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Laboratory Investigation

Radiation enhances the invasive potential of primary glioblastoma cells via activation of the Rho signaling pathway

Gary G. Zhai[1,*], Rajeev Malhotra[2,*], Meaghan Delaney[1], Douglas Latham[1], Ulf Nestler[1], Min Zhang[1], Neelanjan Mukherjee[1], Qinhui Song[1], Pierre Robe[3] and Arnab Chakravarti[1,3]
[1]*Department of Radiation Oncology, Massachusetts General Hospital/Harvard Medical School;* [2]*Department of Medicine, Massachusetts General Hospital/Harvard Medical School;* [3]*Department of Neurosurgery, Brigham and Women's Hospital/Harvard Medical School, Boston, MA, USA*

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Summary

Glioblastoma multiforme (GBM) is among the most treatment-refractory of all human tumors. Radiation is effective at prolonging survival of GBM patients; however, the vast majority of GBM patients demonstrate progression at or near the site of original treatment. We have identified primary GBM cell lines that demonstrate increased invasive potential upon radiation exposure. As this represents a novel mechanism by which radiation-treated GBMs can fail therapy, we further investigated the identity of downstream signaling molecules that enhance the invasive phenotype of irradiated GBMs. Matrigel matrices were used to compare the extent of invasion of irradiated vs. non-irradiated GBM cell lines UN3 and GM2. The *in vitro* invasive potential of these irradiated cells were characterized in the presence of both pharmacologic and dominant negative inhibitors of extracellular matrix and cell signaling molecules including MMP, uPA, IGFR, EGFR, PI-3K, AKT, and Rho kinase. The effect of radiation on the expression of these signaling molecules was determined with Western blot assays. Ultimately, the *in vitro* tumor invasion results were confirmed using an *in vivo* 9L GBM model in rats. Using the primary GBM cell lines UN3 and GM2, we found that radiation enhances the invasive potential of these cells via activation of EGFR and IGFR1. Our findings suggest that activation of Rho signaling via PI-3K is required for radiation-induced invasion, although not required for invasion under physiologic conditions. This report clearly demonstrates that radiation-mediated invasion is fundamentally distinct from invasion under normal cellular physiology and identifies potential therapeutic targets to overcome this phenomenon.

Introduction

Glioblastoma multiforme (GBM) is among the most lethal of all human tumors, with median survivals of approximately 12 months in most studies [1,2]. While involved-field radiotherapy has been found to significantly prolong survival for GBM patients, it is not curative for most GBM patients. GBMs possess a number of 'defense mechanisms' that are known to enhance survival. These include the activation of signal transduction pathways that suppress apoptosis, as well as pathways that enhance proliferation and angiogenesis [3].

Invasive potential has been associated with increasing grade in gliomas, creating a problematic situation in the treatment of glioblastomas. Frequently, these tumors invade adjacent normal brain parenchyma, making complete surgical resection impossible in most cases. Upregulation of matrix metalloproteinases (MMP) such as MMP-2 and MMP-9 has previously been reported to be associated with increased invasive potential in GBMs under physiologic conditions [4–8]. Recent reports identified that radiation can promote invasive behavior in GBM cells [9–11]. The implications of this finding are quite substantial for fractionated radiotherapy, as tumor cells can migrate out of the fixed radiation portal before the subsequent treatment, resulting in a geographic 'miss'. We have confirmed that radiation can enhance the invasive potential in the primary GBM cell lines, UN3 and GM2. We demonstrate here a novel mechanism underlying this phenomenon, which appears to be secondary to EGFR- and IGFR1-mediated activation of PI3K with consequent activation of Rho signaling. We further demonstrate several novel strategies to abrogate this phenomenon in order to improve therapeutic efficacy of radiation in the treatment of GBMs.

Materials and methods

Pharmacological agents and vector constructs

Pharmacological agents were obtained as follows: AG1024, a highly potent and selective IGFR-1 tyrosine kinase inhibitor; LY294002, a PI-3K inhibitor; serine protease urokinase-type plasminogen activator (uPA) inhibitor (PAI-1); matrix metalloproteinase inhibitor-2 and -9 (MMPIs); Y27632, a Rho-associated kinase (ROCK kinase) inhibitor were all purchased from Calbiochem (La Jolla, CA). PAI and MMPI were used

[*] These two authors contributed equally to this work.

Figure 1. Radiation therapy (RT) enhances the in vitro potential for cellular invasion and motility of primary glioblastoma (GBM) cells through IGFR-1 signaling. (a) UN3 and GM2 cell invasion was measured in vitro using Matrigel invasion chambers as described in Material and methods. Invasion index is defined here as the ratio of the percentage of invasive cells in the various RT-treated groups compared to the percentage of invasive cells in the untreated, non-radiated group. Cells were irradiated with different doses ranging from 0 Gy and 8 Gy. Invasive potential in vitro increased with increasing radiation dose up to 6 Gy, at which point this value peaked and declined beyond this dose. Upon inhibition of the IGFR signaling pathway with AG1024 (25 μM in each assay), there was a significant reduction in invasive potential upon radiation exposure at all dose levels. -●-: RT + AG1024, -□-: RT − AG1024. (b) Radiation also increases in vitro cellular motility in UN3 and GM2 GBM cells, as measured using CoStar motility chambers. Motility index is defined here as the relative motility of cells to that of untreated, non-radiated cells. As with invasion, the small molecule inhibitor of IGFR-1 signaling, AG1024, at 25 μM also inhibited this increase in radiation-induced motility, suggesting that increased signaling through IGFR-1 may underlie both increased cellular motility and invasion upon RT exposure in GBM cells. -●-: RT + AG1024, -□-: RT − AG1024. (c and d) Similar assays were performed with UN3 and GM2 cells while grown in serum-free media with increasing amounts of exogenous IGF1 (ranging from 0 to 50 μM). Cell invasion and motility were also investigated with increasing amounts of exogenous EGF (ranging from 0 to 50 μM) for GM2 cells grown in serum-free media. Cells were irradiated as described in Material and methods. Each invasion or motility assay was performed in triplicate and repeated twice. Data were plotted in the same way as a and b.

in doses of 50 μM as this dose was found to optimally inhibit the invasive capabilities of other primary GBM cell lines under physiologic conditions. It was determined that this dose, as well as higher doses of PAI and MMPI failed to inhibit RT-mediated invasion in any of our primary GBM cell lines tested. Y27632, LY294002, and AG1024 were used in doses of 25 μM, as higher doses failed to further reduce RT-mediated invasion. At the specified doses for each inhibitor, we proceeded to verify that there was minimal impact on other similar targets. For example, at doses of 25 μM, AG1024 was found to have little effect on the insulin receptor. Insulin-like growth factor-1 (IGF1) was purchased from Sigma (St. Louis, MO). Kinase-deficient AKT (AKTkd), myristylated AKT (AKTmyr) and wild type AKT (AKTwt) were obtained as gifts from Dr. A. Bellacosa (Fox Chase Cancer Center, Philadelphia, PA). Var-Rho(DN) and Var-Rho(WT) were obtained as generous gifts from Dr. R. Cerione (Cornell University, Ithaca, New York). PI-3K constructs were kindly provided by Dr. M. Weber (University of Virginia, Charlottesville, VA).

