EphA2 as a Novel Molecular Marker and Target in Glioblastoma Multiforme

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Abstract
We investigated the presence of EphA2, and its ligand, ephrinA1, in glioblastoma multiforme (GBM), a malignant neoplasm of glial cells, and normal brain. We also initially examined the functional importance of the interaction between EphA2 and ephrinA1 in glioma cells. Expression and localization of EphA2 and ephrinA1 in human GBM and normal brain were examined using Western blotting, immunofluorescence, and immunohistochemistry. A functional role for EphA2 was investigated by assessing the activation status of the receptor and the effect of ephrinA1 on the anchorage-independent growth and invasiveness of GBM cells. We found EphA2 to be elevated in ~90% of GBM specimens and cell lines but not in normal brain, whereas ephrinA1 was present at consistently low levels in both GBM and normal brain. EphA2 was activated and phosphorylated by ephrinA1 in GBM cells. Furthermore, ephrinA1 induced a prominent, dose-dependent inhibitory effect on the anchorage-independent growth and invasiveness of GBM cells highly overexpressing EphA2, which was not seen in cells expressing low levels of the receptor. Thus, EphA2 is both specifically overexpressed in GBM and expressed differentially with respect to its ligand, ephrinA1, which may reflect on the oncogenic processes of malignant glioma cells. EphA2 seems to be functionally important in GBM cells and thus may play an important role in GBM pathogenesis. Hence, EphA2 represents a new marker and novel target for the development of molecular therapeutics against GBM. (Mol Cancer Res 2005;3(10):541–51)

Introduction
Glioblastoma multiforme (GBM) is an extremely invasive, well-vascularized tumor believed to be of astroglial origin (1). It is the most prevalent (2) and lethal of all primary malignant brain tumors (3), with a median survival rate of ~12 months (3). Despite the standard treatment of surgical resection of the tumor followed by radiation and/or chemotherapy, the survival rate has increased only slightly over the past three decades (3). It is clear that novel therapies are needed to improve the prognosis and quality of life of patients with GBM. Molecular markers that either are found specifically on tumor cells or are highly overexpressed on malignant cells and nearly absent on normal cells are attractive therapeutic targets for approaches such as targeted drug delivery. Along these lines, we identified previously a receptor for interleukin-13, ILRa2, which is a brain tumor–associated cancer/testis tumor antigen and is a very attractive therapeutic target (4).

The Eph receptors comprise the largest family of tyrosine kinase receptors, a group of well-studied transmembrane proteins that are crucial in mediating important signal transduction pathways in cells such as those controlling growth, migration, and differentiation (5). The 15 members of the Eph receptors are divided into A and B classes based on the homology of their extracellular domains (6), which typically include a globular NH2-terminal ligand-binding domain followed by a cysteine-rich domain and two fibronectin type III repeats (7). The COOH-terminal intracellular domain that is conserved among all Eph receptors contains two tyrosine residues involved in the autophosphorylation activity of the receptor and is followed by a tyrosine kinase catalytic domain (8). The phosphorylation of both the membrane proximal tyrosines and those in the catalytic region controls Eph receptor biological activity (9).

The Eph receptors are unique among the tyrosine kinase receptors in that their endogenous ligands, the ephrins, are bound to the surface of neighboring cells. The ephrins are a family of cell surface–anchored proteins of two classes based on how they are attached to the plasma membrane (5). EphrinA ligands are attached via a glycosylphosphatidylinositol linkage, whereas ephrinB ligands possess a transmembrane sequence with an intracellular domain that mediates attachment to the cell membrane (10). All of the ephrins interact with specific Eph receptors, although there is promiscuity as some ephrins bind to more than one Eph receptor (10).

Eph receptors and ephrin ligands display specific patterns of expression during development (11-15). They have been implicated in the complex process of establishing boundaries between populations of cells during the formation of the body plan (11, 12). In neural development, Eph receptors and their ligands have been shown to play an important role in axon guidance through the mediation of contact-dependent processes between cells (13-15). EphA2 is present in the nervous system...
during embryonic development, but unlike most other Eph receptors it is also expressed on the surface of proliferating adult epithelial cells (16-18). However, this expression is at a relatively low level and most commonly limited to the skin, intestine, lung, and ovary (19). Notably, in these cells, the receptor is localized to points of cell-cell contact and bound by its ligand, ephrinA1 (20).

