student's *t* test: *$P<0.001$ versus rim on laminin; **$P<0.01$ versus rim on laminin. (C) Differential sensitivity to apoptosis is not overcome at high concentrations of cytotoxic agent. SF767 and T98G cells were plated on laminin- (10 µg/ml) coated slides in the migration assay format and allowed to migrate for 24 hours. Cells were treated with the specified concentration of camptothecin or Trail as described in Materials and Methods, fixed and DAPI stained. Cells in the rim and core were scored for total and apoptotic cells. Values represent the mean and standard deviation of four replicate measurements. (D) Camptothecin treatment generates activated caspase 3. SF767 cells in the migration assay format were treated with 1 µM camptothecin as described in Materials and Methods, fixed, stained with DAPI and immunocytochemistry for activated caspase 3 performed. Arrows show cells with condensed fragmented chromatin and positive staining for caspase 3. Bar in A and D, 5 µm.

Fig. 3. Camptothecin and Trail-induced apoptosis is inversely proportional to rate of migration. SF767 and T98G cells were plated onto slides coated with the specified concentration of laminin. Migration was measured 24 hours post seeding (●), cells were then treated with 1 µM camptothecin for 24 hours (SF767) or 100 ng/ml Trail for 16 hours (T98G), stained with DAPI then % apoptotic cells in the rim quantitated as described in Materials and Methods (▼). Values represent the mean and standard deviation of three replicate measurements.

A

Migration Rate (μm/day)

-	+	-	-		-	+	-	-	U73122 [1 μM]
-	-	+	-		-	-	+	-	dazmagrel [0.3 μg/ml]
-	-	-	+		-	-	-	+	β1 integrin Mab [1:150]

SF767   T98G

C

Apoptotic nuclei (% of control)

○ migration inhibitors
● laminin dose response

Migration rate (μm/day)

B

apoptotic nuclei (% of total nuclei)

□ cytotoxic agent

■ cytotoxic agent plus migration inhibitor

-+	-+	-+	-+		-+	-+	-+	-+		--	--	--	--		--	--	--	--	Cpt [1 μM]
--	--	--	--		--	--	--	--		-+	-+	-+	-+		-+	-+	-+	-+	Trail [100 ng/ml]
--	++	--	--		--	++	--	--		--	++	--	--		--	++	--	--	U73122 [1 μM]
--	--	++	--		--	--	++	--		--	--	++	--		--	--	++	--	Dazmagrel [300 μg/ml]
--	--	--	++		--	--	--	++		--	--	--	++		--	--	--	++	Beta 1 integrin Mab [1:150]

SF767 core   SF767 rim   T98G core   T98G rim

Fig. 4. Migration inhibitors sensitize actively migrating but not migration-restricted cells to apoptosis. (A) Cells were seeded onto slides coated with 10 μg/ml laminin and allowed to adhere. The media was exchanged for media containing migration inhibitors, cells were incubated for 24 hours and the migration rate was determined as described in Materials and Methods. (B) Cells were plated onto slides coated with 10 μg/ml laminin, allowed to migrate for 24 hours then pretreated with migration inhibitors or solvent control for 2 hours in serum-free media. Media was replaced with fresh serum-free media containing migration inhibitors or solvent control plus 1 μM camptothecin (Cpt) for SF767 cells or 100 ng/ml Trail for T98G cells. Cells were then incubated with camptothecin plus inhibitors or solvent control for 24 hours or Trail plus inhibitors or solvent control for 16 hours, fixed, stained with DAPI and apoptotic and total nuclei quantitated. Gray bars represent cells treated with camptothecin or Trail alone and black bars represent cells treated with migration inhibitor plus camptothecin or Trail. Significance with unpaired Student's t test: *$P<0.05$ vs camptothecin only treatment of rim cells; **$P<0.001$ vs Trail only treatment of rim cells. (C) Migration rate vs % apoptosis was plotted ($r^2=0.79$) using data from Fig. 3 (○) and Fig. 4A,B (●). Data from anti-β1 integrin treatment of SF767 cells was deleted from this analysis.
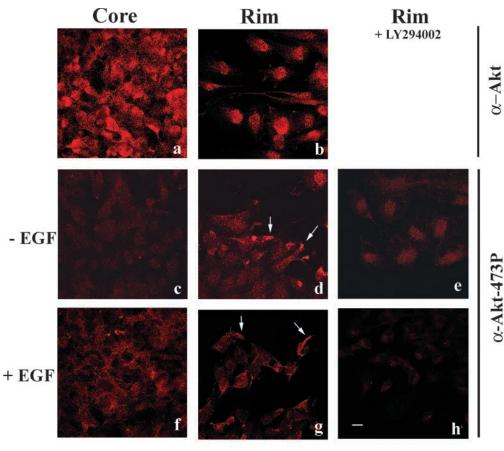
on the same graph as migration rate vs % apoptosis to determine whether migration inhibition by disparate means had quantitatively similar effects on apoptosis induction (Fig. 4C). This shows that pharmacological inhibition of migration is of similar effectiveness as inhibition of migration by plating on less-permissive substrates to increase apoptosis induction.