Cell culture and transfection

The UN3 and GM2 cell lines were established in culture from glioblastoma tumor specimens using techniques described in detail previously [12]. Briefly, the GBM tissues were obtained during open resection and mechanically dissociated, and the dispersed cells and fragments were cultured and passaged as described [12]. Molecular profiling revealed that UN3 had absence of EGFR, PDGFRs, or other RTKs with exception of IGFR-1. Radiation treatments were performed using a 250 kVp photon beams and were delivered using a dose rate of 2 Gy/min. Experiments described in Figure 1c and d were performed using incremental concentrations of exogenous IGF (0–50 μM). Transfection involving PI-3K experiments was based on a previously published method [13] and infection experiments with the varicella viral constructs containing the Rho constructs were conducted as previously reported by Cerione and coworkers [14].

Matrigel invasion assay

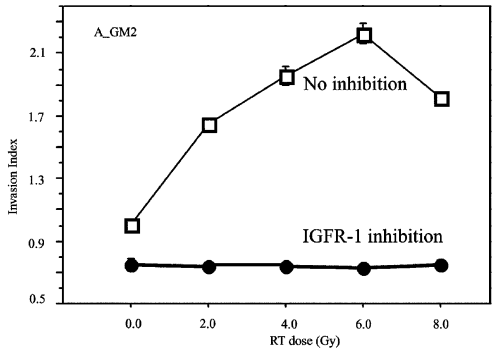
Invasion assays were performed using the Matrigel® (Becton Dickinson) basement membrane chamber assay. Before use, the inserts were rehydrated for 1.5–2 h with 0.5 ml of warm DMEM containing 0.1% BSA. Rehydrated chambers (containing Matrigel® Matrix) were placed into the wells of a 24-well companion plate. Each well contained 0.5 ml of media with 5×10^3 cells plated. The chambers were incubated for 24 h at 37 °C in a 5% CO_2 atmosphere. After 48 h, the chambers were scrubbed with a cotton swab to remove the matrix. Cells on the under surface of the membrane were fixed and stained in solution containing methylene blue dissolved in 70% ethanol and counted on examination by light microscopy. Control chambers included untreated cells. The remaining chambers contained cells treated by specific pharmacological inhibitors. The invasion index was calculated by comparing the number of invasive cells after pharmacological inhibition divided by the number of invasive cells in the control chambers (untreated cells).

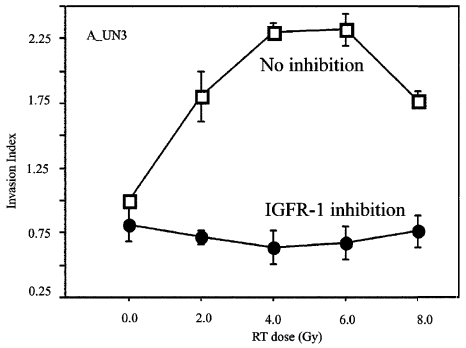
Costar motility assay

Motility assays were performed according to manufacturer's instruction. Briefly, UN3 and GM2 cells were plated at low density onto 35-mm tissue culture dishes (Costar, Cambridge, MA) and incubated overnight in growth medium. The medium was supplemented on the next day with 20 mM *N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic acid) and the remaining procedures were performed with the dishes on a microscope stage maintained at 37 °C. Cell movement was then recorded with a time-lapse video recorder with a 60-fold time compression. The rates of cell motility were calculated by measuring the displacement of individual cells over a 2 h period.

Cell morphology change upon inhibition of Rho-associated kinase

Cell chambers in duplicate were seeded with GM2 cells in density of 1000 cells/cm^2. Rho-associated kinase inhibitor Y27632 was added to overnight growing cells with final concentrations of 50 and 100 μM. Incubation was allowed to proceed for 2 h before medium was removed from the chambers and cells fixed with 5% paraformaldehyde for 10 min, followed by treatment with 100% ice–cold methanol for 20 min. Cells were washed with 1× PBS three times each for 10 min. Phase


A_GM2
No inhibition
IGFR-1 inhibition
Invasion Index
RT dose (Gy)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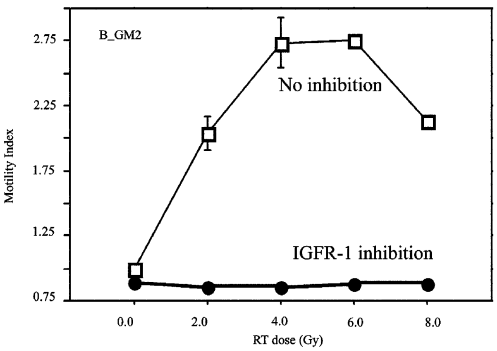
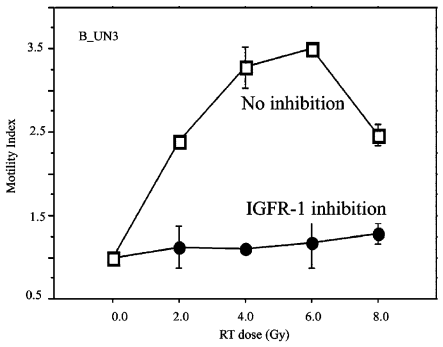
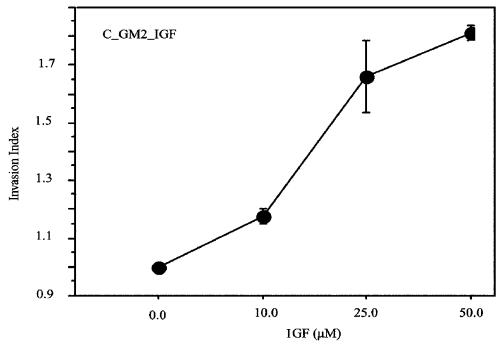
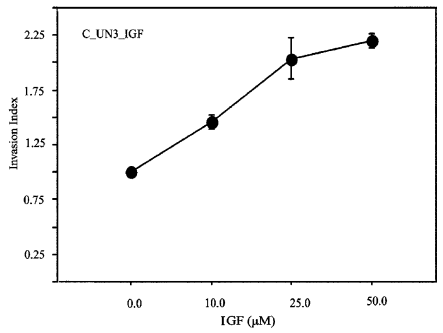
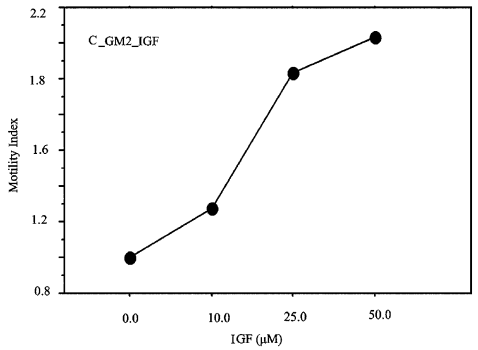
IGFR-1 inhibition
Invasion Index
RT dose (Gy)

