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Ephs and Ephrins in Malignant Gliomas

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Abstract

Eph receptor tyrosine kinases and the corresponding ephrin ligands play a pivotal role in glioma development and progression. Aberrant protein expression levels of the Eph receptors and ephrins are often associated with higher tumor grade and poor prognosis. Their function in tumorigenesis is complex due to the intricate network of possible co-occurring interactions between neighboring tumor cells and tumor microenvironment. Both Ephs and ephrins localize on the surface of tumor cells, tumor vasculature, glioma stem cells tumor cells infiltrating brain and immune cells infiltrating tumors. They can both promote and inhibit tumorigenicity depending on the downstream forward and reverse signalling generated. All the above-mentioned features make the Ephs/ephrins system an intriguing candidate for the development of new therapeutic strategies in glioma treatment. This review will give a general overview on structure and function of Ephs and ephrins, with particular emphasis on the state-of-the-knowledge of their role in malignant gliomas.

Keywords

Eph; Ephrin; Glioma; GBM; Brain Tumor

Introduction

Gliomas account for one third of all primary brain tumors and despite massive effort in developing new therapeutic strategies, they still represent one of the major medical challenges (1, 2). Gliomas are categorized according to their grade: low grade gliomas (WHO grade I-II), like astrocytomas and ependymomas, display benign features and have better prognosis, while more aggressive gliomas (WHO grade III-IV), like oligodendrogliomas or glioblastomas, are usually characterized by anaplastic features and dismal prognosis (3, 4). Standard therapies that include surgery, γ-radiation and temozolomide-based cytotoxic chemotherapy, can only temporarily delay unfavorable prognosis, especially for the higher grades. New therapeutic strategies focus on immunotherapies (5–7), anti-angiogenic agents (8–11), targeted cytotoxins (12–15) or targeting of dysregulated signaling pathways (16–18).

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Ephs form the largest known subfamily of receptor tyrosine kinases (RTKs). Extensive studies focusing on these receptors, first isolated from an Erythropoietin-producing human hepatocellular carcinoma line (19), and the corresponding ligands, ephrins (eph receptor-interacting ligands), have generated a complex body of knowledge helping to decipher their role in physiology and pathology (20–24). Ephs and ephrins have a primary role in embryogenesis and development (25–27); they are present virtually in all the developing tissues regulating a complex pattern of developmental processes like cell adhesion, axon guidance, cell migration, cell sorting, platelet aggregation, and others (25, 28). For example, they are involved in establishing neuronal patterning of the auditory system (29) and in guiding the extension and maturation of cortical dendrites (30). But, their expression is often altered in pathological conditions (26, 31), injuries (32–34) and malignancies (35–39) in adulthood.

Mechanism of Ligand-Receptor Activation

The 16 known members of the Eph family of RTKs are divided into two classes, EphA and EphB (Fig. 1). This classification is based on primary sequence similarity of the ligand binding regions and an affinity for the corresponding ephrin classes A and B, respectively. With few exceptions (40), ligands and receptors of the same subfamily interact with the highest binding affinity and specificity. Both Ephs and ephrins are plasma membrane proteins: ephrin-As are linked to the membrane through a glycophosphatidylinositol (GPI) anchor, while ephrin-Bs contains transmembrane and extended intracellular domains (Fig. 1). The Eph receptors A and B are structurally similar, with an *N*-terminal ligand binding domain, a cysteine-rich region and two fibronectin type III domains on the extracellular side. The intracellular juxtamembrane region contains two conserved tyrosine residues that undergo autophosphorylation (e.g., Tyr-594 and Tyr-772 for EphA2, (41)), a tyrosine kinase domain with two phosphorylation sites, a sterile alpha motif (SAM), and a PDZ-binding motif (Fig. 1).

Signal transduction through Eph receptors is generated bi-directionally upon ligand-receptor binding, initiating a “forward signalling” via receptor phosphorylation and a “reverse signalling” via ligand activation (24, 42) (Fig. 1). The intensity of the signal generated in response to receptor activation greatly depends on the nature of ligand stimulation. In a juxtacrine manner, membrane-attached ligands bind the receptors with high affinity evoking receptor clustering and subsequent phosphorylation. During this process, the glycosylation on the ephrin ligand plays a pivotal role in stabilizing Eph and ephrin heterotetramers on the cellular membrane (43). This has been shown for the interaction between ephrin-A1 and -A5 with EphA2, but it is plausible that this is a common mechanism of ligand-receptor interaction as the glycosylation sites are highly conserved, especially among ephrin-As (43). Eph/ephrin complexes are internalized within a few minutes after receptor activation (44) and the receptors are subsequently degraded (43, 45, 46). It is not well understood whether or not the internalized ligands undergo proteolytic degradation as well. Matrix metalloproteinases (MMPs) are actively involved in the internalization process by cleaving specific residues in the extracellular domain of ephrins of the neighbouring cell and thus allowing internalization of the whole ligand-receptor complex (Fig. 1) (47). MMP-1, -2, -9 and -13 (48) have been shown to be the main players responsible for the cleavage together