Akt is activated in migrating glioma cells

These results suggest that survival signaling is activated differently in migrating cells and migration-restricted cells. We determined whether Akt/protein kinase B (PKB), a phosphoserine kinase involved in growth factor and integrin survival signaling (Vivanco and Sawyers, 2002), is preferentially activated in migrating cells. Akt protein was detected in both core and rim cells by immunofluorescence (Fig. 5a,b). Akt was available for phosphorylation on serine 473 as epidermal growth factor (EGF) treatment resulted in phospho-Akt production in both the rim and the core cells (compare f and g). Note the preponderance of cytoplasmic-like
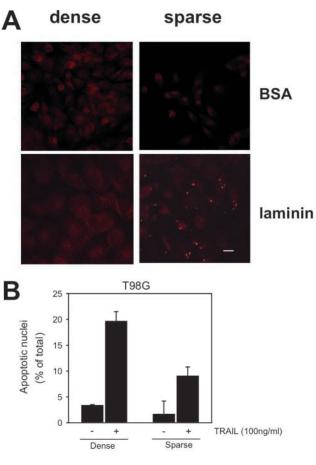
Fig. 5. Phospho-Akt is detected only in migrating cells. T98G cells were plated in the migration format onto laminin-coated slides (10 μg/ml) and allowed to migrate overnight. Cells were then incubated in serum-free media for 4 hours, treated with 100 ng/ml EGF or solvent control for 15 minutes then fixed. For treatment with the PI3-K inhibitor LY294002 (20 μM) was added 2 hours before time of EGF addition. Slides were then processed for total Akt (a and b) or phospho-Akt Ser 473 (c-h) immunocytochemistry as described in Materials and Methods. Arrows in d indicate more intense staining for phospho-Akt at the leading edge type of structures. Arrows in g indicate a different pattern of staining for phospho-Akt following EGF treatment. Bar, 5 μm.

staining. As expected, the PI3-K inhibitor LY294002 blocked the EGF-related phospho-Akt production, indicating that the immunofluorescent signal from the anti-Akt Ser 473 antibody is dependent on PI3-K (compare g and h of Fig. 5). When immunofluorescence for phospho-Akt was performed on cells in serum-free medium in the migration assay format plated onto laminin, phospho-Akt was detected only in actively migrating rim cells and not in migration-restricted core cells (Fig. 5c,d). LY293002 blocked phosphorylation in the migrating rim cells (Fig. 5e), suggesting that PI3-K activity was necessary for phosphorylation of Akt in actively migrating cells. Results were similar for SF767 cells (data not shown). Cell density by itself could not have caused differences in phospho-Akt content because both densely and sparsely plated cells on BSA did not have phosphorylated Akt (Fig. 6A). As reported previously for fibrosarcoma cells (Kim et al., 2001), phospho-Akt was localized to the leading edge in migrating cells (arrows, Fig. 5d).

