Rho/ROCK and MAPK Signaling Pathways Are Involved in Glioblastoma Cell Migration and Proliferation

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Abstract. *Background: Glioblastoma multiforme (GBM) remains the most aggressive and frequently occurring brain neoplasm. Members of the Rho family of small GTP-binding proteins, including Rho, Rac, and Cdc42, have been shown to participate in cell growth differentiation and motility. The mitogen-activated protein kinase (MAPK) pathway, which includes extracellular signal-regulated protein kinases 1 and 2 (ERK1/2), has been shown to regulate cell growth, differentiation and motility. Here, the involvement of the Rho and Rho-associated protein kinase (ROCK) pathway, along with MAPK, was investigated to determine their roles in GBM cell migration and proliferation. Materials and Methods: In vitro studies utilized the human malignant glioblastoma cell line LN-18. The cells were treated with Y-27632, a ROCK inhibitor, and U0126, an upstream MAPK kinase inhibitor (MEK), alone or in combination with one another. Immunoblotting analysis established the levels of phosphorylated ERK1/2. Cell migration was determined by radial migration assay and cell proliferation by MTT. Results: Y-27632 reduced phosphorylation of ERK1/2 at 0.5 and 2 h. U0126 in combination with Y-27632 led to a more pronounced repression of platelet-derived growth factor (PDGF)- or fibronectin (FN)-induced ERK1/2 activation than U0126 treatment alone. Y-27632 treatment for 24 h suppressed GBM cell migration and resulted in a reduction in LN-18 cell proliferation. Furthermore, PDGF and FN-induced cell proliferation was suppressed by pre-treatment with Y-27632 or U0126, with the greatest reduction achieved by a combination of the two inhibitors. Conclusion: Rho/ROCK signaling is involved in GBM cell migration and proliferation, and this pathway may be linked to ERK signaling.*

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Glioblastoma multiforme (GBM), or grade IV astrocytoma, represents the most common primary brain tumor in humans, comprising 12-15% of all intracranial neoplasms and 50-60% of astrocytic tumors. Marked by central necrosis and hemorrhage, neovascularization, and the ability to invade normal brain matter (1), a surgical cure remains impossible, although ample resection has been shown to be associated with increased survival as compared to resection of less than 98% (2). Current treatment options, which often involve a combination of chemotherapy, radiotherapy, and surgery, have proven to be of minor benefit and the clinical course from the time of diagnosis remains catastrophic, with a median survival range of 10-12 months (3). Several genetic alterations, including epidermal growth factor (*EGFR*) amplification, *p53* and phosphatase and tensin homolog (*PTEN*) mutations, and loss of heterozygosity (LOH) on chromosome 10q (4), have been identified in GBM. Elucidating altered signaling pathways in GBM, such as Ras-extracellular regulated kinase (ERK) and Ras-PI3K-Akt, has also been of particular interest for the development of novel therapies.

The Rho family of small monomeric GTPases, which includes Rho, Rac, and Cdc42, is involved in a multitude of cellular events, including cell cycle progression, growth, differentiation, cytoskeletal reorganization, and cell motility. In the context of cellular changes that act to promote cell migration, Rho protein activation has been linked to the formation of focal adhesion complexes and actin stress fibers (5), while Rac and Cdc42 have been associated with lamellipodia and filopodia formation, respectively (6, 7). Furthermore, siRNAs directed against Rac1 or Rac3 have demonstrated that depletion of Rac1 strongly inhibits lamellipodia formation, cell migration, and invasion in SNB19 GBM cells, but has a lower inhibitory effect on cell proliferation (8). Rho target, Ser/Thr p160 Rho-associated protein kinase (ROCK), has been shown to stimulate actin myosin contractility and focal adhesion involved in cell motility. Studies have shown that expression of Rho is increased in human cancer, with increased RhoA expression evidenced in high-grade astrocytomas (9). However, the expression of RhoA and RhoB in human gliomas has been

inversely correlated with tumor grade (10). Some studies have shown that inhibition of ROCK increases motility of GBM cells primarily due to membrane ruffling and collapse of actin fibers (11). Furthermore, recent studies have shown that the suppression of GBM cell motility by olig2 occurs *via* activation of RhoA (12).