with ADAM10 (47, 49, 50) and ADAM12 (51), two members of the ADAM (A Disintegrin And Metalloproteinase) family of sheddases. To add to the complexity of the system, MMPs can indiscriminately cleave ephrins releasing a soluble monomeric form into the extracellular environment, which maintains the ability to bind and activate Eph receptors. Soluble monomeric ephrin-A1, found in the conditioned media of U-251 MG GBM cells, was able to induce EphA2 receptor internalization and down-regulation (45); the same was observed for recombinant monomeric forms of ephrin-A1 and ephrin-A5 (43). Even a GPI-linked ephrin-A1 initiates the contact with the EphA2 receptor in a monomeric form (52). MMPs-dependent cleavage of Eph receptors has also been reported (53). In addition, due to the plasticity of the cellular membrane, Eph receptors can be cis-or trans-activated depending on the relative composition and density of the Eph/ephrin family members on the lipids rafts. Eph receptors cluster after activation in trans by ligand binding or in cis by ligand-independent receptor-receptor interactions (54).

The activation of Eph receptors is usually coupled with dramatic morphological changes influencing the interactions with the extracellular matrix (ECM) (55) and cellular migration (56). A typical cell-rounded shape that is quickly assumed upon stimulation of the Eph receptors is due to the activation of Src and focal adhesion kinase (FAK), and the subsequent Rho-mediated phosphorylation of myosin light chain II that induces then the contraction of the cell cytoskeleton (50, 57) (Fig. 1). Both Src and FAK are involved in cellular motility, angiogenesis and cancer invasion (58).

Eph and ephrins in Gliomas

Prognosis and Survival

Because of their altered expression, Eph and ephrins were suggested as possible molecular markers in gliomas (59–61). Zelinski *et al.* first showed that EphA2 overexpression was sufficient to transform mammary epithelial cells (62). Since then, EphA2 overexpression was associated with several malignancies like ovarian carcinoma (63), pancreatic cancer (64), and several others (24). This receptor is also expressed in astrocytomas and its expression markedly increases with an increasing pathologic grade (65). About 60% of GBMs overexpress EphA2, while it is not found in normal brain and its overexpression correlated directly with poor prognosis and inversely with patient survival (59, 66, 67). Ephrin-B2 has also been suggested as a strong predictor of short-term survival in malignant astrocytomas because patients with high Ephrin-B2 tumor levels had significantly shorter survival than patients with low levels of this ligand (68). Another clinical study showed that Ephrin-B2 and EphB4 expressions increased according to a histopathological grade of gliomas, and the expression levels were related to progression-free survival in glioblastoma patients (69).

Other Eph receptors were detected in gliomas and were linked to patients' outcome. Immunohistochemical studies on 32 GBM specimens suggested EphA7 as a new prognostic marker in GBM. The receptor was found to be overexpressed in about 45% of the samples analyzed and was predictive of the adverse outcome in GBM patients. EphA7 stained both tumor and endothelial cells, but not the surrounding connective tissue (60). Moreover, *EphA5* expression was detected by semiquantitative PCR in normal brain tissues (61).

However, EphA5 expression decreased in low-grade glioma specimens and was further reduced in high-grade gliomas (61). This observation indicates that a decrease in EphA5 expression could be used as a prognostic biomarker of glioma progression and highlights a possible role of EphA5 as tumor suppressor (61). Furthermore, high expression levels of EphB1 appear to be a good prognostic indicator. From the expression profile of 171 glioma specimens, Teng *et al.* showed that EphB2, B3, and B4 expression levels were significantly higher in GBM than in normal brain whilst EphB1 expression did not vary across tumor grades (70). However, based on Kaplan–Meier survival curves, patients with high EphB1 tumor levels had significantly longer survival than patients with low EphB1 tumor levels, suggesting that high EphB1 expression levels correlate with better patient outcome (70).

Proliferation, Invasion and Migration

Cell division is a tightly regulated mechanism preserving physiological number of cellular divisions and thus preventing uncontrolled cellular proliferation, invasion of the surrounding tissue and migration to distant sites (71). Ephs and ephrins, as membrane proteins, are cellular sensors of the environment and they can modify the cellular behavior. In the developing human brain, Ephs and ephrins are mainly known for their role in axon guidance (72, 73). However, in the adult brain Eph receptors are involved in the regulation of structure and function of excitatory synapses (74). In addition, the subventricular germinal zone of the lateral ventricles expresses Eph receptors B1, B3 and A4, and ephrin ligands B2 and B3. Evidence suggests that EphB2 and ephrin-B2 are involved in the migration of neuroblasts and in the cellular proliferation in the subventricular zone (75).