Previously, EphA2 has been found significantly overexpressed in several human epithelial malignancies, such as breast (21, 22), colon (21, 23, 24), ovarian (25), prostate (26, 27), and pancreatic (28) carcinomas. This elevation in the level of EphA2 is thought to be due in part to a decrease in the amount of ligand-mediated receptor degradation (18). For example, the unstable cell-cell contacts within cancer tissue may hinder the ability of EphA2 to interact with ephrinA1 on neighboring cells (20). Consequently, receptor activation and subsequent degradation of EphA2 are markedly decreased. Interestingly, this produces a situation in transformed epithelial cells in which EphA2 is significantly less activated and simultaneously highly overexpressed (18, 20, 22). The activation of EphA2 has been observed to negatively regulate integrin-mediated adhesion, cell spreading, and migration (29). Activated EphA2 has also been shown to suppress the function of integrins and directly cause the dephosphorylation of and subsequent dissociation from focal adhesion kinase (29). Furthermore, EphA2 activation inhibits cell growth (20) and proliferation (30) and decreases cell-extracellular matrix contacts (20). Hence, when EphA2 is present in its inactive state, the cells on which it is expressed may have a tendency to be more motile, invasive, and faster growing. Not surprisingly, therefore, many of the invasive and aggressive phenotypes of the aforementioned cancers have been correlated with the overexpression of inactive EphA2 both in vitro and in vivo (22, 25, 28).

EphA2 also plays an important role in angiogenesis (31) and tumor neovascularization (21) through association with its endogenous ligand, ephrinA1. EphrinA1 was originally described as a tumor necrosis factor-α–inducible endothelial gene product (32), and a role for ephrinA1 as an important factor in angiogenesis has been proposed (33). In contrast to the situation in epithelial cells, the failure of ephrinA1 to activate EphA2 in normal endothelial cells inhibits vascular endothelial growth factor–induced angiogenesis (31). In fact, both EphA2 and ephrinA1 have been shown to be expressed in malignant cells and the associated endothelial cells of the tumor neovasculature (21).

These findings, together with gene expression patterns observed on cDNA microarrays done previously (34), prompted us to investigate the presence and role of EphA2 and its interaction with ephrinA1 in malignant gliomas. Our results suggest that EphA2 is important not only in malignancies of epithelial origin but also in those of glial origin.

**Results**

**Expression of EphA2 and EphrinA1 in Established Human Glioblastoma Multiforme Cell Lines**

The level of EphA2 and ephrinA1 proteins in several GBM cell lines and normal brain was first determined by Western blotting. Five different GBM cell lines (A-172 MG, DBTRG-05 MG, U-251 MG, and G48a) displayed highly elevated levels of EphA2 protein, which migrated as an immunoreactive band at the expected size of 130 kDa (Fig. 1A). The exception was U-87 MG cells, which expressed much less EphA2 than the other GBM cell lines tested (Fig. 1A). Normal brain protein medleys isolated from the frontal lobe of a normal brain (Fig. 1A, Normal brain I) as well as those obtained from Clontech Laboratories, Inc. (Palo Alto, CA; Fig. 1A, Normal brain II) and Chemicon International, Inc. (Temecula, CA; data not shown) showed either a very faint immunoreactive band or the absence of any band at 130 kDa (Fig. 1A). Notably, a nontumorigenic malignant glioma cell line, H4, displayed levels of immunoreactive EphA2 similar to that observed in normal brain (Fig. 1A).