The mitogen-activated protein kinase (MAPK) pathways, of which the ERK pathway is the best studied, are involved in a variety of cellular functions such as growth, proliferation, differentiation, migration and apoptosis (13). The activation of ERK, upon binding of receptor tyrosine kinases by growth factors and mitogens, leads to a downstream chain of phosphorylations involving Ras, Raf and MEK, and has particular importance for understanding the pathogenesis of cancer. Constitutive ERK signaling results in the expression of transcriptional products, such as cyclin D1, which allow entry into the cell cycle, as well as repressing the expression of genes that inhibit proliferation (14). Furthermore, ERK effectors function in angiogenesis, migration, invasion and metastasis (15, 16). In the present study, the involvement of Rho/ROCK and MAPK signaling pathways in GBM cell progression and dissemination was examined.

Materials and Methods

Glioblastoma cell line and culture. The human glioblastoma cell line LN-18, with intact *PTEN* and mutant *p53* at codon 238, was acquired from ATCC (American Type Culture Collection, VA, USA). The cells were grown in minimum DMEM with 10% fetal bovine serum, 1% penicillin/streptomycin and 1% glutamine, in a humidified 5% CO_2 atmosphere. The cells were made quiescent by serum-deprivation in DMEM for 24 h prior to treatments.

Western blotting. The treatments consisted of ROCK inhibitor Y-27632 (30 μM; Calbiochem, San Diego, CA, USA) alone for either 30 min or 2 h, or for 2 h followed by stimulation with platelet-derived growth factor (PDGF) (10 ng/ml; Calbiochem, San Diego CA, USA) or Fibronectin (FN) (20 μg/ml; Sigma; St Louis, MO, USA) for 30 min. Treatments with MEK inhibitor U0126 (10 μM; Calbiochem, CA) for 1 h, alone or in combination with Y-27632 for 30 min, prior to stimulation by PDGF or FN, were also conducted.

After treatment, the cells were harvested in a buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, and 1% (v/v) Triton® X-100 plus protease and phosphate inhibitors (Sigma). The protein content was measured by the Bradford procedure, with bovine serum albumin as a standard. The cell lysate, 50 μg protein, was separated on a 10% sodium dodecyl sulfate (SDS)-PAGE. The membranes were incubated with anti-phospho-ERK1/2 antibodies (phospho-p44/42 MAPK antibody-Thr 202/Tyr 204) and total ERK1/2 antibody following the manufacturer's instructions (Cell Signaling Technology, Beverly, MA USA).

Cell migration assay. A radial monolayer migration assay (17) was used to quantify glioblastoma cell migration. Briefly, 10-well Teflon slides were coated with laminin in PBS at 100 μl/well for 1 h at 37˚C. The wells were then washed twice with PBS. About 3,000 LN-18 cells/well were seeded through a sedimentation manifold (CSM Inc., Phoenix, AZ, USA) and allowed to attach overnight. Following attachment, the cells were either treated with vehicle or media containing epidermal-derived growth factor (EGF) or Y-27632 (30 μM). The circle circumscribing the attached cells was measured at this time for the 0 h reading. The cells were allowed to migrate over a 24 h period, after which another circle circumscribing the cells was recorded and measured. Migration results were reported as the change in the radius of the circles over the 24 h period (μm/day). Measurements were taken using an inverted Zeiss microscope (Axiovert 200) and digitized using a camera.

Cell proliferation assay. Approximately 3,000 LN-18 cells/well were seeded in 96-well culture plates. Quiescent cells were given PDGF or FN, and Y-27632 (30 μM) or U0126 (10 μM) for 24 h. At the end of the experiments, all the media were replaced with 100 μl/well fresh serum-free DMEM. Cell proliferation was evaluated by an MTT Cell Growth Assay Kit (Chemicon International, Temecula, CA, USA) following the manufacturer's instructions. This assay is based on the conversion of the yellow tetrazolium dye MTT to purple formazan crystals by metabolically active cells. Absorbance was measured within 1 h on an ELISA plate reader at 570 nm and data were recorded.

