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Brain Tumor Stem Cells and Anti-Angiogenic Therapy

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1. Introduction

The hypothesis that tumor growth may be sustained by a rare subpopulation of cells, termed cancer stem cells, is currently demonstrated in different types of cancer (Al-Hajj *et al.*, 2004; Jordan *et al.*, 2006; Reya *et al.*, 2001). Brain tumor stem cells were isolated from primary brain tumors, such as malignant glioma (Galli *et al.*, 2004; Hide *et al.*, 2008). Glioma stem cells share some characteristics with normal neural stem cells, including the expression of neural stem cell markers, such as CD133 and Nestin (Hadjipanayis and Van Meir, 2009; Singh *et al.*, 2004). Brain tumor stem cells possess the capacity for self-renewal and multipotency (ability to differentiate into neurons, astrocytes, and oligodendrocytes) and the proliferative ability for generation of many progeny. Furthermore, they are able to initiate new tumors in vivo when transplanted into immunocompromised mice even at low cell numbers (Galli *et al.*, 2004).

Glioma stem cells play an important role in tumor invasion and therapy resistance (Bao *et al.*, 2006a; Calabrese *et al.*, 2007; Dean *et al.*, 2005; Hirschmann-Jax *et al.*, 2004). Complete surgical resection is almost impossible because of the deep invasion to the normal brain parenchyma. Glioma stem cells have the ability to divide slowly and infinitely, which leads to the resistance to chemotherapy and radiotherapy. These stem cell-like properties allow glioma stem cells to survive selectively and initiate recurrence (Hide *et al.*, 2008). Therapeutic strategy that target glioma stem cells may improve the prognosis of malignant glioma. Recent evidence has revealed that glioma stem cells are located in the highly vascular region, and glioma stem cells' properties are tightly regulated by the microenvironment, so called vascular niche, similar to normal neural stem cells (Gilbertson and Rich, 2007; Yang and Wechsler-Reya, 2007).

The development of new therapeutic strategies that target the glioma stem cells and vascular niche may result in more effective treatment of malignant glioma. In this article, we review the recent evidence on the biology of glioma stem cells associated with vascular endothelial growth factor (VEGF)- vascular endothelial growth factor receptor (VEGFR) signalling pathways for anti-angiogenic therapy.

2. Brain stem cells and vascular niche

Recent studies reveal a close relationship between the stem cells and the vascular niche. In the adult brain, neural stem cells were demonstrated to be concentrated around blood

vessels where they had access to signalling molecules, nutrition, and nascent vasculature for migration (Shen *et al.*, 2004). Similarly, glioma stem cells were shown to be located around vascular niches (Calabrese *et al.*, 2007). Vascular niche supplies oxygen and nutrition, and at the same time regulates the glioma stem cells' characteristics, such as self-renewal and differentiation. In addition, glioma stem cells not only receive the signals from the surrounding niche but also modulate the signals through the secretion of VEGF (Bao *et al.*, 2006b; Folkins *et al.*, 2009; Oka *et al.*, 2007). Glioma stem cells and vascular niche represent integral factors for invasion and expansion. Therefore, comprehension of the interactive structural units may lead to development of therapeutic innovations.

2.1 Neural stem cells

Reynolds and Weiss were the first to isolate neural stem cells from the adult striatum that could proliferate and generate multipotent cells *in vitro,* termed neurospheres (Reynolds *et al.*, 1992; Reynolds and Weiss, 1992). Neurosphere culture relies on a serum-free, selective growth factor (epidermal growth factor and fibroblast growth factor 2) system. Neural stem cells are responsive to the growth factor, and they can be passaged and expanded indefinitely with little change in characteristics. Removal of growth factors induces the differentiation of the progeny of neural stem cells into neurons, astrocytes, and oligodendrocytes. Neural stem cells have been isolated also from the subventricular zone lining of the lateral ventricles, dentate gyrus within the hippocampus, and subcortical white matter (Ayuso-Sacido *et al.*, 2008; Eriksson *et al.*, 1998; Nunes *et al.*, 2003; Sanai *et al.*, 2004).
The subventricular zone is the largest of the germinal regions in humans. It is located between the ependymal layer of the lateral ventricles and parenchyma of the striatum. This zone is also thought to be the likely source of glioma stem cells (Sanai *et al.*, 2005).

2.2 Glioma stem cells

Glioma stem cells are thought to originate from transformed neural stem cells and progenitor cell populations (Hadjipanayis and Van Meir, 2009). Clonogenic, neurosphere-forming precursor cells were isolated from glioblastoma specimens by applying the same conditions used for the isolation of human neural stem cells. However, only 0.01%-1.0% of cells even in this selected population from malignant glioma can reinitiate tumors in immunodeficient mice (Hide *et al.*, 2008). There are two major techniques for enrichment of glioma stem cells.
One technique is cell sorting using a specific cell-surface antigen or a combination of the cell surface antigens. Purification of CD133-positive cells from human gliomas by flow cytometry can allow the isolation of glioma stem cells. CD133 was first reported as a marker for hematopoietic stem/progenitor cells (Yin *et al.*, 1997). This cell-surface antigen was later reported as a marker for neural stem/progenitor cells (Uchida *et al.*, 2000). Singh et al. demonstrated that as few as 100 CD133-positive glioma cells could initiate tumors in vivo when transplanted into immunocompromised mice; whereas, the injection of 5×10^4 to 1×10^5 CD133-negative cells is not capable of tumor initiation (Singh *et al.*, 2004). Recently, several reports have suggested that there is no difference in the ability of CD133-positive and CD133-negative cells to form orthotopic tumors (Zheng *et al.*, 2007). CD133-negative cells isolated from glioblastoma were reported to form orthotopic tumors similar to CD133-positive subpopulation (Chen *et al.*, 2010; Wang *et al.*, 2008). This may reflect the presence of other types of glioma stem cells. Further researches are needed to identify more specific cell-surface antigen.