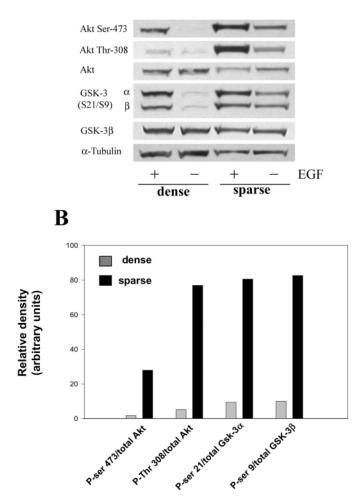Western analysis was also used to evaluate the phospho-Akt

Fig. 6. Cells plated sparsely on laminin have increased staining for phosphorylated Akt and suppress Trail-induced apoptosis. T98G cells were plated sparsely (10,000 cells/cm^2) or densely (50,000 cells/cm^2) onto slides coated with BSA or laminin as indicated and incubated overnight (A and B). (A) Cells were then incubated 4 hours in serum-free media and fixed, and immunocytochemistry for p-Ser-473 Akt was performed as described in Materials and Methods. Bar, 5 μm. (B) Some slides were then treated with Trail for 16 hours as described in the method section, fixed, stained with DAPI and % apoptotic cells determined.

content in actively migrating and migration-restricted cells. Because it was difficult to harvest cell lysates in the core and rim of the migration assay, we determined whether cells plated sparsely (7000 cells/cm^2) or densely (55,000 cells/cm^2) on laminin replicated key characteristics of cells at the core and rim of the migration assay. Both the decreased susceptibility to undergo apoptosis and the increased phospho-Akt immunostaining was replicated when cells were plated sparsely on laminin relative to the densely plated cells on laminin (Fig. 6). This was not due to cell density by itself as cells plated sparsely and densely on BSA did not show these effects.

Complete activation of Akt requires dual phosphorylation at serine 473 and threonine 308 (Bellacosa et al., 1998). Western analysis shows that the sparsely plated cells on laminin have tenfold greater phospho-Akt Ser 473 and phospho-Akt Thr 308
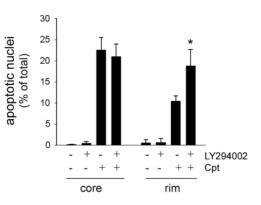
content than the densely plated, migration-restricted cells on laminin (Fig. 7). Treatment of cells with the PI3-K inhibitor reduced phosphorylation of Akt on Ser 473 in actively migrating cells by eightfold (data not shown), confirming immunofluorescent results that LY294002 blocks the activation of Akt. Glycogen synthase kinase-3 (GSK-3) is a downstream substrate of Akt that participates in apoptosis in several cell types (Bijur et al., 2000; Loberg et al., 2002). Fig. 7 shows that GSK-3 is phosphorylated in actively migrating cells but not in migration-restricted cells. Sparsely plated cells on laminin had an eightfold increase in phosphorylation of GSK-3 α and β on serines 21 and 9, respectively (Fig. 7). This provides further evidence that Akt is activated on adoption of the migratory phenotype.

Inhibition of PI3-K suppresses the survival advantage of migrating glioma cells

We investigated whether the PI3-K inhibitor LY294002, which blocked phosphorylation of Akt on Ser 473 in migrating cells (Fig. 5e), also increased apoptosis induction exclusively in migrating cells. LY294002 by itself had little effect on basal rates of apoptosis or on camptothecin-induced apoptosis in migration-restricted cells at the core (Fig. 8). By contrast, LY294002 increased camptothecin-induced apoptosis in migrating rim cells. Akt phosphorylates and activates mTOR (Nave et al., 1999). mTOR and its downstream target, ribosomal S6 kinase, has been reported to be an important regulator of apoptosis in some cells (Castedo et al., 2002). The mTOR inhibitor, rapamycin, had little effect on apoptosis induction of either actively migrating or migration-suppressed glioma cells (data not shown). This indicates that the protective effects of PI3-K in migrating cells are not mediated by mTOR. Taken together, these data support a role for the PI3-K survival pathway in apoptosis suppression of actively migrating glioma cells, potentially through activation of Akt.