Ephs and ephrins patterning is often compromised in brain tumors, therefore cellular proliferation and migration are commonly affected in gliomas biology. *In vitro* studies showed different effects after Eph receptors stimulation by the corresponding ligands. EphA2-overexpressing U-251 MG GBM cells treated with recombinant dimeric ephrin-A1 showed a decrease in migration and proliferation potential (43, 45). Similarly, ligand-dependent EphB1 phosphorylation suppressed migration and invasion in Snb19 and U-251 MG GBM cells (70). This kind of influence of Eph receptors on cellular behavior is not ubiquitous, because phosphorylation of EphA5 did not induce any significant decrease in cell proliferation in U-118 MG GBM cells (44). EphB2 has also been related to invasion and proliferation in glioma cells. EphB2 overexpression in U-251 MG cells stimulated cellular migration and invasion, while reducing cell adhesion (76). GBM neurospheres overexpressing EphB2, when injected intracranially into mice brains, displayed an invasive phenotype at lower proliferative potential. However, EphB2-overexpressed in non-stem-like GBM cells U-87 failed to promote tumor invasion (77).

The importance of ephrin-Bs reverse signaling in glioma cells invasion and migration has been also demonstrated. Invading cells from 19 GBM specimens were collected using laser capture microdissection and EphB/ephrin-B system was identified as the most tightly linked to the invading cell phenotype (68). The protein levels and tyrosine phosphorylation of ephrin-B2 were increased in GBM tissue relative to normal brain (68). Also, in U-87 and U-251 MG GBM cells, ephrin-B2 phosphorylation, induced by addition of a recombinant EphB2 receptor, enhanced cell migration and invasion suggesting ephrin-B2 signaling as a

positive regulator of glioma cell migration (68). Overexpression of EphB2 and its corresponding ephrin-B1 ligand were also shown in medulloblastoma, the most frequent childhood malignant brain tumor (78). EphB2 receptor activation by ephrin-B1 resulted in a decrease in cellular adhesion and an increase in invasion in DAOY and Uw-402 medulloblastoma cell lines. Knockdown of EphB2 abolished ephrin-B1 effects on adhesion and invasion in these cells (78).

Tumor Microenvironment, Angiogenesis and Cancer Stem Cells

The development of a tumor depends on the acquisition of several features, collectively known as “the hallmarks of cancer” (79, 80). Tumor/cancer-associated fibroblasts (TAFs/CAFs), tumor-associated macrophages (TAMs), tumor vessels, tumor infiltrating lymphocytes, and extracellular matrix (ECM) all collectively form the tumor “organ” (81–85) (Fig. 2). The mode of interaction between Eph/ephrins and the tumor microenvironment has been relatively less studied (86). In a recent report Jellinghaus *et al.* showed EphA4 and ephrin-A1 co-localization with CD68, a cellular marker of the macrophage lineage, in advanced human atherosclerotic plaques (87). They also showed that stimulation of human umbilical vein endothelial cells (HUVECs) with soluble ephrin-A1 increased EphA4 receptor tyrosine phosphorylation, which enhanced subsequent adhesion of both the THP-1 monocytic cells and an enriched fraction of CD14+ primary human monocytes. Being that the same was observed for Human Aortic Endothelial Cells (HAEC) and Human Coronary Artery Endothelial Cells (HCAECs), it was concluded that this is possibly a general effect of ephrin-A1 on different types of endothelial cells (87). The increased adhesion induced by EphA4 ligand-dependent forward signaling was dependent on RhoA signaling pathway, which induced significant cytoskeletal changes without affecting transcriptional activity (87). High levels of ephrin-B2 expression were also detected in a murine thymus and spleen suggesting possible role in T cells stimulation (88).

Eph receptor A3 is emerging as a potential candidate for regulating the tumor microenvironment. The receptor is overexpressed in ~ 40% of GBM specimens, mainly in the mesenchymal genomic subtype (89). Vey recent findings localized EphA3 predominantly to the stromal tumor microenvironment of lung, prostate and colon cancers and mouse tumor xenografts. The chIIIA4 α-EPHA3 mAb (89) was specific in targeting tumor stroma and vasculature and inhibited tumor growth by disrupting the tumor stromal architecture (90). In GBM specimens, EphA3 co-localized with cells of myeloid origin in the tumor stoma, and in the perivascular regions in particular (Ferluga *et al.*, unpublished data).

During tumor development, pre-existing vasculature can be initially utilized for oxygen and nutrients supply whether the tumor forms. While tumor size is increasing, a pro-angiogenic environment is then generated by tumor cells, tumor-associated cells and the release of growth factors and MMPs, thus creating a chemotactive gradient to recruit endothelial cells and pericytes to form tumor neovasculature (91, 92). Vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and Tie receptors are all RTKs involved in the communication between host endothelial cells and pro-angiogenic signals produced by tumor cells (93). Eph receptors and ephrins have been

also identified as key regulators of angiogenesis during embryonic development and in postnatal vascular remodelling (94).