The other technique is side population technique using the ability to efflux Hoechst 33342 dye (Hirschmann-Jax *et al.*, 2004). The side population technique is a method to identify cancer stem cells in various cancers, including the brain (Hide *et al.*, 2008). Cancer stem cells are thought to maintain their drug efflux ability, which makes it possible to separate cancer stem cells in unstained cell fractions. These cells appear on the lower left side of dot graphs analyzed by a cell sorter; hence, they are called side population cells.
The current lack of a single marker to identify all cancer stem cells in malignant glioma may suggest the molecular heterogeneity among these cells.

2.3 Glioma vascular niche

Excessive and grossly disorganized blood vessel formation is a hallmark of glioblastoma. This aberrant vascularity has been presumed to be important for satisfying the demand for nutrition of the rapid tumor growth. Normal neural stem cells within the subventricular zone and hippocampus are concentrated in regions of the brain that are rich in blood vessels, called the vascular niche (Palmer *et al.*, 2000). This organization places the stem cells in a close relationship with endothelial and other vascular cells, which facilitate communication among these cell types. The vascular niche protects neural stem cells from apoptotic stimuli to maintain a good balance between self-renewal and differentiation. Similarly, glioma stem cells were intimately associated with the vascular niche in the tumor. CD133-positive/nestin-positive glioma stem cells were frequently discovered close to capillaries within glioblastoma (Calabrese *et al.*, 2007). Endothelial cells have been shown to be one of the most important components in the vascular niche. Endothelial cells secrete paracrine factors that promote normal stem cell survival and self-renewal (Shen *et al.*, 2004). In the same manner, CD133-positive glioma stem cells when transplanted with endothelial cells grew more rapidly than when transplanted alone (Calabrese *et al.*, 2007). In addition, tumors established in the presence of endothelial cells contained up to 25 times more CD133-positive cancer stem cells. Thus, endothelial cells are demonstrated to develop the self-renewal capacity of CD133-positive glioma stem cells.
Recent evidence has suggested that a functional relationship between the glioma stem cells and vascular niche may be bidirectional; such that, the glioma stem cells may maintain the vascular niche just as the vascular niche helps in the proliferation and self-renewal of glioma stem cells. Bao et al. showed that high-level production of VEGF by CD133-positive glioma stem cells could develop their tumor-initiating capacity (Bao *et al.*, 2006b). The authors demonstrated that freshly resected CD133-positive glioma cells increased endothelial migration and tube formation by producing VEGF, leading to vascular-rich and hemorrhagic tumors in the brains of immunocompromised mice, but not for the CD133-negative glioma cells.

3. Angiogenesis and VEGF family

Angiogenesis is a tightly regulated process in which the development of new blood vessels arises from a pre-existing vascular network. It is regulated by endogenous activators and inhibitors during development and by normal physiological processes, such as wound healing. However, angiogenesis is also involved in tumor growth, progression, and metastasis (Hoeben *et al.*, 2004). As a key step in tumor development, the angiogenic switch occurs when endogenous activators of angiogenesis exceed endogenous inhibitors (Hanahan and Folkman, 1996). This phenomenon results in increasing blood vessel

formation and supplying tumors with oxygen and nutrition for growth. However, tumor vasculature is disorganized and poorly structured; but is nonetheless essential for continuous tumor growth. Folkman et al. firstly proposed that tumor growth was restricted in size because there was limitation in the diffusion of oxygen without the blood supply, and so tumor angiogenesis could be a potential therapeutic target (Folkman, 1971; Folkman, 1972; Folkman *et al.*, 1971). Of the angiogenic factors, VEGF has been demonstrated to play a crucial role in angiogenesis and progression of malignant glioma (Plate *et al.*, 1992).

3.1 New blood vessel formation in glioma

Formation of new blood vessels is classified into three models: angiogenesis, vasculogenesis, and arteriogenesis (Tate and Aghi, 2009). Angiogenesis is the formation of new blood vessels by rerouting or remodeling pre-existing vessels, and it is thought to be the primary method of vessel formation in gliomas. Vasculogenesis is classically considered an embryonic process, but recently it has been identified in adults, too. Vasculogenesis in tumors has been demonstrated to be the formation of blood vessels from circulating marrow-derived endothelial progenitor cells that are recruited to the tumor. Finally, arteriogenesis is a process in which arteriolar networks hypertrophy in order to sustain increased oxygen demands. A current model of tumor vessel formation suggests that this process involves recruitment of sprouting vessels from existing blood vessels and incorporation of endothelial progenitors into the growing vascular bed. Thus, only angiogenesis and vasculogenesis were thought to play important roles in tumor biology. The necrotic and hypoxic nature of glioblastoma is thought to cause angiogenesis and vasculogenesis through the induction of angiogenic factors including VEGF (Onishi *et al.*, 2011).