Fig. 7. Western analysis for phospho-Akt production in cells plated sparsely on laminin. T98G cells were plated onto laminin-coated dishes at 7000 (sparse) or 55,000 (dense) cells/cm^2 and lysates collected. (A) Total Akt, Akt phosphorylated on Ser-473, Akt phosphorylated on Thr-308, total GSK-3β, GSK-3α phosphorylated on Ser-21, GSK-3β phosphorylated on Ser-9 and tubulin was evaluated by SDS-PAGE and western analysis as described in Materials and Methods. (B) Signals were quantified by densitometry using Gel Expert software by Nucleovision.

Fig. 8. Pharmacological inhibition of PI3-K increases apoptosis sensitivity of migrating cells. SF767 cells were plated onto laminin coated (10 μg/ml) slides in the migration assay format and incubated overnight. Cells were treated with 1 μM camptothecin (Cpt) for 24 hours, or preincubated with LY294002 for 2 hours before camptothecin addition, fixed and stained with DAPI. Cells in rim and core were scored for total cells and cells with condensed, fragmented chromatin. Values represent the mean and standard deviation of five replicate measurements. Significance with unpaired Student's *t* test: *$P<0.001$ vs camptothecin alone.

Discussion

Glioma cells that migrate out of the tumor mass into normal brain tissue exist in a penumbra that affords their escape from tumor resection, chemotherapy and radiation, resulting in treatment failure. Therapies that target migratory glioma cells may increase treatment response. Here, we showed that migration and susceptibility to apoptosis are tightly linked. Migrating glioma cells are two to five times more resistant to Trail-induced and camptothecin-induced apoptosis than migration-restricted cells. There is an inverse relationship between migration and apoptosis susceptibility. Plating cells on increasing concentrations of laminin increases the rate of migration and decreases the levels of apoptosis induction. Multiple migration inhibitors increased apoptosis sensitivity of actively migrating glioma cells. This is in marked contrast to migration-restricted glioma cells, where migration inhibitors had little effect on apoptosis induction. These data argue that apoptosis suppression accompanies acquisition of the migratory phenotype. In addition, these studies indicate that migration inhibitors, while having little affect on migration-suppressed cells, may be used to enhance the response of migrating glioma cells to cytotoxic therapy.

Cell attachment to substrate triggers integrin-mediated survival signaling (Frisch and Ruoslahti, 1997). Here we see that apoptosis suppression is associated more closely with engagement of the migratory phenotype than attachment. For cells in the migration assay plated onto laminin, both core and rim cells are exposed to laminin, but there is a distinct difference in their susceptibility to apoptosis. The actively migrating rim cells are two to five times less susceptible to camptothecin-induced or Trail-induced apoptosis relative to migration-restricted cells in the core. In addition, for core and rim cells plated onto laminin, only the rim cells display phosphorylated Akt and are sensitized to apoptosis with a PI3-K inhibitor. This suggests that the increased survival of migrating rim cells relative to the core cells is not due to attachment-induced integrin survival signaling alone, but rather is associated with migration.

Cell density can affect susceptibility to apoptosis induction (Preobrazhensky et al., 2001). The effects seen here cannot be due to cell density alone because core and rim cells in the migration assay plated onto BSA did not show large differences in susceptibility to camptothecin-induced apoptosis. In addition, there was no difference in Akt phosphorylation between sparse and dense cells plated onto BSA.

Another possible confounding factor is differences in exposure to cell-derived extracellular matrix (ECM) in the core and rim cells. Cells in the densely packed core may lay down more ECM than cells at the rim. However, the same results were obtained for cells plated in the migration assay format onto cell-derived ECM. The actively migrating rim cells were less susceptible to apoptosis-induction than the migration-restricted core cells (data not shown). Therefore, differences in ECM content in core and rim cannot be responsible for the differences seen in apoptosis induction.