Ephrin-B2 and EphB4 are primary markers for arterio-venous differentiation, with ephrin-B2 expressed exclusively by arterial endothelial cells and EphB4 by venous endothelial cells (95, 96). An *in vivo* study identified ephrin-B2 reverse signalling as a positive regulator inducing VEGF receptor-2 internalization and activation of the downstream signalling pathway, eventually controlling endothelial filopodia-mediated vessel sprouting. Ephrin-B2 reverse signalling has been suggested as a therapeutic target in combinatorial anti-angiogenic treatments (97). In agreement with these findings, EphB4 displayed a proangiogenic role by activating ephrin-B2 reverse signalling in the vasculature of a breast cancer mouse model, promoting tumor progression (98). It is noteworthy that EphB4 and ephrin-B2 expression levels increase in clinical glioma samples according to the grade and thus status of neovascularization (20, 69).

EphA2 is also a major player involved in tumor angiogenesis. It was found highly present in GBM tumor vasculature (59). EphA2-deficient endothelial cells displayed impaired survival and tumor-mediated migration, and failed to incorporate into tumor microvessels *in vivo* (99). Similarly, ephrin-A1 stimulation of EphA2-expressing endothelial cells induced cellular migration and increased survival. In addition, EphA2 ligand-dependent activation was necessary to induce VEGF-dependent angiogenesis (100). In SCID mice *in vivo* model, EphA5 was present in plasma and platelets. Interestingly, EphA5 levels significantly decreased in plasma of mice bearing angiogenic, fast-growing glioblastoma tumors and increased in mice bearing microscopic dormant glioblastoma (61). Thus, the evidence suggests an active role that Eph/ephrins play in the tumor microenvironment, an avenue that still needs to be better understood (Fig. 2).

Glioma stem-like cells (GSCs) are a small portion of self-renewing glioma tumor cells with high tumorigenic potential and low proliferation rate. GSCs are particularly resistant to chemo-and radiation therapy, being potentially responsible for tumor recurrence after treatment (101–104). Several studies focused on the role of Ephs and ephrins in cancer stem cells (20); however, less is known about their role and expression in GSCs. Binda *et al.* showed that EphA2 has a regulatory role in tumor-propagating cells (TPCs) with stem-like characteristics (105). By cell sorting they showed that high EphA2 levels are a hallmark of TPCs. EphA2 was prominently down-regulated when TPCs were differentiated, losing stemness and tumorigenicity. Moreover, ephrin-A1-Fc treatment depleted the TPC pool, inhibiting self-renewal and inducing astroglial differentiation (105). A recent study also confirmed that EphA2 knockdown suppressed stem cell properties of GSCs, causing diminished self-renewal, reducing stem marker expression and decreasing tumorigenicity, while its overexpression had opposite effects (106). Similar studies showed that the loss of EphA3 in GBM cells prevents tumorsphere formation, inducing neuronal and glial cell differentiation (89). So, EphA3 appears to be important in maintaining the de-differentiated and tumorigenic state of GSCs (89) (Fig. 2).

Downstream Signalling

Eph receptors dimerize after ligand stimulation, with subsequent phosphorylation of tyrosine and serine residues in the juxtamembrane region, allowing the intracellular tyrosine kinase to convert the receptor into its active form and subsequently activate or repress the downstream signalling (107). The variety of partners that can be regulated by activated-Eph receptors give reason to the complexity of the signalling network generated.

Genome analyses of 22 GBM samples revealed major alterations in genes encoding components of TP53, RB1, and PI3K pathways (108). The tumor suppressor protein p53 is mutated in more than 50% of all human cancers; p53 is activated in response to cellular stresses such as DNA damage, heat, hypoxia and nutrient depletion and is a critical regulator of cell cycle arrest and/or apoptosis (109). Early *in vitro* studies identified both EphA2 and its ligand ephrin-A1 as targets of p53 (110). In particular, p53, p73, and p63 (two p53 homologues) responsive elements are located within the *ephA2* promoter region. EphA2, but not EphA3 or EphB2, expression and protein levels increased upon p53 activation (110). Of interest, ephrin-A1 and EphB4 were up-regulated by p53 in DLD-1 colorectal carcinoma cells (111).

The RAS-RAF-mitogen-activated protein kinase (MAPK)/ERK pathway leads to uncontrolled cellular growth, a necessary step in the development of cancer, and it is commonly activated by RTKs. Pratt *et al.*, first showed that ligand-activated EphA2 signalling starts from the interaction of tyrosine-phosphorylated EphA2 with the SHC adaptor protein (112). SHC bridges EphA2 to GRB2, which facilitates activation and nuclear translocation of the ERK kinases, eventually destabilizing cellular attachment to the ECM (112). Later studies in breast cancer cells demonstrated that EphA2 is a direct transcriptional target of the RAS-RAF-MAPK pathway (94). The authors proposed that EphA2 signalling contributes to a negative feedback loop that negatively regulates RAS activity in a ligand-dependent manner. Briefly, the activated MAPK pathway contributes to the suppression of ephrin-A1 expression, thus increasing the levels of EphA2 and the proliferative and migratory potential of cells (45, 113). On the other hand, the inhibition of the pathway induces ephrin-A1 expression whilst reducing EphA2 levels (113). Similarly, ephrin-B1 binding to EphB2 caused a decrease in the levels of active GTP-bound Ras as well as a decrease in MEK1 and ERK1/2 phosphorylation. The inhibition of the MAPK cascade requires phosphorylation at the conserved juxtamembrane tyrosine residues of EphB2 (114). In NG108 neuronal cells, activated EphB2 was shown to down-regulate the RAS-RAF-MAPK pathway and neurite retraction by recruiting p120RasGAP (115).