3.2.1 VEGF family

The vascular endothelial growth factor (VEGF) family consists of five members: VEGF-A (thereafter called VEGF), VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF) (Kowanetz and Ferrara, 2006). In addition, multiple isoforms of VEGF, VEGF-B, and PIGF are generated through alternative splicing of pre-mRNA (Sullivan and Brekken, 2010). VEGF family ligands show different affinities for the three VEGF tyrosine kinase receptors: VEGF receptor (VEGFR)-1, VEGFR-2, and VEGFR-3. Moreover, several co-receptors, such as neuropilins (Nrp)-1 and Nrp-2, also regulate VEGF family interaction with VEGFRs.

3.2.2 VEGF(-A)

The *VEGF* gene contains eight exons and seven introns. VEGF binds to VEGFR-1, VEGFR-2, Nrp-1, and Nrp-2. VEGF induces vascular permeability and also functions as an endothelial cell mitogen and survival factor, and an inducer of endothelial cell and monocyte migration (Kowanetz and Ferrara, 2006). Knock out studies in mice showed that homozygous or heterozygous deletion of the *VEGF* gene was embryonically lethal, resulting in defects in vasculogenesis and cardiovascular abnormalities. These studies have demonstrated that VEGF is essential for development. VEGF is important to postnatal angiogenic processes such as wound healing, ovulation, and pregnancy. VEGF is also involved in tumor angiogenesis, arthritis, macular degeneration, and diabetic retinopathy. VEGF is considered to be a strong angiogenic effector under most physiological and pathological conditions (Hicklin and Ellis, 2005; Sullivan and Brekken, 2010).

3.2.3 VEGF-B

VEGF-B binds to both VEGFR-1 and Nrp1. The function of VEGF-B is still controversial. *VEGF-B* null mice were viable and largely healthy, except for some abnormalities in cardiac conduction (Sullivan and Brekken, 2010). VEGF-B plays some roles in heart function in adults, but not in developmental angiogenesis or cardiovascular development (Sullivan and Brekken, 2010). Therefore, VEGF-B has been thought to have a negligible role as an angiogenic factor even under pathological conditions. However, recent studies revealed that VEGF-B was a potent survival factor for blood vessels, and the inhibition of VEGF-B lead to pathological angiogenesis by abolishing blood vessel survival in animal models (Hicklin and Ellis, 2005).

3.2.4 VEGF-C

VEGF-C binds to VEGFR-2 and VEGFR-3. It is involved in developmental lymphangiogenesis and in the maintenance of adult lymphatic vasculature (Sullivan and Brekken, 2010). *VEGF-C* null mice were embryonic lethal and heterozygous *VEGF-C* loss was characterized by lymphedema because of defective development of the lymphatic vasculature. VEGF-C is not necessary for blood vessel development because vessels appear normal in *VEGF-C* null animals (Sullivan and Brekken, 2010). Although VEGF-C is not expressed in a normal brain, recent reports that show the high expression of VEGF-C in malignant glioma suggest the ligands' role in glioma angiogenesis (Grau *et al.*, 2007; Jenny *et al.*, 2006; Witmer *et al.*, 2001).

3.2.5 VEGF-D

VEGF-D binds to both VEGFR-2 and VEGFR-3. VEGF-D is also involved in developmental lymphangiogenesis and adult lymphatic vasculature (Sullivan and Brekken, 2010). *VEGF-D* null mice were viable and had a normal lymphatic vasculature during development and in adults, which suggests that VEGF-C and other growth factors may substitute for VEGF-D function. VEGF-C and VEGF-D bind to VEGFR-2 and they might also play a role in angiogenesis as well, especially during pathological states such as tumor growth. However, the role of these ligands in tumor angiogenesis is unclear. Similar to VEGF-C, VEGF-D is shown to be expressed highly in malignant glioma but not in the normal brain, which suggests the ligands' contribution to glioma angiogenesis (Grau *et al.*, 2007; Witmer *et al.*, 2001).

3.2.6 VEGF-E

VEGF-E is not a mammalian VEGF homolog, but rather a viral protein encoded by the parapoxvirus Orf virus (Shibuya, 2003). VEGF-E binds to only VEGFR-2. It is also involved in angiogenesis like VEGF-A but its role still remains unclear.

3.2.7 Placental growth factor

Placental growth factor (PlGF) binds to only VEGFR-1 and is also involved in angiogenesis (Hicklin and Ellis, 2005). PlGF is also suggested to play the role in recruitment of monocyte and vascular progenitor cells from bone marrow to tumors. This ligand is primarily expressed in the placenta, and also in other organs such as the heart, retina, and muscle (Sullivan and Brekken, 2010). Although *PlGF* null mice were viable and displayed no defect in embryonic angiogenesis or developmental abnormalities, the loss of PlGF impaired angiogenesis, plasma extravasation, and collateral growth during ischemic conditions,

inflammation, wound healing, and tumor growth (Hicklin and Ellis, 2005). Lastly, PlGF may play important roles on pathologic states in adult.