The tight linkage between migration activation and decreased susceptibility to apoptosis indicates that migration may activate survival signaling. Akt is an important component of PI3-K survival signaling from growth factor and integrin receptors (Vivanco and Sawyers, 2002). Generation of phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P_3] by PI3-K causes recruitment of Akt to the plasma membrane, where it interacts directly with PtdIns(3,4,5)P_3 through its PH domain (Franke et al., 1997; Vivanco and Sawyers, 2002). Akt at the plasma membrane is phosphorylated and activated by 3-phosphoinositide-dependent protein kinase-1 (PDK1) and an unidentified kinase termed PDK2 (Vanhaesebroeck and Alessi, 2000; Alessi et al., 1997). Here we obtain evidence that migrating glioma cells activate the PI3-K/Akt pathway. Migrating glioma cells have increased levels of phosphorylated Akt and the Akt substrate, GSK-3, relative to migration-restricted cells. Consistent with activation of PI3-K survival signaling in migrating cells, both Akt phosphorylation and reduced apoptosis in migrating cells is blocked by the PI3-K inhibitor LY294002. Because LY294002 had little effect on apoptosis induction in migration-restricted core cells, this supports the preferential activation of the PI3-K survival pathway in migrating cells.

The localization of phosphorylated Akt in migrating glioma cells was distinctly different to that of phosphorylated Akt produced following EGF treatment. Phosphorylated Akt in migrating cells was localized at the leading edge, as has previously been described for Akt in a human fibrosarcoma cell line (Kim et al., 2001). There are other reports of a localization to or change in activity within the leading edge of apoptosis-modulating proteins. Rac protein and activity was increased in growing pseudopodia but not the cell body or retracting pseudopodia in invading NIH 3T3 cells (Cho and Klemke, 2002a), and Rac has also been reported to be a crucial survival signaling molecule in glioma cells (Senger et al., 2002). The adaptor proteins CAS and Crk physically interact in growing pseudopodia but not in the cell body (Cho and Klemke, 2002b). Coupling of CAS and Crk has been reported to suppress apoptosis as well as activate migration (Cho and Klemke, 2000a). The translocation and/or change in activity of proteins that regulate both migration and apoptosis within the leading edge may underlie the decreased susceptibility of actively migrating glioma cells to apoptosis-induction.

Taken together, these data indicate that engagement of the migratory phenotype decreases susceptibility to apoptosis by both activation of a death receptor and a DNA damaging agent. Suppression of migration by pharmacological inhibitors of migration, by restriction of movement by high cell density or by plating cells on a substrate that does not support migration, all result in increased susceptibility to apoptosis induction. Because treatment of migrating glioma cells with migration inhibitors has little effect on migration-restricted cells yet increases the sensitivity of migrating cells to apoptosis induction, this may lead to the development of treatments that specifically target migrating glioma cells.

References

Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B. and Cohen, P. (1997). Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. *Curr. Biol.* **7**, 261-269.

Bellacosa, A., Chan, T. O., Ahmed, N. N., Datta, K., Malstrom, S., Stokoe, D., McCormick, F., Feng, J. and Tsichlis, P. (1998). Akt activation by growth factors is a multiple-step process: the role of the PH domain. *Oncogene* **17**, 313-325.

Berens, M. E., Rief, M. D., Loo, M. A. and Giese, A. (1994). The role of extracellular matrix in human astrocytoma migration and proliferation studied in a microliter scale assay. *Clin. Exp. Metastasis* **12**, 405-415.

Bijur, G. N., de Sarno, P. and Jope, R. S. (2000). Glycogen synthase kinase-3beta facilitates staurosporine- and heat shock-induced apoptosis. Protection by lithium. *J. Biol. Chem.* **275**, 7583-7590.

Castedo, M., Ferri, K. F. and Kroemer, G. (2002). Mammalian Target of Rapamycin (mTOR): Pro- and anti-apoptotic. *Cell Death Diff.* **9**, 99-100.

Cho, S. Y. and Klemke, R. L. (2000a). Extracellular-regulated kinase activation and CAS/Crk coupling regulate cell migration and suppress apoptosis during invasion of the extracellular matrix. *J. Cell Biol.* **149**, 223-236.

Cho, S. Y. and Klemke, R. L. (2002b). Purification of pseudopodia from polarized cells reveals redistribution and activation of Rac through assembly of a CAS/Crk scaffold. *J. Cell Biol.* **156**, 725-736.

Franke, T. F., Kaplan, D. R., Cantley, L. C. and Toker, A. (1997). Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate. *Science* **275**, 665-668.

Frisch, S. M. and Ruoslahti, E. (1997). Integrins and anoikis. *Curr. Opin. Cell Biol.* **9**, 701-706.