Immunoblotting for the ephrinA1 ligand in GBM cell lines revealed strikingly different results than those observed for EphA2. There was no indication of an immunoreactive band migrating at the expected size of ephrinA1 (~25 kDa) in most of the same cell lines in which EphA2 was abundantly overexpressed, such as A-172 MG, DBTRG-05 MG, and G48a (Fig. 1A). In U-251 MG cells, however, we observed a faint ephrinA1 immunoreactive band (Fig. 1A). In addition, normal brain tissue possessed very low levels of immunoreactive ephrinA1 similar to the level of EphA2 observed in the same samples (Fig. 1A). Because ephrinA1 is a tumor necrosis factor-α–inducible gene product (33), human umbilical vein endothelial cells stimulated with tumor necrosis factor-α were included as a positive control for ephrinA1 detection in cell lysates (Fig. 1B).

Next, we performed immunofluorescence to further investigate the expression and localization of EphA2 and ephrinA1...
in GBM cells. Abundant, specific staining for EphA2 was observed in all GBM cell lines examined using both monoclonal and polyclonal EphA2 antibodies (Fig. 2A). In confluent cells, a distinct, honeycomb pattern of staining was evident (Fig. 2A, U-251 MG cells), which is indicative of the expected membrane-localized expression of the receptor. Some cytoplasmic and perinuclear staining was also seen. In one GBM cell line, U-87 MG, we observed the level of EphA2 immunofluorescence to be visibly lower compared with all other GBM cells (Fig. 2A). This finding is consistent with the low level of immunoreactive EphA2 protein found in U-87 MG cells by Western blotting (Fig. 1A).

To further investigate the expression of EphA2 and ephrinA1 in GBM, we performed immunofluorescence for ephrinA1 in four GBM cell lines (U-251 MG, DBTRG-05 MG, A-172 MG, and G48a). Red, polyclonal EphA2 antibody; green, monoclonal EphA2 antibody. 4',6-Diamidino-2-phenylindole (DAPI) staining reveals nuclei of cells stained for ephrinA1. Scale bar represents 100 μm.


Expression of EphA2 and EphrinA1 in Human Glioblastoma Multiforme Specimens

Next, we investigated the expression of the receptor and its ligand in GBM specimens and normal brain tissue by Western blotting and immunohistochemistry. For Western blotting, whole tissue lysates were prepared from snap-frozen human GBM, prepared from the frontal lobe of a normal brain, or commercially purchased. We found that immunoreactive EphA2 was elevated above that in normal brain in 13 of 14 GBM tumors examined, whereas 6 tumors exhibited large overexpression of EphA2 (Fig. 3). In 9 of 13 samples in which EphA2 was elevated, immunoreactive ephrinA1 was present at markedly lower levels (Fig. 3, GBM 6, 12, 14, 121, 24, 117, and 105). The remaining 4 samples all displayed an increase in both EphA2 and ephrinA1 immunoreactivity above that seen in normal tissue (Fig. 3, GBM 102, 135, 45, and 112). Only one tumor, GBM 99, seemed to have low levels of both the receptor and the ligand (Fig. 3).

To further assess the expression of EphA2 and ephrinA1, we performed immunofluorescence on several frozen sections of
human GBM tissue and nonmalignant brain. These experiments revealed abundant staining for EphA2 in GBM and a very low level of detectable immunoreactive receptor in normal brain (Fig. 4A). Several different samples of normal brain were stained for EphA2 with consistently negative results (data not shown). Immunofluorescent ephrinA1 was present at near background levels in all the same sections with the exception of GBM 102 (Fig. 4B), which also displayed an elevated level of ephrinA1 by Western blotting (Fig. 3). Notably, the level of EphA2 and ephrinA1 immunofluorescence observed in frozen sections was consistent with the amount of immunoreactive protein seen by Western blotting (Figs. 3 and 4).

Immunohistochemistry on paraffin-embedded sections of human GBM revealed the same high degree of staining for EphA2 as was seen by Western blotting and immunofluorescence (Fig. 5A). In four different neuropathologist-verified GBM tumor specimens, including giant cell GBM, we observed dense, specific EphA2 staining throughout the sections, including glioma cells and tumor vascular endothelial cells (Fig. 5A). EphA2 staining was again near the detection limits of the assay in nonmalignant brain tissue (Fig. 5A). Unlike the high degree of specific staining observed for EphA2, ephrinA1 was found at low levels throughout the GBM specimens (Fig. 5B). In nonmalignant brain, we observed ephrinA1 staining specifically and intensely localized to the endothelial cells of vessels (Fig. 5B), a phenomenon also seen to a small degree for EphA2 (Fig. 5A). Interestingly, this specific vascular endothelial cell localization of ephrinA1 in normal brain seemed less apparent in GBM (Fig. 5B). In addition, a tissue microarray composed of 61 paraffin-embedded sections of various brain tumors and normal brain revealed the strongest staining for EphA2 consistently among the high-grade (WHO grades 3 and 4) astrocytomas; ephrinA1 staining of these sections revealed the same uniform low levels of the ligand.1