3.2.8 VEGF family expressions in glioma cells

The over-expression of VEGF in glioma was demonstrated (Plate *et al.*, 1992), and recent studies also revealed the over-expression of VEGF in glioma stem cells by evaluating expression levels of VEGF in conditioned media from matched CD133+ and CD133- glioma cultures (Bao *et al.*, 2006b). High expression of VEGF-B mRNA was shown in low and high grade gliomas (Gollmer *et al.*, 2000). Grau SJ et al. analyzed expressions of VEGF-C, VEGF-D, and VEGFR-3 in glioblastomas, and showed strong protein expression of VEGFR3 on tumor endothelium, while VEGF-C and VEGF-D were expressed on numerous cells in areas of high vascularization (Grau *et al.*, 2007). Although VEGF-C, VEGF-D, and VEGFR-3 are not expressed in normal brain tissue, expressions of VEGFR3, VEGF-C and VEGF-D were found on the protein and RNA levels. Nomura et al. investigated the relationship between PlGF and primary brain tumor angiogenesis. PlGF mRNA was expressed in all the hypervascular primary brain tumors (Nomura *et al.*, 1998). In addition, they conducted hypoxic experiments with cultured U-251MG human glioma cells to determine the mechanism of PlGF gene regulation. As the atmospheric oxygen concentration was decreased, the PlGF mRNA level in the U-251MG cells was markedly increased. These results suggested that PlGF may contribute to the pathogenesis of brain tumor angiogenesis.
Until now, it has been demonstrated that only VEGF is expressed highly in glioma stem cells.

3.3.1 VEGF receptors

There are three receptor tyrosine kinases that mediate the angiogenic functions of VEGF family members (Kowanetz and Ferrara, 2006). They are structurally very similar. The VEGF receptors contain a seven immunoglobulin-like domain extracellular region, a single transmembrane domain segment, a juxtamembrane segment, a split intracellular protein-tyrosine kinase domain, and a carboxyterminal tail. Unlike other VEGFR genes, alternative splicing of *VEGFR-1* produces a soluble form of the receptor (sVEGFR-1) that contains only extracellular domain (Sullivan and Brekken, 2010). This receptor does not possess the activity of tyrosine kinase in intracellular signaling, and inhibits the function of VEGF. VEGFR-1 and VEGFR-2 were originally identified on endothelial cells, and VEGFR-3 was identified on lymphatic endothelial cells (Kowanetz and Ferrara, 2006).

3.3.2 VEGFR-1

VEGFR-1 is a receptor for VEGF, VEGF-B, and PlGF. It binds VEGF with at least 10-fold higher affinity than VEGFR-2, but the kinase activity is weaker than that of VEGFR-2 (Sullivan and Brekken, 2010). Although *VEGFR-1* null mice were embryonic lethal, the mice that did not express the tyrosine kinase domain of VEGFR-1 but retained the ligand-binding extracellular domains and transmembrane segment (*VEGFR1-TK-/-*) were viable. Thus, VEGFR-1 was initially considered to be a negative regulator of VEGF activity by acting as a decoy receptor for VEGF. VEGFR-1 was also expressed by monocytes, macrophages, and other bone marrow-derived progenitor cells (myeloid cells) with cell-surface marker of CD11b (Kaplan *et al.*, 2005). VEGFR-1 was thought to mediate the migration of bone marrow-derived cells into cancerous tissues and recruitment of endothelial progenitor cells

as another function resulted in tumor growth and angiogenesis (Sullivan and Brekken, 2010). *VEGFR-1* null mice were embryonic lethal because of excessive hemangioblast proliferation and poor organization of vascular structures, which seemed to be due to the inhibition of the function as a negative regulator for VEGF signaling and the inhibition of angiogenesis via recruitment of endothelial progenitor cells. In addition, VEGFR-1 was also shown to be expressed by the subsets of liquid and solid tumor stem cells, which resulted in tumor cell survival and growth (Kowanetz and Ferrara, 2006). Although VEGFR-1 must be critical for physiologic and developmental angiogenesis, the precise function of VEGFR-1 remains unclear. Recent studies have shown that during pathologic conditions such as tumorigenesis, VEGFR-1 is a potent, positive regulator of angiogenesis (Hicklin and Ellis, 2005). Hence, current evidence has suggested that the function of VEGFR-1 differs with each stage of development, various states of physiologic and pathologic conditions, and cell types in which it is expressed.

3.3.3 VEGFR-2

VEGFR-2 is the major mediator of VEGF-induced angiogenic signalling. The functions of VEGFR-2 include endothelial cell survival, migration, proliferation, and vascular permeability. This receptor has the most important role in vessel formation during both physiologic and pathologic conditions (Hicklin and Ellis, 2005). Recent studies have shown that VEGFR-2 is also expressed in subsets of liquid and solid tumor cells in addition to endothelial cells, which imply the additional role of VEGF in cancer through stimulation of VEGFRs on tumors cells (Hicklin and Ellis, 2005; Kowanetz and Ferrara, 2006). VEGFR-2 is a receptor for VEGF, VEGF-B, VEGF-C, VEGF-D, and VEGF-E. VEGFR-2 has a lower affinity for VEGF than VEGFR-1, but it has a stronger kinase activity. *VEGFR-2* null mice were embryonic lethal (Sullivan and Brekken, 2010). These animals had severe defects in endothelial and hematopoietic cell development with no organized blood vessel found at any point within the developing embryo or the yolk sac.

