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Phenotypic Transition as a Survival Strategy of Glioma

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Abstract

Malignant glioma is characterized by rapid proliferation, invasion into surrounding central nervous system tissues, and aberrant vascularization. There is increasing evidence that shows gliomas are more complex than previously thought, as each tumor comprises considerable intratumoral heterogeneity with mixtures of genetically and phenotypically distinct subclones. Heterogeneity within and across tumors is recognized as a critical factor that limits therapeutic progress for malignant glioma. Recent genotyping and expression profiling of gliomas has allowed for the creation of classification schemes that assign tumors to subtypes based on similarity to defined expression signatures. Also, malignant gliomas frequently shift their biological features upon recurrence and progression. The ability of glioma cells to resist adverse conditions such as hypoxia and metabolic stress is necessary for sustained tumor growth and strongly influences tumor behaviors. In general, glioma cells are in one of two phenotypic categories: higher proliferative activity with angiogenesis, or higher migratory activity with attenuated proliferative ability. Further, they switch phenotypic categories depending on the situation. To date, a multidimensional approach has been employed to clarify the mechanisms of phenotypic shift of glioma. Various molecular and signaling pathways are involved in phenotypic shifts of glioma, possibly with crosstalk between them. In this review, we discuss molecular and phenotypic heterogeneity of glioma cells and mechanisms of phenotypic shifts in regard to the glioma proliferation, angiogenesis, and invasion. A better understanding of the molecular mechanisms that underlie phenotypic shifts of glioma may provide new insights into targeted therapeutic strategies.

Key words: angiogenesis, glioma, invasion, phenotype

Introduction

Malignant glioma is characterized by rapid proliferation, invasion into surrounding central nervous system (CNS) tissues, and aberrant vascularization.[1)] The tumors consist of a core mass and a penumbra of invasive, single cells, decreasing in numbers towards the periphery. Two major aspects of glioma biology are the formation of new blood vessels through angiogenesis and the invasion of glioma cells via white matter tracts, which are the hallmarks of glioblastoma (GBM). The diffusely infiltrative nature of GBM is the main obstacle for the development of effective treatments. There is accumulating evidence that invasive glioma cells show a decreased proliferation rate and a relative resistance to apoptosis, which may contribute to chemotherapy and radiation resistance. Thus, deep infiltration and resistance to irradiation and chemotherapy remain a major cause of patient mortality. The standard therapy for GBM is maximal safe resection and adjuvant irradiation of the tumor bed with concomitant temozolomide.[2)] Despite recent advances in treatment with surgery, radiation, and chemotherapy, less than 10% of patients with GBM survive beyond 5 years after diagnosis.

Heterogeneity within and across tumors is recognized as a critical factor that limits therapeutic progress for malignant glioma. Also, malignant gliomas frequently shift their biological features upon recurrence and progression. Therefore, elucidation of mechanisms underlying phenotypic heterogeneity and shift is necessary for the development of curative therapies for malignant glioma. In this review, we discuss molecular and phenotypic heterogeneity of glioma cells and mechanisms of phenotypic shift in regard to glioma proliferation, angiogenesis, and invasion.

Clinical Evidence of Heterogeneity in Glioma

As the term glioblastoma "multiforme" indicates, the histopathology of this tumor type is extremely