Results

Effect of ROCK and MEK inhibition on downstream MAPK signaling pathway in glioblastoma cells. LN-18 cells were utilized to assess the effect of Rho GTPase on the MAPK pathway proteins in cellular growth and viability. Activation of ERK was determined following treatments with ROCK inhibitor Y-27632 (30 μM) for 30 min and 2 h, either alone or before stimulation by FN or PDGF (30 min). Immuno-analysis revealed that treatment of the GBM cells with Y-27632 for 2 h reduced the expression of p-ERK more than treatment for 30 min (Figure 1A). Furthermore, exposure of the cells treated with Y-27632 for 2 h, prior to FN or PDGF treatment for 30 min, failed to re-establish the levels of activated p-ERK seen in the cells treated with FN or PDGF alone (Figure 1A). Additionally, ROCK inhibition for 30 min and MEK inhibition for 1 h, together led to a pronounced decrease in levels of activated p-ERK, as compared to MEK inhibition alone (Figure 1B). Furthermore, FN- and PDGF-induced stimulation of ERK was more noticeably suppressed by combined pre-treatment withY-27632 and U0126 (Figure 1B, top panel). The bottom panel of Figure 1A and 1B represents the levels of total ERK1/2, which appeared to be similar in all lanes.

Effect of ROCK inhibition on radial migration of glioblastoma cells. The cells exposed to Y-27632 were dispersed over a smaller radius from the center of the confluent cell monolayer as compared to cells exposed to either vehicle or EGF (Figure 2A). The radial migration technique revealed that EGF (5 ng/ml) treatment for 24 h resulted in dispersion of LN-18 cells, as compared to control. Quantitative analysis revealed that ROCK inhibition virtually abolished any migration of the

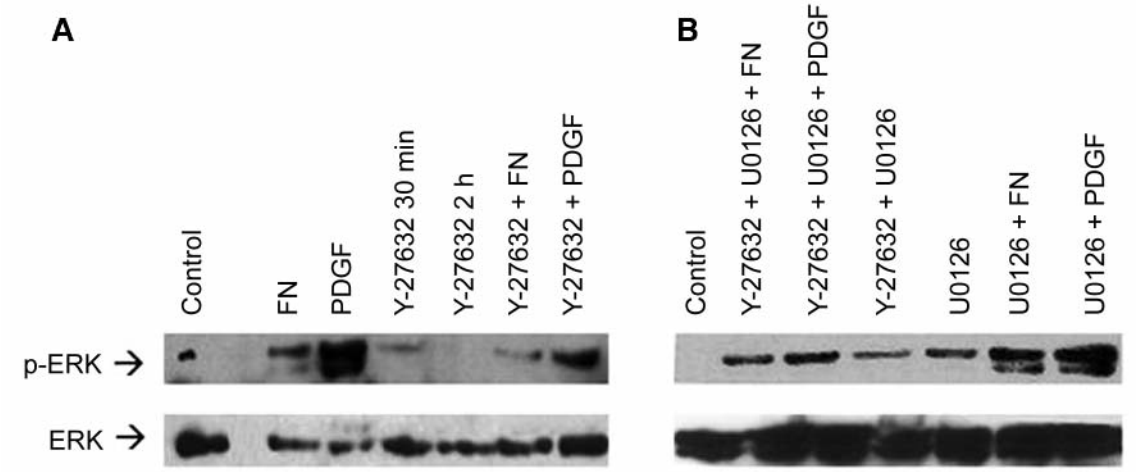

Figure 1. *Effect of treatment with ROCK inhibitor Y-27632 (A) or MEK inhibitor, U0126, in combination with Y-27632 (B) on levels of activated ERK in LN-18 glioblastoma cells with or without stimulation by PDGF or FN.*