3.3.4 VEGFR-3

VEGFR-3 binds both VEGF-C and VEGF-D and is a key regulator of normal and tumor lymphangiogenesis (Hicklin and Ellis, 2005; Kowanetz and Ferrara, 2006; Sullivan and Brekken, 2010). During development and in adulthood, its expression is limited to lymphatic endothelial cells. VEGFR-3 is also expressed in the embryonic vasculature. *VEGFR-3* null mice were embryonic lethal and displayed cardiovascular failure as a result of the abnormal structure and organization of large vessels. VEGFR-3 is not expressed in the brain, but recent studies have shown that VEGFR-3 is expressed highly with VEGF-C and VEGF-D in malignant glioma endothelium, which may suggest that VEGFR-3 and these ligands contribute to glioma angiogenesis (Grau *et al.*, 2007; Jenny *et al.*, 2006).

3.3.5 VEGFRs expressions in glioma cells

The expression of VEGFRs on liquid and solid tumor cells has been reported already (Dias *et al.*, 2001; Dias *et al.*, 2000; Ferrer *et al.*, 1999). VEGF may also have another role in cancer through the stimulation of VEGFRs on tumor cells. Although the significance of this expression is still under investigation, it has been hypothesized that VEGF ligands promote tumor growth not only in a paracrine manner, but also in an autocrine manner. Rafii et al. showed that functional VEGF/VEGFR-2 autocrine loop was present in subsets of human

leukemias and supported *in vivo* leukemic cell survival and migration (Dias *et al.*, 2000). Fan F et al. demonstrated that VEGFR-1 activation by VEGF or VEGF-B led to activation of the MAPK pathway in tumor cells and phenotypic changes including an increase in cell migration and invasion (Fan *et al.*, 2005).
Knizetova et al. demonstrated that the expression of VEGFR-1 and VEGFR-2 genes in glioma cells was shown by PCR (Knizetova *et al.*, 2008). They reported autocrine regulation of glioblastoma cell proliferation. Lucio-Eterovic et al. showed the mRNA and protein levels of VEGFRs in glioma stem cell line, suggesting that autocrine VEGF signalling blockade played an important role in glioma invasion (Lucio-Eterovic *et al.*, 2009).
The expression of VEGFR-3 in glioma cells, including glioma stem cells, is still unreported.

3.4 VEGFR signaling molecular pathways

Receptor tyrosine kinases (RTKs) are transmembrane proteins that mediate the transmission of extracellular signals to the intracellular environment. RTKs are activated through the binding of a growth factor ligand to the extracellular domain, leading to receptor dimerization and subsequent autophosphorylation of the receptor complex by the intracellular kinase domain, using ATP. The phosphorylated receptor then interacts with a variety of cytoplasmic signaling molecules, leading to signal transduction.
In VEGFR-2 intracellular signaling pathways, main signalling cascades include the phospholipase Cγ(PLCγ)-protein kinase C-Raf kinase-mitogen-activated protein kinase kinase (MEK)-MAPK pathway and Phosphatidylinositol-3-Kinase(PI-3K)/AKT pathway. The PLC-γ pathway regulates cell proliferation and cell migration. The PI-3K pathway regulates cell migration and cell survival via anti-apoptosis effect. The PI-3K pathway also regulates vascular permeability via heat shock protein 90 (Hsp90) and endothelial nitric oxide synthase (eNOS), but the signal is still not well understood (Kowanetz and Ferrara, 2006). Unlike other RTKs, such as epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR), the Ras pathway is not involved so strongly in the VEGFR-2 signalling pathway.
In VEGFR-1 and VEGFR-3 intracellular signaling pathways, a limited number of signalling effectors have been shown to act downstream of these receptors. Further investigation for these receptors in cancer cells is required.

4. VEGF/VEGFR-related molecular biology of malignant glioma

4.1 Glioma invasion and angiogenesis

Marked proliferation, angiogenesis, and invasion are hallmarks of malignant gliomas. Magnetic resonance imaging (MRI) showed two characteristic regions of malignant glioma; the central region with gadolinium-enhanced mass and the marginal region with high-intensity signals on T2-weighted images. Histopathological analysis of malignant glioma invasion showed that the clusters of glioma cells and necrotic tissue were seen in the central region with gadolinium contrast enhancement. In the marginal region with high-intensity signals on T2-weighted images, diffuse infiltrating glioma cells were seen around and inside the normal brain parenchyma. Vascular proliferation was seen in both regions. (Onishi *et al.*, 2011) Glioma cells, including glioma stem cells, are beside newly developed vessels and had a close interaction with vascular niche especially in the paracrine manner. High-level expression of VEGF family and VEGFRs has been shown in glioma vasculature and glioma

cells, including glioma stem cells (Hicklin and Ellis, 2005). Glioma stem cells expressed higher levels of VEGF than the matched non-stem glioma cells and displayed greater angiogenic potential *in vitro and in vivo (Bao et al., 2006b).* Furthermore, recent studies have demonstrated that the relationship between glioma stem cells and vasculature is complex and bi-directional. Therefore, anti-angiogenic therapy has the potential to function as an anti-glioma stem cell therapy (Folkins *et al.*, 2009; Oka *et al.*, 2007). Calabrese et al. have demonstrated that the treatment of mice with VEGF inhibitor after glioblastoma implantation resulted in a large reduction in the number of glioma stem cells and blood vessels of the tumors. In contrast, VEGF inhibitor had minimal effect on the proliferation or survival of most cells in the tumors, suggesting that the glioma stem cells were targeted (Calabrese *et al.*, 2007).