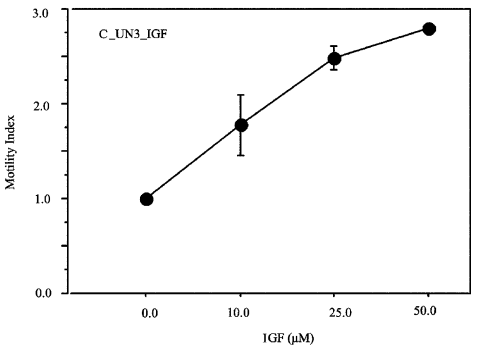

B_GM2
No inhibition
IGFR-1 inhibition
Motility Index
RT dose (Gy)

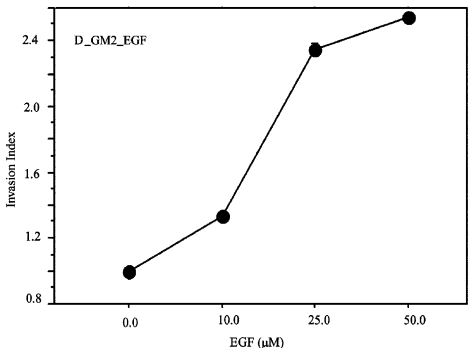

B_UN3
No inhibition
IGFR-1 inhibition
Motility Index
RT dose (Gy)

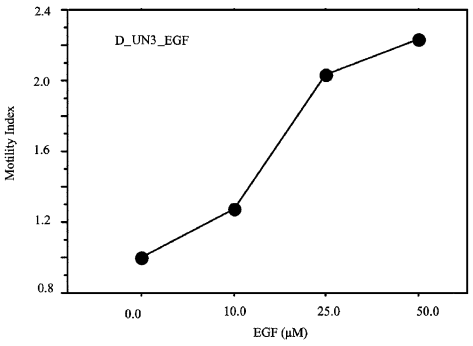

C_GM2_IGF
Invasion Index
IGF (μM)


C_UN3_IGF
Invasion Index
IGF (μM)


C_GM2_IGF
Motility Index
IGF (μM)


C_UN3_IGF
Motility Index
IGF (μM)


D_GM2_EGF
Invasion Index
EGF (μM)


D_UN3_EGF
Motility Index
EGF (μM)

images of the cells were acquired using microscopy equipped with a Nikon camera.

Immunoblot analysis

Lysates were generated by placing these cells in RIPA lysis buffer. Bradford assays were performed to determine total protein concentrations, which were normalized to 1 μg/μl for all samples. Samples were then prepared in sample buffer and heated to 95 °C for 5 min. Samples were run on 10–12% polyacrylamide gels. 15 μl of protein lysate in sample buffer from each tissue were loaded within each well. Positive controls were loaded on each gel and represented lysates from UN3 and GM2 cells for phospho-ERK1/2, phospho-PI-3K, phospho-PKC and phospho-AKT. Gels were run at constant current (30 mA) for 2 h for maximum separation. Semidry transfer was then performed. The membrane was then blocked for 1 h in 5% milk in 0.2% TBST (Tris buffer solution with 0.2% Triton X). The membranes were then washed in 0.2% TBST three times for 15 min each. The membranes were then incubated overnight with primary antibody directed against phospho-ERK1/2 (Cell Signaling Technology), phospho-AKT (Cell Signaling Technology), phospho-PI-3K (Cell Signaling Technology), and phospho-Protein Kinase C (Cell Signaling Technology). Antibodies against Rac-GTP, cdc42-GTP, and Rho-GTP (Calbiochem) were used for Western analysis after the pull-down experiments, which were carried out according to the manufacturer's protocol (New England Biolabs, Beverly, MA), to detect protein expression levels. All antibodies were used in 1:1000 concentrations. Subsequently, the membranes were washed in 0.2% TBST three times for 15 min each. Membranes were then incubated with secondary antibody for 45 min and subsequently washed. Chemiluminescent (Bio-Rad) detection was then used to detect expression of these proteins and phospho-proteins, the levels of which were quantitated using densitometry.

In vivo *studies*

The *in vivo* studies were undertaken according to previously published methods [9]. 12-week-old male Fisher rats (Charles River Laboratories, Wilmington, MA) were first anesthetized with brevimytal (500 mg in 10 ml 0.9% NaCl), and subsequently stereotactic injections of 10,000 logarithmic-phase 9L cells into the striatum were performed. Cells were pretreated with inhibitors (25 μM each), irradiated with 3 Gy, and dissolved in PBS prior to implantation. At 21 days, the rats were sacrificed. The brains were shock frozen in liquid nitrogen and dissected in 8-μm sections for histology or 15-μm sections for volumetry. Tumor volumes were determined on HandE-stained sections ($n = 15$). For measurement of invasion index, brain sections were stained with a nestin antibody [15] that specifically targets the 9L tumor cells and not the surrounding brain. To describe briefly, slides were incubated with acetone for 10 min, air dried, incubated with 1% H_2O_2 for 30 min, washed with PBS, and blocked with BSA (10 mg/10 ml PBS) for 10 min. Nestin antibody (1:1000; BD Biosciences, San Jose, CA) was added overnight, labeled with biotinylated anti-mouse IgG antibodies, incubated with avidin–biotin reagent Vectastain Elite ABC peroxidase (Vector Laboratories, Burlingame, CA), and developed in diaminobenzidine (Sigma, St Louis, MO). As a control, preimmune serum was used in place of the primary antibody. Nestin-positive tumor cell clusters (with clusters being defined as tumor cell aggregates >10 cells) distant from the bulk tumor mass were counted on six independent 8-μm sections/tumor.

Results

Radiation-mediated enhancement of invasion and motility

Matrigel and Costar assays were performed *in vitro* to examine the invasive potential and motility associated with UN3 and GM2 GBM cell lines. Our data demonstrated enhanced invasive potential and motility with increasing radiation doses up to 6 Gy in a dose-dependent manner, at which point this value plateaued and declined (Figure 1a for invasion, Figure 1b for motility). The data from this *in vitro* assay confirmed previous observations that radiation can enhance both invasion and motility of GBM and other cells [9–11].