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variable. The heterogeneity can vary both across patients as well as spatially in each tumor. Intertumoral heterogeneity is remarkable upon pathologic evaluation. While some lesions show a high degree of cellular pleomorphism with numerous multinucleated giant cells, others are higher in cellular number but rather monotonous. Intratumoral regional heterogeneity is also remarkable[3] (Fig. 1A). The center of the tumor comprises an area of high-density tumor cells (Fig. 1B). Necrosis and pseudopalisading glioma cells are seen in the core of the tumor. Marked angiogenesis, which is characterized by thick endothelial proliferation, is seen in and around the core. These new tumor-induced vascular channels are structurally abnormal and to varying degrees lack the normal blood-brain barrier (BBB). At the borders, clusters of tumor cells are observed around dilated neovasculatures (Fig. 1C). Diffuse single cell infiltration from the tumor core to the surrounding normal brain parenchyma is observed, thus rendering the border between the tumor and normal brain tissue indistinct. Scherer showed an infiltrative growth pattern that was associated with distinct anatomic structures, that is, tumor cells followed myelinated axons and the basement membranes of blood vessels.[4] Infiltration by single cells distribute far beyond the tumor core (Fig. 1D). In distant areas, such as the cerebral cortex overlaying the tumor mass, scattered tumor cells are also found; however, neither dilated vessels nor increased vascular density is seen in this area. We have shown that there are at least two invasive phenotypes: cluster formation around neovascular vessels, and single cell infiltration into normal parenchyma.[3]

Many generalized associations have been established linking anatomical imaging traits with underlying histopathology. Magnetic resonance (MR) imaging findings of malignant glioma reflect their pathologically heterogenic features as well.[5] The most common imaging appearance of GBM is a large heterogeneous mass that exerts considerable mass effect. Remarkable gadolinium enhancement is seen in the main mass where tumor cells actively proliferate and induce angiogenesis (Fig. 2A). Contrast enhancement is a result of extravasation of contrast media through leaky neovasculature where the BBB is disrupted. Large necrotic areas usually occupy the tumor center, while viable tumor cells and abnormal vessels tend to accumulate in the periphery corresponding to the contrast-enhancing ring seen radiographically. In the advancing edge, tumor cells invade into normal parenchyma, resulting in a vague border of enhancement. On T_2-weighted MR images, a broader area of