**FIGURE 3.** EphA2 and ephrinA1 immunoreactivity in human glioblastoma multiforme and normal brain tissue. Western blot of EphA2 and ephrinA1 immunoreactivity done in glioblastoma multiforme tumor lysates and normal brain. Normal brain was obtained from the same sources as in Fig. 2.

Effect of EphrinA1 on Anchorage-Independent Growth of Glioblastoma Multiforme Cells

Due to the trend of differential expression of EphA2 and ephrinA1 observed in GBM cell lines and tumor tissue, we next investigated a possible functional relationship between the receptor and the ligand in GBM. We first examined the effect of ephrinA1 on the anchorage-independent growth of high EphA2-expressing GBM cell lines by assessing the ability of U-251 MG and DBTRG-05 MG cells to form colonies in soft agar in the presence or absence of ephrinA1. We found a dose-dependent inhibitory effect of ephrinA1 on the anchorage-independent growth of the two cell lines (Fig. 6A). Because soluble ephrinA1 has been shown previously to activate EphA2 receptors in vitro (10, 35), we suggest that ephrinA1-mediated activation of EphA2 has a negative effect on the anchorage-independent growth of GBM cells. To confirm that this phenomenon specifically involves ephrinA1-mediated activation of EphA2, the same experiment was done using two cell lines that express low levels of EphA2: H4 and U-87 MG (Fig. 1A). We failed to observe any significant dose-dependent inhibition of anchorage-independent growth with increasing concentrations of ephrinA1 in both cell lines studied (Fig. 6B). In general, these cells formed smaller colonies in soft agar than the cells that highly overexpress EphA2.

**Effect of Ephrin A1 on Glioblastoma Multiforme Cell Invasion**

Another enhanced malignant feature of GBM cells that may result in part from a lack of EphA2 receptor activation is invasion. Therefore, we investigated the effect of exogenous ephrinA1 on the invasiveness of three different GBM cell lines. The U-251 MG and A-172 MG cells, high overexpressors of EphA2, exhibited a substantial dose-dependent decrease in invasiveness with increasing concentrations of ephrinA1 (Fig. 7). Treatment with 1.0 μg/mL ephrinA1 caused an 80% decrease in invasion in A-172 MG cells and a 52% decrease in U-251 MG cells (Fig. 7). In contrast, the U-87 MG cells were less invasive than the high EphA2-expressing cell lines, and we observed only a 20% decrease in invasion from nontreated control to treatment with 1.0 μg/mL ephrinA1 (Fig. 7).

1 Wykosky et al., in preparation.
Activation Status of the EphA2 Receptor in Glioblastoma Multiforme Cells

The low levels of ephrinA1 expression in GBM suggested that the EphA2 receptors in these tumors are likely not activated and thus are present in the non-tyrosine-phosphorylated state (9, 21). To investigate this possibility, we performed immunoprecipitation of the lysates from several GBM cell lines and tumors using a monoclonal EphA2 antibody. As seen in Fig. 8A, we detected little to no tyrosine-phosphorylated EphA2 in GBM cell lines and tumors, whereas immunoreactive EphA2 was readily observed. Notably, the amount of immunoreactive EphA2 immunoprecipitated from the two GBM tumors correlates directly with that found by Western blotting (Fig. 3). The lack of detectable tyrosine phosphorylation suggests that the EphA2 receptor is inactive possibly due to lack of stimulation by ephrinA1 originating from neighboring cells. On treatment with ephrinA1, the levels of tyrosine-phosphorylated EphA2 increased dramatically over time in U-251 MG cells, an indication of ephrinA1-mediated receptor activation (Fig. 8B). There was no increase in phosphorylated EphA2 with IgG1-treated samples (Fig. 8B). EphrinA1-induced EphA2 phosphorylation was found to occur as early as 10 minutes following treatment and lasted for up to 60 minutes. The activation of the receptor seemed to be reversible, as phosphorylated EphA2 was no longer detected 2 hours following ephrinA1 stimulation (Fig. 8B). In addition, the levels of total immunoreactive EphA2 in ephrinA1-treated cell lysates began to decrease after 60 minutes of treatment (Fig. 8C).