Giese, A., Rief, M. D., Loo, M. A. and Berens, M. E. (1994). Determinants of human astrocytoma migration. *Cancer Res.* **54**, 3897-3904.

Giese, A., Loo, M. A., Norman, S. A., Treasurywala, S. and Berens, M. E. (1996). Contrasting migratory response of astrocytoma cells to tenascin mediated by different integrins. *J. Cell Sci.* **109**, 2161-2168.

Hao, C., Beguinot, F., Condorelli, G., Trencia, A., van Meir, E. G., Yong, V. W., Parney, I. F., Roa, W. H. and Petruk, K. C. (2001). Induction and intracellular regulation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) mediated apoptosis in human malignant glioma cells. *Cancer Res.* **61**, 1162-1170.

Khoshyomn, S., Penarp, P. L., Rossi, J., Wells, A., Abramson, D. L. and Bhushan, A. (1999). Inhibition of phospholipase C-gamma1 activation blocks glioma cell motility and invasion of fetal rat brain aggregates. *Neurosurgery* **44**, 568-577.

Kim, D., Kim, S., Koh, H., Yoon, S. O., Chung, A. S., Cho, K. S. and Chung, J. (2001). Akt/PKB promotes cancer cell invasion via increased motility and metalloproteinase production. *FASEB J.* **15**, 1953-1962.

Loberg, R. D., Vesely, E. and Brosius, F. C. (2002). Enhanced glycogen synthase kinase-3ß activity mediates hypoxia-induced apoptosis of vascular smooth muscle cells and is prevented by glucose transport and metabolism. *J. Biol. Chem.* **277**, 41667-41673.

Mariani, L., Beaudry, C., McDonough, W. S., Hoelzinger, D. B., Demuth, T., Ross, K. R., Berens, T., Coons, S. W., Watts, G., Trent, J. M. et al. (2001). Glioma cell motility is associated with reduced transcription of proapoptotic and proliferation genes: a cDNA microarray analysis. *J. Neurooncol.* **53**, 161-176.

Nave, B. T., Ouwens, M., Withers, D. J., Alessi, D. R. and Shepard, P. R. (1999). Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation. *Biochem. J.* **344**, 427-431.

Nobes, C. D. and Hall, A. (1995). Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* **81**, 53-62.

Preobrazhensky, S., Malugin, A. and Wentz, M. (2001). Flow cytometric assay for evaluation of the effects of cell density on cytotoxicity and induction of apoptosis. *Cytometry* **43**, 199-203.

Ridley, A. J., Allen, W. E., Peppelenbosch, M. and Jones, G. E. (1999). Rho family proteins and cell migration. *Biochem. Soc. Symp.* **65**, 111-123.

Senger, D. L., Tudan, C., Guiot, M. C., Mazzoni, I. E., Molenkamp, G., LeBlanc, R., Antel, J., Olivier, A., Snipes, G. J. and Kaplan, D. R. (2002). Suppression of Rac activity induces apoptosis of human glioma cells but not normal human astrocytes. *Cancer Res.* **62**, 2131-2140.

Strasser, A., Harris, A. W., Huang, D. C., Krammer, P. H. and Cory, S. (1995). Bcl-2 and Fas/APO-1 regulate distinct pathways to lymphocyte apoptosis. *EMBO J.* **14**, 6136-6147.

Vanhaesebroeck, B. and Alessi, D. R. (2000). The PI3K-PDK1 connection: more than just a road to PKB. *Biochem. J.* **346**, 561-576.

Vivanco, I. and Sawyers, C. L. (2002). The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat. Rev. Cancer* **2**, 489-501.

Wick, W., Wagner, S., Kerkau, S., Dichgans, J., Tonn, J. C. and Weller, M. (1998). BCL-2 promotes migration and invasiveness of human glioma cells. *FEBS Lett.* **440**, 419-424.

Yoshizato, K., Zapf, S., Westphal, M., Berens, M. E. and Giese, A. (2002). Thromboxane synthase inhibitors induce apoptosis in migration-arrested glioma cells. *Neurosurgery* **50**, 343-354.