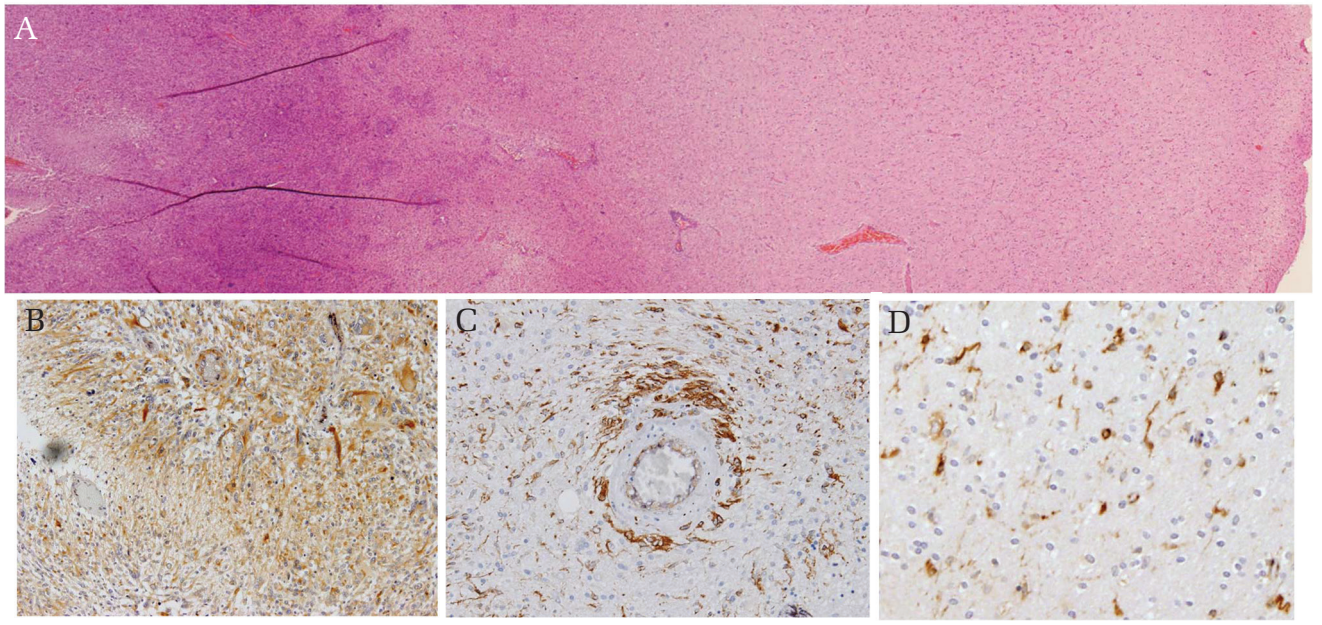

Fig. 1 Pathologic photograph of human glioblastoma samples showed intratumoral regional heterogeneity. The tumors consist of a core mass (*left side*) and a penumbra of invasive, single cells, decreasing in numbers towards the periphery (*right side*) (hematoxylin and eosin) (A). The center of the tumor comprises an area of high-density tumor cells (immunohistochemical staining with glioma-specific MAP2e antibody) (B). At the borders, clusters of tumor cells were observed around dilated neovasculatures (C). In distant areas, scattered tumor cells were also found; however, neither dilated vessels nor increased vascular density were seen in this area (D).

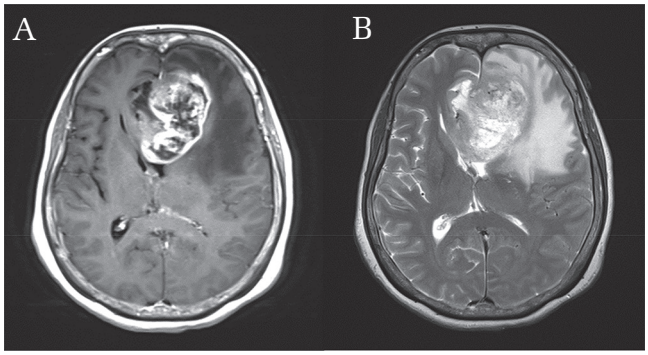

Fig. 2 Magnetic resonance imaging findings of GBM reflect their pathologically heterogenic features. On gadolinium-enhanced T_1-weighted images (A), remarkable enhancement is seen in the main mass, a result of extravasation of contrast media through BBB-disrupted neovasculature. Large necrotic areas occupy the tumor center, while viable tumor cells and abnormal vessels tend to accumulate in the periphery. On T_2-weighted MR images (B), a broader area of high intensity reflects increased water mobility in areas of both edema and invasive tumor.

high intensity that overlays the contrast enhancing area is usually seen (Fig. 2B). These areas reflect increased water mobility in areas of both edema and invasive tumors.[6] Gadolinium enhancement on the T_1-weighted image is not seen in this area since BBB-disrupted neovasculature is absent.

Molecular Heterogeneity of Glioma Cells

Although morphological heterogeneity in GBM is observed pathologically, the inability to define different patient outcomes on the basis of pathologic features illustrates a larger problem in our understanding of the classification of GBM. Recent genotyping and expression profiling of human gliomas has allowed for the creation of classification schemes for high-grade gliomas that assign tumors to subtypes based on similarity to defined expression signatures. Malignant gliomas can be categorized into four subtypes: proneural, neural, classical, and mesenchymal based on The Cancer Genome Atlas (TCGA) study.[7–9] The proneural subtype, which shows high expression of the genes implicated in neurogenesis, is associated with better clinical outcome, especially with IDH-1 mutation and PDGFRA expression. In contrast, the mesenchymal subtypes are characterized by more aggressive phenotypes, presumably due to high expression of genes related to cellular proliferation and angiogenesis.[10] There is increasing evidence that gliomas are more complex than previously thought, as each tumor comprises considerable intratumoral heterogeneity with mixtures of genetically and phenotypically distinct subclones. Recent studies have uncovered genetic diversity in single cells of an individual GBM patient.[11,12] Although the original classification scheme established by TCGA was established from bulk tumor profiles, they showed that individual cells within a tumor vary in classification. Understanding the links between genetic and functional behavior of individual GBM clones, derived from single patient samples, will be essential to decipher patient-specific molecular mechanisms of GBM progression and therapeutic resistance.