Discussion

We have shown that EphA2 is abundantly and specifically overexpressed in GBM cell lines as well as in human GBM tumor tissue. Important in the consideration of EphA2 as a potential molecular target for medical intervention, the expression of this receptor is detected at very low levels in normal brain. In addition, we documented that in cells and tumors that abundantly overexpress EphA2, ephrinA1 is, on average, present at much lower levels. Furthermore, our findings suggest the existence of a functional relationship between EphA2 and ephrinA1 in GBM. We found that ephrinA1 activates EphA2 and elicits signals through tyrosine phosphorylation of the receptor that result in the negative regulation of oncogenic properties, such as anchorage-independent growth and invasion.

To our knowledge, this is the first study investigating the presence and functional significance of the EphA2 receptor and its ligand, ephrinA1, in a human cancer of astroglial origin. EphA2 has been shown previously in murine glioma (36).
FIGURE 5. EphA2 and ephrinA1 expression in human glioblastoma multiforme and normal brain. EphA2 (A) and ephrinA1 (B) immunohistochemistry of paraffin-embedded sections of glioblastoma multiforme and normal brain tissue. Three representative areas of each section are shown.
In addition, another member of the Eph receptor family, EphB2, has been found to play a role in the migration and invasion of human glioma cells (37). The abundant over-expression of EphA2 in GBM may result from a decrease in the amount of ligand-induced receptor degradation (18, 20, 38). In normal tissues with stable cell-cell contacts, ephrinA1 binds and activates EphA2, causing the internalization of the receptor-ligand complex (20, 38). Subsequently, EphA2 is degraded due to interaction with c-Cbl (38, 39) and is found expressed at low levels in its activated state in normal adult epithelial tissue (20-22). In contrast, the overexpressed EphA2 in epithelial cancers is found predominantly in the nonactivated state, in which the tyrosines of the catalytic kinase domain remain unphosphorylated (20, 22). As our current results suggest, this situation holds true for malignant gliomas. We failed to detect any significant amount of EphA2 receptor tyrosine phosphorylation in both GBM cell lines and tumors, showing that EphA2, although overexpressed, is present in the biologically inactive state. The very low levels of ephrinA1 in GBM cells and most tumor tissue may, at least partially, explain the lack of EphA2 receptor activation and resultant persistent overexpression. In addition, the gene expression for the receptor is also increased in GBM, which may contribute to the presence of elevated gene product in this disease.

The pattern of EphA2 and ephrinA1 differential expression was evident not only in cell lines but also in GBM tumors. There were few exceptions, likely owing in part to the heterogeneous nature of tumors. One property of neoplastic transformation is a decrease in the amount of stable cell-cell contacts (40). Therefore, it is plausible that ephrinA1 is indeed present in some tumors but can bind to EphA2 only in a short-lived manner that is insufficient to elicit receptor internalization and degradation. Alternatively, the majority of ephrinA1 may no longer exist in a form that is capable of successfully binding to and/or activating EphA2 in malignant tissue. For example, it is possible that ephrinA1 is cleaved from the surface of the cell and is present in the extracellular environment in a soluble, monomeric form, which does not effectively engage the EphA2 receptor (10). Both scenarios would promote the overexpression of EphA2.