There is evidence suggesting the opposite effect on the Ras pathway. Vindis *et al.* demonstrated that ligand-activated EphB1 activates ERK 1/2 and thus chemotaxis in primary human renal microvascular endothelial cell (97). The proposed model suggests that ligand-activated EphB1 recruits auto-phosphorylated c-Src, which phosphorylates $p52^{Shc}$, allowing the binding of the SHC PTB domain to Tyr778 of EphB1. Grb2 is also recruited probably through the intermediary of $p52^{Shc}$. The resulting activation of the ERK cascade enhances cellular migration (116). Therefore, the downstream effect on the MAPK/ERK pathway following Eph ligand-dependent activation is very tissue and cell type dependent.

Of interest, Eph receptors can activate downstream signalling also by a crosstalk with the receptors derived from a different family. One example of such a phenomenon has been documented by Fukai and co-workers; they demonstrated that EphA4 forms a heteroreceptor complex with the fibroblast growth factor receptor 1 (FGFR1) in U-251 MG GBM cells. EphA4 promoted FGFR1-mediated cellular proliferation and migration by activating the MAPK pathway and inducing Akt phosphorylation (117).

The epidermal growth factor receptor (EGFR) acts as an oncoprotein in gliomas. Li *et al.* showed that ephrin-A5 acts as a tumor suppressor in gliomas. Ephrin-A5 forced expression reduced tumorigenicity of human glioma U373 cells by promoting ubiquitylation and degradation of the EGFR (118).

Upstream activation of RTKs and/or loss of the negative regulator, Phosphatase/tensin homolog deleted on chromosome 10 (PTEN), are the major causes for PI3K/AKT activation, particularly in primary GBM (119). In early studies, ephrin-A1 was demonstrated to stimulate PI3K activity via direct interaction of EphA2 with the p85 subunit of PI3K (120). Migratory glioma cells are known to have high AKT activity (121). Miao and colleagues showed that AKT was highly phosphorylated at both T308 and S473 sites upon serum stimulation in highly migratory U373 MG cells, however co-treatment with ephrin-A1 completely blocked AKT activation, suggesting a direct crosstalk between AKT and EphA2 (122). EphA2 was suggested to be a substrate for AKT, which in turn is negatively regulated by the ligand-activated EphA2. They also demonstrated that AKT-phosphorylated EphA2 on S897 was necessary for ligand-independent promotion of cell migration and invasion and the site became dephosphorylated upon ligand stimulation (122). This offers a possible explanation for the contradictory behaviour of this receptor, which can act as either oncoprotein or tumor suppressor (24). Indeed, later Yang *et al.*, showed that the ligand-dependent activation of EphA2 decreased the growth of PC3 prostate cancer cells and inhibited the AKT-mTORC1 pathway, which was hyperactivated due to loss of PTEN (123). The same pathways have been highlighted by a recent publication, demonstrating that alterations in EphB2 activity induced several changes among the members of the PI3K–AKT–mTOR pathway and the RAS–RAF–MEK–ERK pathway in DAOY medulloblastoma cells (78).

Therapeutic strategies

Standard treatments of glioma patients include surgery, radiation therapy, and chemotherapy (124, 125). Surgery, or radio-surgery, allows total or partial resection of the tumor depending on the location. However, the treatment does not lead to cures nor to long-survival benefits. Brain tumors are also poorly accessible to circulating drugs because of the blood-brain barrier (BBB) or blood-brain tumor-barrier (BBTB), further increasing the obstacles facing effective treatment. New therapeutic directions focus on the employment of agents specifically targeting tumors while preserving the surrounding healthy brain. In general, the strategies can be divided into active and passive immunotherapies, cytotoxic agents and targeted agents, and small molecule inhibitors. Convection-enhanced delivery (CED) seems to be the most promising approach to deliver drugs locally into the tumor or tumor resection cavity, because it bypasses both BBB and BBTB (15, 126–128).

General RTKs inhibitors, like Dasatinib and Regorafenib, have been tested in brain tumor patients. Dasatinib (Sprycel, BMS-354825, Bristol-Myers Squibb) is an oral inhibitor of multiple targets, including c-KIT, Src, and PDGFRA and B (129). Dasatinib inhibits EphA2 directly with an IC_{50} of 17 nmol l^{-1} in sensitive breast cancer cells (130) and EphB2 at similar concentrations (131). It was shown to inhibit ligand-induced EphA2 internalization and subsequent degradation in pancreatic cell lines (132). Of interest, dasatinib inhibited the Src family of kinases, involved in the Eph downstream signalling, significantly suppressing proliferation of primary glioma cells, but it had no measurable inhibitory effect on the growth of glioma stem-like cells (133). The results of a phase I clinical trial of vandetanib, a VEGFR-2 inhibitor, combined with dasatinib, during and after radiotherapy, in children with newly diagnosed diffuse intrinsic pontine glioma (DIPG), were recently published. Unfortunately, the combinatorial therapy did not change the poor prognosis for children with DIPG (134).