Evidence of Phenotypic Shift

It has been reported that malignant gliomas frequently shift their biological features upon recurrence and progression. For example, malignant gliomas shift towards the mesenchymal subclass upon recurrence,[7,13,14] although the underlying molecular mechanisms have not yet been elucidated. In some patients with recurrent disease, such phenotypic shift is assumed to be induced by therapy.[7]

Radiotherapy has been found to promote the invasion of various kinds of cancer cells including GBM.[15–17] Fractionated sublethal doses of radiation activate multiple signaling pathways in tumor cells, which modulate several cellular functions and induce the secretion of growth factors and chemokines, resulting in increased migration and invasiveness of cancer cells.[18–21] Also, radiation contributes to the acquisition of radioresistance of the tumor cells.[22]

GBM is characterized by extensive microvascular proliferation and high expression levels of proangiogenic cytokines, highlighting the potential value of treatments targeting angiogenesis. Antiangiogenic treatment likely achieves a beneficial impact through multiple mechanisms of action. Although the addition of bevacizumab, a humanized monoclonal antibody against vascular endothelial growth factor (VEGF) to the conventional standard therapy (chemoradiotherapy with temozolomide) for newly diagnosed GBM prolonged the progression-free survival time and the performance status of patients, it provides only limited overall survival benefits.[23] Also, current anti-angiogenic therapies have revealed several unanticipated problems. de Groot et al. showed that glioma cases developed an apparent phenotypic shift to a predominantly infiltrative pattern of tumor progression after treatment with bevacizumab.[24]

Mechanisms of Phenotypic Shift

The ability of cancer cells to resist adverse conditions such as hypoxia and metabolic stress is necessary

for sustained tumor growth and strongly influences tumor behaviors, including proliferation, survival, angiogenesis, and migratory capacity. There is growing evidence that antiglioma treatments may induce the phenotypic shift of a tumor by selecting highly invasive tumor cells or hypoxia-resistant cells, by up-regulating alternative pathways resistant to treatment, or by up-regulating genes triggering new invasive programs.

Glioma cells are known to shift their phenotype to survive metabolic stress and seek out favorable growth conditions. Glioma cells have two strategies to escape from hypoxic conditions: to induce angiogenesis, or to migrate to normoxic normal parenchyma. Glioma cells near starvation and hypoxia increase their movement intensity and diffusiveness in the search for nutrients and oxygen when compared to their foraging activity under normal conditions. Under scarce environmental conditions, glioma cells presenting such behavior can have an adaptive advantage.

Hypoxia is a common feature of most cancers. Recently, several biological studies have linked hypoxia to the invasive behavior of tumors. In particular, it has been observed that hypoxia is responsible for down-regulation of cadherins, resulting in the disruption of cell–cell adhesive interactions, the promotion of invasive and metastatic behavior,[25] and the reduction of proliferative activity.[26] These biological observations support the hypothesis that hypoxia triggers the switch from a proliferative to an invasive phenotype. The invasion/proliferation switch depends on the oxygen level.

To sustain tumor growth, cancer cells adapt to fluctuations in energy availability. The metabolic capacity to use glycolysis as an energy source under aerobic conditions, known as the Warburg effect, is characteristic of cancer cells. Glioma cells also tend to present with a metabolic preference for glycolysis. However, recent studies have revealed that the metabolic characteristics of glioma cells are not as uniform as initially thought. Saga et al. showed that there are at least two types of glioma-initiating cells different in glucose consumption.[27] They isolated two clones: clone A relies mainly on glycolysis for energy production, and clone B relies more on mitochondrial respiration. These clones can switch metabolic preference. Godlewski et al. showed that phenotypic shift is regulated by metabolic stress.[28] They claim that an abundance of nutrients allows for high expression of miR-451, which promotes high proliferation. On the other hand, in scarce environments miR-451 levels are decreased, slowing the proliferation and enhancing the migration of the glioma cells. miR-451 is a regulator of the LKB1/AMPK pathway, and this may represent a fundamental mechanism that contributes to cellular adaptation in response to altered energy availability.