Inhibition of the MMP and uPA pathways does not influence RT-mediated invasion

In view of the strong correlation reported between increased matrix metalloproteinase (MMP) levels and tumor cell invasiveness in human gliomas [4,8,16] and the demonstrated roles of the serine protease urokinase-type plasminogen activator (uPA) in glioma cell invasion and neovascularization [17–20], we investigated the roles of these two pathways in RT-mediated invasion using specific plasminogen activator inhibitor (PAI) and MMP inhibitor (MMPI). We found that neither played a detectable role for radiation-mediated cellular invasion (Figure 2a and b) as opposed to the 50% reduction in invasive potential observed with PAI and MMPI under physiologic conditions for the same cell lines (see below for Discussion and Figure5 f and g). The results underscore the mechanistic differences between invasion under physiologic conditions vs. in the presence of radiation.

Role of IGFR-1 in radiation-mediated enhancement of invasion and motility of glioma cells

As MMPs and uPAs were not found to impact radiation-mediated invasion with glioma cell lines, we next examined the role of growth factors, as receptor tyrosine kinase (RTK) activation has been previously associated with increased invasive potential [3,13,21]. Since the UN3 cell line does not express EGFR,

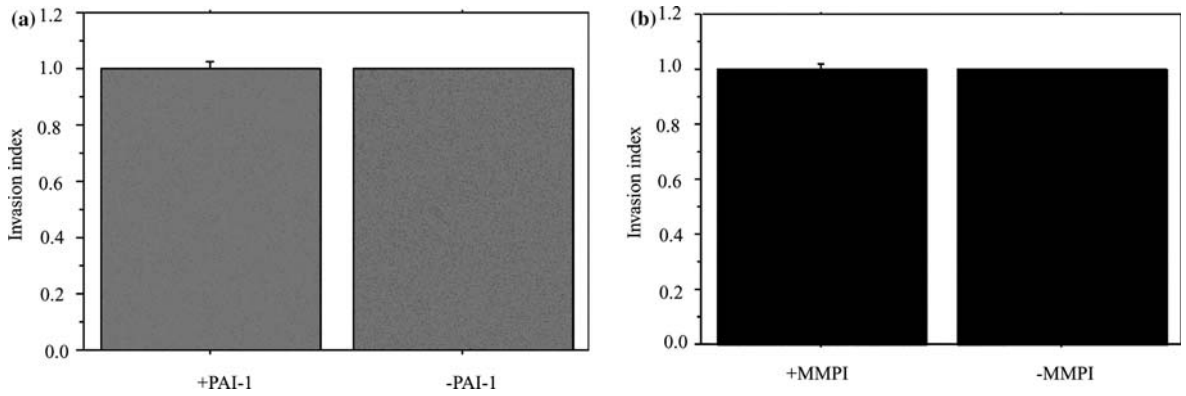


Figure 2. In vitro demonstration that inhibition of urokinase plasminogen activator (uPA) or matrix metalloproteinase has no impact on RT-induced invasion of UN3 cells. (a) Invasion assay with irradiated UN3 cells (6 Gy) was performed using either untreated cells or cells treated with PAI at 50 μM. (b) The same assays as in (a) were also performed to study the invasive potential of irradiated cells using MMP inhibitor at 50 μM. The increased invasion observed after RT exposure in UN3 cells cannot be abrogated through the use of matrix metalloproteinase inhibitors (MMPI's), selective for MMP 2 and 9.

PDGFRs, or other RTKs with exception of IGFR-1 (data not shown), we proceeded to investigate whether IGFR-1 plays a role in mediating cell invasion and motility. Inhibition of the IGFR-1 signaling pathway with compound AG1024 gave rise to a significant reduction in invasive potential upon radiation exposure at all dose levels (Figure 1a). This inhibition reduced the invasion index to levels equivalent to, or even below that, of untreated cells regardless of the dose of radiation administered. The same effect was observed with cell motility upon radiation treatment with or without the inhibitor, indicating the dependence of both RT-mediated cell invasion and motility on the activation of IGFR-1 signaling (Figure 1b). To further assess this dependence, we tested the cellular effect of exogenous insulin-like growth factor 1 (IGF1) on invasion and motility in glioma cells. Irradiated cells grown in serum-free media and increasing amounts of exogenous IGF1, with concentrations ranging from 10 to 50 μM, exhibited increasing invasion potential and motility in a dose-dependent manner, confirming the role of the IGFR-1 pathway in RT-mediated invasion (Figure 1c). Curiously, these cells did not experience a change in motility or invasive potential in the presence of IGF1 in the absence of radiation (data not shown). These results were confirmed in the GM2 cell line, which has expression of both IGFR1 and EGFR. As the GM2 cell line expresses both EGFR and IGFR1, we examined the roles of both RTK's in radiation-mediated invasion and enhancement of cellular motility. Both were found to have important roles in this regard (Figure 1c and d).

Identification of IGFR-1 and EGFR-dependent downstream pathways upregulated by radiation treatment

Since the IGFR-1 and EGFR pathways were demonstrated to be critical for radiation-mediated cellular invasion and motility, we next addressed the mechanistic roles of individual downstream signaling pathways. The expression levels of phosphorylated PI-3K, AKT, ERK1/2, and PKC were measured in radiated UN3 and GM2 cells grown in serum-free media (Figure 3a and b) with or without exogenous IGF1 (and EGF in GM2 cells) through quantitative Western blot analysis. We found that phosphorylated PI-3K, AKT and ERK1/2 expression levels were significantly increased in the presence of IGF1 (and EGF in GM2 cells); however, expression levels of phosphorylated PKC did not change substantially with either exogenous IGF1 or EGF (Figure 3a). We also examined the effect of radiation on the Rho signaling pathway, which is known to be activated downstream of IGFR-1 and EGFR. Expression of GTP-bound members of the Rho family, including RhoA, Rac1, and Cdc42 were all upregulated in the presence of exogenous IGF1 upon radiation treatment of UN3 cells (Figure 3b) and in the presence of either IGF1 or EGF upon radiation treatment of GM2 cells. However, in the absence of radiation, expression of each of these three members was unaffected by exogenous IGF1 (or EGF in the case of GM2 cells) (Figure 3b), indicating that these growth factors can act synergistically with radiation in activating these critical downstream signaling pathways.