EphA2 has been shown previously to be important in tumor neovascularization (21, 31). Malignant gliomas are inherently highly vascular tumors. Angiogenic factors, such as vascular endothelial growth factors, are present at variable levels in the tumor microenvironment (31). The level of ephrinA1 present in a GBM tumor, therefore, may be in part related to tumor cell response to angiogenic factors. Alternatively, the presence of ephrinA1 in tumor tissue may be attributable to that expressed in the normal vasculature. Notably, we found ephrinA1 in normal brain tissue specifically localized to vascular endothelial cells, which was less apparent in GBM specimens. In addition, the exact location from which the tumor tissue samples originated is unknown. It is possible that different regions of a tumor (i.e., the core versus the invading edge; ref. 41) have varying expression profiles of EphA2 and ephrinA1. EphA2, when activated by ephrinA1, signals through pathways involved in the negative regulation of cell growth, migration, proliferation, and invasion (20, 22, 30, 42, 43). We found that not only does ephrinA1 cause the phosphorylation of EphA2 but also that this change in the receptor status correlates with a decrease in anchorage-independent growth and invasion of GBM cells. Furthermore, these changes in cellular behavior caused by ephrinA1 seem to be related to the level of EphA2 expression, because they are not seen in low-level EphA2-expressing cell lines. Hence, the EphA2/ephrinA1 system has the potential to play a highly significant role in GBM, as EphA2...
overexpressed in an inactive form may allow unwarranted intracellular signals facilitating tumor progression and/or maintenance. Studies investigating these possibilities are ongoing in our laboratory.

We have shown that EphA2 is highly overexpressed and functionally important for the oncogenic properties of GBM. This work forms the basis for future studies investigating EphA2/ephrinA1 system as a target for the development of molecular-based interventions for high-grade gliomas.

**Materials and Methods**

**Cell Lines and Tissues**

Cell lines derived from human GBM (U-87 MG, U-251 MG, DBTRG-05 MG, and A-172 MG), human malignant glioma (H4), and human umbilical vein endothelial cells were obtained from American Type Culture Collection (Manassas, VA). G48a cells were primary GBM human explant cell cultures isolated in this laboratory (44). The 6B53-11 GBM cells were obtained as a generous gift from Dr. David James (Mayo Clinic, Rochester, MN). The 6B53-11, A-172 MG, and H4 malignant glioma cells were grown in DMEM with 10% FCS (Life Technologies, Rockville, MD). The U-87 MG cells were grown in Earle’s MEM, 10% FCS, 0.1 mmol/L nonessential amino acids, 2 mmol/L glutamine (Life Technologies), and 100 µg/mL sodium pyruvate. G48a cells were grown in RPMI 1640 (Life Technologies), 10% FCS, 100 µg/mL sodium pyruvate, 100 µg/mL l-cysteine (Life Technologies), 20 µg/mL l-proline (Sigma, St. Louis, MO), 1× HT supplement consisting of 0.1 µmol/L sodium hypoxanthine and 0.016 µmol/L thymidine, 5 units/mL penicillin G, and 5 units/mL streptomycin sulfate (Life Technologies). Tumor necrosis factor-α was obtained from R&D Systems (Minneapolis, MN). Tissue samples from human GBM and normal brain were obtained from the operating room, formalin fixed, or frozen immediately and stored at −80°C. Sections (10 µm) of GBM were thaw mounted onto slides, which were stored at −80°C until assayed.