Regorafenib (BAY 73–4506, commercial name Stivarga) shows anti-angiogenic activity due to the targeting of VEGFR2 and TIE2 tyrosine kinase inhibition (135). Sorafenib/regorafenib, in combination with lapatinib, killed multiple primary human GBM tumor isolates in a greater than additive manner, in a process that involves induction of endoplasmic reticulum stress, autophagy, and intrinsic and extrinsic apoptotic pathways (136). To our knowledge there are no published studies aiming to decipher possible relationship between Regorafenib and the Eph/ephrin system. Of interest, a recent study identified and characterized doxazosin as a novel small molecule agonist specifically for EphA2 and EphA4. Similarly to ephrin-A1, doxazosin inhibited Akt and ERK kinase activities in an EphA2-dependent manner. Treatment with doxazosin induced receptor internalization suppressing cellular migration of prostate, breast and glioma cancer cells (137).

Different therapeutic strategies have been developed to specifically target Eph receptors that are overexpressed in gliomas. Being that EphA2 overexpression was found in 60% of GBMs and was associated with poor prognosis, Debinski and colleagues produced an ephrin-A1-based cytotoxin to specifically target EphA2 overexpressing cells. The cytotoxin was generated by chemically conjugating dimeric ephrin-A1-Fc with a modified version of the *Pseudomonas* exotoxin A. The cytotoxin killed GBM cells *in vitro* with an IC_{50} of 10^{-11} mol l^{-1}, and *in vivo* (12). Because of the very promising *in vitro* and *in vivo* preclinical data, the cytotoxin in combination is now in Phase I clinical trial in dogs with spontaneous gliomas.

EphA3 is overexpressed in about 40% of the clinical specimens, on tumor cells, stroma and vasculature. This receptor is specifically targeted by a monoclonal antibody (mAb IIIA4). Recently, Day *et al.* showed that the *in vivo* targeting of EphA3 with radiolabelled mAb IIIA4 with the beta-emitting radionucleotide lutetium (^{177}Lu) was very effective in preventing tumor formation possibly by targeting the tumor-initiating cells, with minimal toxicity to normal tissues (89).

A humanized anti-EphB4 monoclonal antibody has also been produced (hAb47), and conjugated to Cy5.5 to produce Cy5.5-hAb47; future clinical applications are envisioned in

the near-infrared (NIR) fluorescence imaging of EphB4 expression in tumors (138). Mingyue and colleagues identified an EphB6 variant (EphB6v) analyzing a panel of brain tumor cell lines and GBM specimens. EphB6v has a unique 54 amino acid sequence at the *C*-terminus that was not found in normal EphB6. EphB6v is preferentially expressed in malignant brain tumors, such as GBM, and anaplastic astrocytomas (139). Two EphB6v-derived peptides have been identified to specifically recognize cytotoxic T lymphocytes (CTLs) *in vitro*, in the peripheral blood mononuclear cells of HLA-A2(+) glioma patients, suggesting a possible future applications in peptide-based vaccine therapy in glioma patients (139).

High grade gliomas possess immunoediting properties, which refers to the two-faced effect of the immune system: host-protective and tumor-promoting (140). For example, an immunosuppressive environment is generated by secreting immune-inhibitory molecules (e.g., TGF-β, IL-10, VEGF, and others) (141, 142). The failure of an effective immune response is recognized as one of the major reasons for uncontrolled tumor growth (143), generating the rationale for the development of anti-cancer immunotherapies (7, 144).

Tumor-associated antigens (TAAs) are immunogenic, tumor-specific or tumor-associated molecules that are minimally expressed or absent in normal tissue (145). Active and passive immunotherapies are two different strategies used to generate anti-tumor activity. Active immunotherapy employs tumor-specific vaccines, like TAAs, administered in the context of a non-specific immune co-stimulation. These vaccines induce an immune response mainly by activating cytotoxic T lymphocytes (CTLs) that are able to recognize endogenous tumor antigens (146). Passive immunotherapy is also referred to as adoptive immunotherapy. Adoptive immunotherapy, or adoptive cell transfer (ACT), involves infusion of autologous lymphocytes, T lymphocytes and natural killer (NK) cells, into patientsfollowing *ex vivo* expansion (147). In particular, chimeric antigen receptor (CAR) T cells can be engineered with glioma-specific antigens that provide targets for CAR-based immunotherapy. Among them, the most promising appear to be IL-13 receptor alpha 2 (IL-13RA2), human epidermal growth factor receptor 2 (HER2), and EphA2 (143, 148–151).

Promising results have been obtained using an Eph-dendritic cells (DC)-based vaccine to induce both tumor antigen-specific CTLs and helper-T cells. Human leukocyte antigen (HLA) A2+ peripheral blood mononuclear cells (PBMCs), from healthy donors and glioma patients, have been stimulated with autologous dendritic cells (DCs) loaded with the synthetic $EphA2_{883–891}$ (TLADFDPRV) peptide. The stimulation induced an antigen-specific, anti-glioma CTL response in HLA-A2+ patient-derived PBMCs (152).