Activation of RhoA and Rac1 is mediated by IGFR-1 and EGFR through PI-3K

It has been well established that the Rho signaling pathway can be activated downstream of PI-3K signaling and that activation of the Rho pathway can affect tumor cell motility [22–27]. To demonstrate whether the activation of Rho family members is regulated by the PI-3K pathway upon radiation exposure, we transfected UN3 and GM2 cells with either a wild-type (wt) PI-3K construct or a dominant-negative form (DN). Both RhoA–GTP and Rac1–GTP protein expression were elevated in the PI-3K (wt)-transfected cells in UN3 and GM2 cells upon radiation exposure compared to that observed in the PI-3K(DN)-transfected cells (Figure 4). Cdc42 expression was less affected by transfection with the dominant-negative PI-3K construct in UN3 cells, but did show a reduction

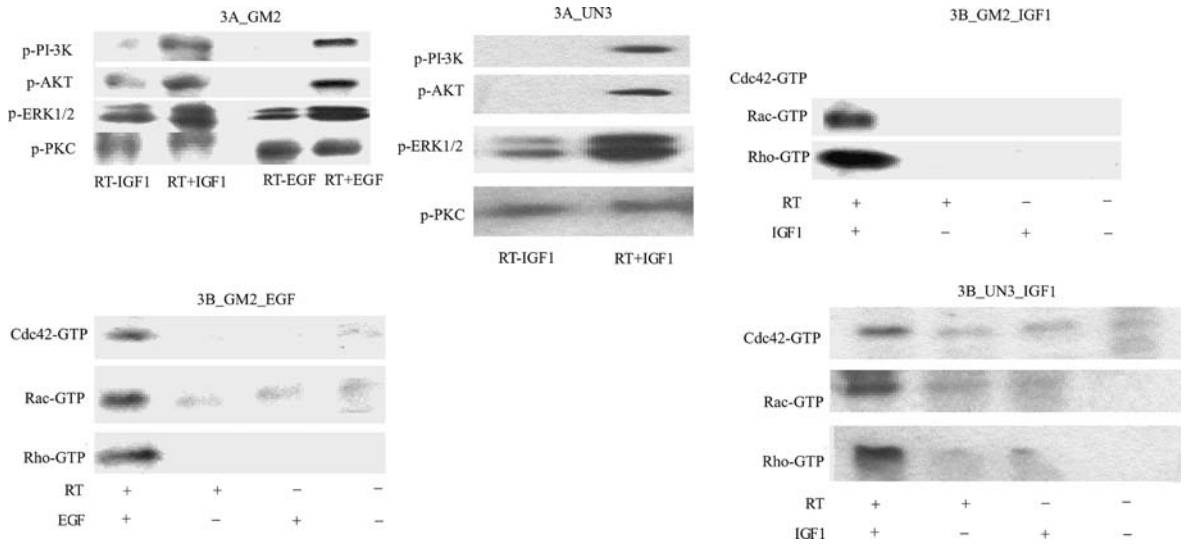


Figure 3. Identification of critical downstream signaling pathways that are activated upon RT exposure in an IGF1 and EGF-dependent manner, using phospho-specific antibodies. (a) Western blotting experiments were carried out using cell lysates prepared both from irradiated GM2 cells (2 Gy) with or without IGF1 and EGF at 50 μM, and from UN3 cells (2 Gy) with or without IGF1 at 50 μM. The cells were otherwise grown in serum-free medium. Phospho-PI-3K, phospho-AKT, phospho-ERK1/2, and phospho-PKC antibodies were used to detect radiation-induced activities of these kinases in the presence or absence of growth factor. (b) Activation of Rho-family members upon RT-exposure. Antibodies against Cdc42-GTP, Rac-GTP, and Rho-GTP (Calbiochem) were used for Western analysis, which were carried out according to the manufacturer's protocol to detect protein expression levels under different conditions: (1) when irradiated GM2 cells were treated with or without IGF1 and EGF, (2) when irradiated UN3 cells were treated with or without IGF1, (3) when non-irradiated GM2 cells were treated with or without IGF1 and EGF, (4) when non-irradiated UN3 cells were treated with or without IGF1. All antibodies were used in 1:1000 concentrations. Activation of RhoA, Rac, and Cdc42, as measured by levels of GTP-associated protein, is also dependent upon IGF1 and EGF signaling in irradiated glioma cells.

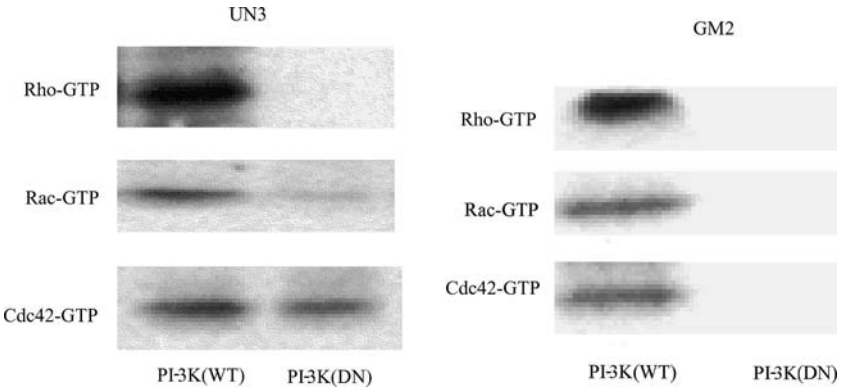


Figure 4. Activation of RhoA and Rac1 is mediated by IGFR-1 through PI-3K in irradiated glioma cells. UN3 and GM2 cells were transfected with either wild-type or dominant-negative PI-3K constructs to determine whether PI-3K signaling mediates activation of Rho family members upon exposure to RT. Both RhoA–GTP and Rac1–GTP protein expression were elevated at least 5 times in the PI-3K (wt)-transfected cells upon radiation exposure compared to that observed in the PI-3K(DN)-transfected cells. Cdc42 expression was unaffected by transfection with the dominant-negative PI-3K construct in UN3, but affected significantly in GM2.

in expression in GM2 cells. Curiously, in the absence of RT, there was no significant effect of PI-3K inactivation on the activated state of Rho-family members (data not shown).

Inhibition of EGFR, IGFR-1, PI-3K, and Rho signaling reduces the invasive potential of glioma cells

To assess the impact of signal transduction inhibitors in reversing the RT-mediated invasion of UN3 and GM2 cells, the efficacy of pharmacologic and biologic inhibitors of EGFR, IGFR-1, PI-3K, AKT, and Rho-associated kinases were further examined. Inhibition of PI-3K via the pharmacologic inhibitor LY294002 led to a ~2.5-fold reduction ($P < 0.0001$) of RT-mediated invasion for both UN3 and GM2 cells (Figure 5a). However, inactivation of AKT activity (Figure 5b) through the use of dominant-negative forms of the protein did not lead to any detectable reduction of RT-mediated invasion. Inhibition of IGFR-1 through the small molecule inhibitor AG1024 led to a significant twofold reduction in RT-mediated (4 Gy) invasion in UN3 cells *in vitro* ($P < 0.0001$, Figure 5c). The reduction of RT-mediated invasion was more significant in

the presence of dual EGFR and IGFR1 inhibition in GM2 cells, which co-express both receptors, compared to inhibition of either RTK alone ($P < 0.0001$). This data suggests that in GBM cells that co-express multiple RTKs, inhibition of individual RTKs may be of limited benefit in reducing RT-mediated invasion. Inhibition of Rho signaling also resulted in a significant reduction in cellular invasion. First, inhibition through infection of UN3 and GM2 cells via a varicella virus containing a dominant-negative form of Rho (RhoN19), resulted in significant reductions in invasion ($P < 0.0001$, Figure 5d). This was confirmed by experiments using pharmacologic inhibition of Rho kinase with the agent Y27632, which also resulted in a significant reduction of RT-mediated invasion ($P < 0.0001$, Figure 5e). Neither pharmacologic inhibition of PKC nor the inhibition of Rac or that of Cdc42 using varicella virus containing the respective dominant-negative constructs RacN17 and cdc42N17 produced any significant change in RT-mediated invasion in UN3 or GM2 cells (data not shown).