**Western Blots**

Cell lysates were prepared from subconfluent cultures. Cells were washed with PBS and lysed in radioimmunoprecipitation assay buffer (PBS, 0.5% sodium deoxycholate, 0.1% SDS, and 0.5% Igepal) containing mammalian protease inhibitor cocktail (Sigma). Nonmalignant brain and pathologist-verified GBM tumor tissue were minced into small pieces.
while frozen and homogenized in radioimmunoprecipitation assay buffer with mammalian protease inhibitor cocktail. Lysates were passed through an 18-gauge needle to shear the DNA and were incubated on ice for 60 minutes. Nonsoluble debris was pelleted at 10,000 rpm for 10 minutes and the supernatant was collected and stored at −80°C until use. Normal human brain lysates were purchased from Chemicon International and Clontech Laboratories. Lysates were separated by SDS-PAGE using 10% or 15% acrylamide or with 4% to 15% Tris-HCl gradient gels (Bio-Rad Laboratories, Hercules, CA). Proteins were then transferred to a polyvinylidene difluoride membrane (Pierce, Rockford, IL) and blocked for at least 1 hour with blotto (5% milk in PBS/0.05% Tween 20). Membranes were incubated with primary antibody diluted in blotto overnight at 4°C while shaking. Rabbit polyclonal EphA2 (1:100) and ephrinA1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal EphA2 clone D7 (1:500), phosphotyrosine clone PY20 (1:1,000), and β-actin (1:50,000) antibodies were purchased from Sigma. Following three 5-minute washes in PBS/0.05% Tween 20, membranes were incubated with secondary antibody conjugated with horseradish peroxidase (goat anti-mouse IgG or goat anti-rabbit IgG) at a dilution of 1:5,000 in blotto for 1 hour. Membranes were washed thrice for 5 minutes each in PBS/0.05% Tween 20 and detection was done using the Enhanced Chemiluminescence Plus Western Blotting Detection System (Amersham Biosciences, Little Chalfont, United Kingdom). Membranes were exposed to autoradiographic film X-OMAT AR for various times. Films were scanned at 600× dpi and images were compiled using Jasc Paint Shop Pro version 6.0.

Immunofluorescence and Immunohistochemistry

For immunofluorescence, GBM cell lines and human explant cells were grown overnight on sterile glass slides in the appropriate medium. Slides were washed twice in PBS and fixed for 2 minutes in acetone at −20°C. Slides were then washed twice in PBS and either used immediately or stored at −80°C until use. Frozen sections were thawed and subsequently fixed for 10 minutes in acetone at −20°C. Slides were washed twice in PBS and blocked for 1 hour in 10% normal goat serum at room temperature. Primary antibodies EphA2 polyclonal (1:200) or monoclonal (1:1,000) or ephrinA1 polyclonal (1:200) were diluted in 1.5% normal goat serum and incubated overnight at 4°C. No antibody control slides were incubated with 1.5% normal goat serum. Slides were washed twice in PBS for 5 minutes each and incubated with secondary antibody for 45 minutes at room temperature. Secondary antibodies included goat anti-rabbit rhodamine (1:200), donkey anti-rabbit rhodamine (1:200; both from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) or goat anti-mouse IgG Oregon Green (1:200; Molecular Probes, Eugene, OR). Slides were counterstained with Hoescht 33258 nuclear counterstain (4′,6-diamidino-2-phenylindole). Slides were washed twice for 5 minutes each in PBS and mounted with Gel-Mount (Biomedia Corp., Foster City, CA).

For immunohistochemistry, tumor specimens were fixed in buffered formalin and embedded in paraffin. Sections (5 μm) were cut and mounted on chrom-alum slides. Tissue microarrays were obtained from Cybrdi, Inc. (Gaithersburg, MD). After completely dry, slides were baked at 65°C until the paraffin was melted. Slides were deparaffinized in xylene and rehydrated through alcohol. Antigen retrieval was done with 10 mmol/L sodium citrate buffer (pH 6.0) by microwaving twice on medium for 5 minutes each. Once cooled, endogenous peroxidase activity was quenched by incubating slides for 30 minutes in a peroxide/methanol bath. Slides were washed with three changes of PBS over 5 minutes. Staining was done using the SensiTek Horseradish Peroxidase Anti-Polyvalent kit (SekiTek Laboratories, Logan, UT). Background staining was blocked with ScyTek Superblock for 5 minutes. Slides were washed with PBS and incubated with primary antibody (EphA2 polyclonal 1:200 or ephrinA1 polyclonal 1:200) made in PBS overnight at 4°C. No antibody control slides were incubated with PBS. Excess antibody was removed by washing with PBS. Slides were then blocked in diluted Superblock (1:10 in PBS) for 15 minutes and washed with PBS. Slides were incubated in ScyTek biotinylated secondary antibody for 15 minutes and then washed with PBS. ScyTek avidin-horseradish peroxidase was applied to the slides and allowed to incubate for 20 minutes. Slides were rinsed in distilled water. Visualization with ScyTek AEClchromagen was done and allowed to proceed for 8 to 10 minutes. Slides were rinsed with tap water and counterstained in hematoxylin for 1 minute. Slides were given a final rinse in tap water and mounted with Crystal-Mount (Biomedia).