Necrotic tissue in the central region of malignant glioma was surrounded by pseudopalisading cells. Recently, Rong et al. demonstrated that pseudopalisading cells were present in severely hypoxic regions, over-expressed hypoxia-inducible factor, and secreted VEGF (Rong *et al.*, 2006). Pseudopalisading cells were cell populations composed of a series of actively migrating glioma cells, moving away from a central hypoxic region. Hypoxia activated the VEGF promoter and transcription in glioma cells, which could have led to the cell activation of a migrating phenotype toward the viable vessels (Brat *et al.*, 2004). Both *in vitro* and *in vivo* models have demonstrated that tumor hypoxia results in increased glioma cell migration (Elstner *et al.*, 2007).

The autocrine function of VEGF in cancer invasion was first shown in invasive breast cancer cell lines (Bachelder *et al.*, 2002; Price *et al.*, 2001). In malignant glioma, tumor hypoxia may also increase tumor invasion in a VEGF/VEGFR autocrine manner independent of angiogenesis. Autocrine stimulation of VEGFRs on glioma cells including glioma stem cells, has been shown to be important for cell survival, proliferation and invasion (Knizetova *et al.*, 2008). In addition, hypoxia can promote the expansion of glioma stem fraction and regulate the expression of stem cell markers (Heddleston *et al.*, 2009; McCord *et al.*, 2009; Soeda *et al.*, 2009). Hypoxic condition induced VEGF expression in both glioma stem cells and non-stem glioma cells, but the VEGF levels were consistently higher in glioma stem cells (Li *et al.*, 2009). Under normoxic condition, glioma stem cells also expressed a higher level of VEGF than non-glioma stem cells.

In addition, other histopathological findings as glioma invasion models were reported; such that glioma cell infiltrations into normal brain parenchyma independent of vasculature (Onishi *et al.*, 2011). Cancer stem cells have been shown to promote metastasis (Hermann *et al.*, 2007; Li *et al.*, 2007). Although malignant gliomas rarely metastasize beyond the central nervous system, MRI sometimes shows glioma cells infiltration to contralateral hemisphere. This infiltrative feature away from tumor vascular niche may be equivalent to metastasis. Recent evidences in lung cancer have demonstrated that immature myeloid cells can be recruited to the metastatic sites and immature myeloid cells prepare to make the pre-metastatic state for cancer cells (Kaplan *et al.*, 2005). Immature myeloid cells express VEGFR-1, and a neutralization antibody for VEGFR-1 has been shown to significantly suppress the metastasis in lung cancer (Hiratsuka *et al.*, 2002). This evidence may suggest that bone marrow-derived cells, such as immature myeloid cells, are other components of the niche, and these cells are associated with glioma cells invasion via VEGF signalling pathways. Folkins et al. investigated that bone marrow-derived cells recruitments in mice with glioma stem cell-rich xenograft tumor (Folkins *et al.*, 2009). Glioma stem cells were shown to

increase the mobilization of endothelial progenitors, but not myeloid cells. Further researches are required to show the possibility that the myeloid cells may be associated with glioma cells invasion.

In summary, the VEGF paracrine signals between glioma cells and vascular niche enhance glioma cells invasion in the vascular-rich regions. In addition, the VEGF autocrine signals on glioma cells also enhance self-invasion in the hypoxic regions. As another component of the niche, myeloid cells may be involved in glioma cells invasion via VEGF signalling pathways. In particular, glioma stem cells have stronger signals of VEGF under various situations.

4.2 Therapeutic resistance of glioma stem cells

As well as regulating stem cell proliferation and survival, niche may also play a protective role of shielding stem cells from environmental insults (Moore and Lemischka, 2006). It was demonstrated that vascular niche could protect glioma stem cells from chemotherapy and radiotherapy (Huang *et al.*, 2010). In fact, it was shown that the postoperative first-line chemotherapy for malignant glioma, temozolomide, was not so effective for glioma stem cells (Liu *et al.*, 2006).

Anti-angiogenic therapy is thought to have the potential to improve such therapy resistance. VEGF was one of the most characteristic permeability factors and it was demonstrated to contribute to BBB breakdown in gliomas directly (Tate and Aghi, 2009). Increased permeability of tumor blood vessels induced by VEGF resulted in elevated interstitial pressure and significant intracerebral edema. The elevated interstitial pressure decreases the transport of medication to tumor cells. In addition, chemotherapy and radiotherapy are less effective in the hypoxic area. Jain et al. proposed that the normalization of tumor vasculature by anti-angiogenic therapy could decrease brain edema, enhance drug delivery and increase radiation sensitivity (Jain, 2005). Vredenburgh et al. has reported that bevacizumab (VEGF inihibitor) in the combination with irinotecan (cytotoxic drug) was an effective treatment for recurrent malignant glioma (Vredenburgh *et al.*, 2007). The normalization of tumor vasculature resulted in decresed interstitial pressure, less hypoxia, and increased delivery of irinotecan to tumor. The auther concluded that the efficacy seen with the combination of bevacizumab and irinotecan could be explained by the anti-tumoral stem cell effect of bevacizumab and by the anti-differentiated glioma tumor cell effect of irinotecan.