More recently, a phase I/II trial was performed using polarized dendritic cells (αDC1) loaded with synthetic peptides for glioma-associated antigen (GAA) and stabilized by lysine and carboxymethylcellulose (poly-ICLC) (153). GAAs used are EphA2, IL-13Rα2, YKL-40, and gp100. The trial demonstrated not only safety of the vaccination therapy, but also the induction of an anti-glioma immune response, which resulted in GBM progression free for at least 12 months in around 40% of the enrolled patients (153).

Concluding remarks

Ephs and ephrins are certainly one of the most intriguing families of RTKs and corresponding ligands, and one of the most promising candidates in targeted therapies of malignant gliomas (154, 155). It is not surprising to detect Ephs and ephrins abnormal expression in tumor cells, as these proteins are usually expressed during developmental processes. Moreover, progenitor or tumor-initiating cells also rely on the presence and function of these proteins., However, overexpression and downstream signaling of Ephs and ephrins in cancer are highly tissue and cell type specific, generating tremendous biological variability; the same Eph receptor can act as an oncoprotein or as a tumor suppressor. In addition, a crosstalk between Ephs and the receptors of different families potentiate the complexity of the signaling cascade. The major challenge is to contextualize the aberrant expression and signaling of these receptors and ligands within the tumor environment and different subpopulations of cells present within the tumors. EphA2 and EphA3 in particular are the receptors overexpressed not only in glioma tumor cells, but also in tumor vasculature tumor cells infiltrating normal brain and immune cells infiltrating tumors. New therapeutics specifically targeting these receptors showed preliminarily promising results. Moving towards that direction will help to deepen our understanding of the relationship between this ligand-receptor system and the pathobiology of malignant gliomas, and possibly offer urgently needed more effective therapeutic strategies.

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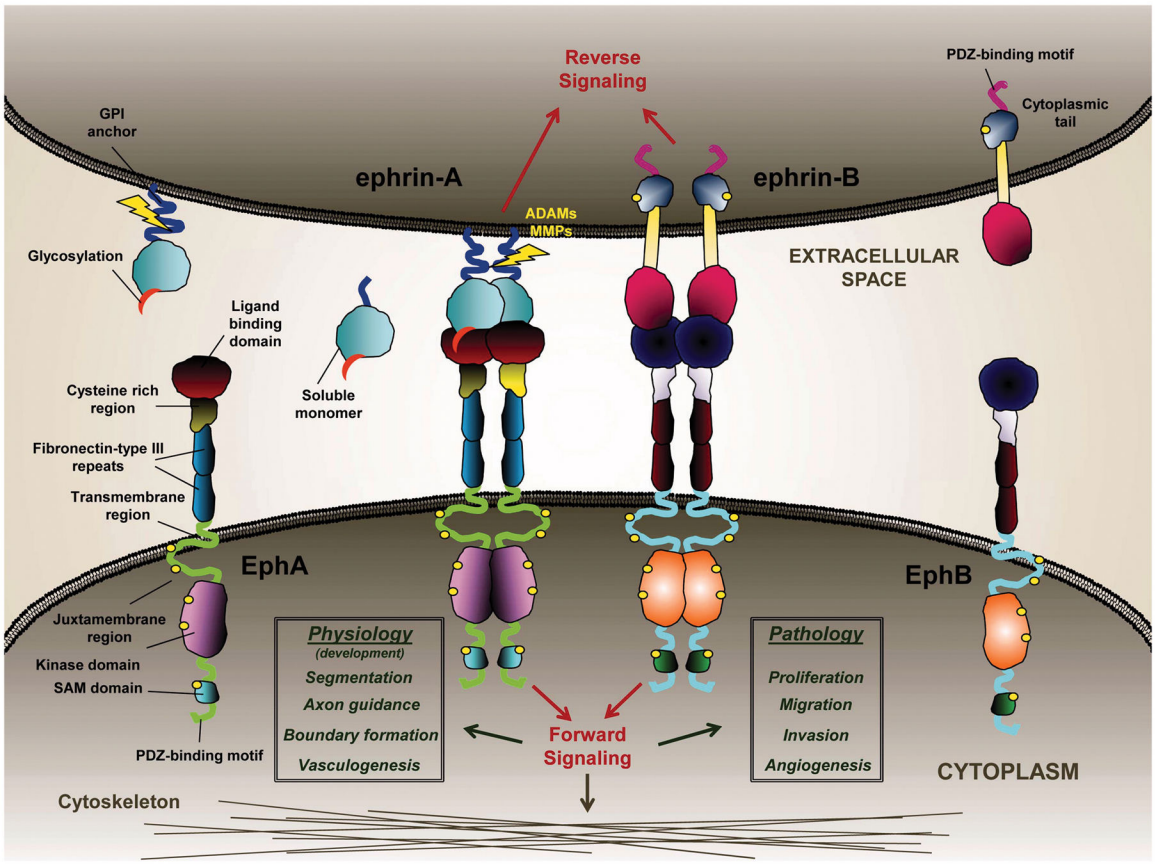

Figure 1.