We next investigated the efficacy of these signaling antagonists in inhibiting invasion in the absence of radiation (under physiologic conditions) in UN3 and GM2 cells (Figure 5f). It becomes strikingly apparent that the mechanisms of invasion in the setting of radiation vs. under physiologic conditions may, indeed, be quite different. For both cell lines, inhibition of MMP and uPA resulted in greatest suppression of invasion under physiologic conditions in contrast to their negligible role in the setting of radiation (Figure 2a and b). Inhibition of RTKs including IGFR1 and EGFR as well as inhibition of PI-3K resulted in a significant suppression of invasion under physiologic conditions, albeit more modestly than in the setting of radiation. The mediators downstream of the RTKs and PI-3K may be one major source of divergence between the two phenomena. Perhaps most surprisingly, pharmacologic inhibition of Rho kinase had no effect on reducing invasion under physiologic conditions, as opposed to its seemingly critical role in mediating invasion in irradiated UN3 and GM2 cells. This data strongly suggests that the underlying mechanisms of invasion differ in the presence vs. absence of radiation.

Morphological changes associated with radiation treatment are prevented by Rho inhibition in glioma cells

Previous studies have established a correlation between the invasive potential of tumor cells and cell morphology [28–36]. In order to investigate the potential changes in morphology induced by radiation of glioma cells, cell morphology experiments were carried out using the primary GM2 glioma cell lines. Radiation-treated GM2 cells on average exhibited more elongated cell morphology relative to non-radiated cells. However, treatment of these irradiated cells with the Rho kinase inhibitor (Y27632) resulted in a cell morphology that appeared to resemble a more differentiated, non-radiated appearance (Figure 5g). Results with UN3 cells appeared similar.

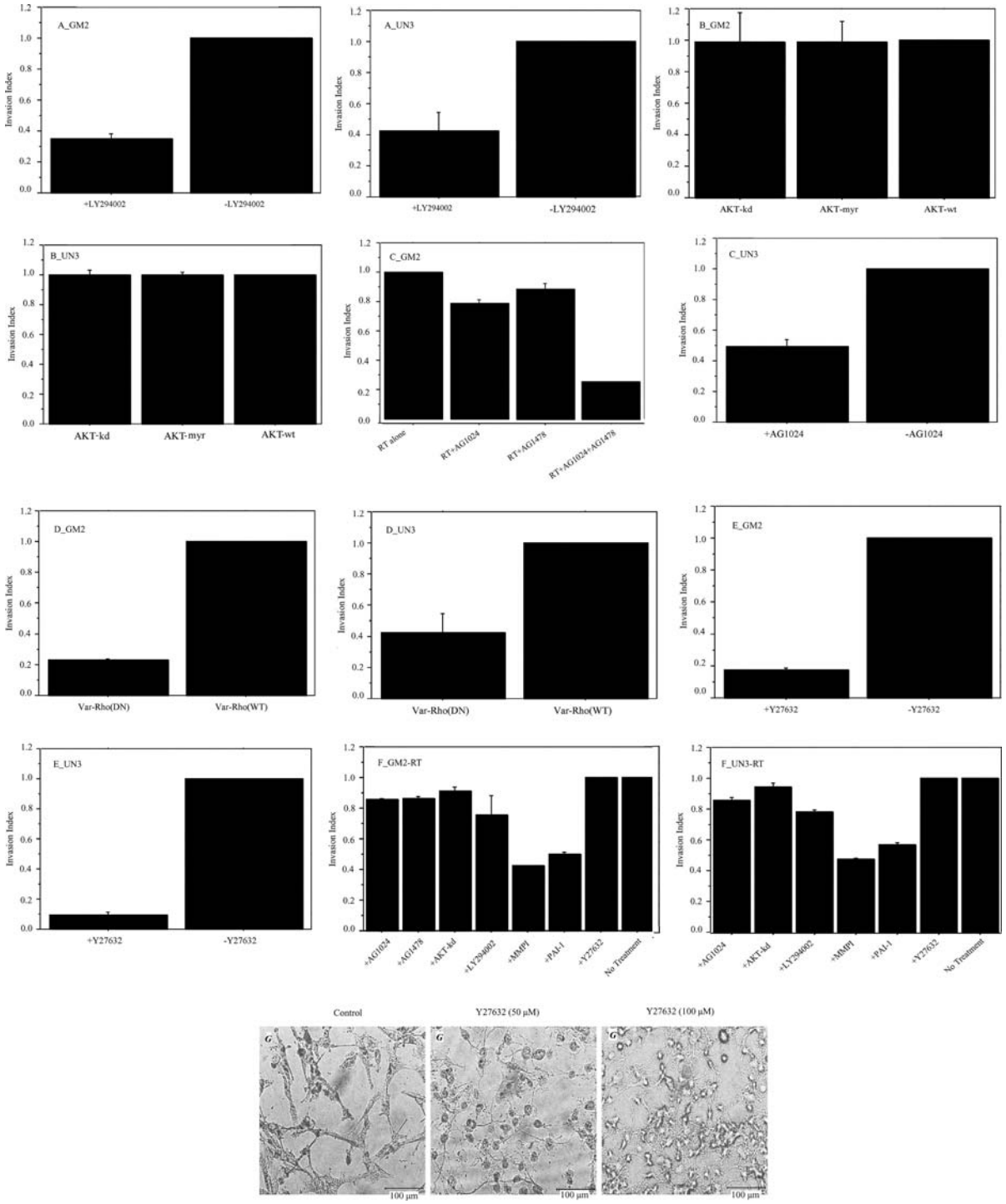
In vivo assessment of RT-mediated invasion in glioma cells

As these primary GBM cells could not be grown as xenografts, we investigated whether 9L cells, which can be grown *in vivo*, behaved similarly to GM2 and UN3 *in vitro*, with respect to RT-mediated invasion. Figure 6a demonstrates that radiation-treated 9L cells had increased invasive potential with increasing radiation doses up to 6 Gy. To investigate the impact of radiation exposure on cell invasion in the *in vivo* setting, radiated and non-radiated 9L glioma cells were implanted intracranially into Fisher rats and the degree of tumor invasion was assessed histologically. An invasion index was determined based upon the number of clusters of tumor cells located distant from the primary tumor site at 21 days, revealing that radiation treatment increases the invasion index twofold using this *in vivo* model of glioma cell migration. In a subsequent set of experiments, the cells were treated by radiation in combination with pharmacologic and biologic inhibitors of the same pathways studied in the previous *in vitro* experiments. Results demonstrated that inhibition of IGFR-1, PI-3K, and Rho (both pharmacologically and biologically) led to a significant five-to-six-fold reduction of RT-mediated invasion *in vivo* (Figure 6b). This confirms our *in vitro* findings that IGFR-1 and its downstream signaling pathways (PI-3K and Rho, in particular) are important mediators of radiation-mediated invasion in gliomas and may represent important targets to improve radiation treatment response in the clinical management of these tumors (Figure 7).