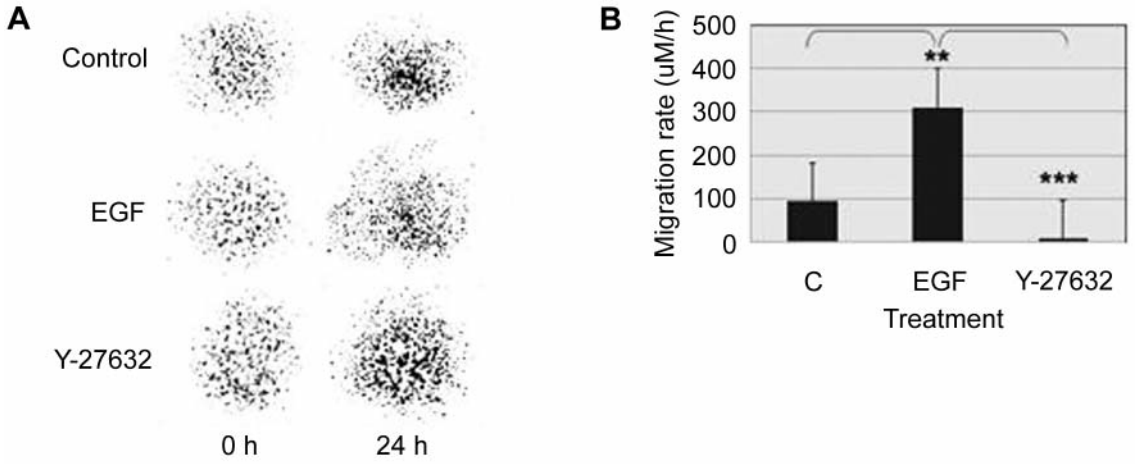

Figure 2. *Radial migration of LN-18 glioblastoma cells. A, Photomicrograph of cells 0 h (after 24 h seeding) and 24 h (after further incubation with various agents). B, Quantitative analysis of GBM cell migration.*

GBM cells, as compared to the control and EGF-treated cells (Figure 2B). The cells treated with EGF showed an increased in migration (Figure 2B).

Effect of ROCK inhibition on glioblastoma cell growth and proliferation. LN-18 cells that had been exposed to Y-27632 over a period of 24 h revealed a 30% reduction in proliferation, as compared to the control (Figure 3). Similar results were achieved with the application of U0126 alone. However, the most effective treatment was achieved by a combination of Y-27632 and U0126, which yielded a 40% decline in proliferation, as compared to the control. Whereas both PDGF and FN enhanced proliferation of GBM cells, pre-treatment with either Y-27632 or U0126 alone, or in combination, prevented cell proliferation (Figure 3).

Discussion

This study demonstrated that inhibition of the Rho-associated protein kinase, ROCK, decreased activation of ERK1/2 as well as suppressing PDGF- or FN-induced activation of ERK1/2. Moreover, combined inhibition of ROCK and MAPK kinase potentiated the repression of ERK1/2 phosphorylation. This study also established that inhibition of ROCK suppressed GBM cell migration and GBM cell proliferation was reduced by inhibition of ROCK with MAPK. PDGF- or FN-induced proliferation was abrogated by pre-treatment with ROCK or MAPK inhibitor and combined treatment with these two inhibitors resulted in a pronounced decrease in cell proliferation.

Several investigations have linked the Rho and ERK signaling pathways. Specifically, urokinase-type plasminogen

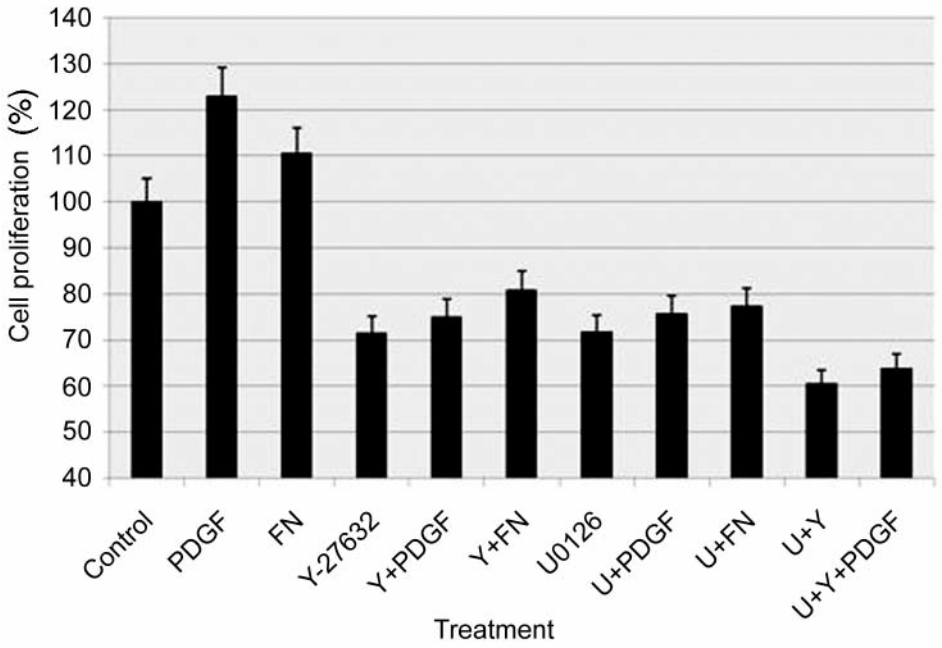

Figure 3. *MTT assay of LN-18 glioblastoma cell proliferation. The level of the control was set to 100.*