Schematic representation of Ephs and ephrins structure and the mode of interaction between neighboring cells. Ligand-dependent activation induces receptor phosphorylation and a downstream signaling cascade, which acts on the cytoskeleton and regulates cellular proliferation, migration and invasion in glioma cells. Activated clusters of receptors internalize into the receptor-expressing cell and are eventually degraded.

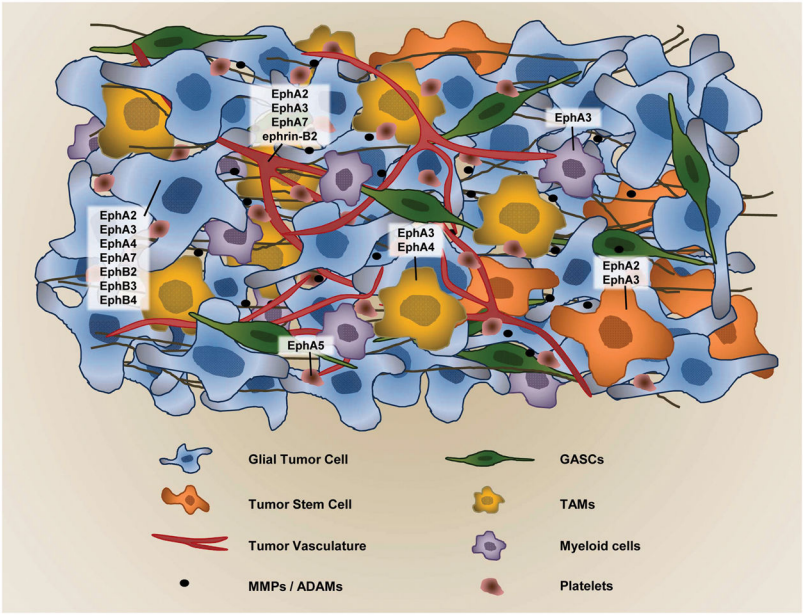

Figure 2.

Ephs and ephrins in glioma tumor environment. Different Eph receptors are overexpressed not only in glioma tumor cells, but also in the surrounding tumor-infiltrating cells like tumor-associated macrophages (TAMs) (84), in tumor vasculature, glioblastoma-associated stromal cells (GASCs)(83), and in the stem-like cell population (103).

Table 1

Summary of Ephs and Ephrins expression, functional role and localization in gliomas.

Ephs/ Ephrins	Expression / Survival	Ligand-Dependent Effect	Downstream Signaling After Ligand-dependent Stimulation	Localization Within the Tumor	Reference
EphA2	↑ / ↓	↓ Migration and proliferation.	↓ The MAPK pathway and blocks Akt activation.	Tumor cells, vasculature and stem cells.	(43, 45, 59, 106, 113, 122)
EphA3	↑ / ↓	↓ Proliferation.	↓ The MAPK pathway.	Tumor cells, vasculature, stem cells and stroma.	(89, 156)
EphA4	↑ / -	↑ Migration and proliferation if FGF stimulated.	↑ The MAPK pathway and phosphorylates Akt.	Tumor cells.	(117)
EphA5	↓ / ↑	No significant decrease in cell proliferation.	-	Plasma and platelets.	(61)
EphA7	↑ / ↓	-	-	Tumor microvasculature.	(60)
ephrin-A1	↓ / -	Down-regulates EphA2.	-	Very low levels on tumor cells.	(45)
ephrin-A5	↓ / -	Down-regulates EphA2 and EphA3.	↓ EGFR	Very low levels on tumor cells.	(45, 118)
EphB1	↓ / ↑	↓ Migration and invasion.	↑ ERK1/2 *	-	(70)
EphB2	↑ / -	↑ Invasion.	↓ The MAPK and the PI3K–Akt– mTOR pathways.	Tumor vasculature.	(76–78)
EphB3	↑ / -	-	-	Tumor cells.	(70)
EphB4	↑ / ↓	-	Pro-angiogenic effect.	Tumor cells.	(69, 98)
ephrin-B1	↑ / -	↑ Invasion.	-	-	(68)
ephrin-B2	↑ / ↓	↑ Migration and invasion.	Induce VEGFR2 internalization and the downstream pathways and vessel sprouting.	-	(68, 69, 98)
ephrin-B3	↑ / -	↑ Invasion.	-	-	(68)

Abbreviations: ↑= Increased; ↓= Decreased; - = No data; * = not shown in gliomas; MAPK= mitogen-activated protein kinase; PI3K= phosphatidylinositol-4,5-bisphosphate 3-kinase; EGFR= epidermal growth factor receptor; mTOR= mammalian target of rapamycin; ERK= extracellular signal-regulated kinases; VEGFR2= vascular endothelial growth factor receptor-2.