Discussion

Increased invasive potential has been associated with increasing tumor grade in human gliomas [3]. Under physiologic conditions, several studies have suggested that MMPs contribute to cancer cell invasion of surrounding normal tissues and metastasis through cell-surface ECM degradation. Strong correlations have been reported between elevated MMP levels and tumor cell invasiveness in human gliomas [37]. Moreover, the urokinase-type plasminogen activator receptor (uPAR) has been demonstrated to play a critical role in the regulation of cell-surface plasminogen activation in several physiological and pathological conditions. For example, antisense vectors used to downregulate uPAR expression at the level of mRNA and protein in glioblastoma cells was found to inhibit tumor formation in nude mice [38]. Strong correlations found between elevated uPA levels in glioblastoma cells and tumor invasiveness have sparked interest in uPA as a potential target for therapy. Furthermore, previous study has shown that MMP's can mediate GBM cellular invasion (6).

In the present study, we have not only confirmed that radiation can enhance the invasive potential of GBM cells, but we have demonstrated that the underlying mechanism may be distinct to that previously reported under physiologic conditions. Initial investigations into



whether MMPs and uPA play a significant role in this radiation-mediated phenomenon yielded negative results, although assuming greater significance under physiologic conditions. The conventional wisdom has been that radioresistance in GBMs is a phenomenon secondary to activation of pro-survival, angiogenic, and proliferative molecular pathways. While this may be the case, the findings of this study suggest that radioresistance may be a far more complex phenomenon. This is especially true with fractionated radiotherapy, where ~30 daily fractions of 2 Gy doses are administered for GBM patients. Based on the results of this study, it is not inconceivable that the surviving population of GBM cells after each 2 Gy fraction (which can be as high as 50%) can acquire enhanced invasive potential, enabling these cells to migrate to the periphery or even outside of the radiation portal, resulting in geographic 'misses'. While it is still likely that inherent cellular radioresistance plays a major role in the clinical treatment resistance of GBMs, the ability of radiation to enhance cellular motility and invasiveness of GBM cells may prove even more problematic when more improved therapies are developed to enhance the sensitivies of GBMs to radiation-induced cell death. It can be argued

Figure 5. Inhibition of IGFR-1, PI-3K, and Rho reduces the invasive potential of glioma cells. A. Irradiated UN3 and GM2 cells were treated with LY294002 (a PI-3K inhibitor) in a final concentration of 25 μM for 5 h, followed by invasion assays. An approximate 2.5-fold reduction ($P < 0.0001$) of RT-mediated invasion of GM2 and UN3 cells was observed. (b). Inactivation of AKT activity through the use of dominant-negative forms (kinase-deficient or myristylated) AKT did not lead to any detectable reduction of RT-mediated invasion in both GM2 and UN3 cells. (c) Inhibition of IGFR-1 through AG1024 (25 μM) led to a significant reduction in RT-mediated invasion in UN3 cells in vitro ($P < 0.0001$). For GM2 cells, inhibiting IGFR-1 with AG1024 yielded 25% reduction in invasion index as opposed to that observed with cells treated with irradiation alone. Inhibiting EGFR with compound AG1478 produced a similar effect (~20% reduction) whereas inhibition with both inhibitors yielded roughly an 80% reduction in invasion index. (d) Inhibition of Rho signaling, through infection of UN3 and GM2 cells via a varicella virus containing a dominant-negative form of Rho (RhoN19), resulted in a significant reduction in cellular invasion ($P < 0.0001$). (e) Inhibition of Rho signaling, through pharmacologic inhibition of Rho-associated kinase with compound Y27632, also led to an even greater reduction in cellular invasion ($P < 0.0001$) in both UN3 and GM2 cells. (f) Under physiological conditions (non-irradiated), invasion indices were obtained with various pharmacologic inhibitors on UN3 and GM2 cells, demonstrating that physiologic invasion works through different mechanisms than RT-mediated invasion. Whereas inhibition of IGFR-1, Akt, and PI-3K are shown to have modest effects on physiologic invasion, inhibition of MMP and uPA has much more pronounced effects. Inhibition assays with UN3 and GM2 under physiological condition were performed as those described for the irradiated cells. (g) GM2 cell morphology changes upon inhibition of Rho-associated kinase using pharmacological agent Y-27632 at 50 and 100 μM. Incubation time was 2 h. Control cells were irradiated but did not have inhibitor added. Images were obtained with phase microscopy at 20× magnification.

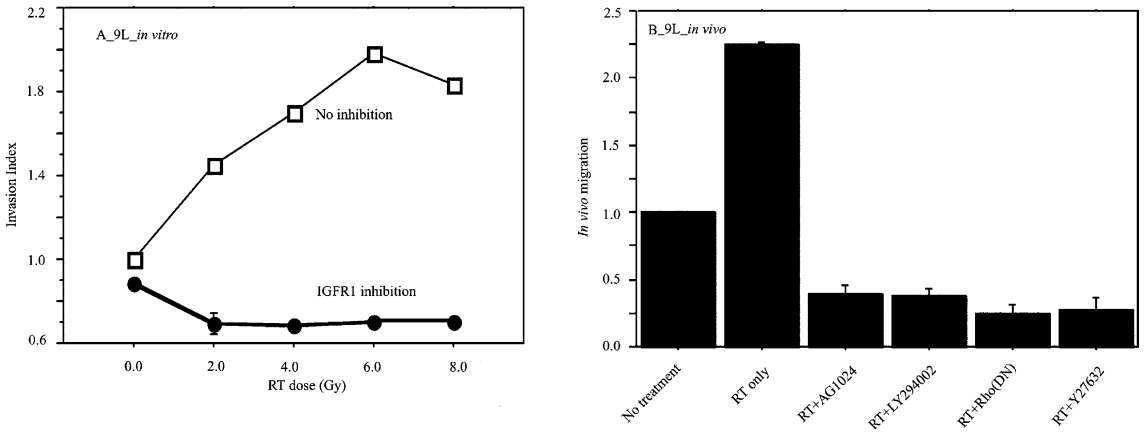


Figure 6. Verification of enhanced invasive potential upon irradiation of glioma cells using a different cell line 9L both *in vitro* and *in vivo*. (a) The invasive potential of 9L cells upon irradiation treatment was measured in the same fashion as that used for UN3 and GM2 described in Figure 1. The data show a similar pattern as for UN3 and GM2 cells. -●-: RT + AG1024, -□-: RT-AG1024. (b) 9L glioma model implanted intracranially in Fisher rats. The cells were treated with 3 Gy RT in combination with pharmacologic and biologic inhibitors. Clusters (> 10 cells) of migrating cells distant from the primary tumor were scored at 21 days. Values are expressed as ratios with the denominator representing migration of untreated cells (for detailed description of the methods, refer to *In vivo* studies in Materials and methods).

that strategies would have to be developed to abrogate this phenomenon or rely on improved cytotoxic or biotherapeutic strategies to treat the cells that have migrated away from the primary tumor mass.