Radiation therapy was demonstrated to induce VEGF expression in glioma cells (Hovinga *et al.*, 2005). It was also demonstrated that VEGF blocked the killing of endothelial cell by radiation (Gorski *et al.*, 1999). The radiation-enhanced VEGF secretion was associated with an increased angiogenic potential of the tumor, which thought to be a factor in radioresistance. Anti-VEGF therapy has the potential to prevent VEGF secretion after radiation and enhance radiation sensitivity. Recent studies have shown that glioma stem cells were more resistant to radiation than the matched non-stem glioma cells (Bao *et al.*, 2006a; Rich, 2007). In response to radiation-induced DNA damage, glioma stem cells preferentially activated several critical DNA damage checkpoint proteins. As a result of the preferential DNA damage checkpoint activation, glioma stem cells were more efficient in repairing the damaged DNA and more rapidly recover from the DNA damage than the matched non-stem tumor cells (Bao *et al.*, 2006a). Thus, anti-angiogenic therapy enhances glioma cells' and glioma stem cells' sensitivity for cytotoxic chemotherapy and radiotherapy.

5. VEGF/VEGFR-related therapeutic target of malignant glioma

An upregulation of VEGF family and the VEGF receptors has been shown in malignant gliomas and that can be a target for cancer therapy. In the anti-angiogenic therapy, drugs targeting anti-VEGF/VEGFR pathways have recently attaracted considerable attention.

5.1 VEGF inhibitor

Bevacizumab is a humanized monoclonal antibody that binds to VEGF-A, preventing it from binding to receptors and activating signaling cascades that lead to angiogenesis. The proof of the concept that targeting VEGF-A could inhibit the growth of tumors was demonstrated in a mouse model in 1993 (Kim *et al.*, 1993). The first clinical trial was performed for the treatment of colorectal cancer. The first line chemotherapy (irinotecan, fluorouracil (5-FU), and leucovorin) with bevacizumab for colorectal cancer significantly increased the progression-free survival (PFS), as well as the median overall survival (OS), leading to FDA approval of bevacizumab as the first drug developed for anti-angiogenesis and anti-cancer use in humans (Hurwitz *et al.*, 2004).

Vredenburgh et al. performed a phase II trial to evaluate the efficacy of bevacizumab in combination with chemotherapy for malignant gliomas (Vredenburgh *et al.*, 2007). Bevacizumab and irinotecan were administrated to 32 patients with recurrent high-grade glioma. The radiographic response was observed in 14 of 23 patients (61%). The median PFS for treated patients was 24 weeks, the 6-month PFS was 30%, and the overall median survival time was 42 weeks. This study suggested that bevacizumab in combination with irinotecan is an effective treatment for recurrent glioblastoma. Recently, Albert Lai et al. reported an open-label, prospective, multicenter single-arm phase II study that combined bevacizumab with radiation therapy (RT) and temozolomide (TMZ) for the treatment of newly diagnosed glioblastoma (Lai *et al.*, 2011). In this study, 70 patients with newly diagnosed glioblastoma were enrolled between 2006 and 2008. Patients received standard RT starting within 3 to 6 weeks after surgery with concurrent administration of daily TMZ and biweekly bevacizumab. After completion of RT, patients resumed TMZ for 5 days every 4 weeks and continued biweekly bevacizumab. OS and PFS were 19.6 and 13.6 months, respectively. Patients treated with bevacizumab and TMZ during and after RT showed an improvement of PFS but not OS compared to the control group.

5.2 VEGF-Trap

Aflibercept (VEGF-Trap, AVE0005) is a soluble fusion protein of the human extracellular domains of VEGFR-1 and VEGFR-2 and the Fc portion of human immunoglobulin (Ig) G. Aflibercept binds to both VEGF-A and PlGF with higher affinity than monoclonal antibodies and prevents theVEGF-A and PlGF ligands from binding and activating cell receptors. In vitro, aflibercept was shown to have an antiproliferative activity and completely blocked the VEGF-induced VEGFR-2 phosphorylation (Sullivan and Brekken, 2010). In xenograft models, tumor growth and tumor-associated angiogenesis were inhibited by aflibercept (Holash et al., 2002). Candelaria Gomez-Manzano et al. reported that treatment of animals bearing human gliomas with VEGF Trap resulted in a significant increase in the mean survival (Gomez-Manzano et al., 2008).

5.3 VEGFR inhibitor

In addition to VEGF inhibitors, small molecule inhibitors of VEGFR have been tested in recurrent malignant gliomas. They often target more than one type of receptor and affect

both endothelial cells and cancer cells because the receptors are expressed on both types of cells. Because the target kinase specificity between inhibitors can vary, different compounds have shown various levels of efficacy and activity between cancers (Sullivan and Brekken, 2010; Kowanetz and Ferrara, 2006). Some of these agents include compounds that bind to the ATP binding site of the RTK, which block receptor activation, or with antibodies that bind to the growth factors or their receptors, which prevent the binding and subsequent activation of receptors.

Cediranib (AZD2171) inhibits all known subtypes of VEGFR and has been evaluated in a phase 2 trial of patients with recurrent glioblastoma (Batchelor *et al.*, 2010; Batchelor *et al.*, 2007). Results were comparable to those reported for bevacizumab, with a response rate of 56% and a 6-month PFS rate of 26%. A striking steroid-sparing effect was observed. The drug was largely well tolerated, with hypertension, diarrhea, and fatigue as the most common adverse effects. Using dynamic contrast-enhanced MRI, it was demonstrated that cediranib therapy reduced blood vessel size and permeability. These are the first clinical data to support the hypothesis that antiangiogenic therapy may transiently "normalize" the dilated, abnormally permeable tumor vasculature. Other VEGFR inhibitors in which clinical studies were conducted for malignant gliomas are listed in Table 1.