activator-stimulated tumor cells were blocked by MEK-specific antagonists of Rho/ROCK (5). PDGF and FN control cellular functions *via* the Ras-ERK pathways. The present study established that Rho/ROCK and Ras-ERK function in a cooperative manner to influence cellular motility and growth (Figures 1, 3). ROCK inhibition caused a time-dependent suppression of p-ERK levels, and PDGF or FN-induced activation of p-ERK was suppressed by pre-treatment with the ROCK inhibitor for 2 h (Figure 1). A combined treatment with inhibitors of these two pathways produced a greater suppression of p-ERK than did U0126 treatment alone (Figure 1B). Such observation places ERK downstream of Rho/ROCK in these GBM cells, and rejects the idea of cross-talk between the two pathways. Y-27632 or U0126 suppressed GBM cell growth, and more importantly, both suppressed PDGF- and FN-induced cell proliferation. As shown in Figure 3, a combined treatment of Y-27632 and U0126 demonstrated a greater suppression of cellular proliferation.

Members of the Rho family of small GTP-binding proteins, including Rho, Rac, and Cdc42, have been shown to influence cell growth and differentiation. Salhia *et al*. (11) showed that Rho-associated protein kinase inhibitor Y-27632 increased the number and length of cell processes, encouraged membrane ruffling and altered actin stress fibers in astrocytoma cells, with a concurrent 2-fold increase in astrocytoma migration and invasion. Using a Rac-GTP pull-down assay, the investigators found that astrocytoma motility stimulated by Rho-kinase inhibition was associated with Rac1 activation. Moreover, suppression of GBM cell motility by olig2 involved RhoA (12). In the present study, the inhibition of ROCK suppressed GBM cell radial migration, which may have been attributable to differences in inhibitor concentrations. Furthermore, a suppression of cell proliferation in Y-27632-treated cells was demonstrated and it is possible that under these conditions, proliferation mechanisms superseded cell migration. At the same time, the possibility that GBM cell lines are genetically distinct and this contributes to disparities within the results, cannot be eliminated.

Rho family proteins and their downstream targets regulate a wide variety of signaling processes that control biological functions and much work has been attempted to elucidate the ramifications of Rho pathway dysregulation on disease processes (18). In particular, many studies have examined Rho signaling as it relates to cancer pathogenesis (19, 20). Our previous investigation revealed a marked down-regulation of levels of ARHGAP26, a Rho GAP that converts Rho to its inactive GDP-bound form, thus allowing Rho to promote cell growth and migration (21). The process of oncogenesis relies on the migratory and invasive phenotype of cells (22). Several previous studies have corroborated our evidence that Rho proteins play a specific role in cancer progression. Studies have shown that RhoA appears to be up-regulated in human carcinomas, specifically in high-grade astrocytomas (9). Similarly, a large series of astrocytomas revealed a significant correlation between WHO grade of malignancy of astrocytoma and the expression of focal adhesion kinase and proline-rich tyrosine kinase (Pyk2), both of which are downstream targets of RhoA (23). Studies of Pyk2 in glioma cells revealed that

silencing of this protein can suppress glioma motility and it appears to play an important role in GBM cell migration. On the other hand, a study of 24 human astrocytic tumors revealed that the expression of RhoA and RhoB decreased in brain tumors and was found to be inversely correlated with tumor grades II-IV (10).

Finally, treatments aimed at prolonging survival time in GBM patients have made use of associations with Rho signaling pathways. Radiotherapy, although effective at prolonging survival, increases the invasive potential of primary GBM cells through activation of Rho signaling *via* PI3K (24). Correspondingly, inhibiting Rho GTPase *in vitro* has been shown to radiosensitize human glioma cells (25). Future studies may prove to be of prognostic significance if they continue to uncover the complexity and inter-relatedness of Rho signaling pathways as they relate to GBM pathogenesis. Moreover, future studies may delineate the interaction of Rho with other molecules such as TORC2 complexes in influencing cell migration. Novel pharmacological agents targeted at these pathways may have therapeutic significance by inhibiting GBM progression.

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