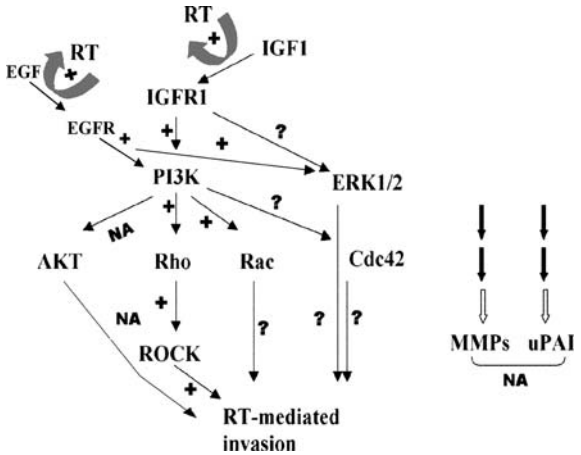


Figure 7. Integrated diagrammatic representation of signaling pathways involved in radiation-mediated invasion of glioma cells. NA = No Association.

Therefore, another potentially critical finding of this study is the mechanism by which radiation can enhance invasive potential of GBM cells. We have previously reported that both EGFR and IGFR1 can enhance invasion of primary GBM cells through a PI3K-dependent mechanism under physiologic conditions. The findings of the present study extend these previously reported findings in several different ways. First, we have demonstrated that although both IGFR1 and EGFR can enhance invasion under physiologic conditions in GBMs, this appears to be the case to a much larger degree after radiation treatment. Further, we have demonstrated possible strategies to antagonize radiation-induced invasion, which is highly dependent on the molecular profile of the tumor. Co-expression of IGFR1 and EGFR requires that both receptors be simultaneously targeted to abrogate radiation-induced invasion to the greatest degree. In cases where only one RTK is expressed, inhibition of that particular RTK may prove sufficient. Other potentially important RTKs such as integrin receptor and PDGFR were not expressed in appreciable amounts in these cell lines; however, these merit further investigation as well.

The observation that Rho-pathway activation may be the central mediator of radiation-mediated invasion is perhaps one of the most significant, as well as novel findings of this study. Rho family proteins are regulators of extracellular stimuli-mediated signaling pathways that control numerous intracellular processes including actin cytoskeletal organization, gene expression, invasion, and cell cycle progression [28,33,39–42]. The GTP-bound forms of RhoA, Rac, and cdc42 are the active states of these small GTPases that regulate specific morphologic change in the actin microfilament-based cytoskeleton. Reorganization of this cytoskeleton facilitates the dynamic changes necessary for cellular adhesion, motility, and tumor metastasis. We demonstrated that activation of RhoA and Rac1 was mediated by IGFR-1 and EGFR signaling through PI-3K in irradiated cells (Figure 4a). Our data support the well-established observation that aberrant expression of Rho family proteins causes tumorigenic, invasive and metastatic transformation in many cell types. Qiang et al. [34] have recently described the critical role of RhoA in Wnt-induced morphologic changes in multiple myeloma cells. Many members of this family of proteins with GTPase activity including RhoA, Rac, and cdc42 have been implicated in changes in cell morphology, formation of stress, focal adhesion, and cell motility. Our measurements of cell dimensions determined that the irradiated GM2 glioma cells on average showed a significant morphological switch upon treatment with Rho kinase (ROCK) inhibitor (Figure 5g). This line of evidence is in agreement with what has been observed in multiple myeloma cells and suggests that the increased cell invasiveness is closely linked to cell morphology. Strategies to inhibit Rho activation include RTK inhibitors of IGFR1 and EGFR, respectively. The problem with this strategy is that our findings suggest that RTKs can compensate for the loss of function of each other. Therefore, in order for this strategy to be effective, multiple RTKs must be simultaneously targeted for inhibition. Perhaps a more attractive strategy would be to inhibit Rho function more centrally using ROCK inhibitors. Our previous data suggest that Rho kinase inhibitors have minimal impact on the clonogenic survival and morphological appearance of normal human astrocytes upon radiation exposure. ROCK inhibitors, however, cause dramatic morphological changes in GBM cells as shown in this study and inhibit their increased invasiveness upon radiation exposure. Interestingly, under normal physiological conditions, ROCK inhibitors only had minimal effect in reducing invasive potential of GBM cells, suggesting that Rho pathway activation may play a more unique role in radiation-mediated invasion, as opposed to invasion under physiologic conditions. As this is the first report suggesting that there may be unique invasion pathways activated by radiation exposure, further investigation of this phenomenon is warranted to pin down other important differences.

In summary, our identification, both *in vitro* and *in vivo*, of signaling pathways involved in the enhancement of cellular invasion potential and motility as a result of irradiation of glioblastoma cell lines bears clinical implications. It provides the molecular mediators of RT-mediated invasion in gliomas, which may prove to be important biotherapeutic targets to enhance RT response in the clinical management of these tumors. Inhibition of the EGFR/IGFR-1/PI-3K/Rho pathway may improve outcome in patients undergoing radiation treatments by decreasing invasive potential. The current investigation suggests that it may ultimately require concurrent administration of specific biotherapeutic agents such as EGFR/IGFR-1 and Rho kinase inhibitors in order to minimize RT-induced cancer cell invasion and to attain a synergistic therapeutic gain in the treatment of malignant gliomas. The novel observations reported in this study suggesting that radiation-mediated invasion differs mechanistically from that under normal physiologic conditions and the specific indictment of Rho signaling in this process warrant further investigation.

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Address for offprints: Arnab Chakravarti, Department of Radiation Oncology, Massachusetts General Hospital/Harvard Medical School, 100 Blossom Street, Founders House, Room 536, Boston, MA 02114, USA; Tel.: +1-617-724-1175; Fax: +1-617-726-2098; E-mail: achakravarti@partner.org