Inhibitor	Primary targets (other targets)	Mechanism of action
Bevacizumab	VEGF-A	Monoclonal antibody
Aflibercept	VEGF-A (VEGF-B, PlGF)	Soluble decoy receptor
Cediranib	VEGFR-2 (other VEGFR, PDGFR-β, c-kit)	Tyrosine kinase inhibitor
Sorafenib	VEGFR-2 (other VEGFR, Raf, PDGFR-β, c-kit, Ras, RET)	Tyrosine kinase inhibitor
Sunitinib	VEGFR-2 (other VEGFR, PDGFR-β, Flt3, c-kit)	Tyrosine kinase inhibitor
Pazopanib	VEGFR-2 (other VEGFR, PDGFR-α,β,c-kit)	Tyrosine kinase inhibitor
Vandetanib	VEGFR-2 (other VEGFR, EGFR)	Tyrosine kinase inhibitor
Vatalanib	VEGFR-1,2 (PDGFR-β, c-kit)	Tyrosine kinase inhibitor
Brivanib	FGFR (VEGFR)	Tyrosine kinase inhibitor
CT-322	VEGFR-2 (other VEGFR)	Adnectin
XL-184	VEGFR-2 (c-Met, RET, c-Kit, Flt3, Tie-2)	Tyrosine kinase inhibitor

Table 1. VEGF/VEGFR related anti-angiogenic drugs in clinical development for high-grade glioma

6. Summary and future outlook

The existence of glioma stem cells prompts us to review the cancer biology. Gliomas appear to have a cellular hierarchy rather than a bulk of equally potent tumor cells. Within this hierarchy, glioma stem cells have an extraordinary capacity for tumor-initiation, and glioma stem cells are thought to be attractive targets for anti-glioma therapies. The development of novel treatments against glioma stem cells is expected as an urgent task. Glioma stem cells'

properties are maintained with vascular niche, and vascular niche is maintained with VEGF secreted by glioma stem cells. Glioma stem cells and vascular niche make the bi-directional functional units. Of anti-angiogenic factors, VEGF family/VEGFRs are one of the most important therapeutic targets for these units.

As the use of anti-angiogenic drugs such as bevacizumab becomes widespread, some problems surfaced. Firstly, the potential side effects that occur in the short-term or long-term use of angiogenesis inhibitors are becoming apparent (Norden *et al.*, 2008). These side effects include gastrointestinal perforations, impaired wound healing, bleeding, hypertension, proteinuria, and thrombosis. These occurrences are unpredictable and further studies are needed to measure the risk for patients, understand the cause of complications, and find prophylactic measures to minimize risk. Secondly, it has been found that most tumors become to be resistant to anti-angiogenic drugs as a consequence of the long-term administration of anti-angiogenic drugs (Norden *et al.*, 2008). Because multiple signaling pathways are involved in angiogenesis, blocking a single pathway may not be highly effective. The administration of single anti-angiogenic agent can lead tumors to acquire the resistance when the tumor cells develop other angiogenesis pathways. The combination drug therapies targeting multiple pathways may be able to overcome this problem. Therefore recently, clinical trials of combination drug therapies are performed such as other anti-angiogenic drugs, cytotoxic drugs or anti-invasion drugs (Van Meir *et al.*, 2010). Thirdly, the further studies are needed to evaluate the administration protocol of anti-angiogenic agents. For example, metronic dosing, which is administration of small doses of drugs in a rapid cycle, has the potential to improve in outcomes over standard dosing (Kesari *et al.*, 2007). Kesari S et al. performed the phase 2 study of metronomic four drugs chemotherapy (etoposide, cyclophosphamide, thalidomide and celecoxib) for reccurent malignant gliomas (Kesari *et al.*, 2008). Although this study did not show a significant improvement of OS in heavily pretreated patients who were generally not eligible for conventional protocols, there were some responders. It was suggested that further studies using metronomic chemotherapy combined with more potent anti-angiogenic agents in patients with less advanced disease may be warranted.

Brain tumor stem cells have a bi-directional relationship with vascular niche. Anti-angiogenic therapy is the strategy targeting for vascular niche, which may result in the strategy targetting for brain tumor stem cells. Accumulated evidence suggests that VEGF family/VEGFRs are strongly related with the biology of brain tumor stem cells. Thus VEGF family/VEGFRs signaling pathway is expected to be one of the most important targets of anti-angiogenic therapies for malignant gliomas.

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Over the last thirty years, the foremost inspiration for research on metastasis, cancer recurrence, and increased resistance to chemo- and radiotherapy has been the notion of cancer stem cells.The twenty-eight chapters assembled in Cancer Stem Cells - The Cutting Edge summarize the work of cancer researchers and oncologists at leading universities and hospitals around the world on every aspect of cancer stem cells, from theory and models to specific applications (glioma), from laboratory research on signal pathways to clinical trials of bio-therapies using a host of devices, from solutions to laboratory problems to speculation on cancersâ€™ stem cellsâ€™ evolution. Cancer stem cells may or may not be a subset of slowly dividing cancer cells that both disseminate cancers and defy oncotoxic drugs and radiation directed at rapidly dividing bulk cancer cells, but research on cancer stem cells has paid dividends for cancer prevention, detection, targeted treatment, and improved prognosis.

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