Photomicrographs were taken with a ×63 magnification oil immersion lens in all cases with a Zeiss Axiosvion camera. Background was normalized to the samples without primary antibody. Images were processed with Jasc Paint Shop Pro version 6.01.

Anchorage-Independent Growth

U-251 MG, DBTRG-05 MG, U-87 MG, or H4 cells (2 × 10^5) were plated in six-well plates in growth medium plus 0.35% agar (Fisher, Fair Lawn, NJ) on a base layer of growth medium plus 0.5% agar. Cells were supplemented with a recombinant mouse ephrinA1/Fc chimera (R&D Systems) at 0.001, 0.01, 0.1, 0.5, or 1 μg/mL or vehicle alone (each concentration point was done in triplicate). EphrinA1 was replenished with fresh medium 3 days after plating, and colonies were counted at low power after 14 days. Clusters of colonies greater than 75 cells for DBTRG-05 MG and U-251 MG or 25 cells for U-87 MG and H4 (these latter two cell lines grow poorly in soft agar) were counted in 10 random fields at low power; each experimental condition was done in triplicate for every assay.

Invasion Assay

BD Biocoat Matrigel invasion chambers, control inserts, and wells (BD Biosciences, Bedford, MA) were rehydrated with 500-μL serum-free medium at 37°C for 2 hours. Cells were pretreated for 1 hour with 0.01, 0.1, 0.5, or 1.0 μg/mL ephrinA1-Fc at 37°C, trypsinized, quenched with PBS plus 0.1% bovine serum albumin, and counted. After removing rehydration medium, 750-μL medium plus 5% fetal bovine serum was added to each well of the 24-well plate followed immediately by the addition of 4 × 10^4 cells in 500-μL serum-free medium plus the appropriate concentration of ephrinA1-Fc.
to each chamber and control insert. Plates were incubated for 20 to 22 hours at 37 °C. Noninvading cells were removed from the upper surface of the membrane by scrubbing the membrane with a cotton swab. Cells on the lower surface of the membrane were stained with the Diff Quik stain kit (IMEB, Inc., San Marcos, CA). Invading cells in five random fields were counted with a x40 lens for the three Matrigel insertions and control insert used for each cell type and treatment condition (each was done in triplicate) to obtain the mean number of cells migrating through the Matrigel membrane and the control insert membrane. Data are expressed as the percent invasion through the Matrigel matrix membrane relative to migration through the control membrane.

Immunoprecipitation

Cell lysates were prepared from subconfluent cultures. Cells were washed with PBS and lysed in radioimmunoprecipitation assay buffer (PBS, 0.5% sodium deoxycholate, 0.1% SDS, and 0.5% Igepal) containing mammalian protease inhibitor cocktail and 1 mMol/L sodium vanadate. Cell lysate (∼ 500 µg) was incubated with 5 µg monoclonal EphA2 (clone D7) overnight at 4 °C. Seventy microliters of a 50% PBS/bead slurry containing ∼ 35 µL packed protein G-Sepharose beads (Sigma) were added and incubated for a minimum of 1 hour at 4 °C. Beads were collected by centrifugation, washed thrice with ice-cold radioimmunoprecipitation assay buffer, and resuspended in 60 µL of 3× SDS sample buffer (New England Biolabs, Ipswich, MA). Samples were heated at 100 °C for 5 minutes. Supernatant was collected and stored at −20 °C until separated using SDS-PAGE for Western blotting.

EphA2 Receptor Phosphorylation

Subconfluent cultures of U-251 MG cells were serum starved overnight in 100-mm dishes. Cells were treated with a recombinant mouse ephrinA1/Fc chimera or mouse monoclonal IgG1 isotype control (R&D Systems) for the indicated times. Cell lysates were prepared at the indicated times and used for immunoprecipitation with EphA2 followed by Western blotting for phosphotyrosine and, subsequently, EphA2 (see above).

References


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