As proposed, malignant gliomas can be categorized into four molecular subtypes and contribute to heterogeneity in this tumor type. However, phenotypic and molecular shifts may blur the boundaries between the proposed subtypes. It is not known if shifting represents the accumulation of genetic changes inherent in the progression of the tumor or if treatment itself can accelerate this transition. The latter scenario highlights the importance of understanding the impact of a treatment on the biologic response and selective pressure within the tumor and its subsequent behavior. There are three major hypotheses that explain phenotypic shift of glioma.[29,30] 1) Selection of coexisting subclones. Each glioma is comprised of mixtures of genetically distinct subclones within the same tumor. Due to selection pressure triggered by antiglioma treatment or environmental changes, certain types of tumor cell lineages, which are naïve to the pressure and constitute the majority of the tumor, may be eliminated. As a result, another cell lineage that is resistant and has different features may dominate the residual tumor. 2) Phenotypic conversion by mutation. It is widely believed that tumor cells change their phenotype due to mutations that are acquired during cancer progression. However, the short time required for the recurrence of malignant glioma after treatment cannot be deduced solely from a mutation-based theory. 3) Molecular switch. A certain molecule acts as a molecular switch to change phenotypes in the absence of genetic change or mutation. To address the mechanisms of phenotypic shifting of glioma, there are several lines of evidence from the molecular analysis of clinical samples and in the experimental setting.

I. Epithelial–mesenchymal transition

Epithelial–mesenchymal transition (EMT) was originally described as a critical mechanism in embryonic development induced by a range of intrinsic and extrinsic factors including transforming growth factor (TGF)-β,[31] epidermal growth factor (EGF),[32] hepatocyte growth factor (HGF),[33] and various other cytokines. All of these transcription factors are indispensable for embryonic development, and they play a spatiotemporally distinct role during embryonic development.[34] Several studies have shown that EMT is also related to wound healing, tissue remodeling, and invasion of cancer including malignant glioma.[35,36] Recent studies have established that TGF-β is a master regulator of EMT in various cancers, such as breast, prostate and lung

cancer, leading to enhanced invasive and metastatic capacities of these cells.[37,38] Similar mechanisms have a major impact on subtype status and tumor invasion in GBM. Joseph et al. identified TGF-β signaling as a strong inducer of EMT in GBM.[39] They also showed TGF-β signaling involves activation of SMAD2 and ZEB1, known transcriptional inducers of mesenchymal transition in epithelial cancers. TGF-β exposure of established and newly generated GBM cell lines was associated with morphological changes, enhanced mesenchymal marker expression, and migration and invasion in vitro and in an orthotopic mouse model. On the contrary, Zhang et al. showed that the blockade of TGF-β signaling using TGF-β receptor (TGFβR) I kinase inhibitor (LY2109761) markedly reduced the expression of mesenchymal markers in the orthotopic GBM model.[40] They also conducted a preclinical study of the antitumor effects of LY2109761 in combination with radiotherapy. Histologic analyses showed that LY2109761 inhibited tumor invasion promoted by radiation, reduced tumor microvessel density, and attenuated mesenchymal transition. TGF-β can be a therapeutic target to suppress tumor progression. The c-Met receptor and its ligand scatter factor/hepatocyte growth factor (SF/HGF) are strongly overexpressed in malignant gliomas. Signaling through c-Met as well as exposure to hypoxia can stimulate glioma cell migration and invasion. Eckerich et al. showed that approximately half of both the cell lines and the primary cultures of human GBM respond to hypoxia with an induction of c-Met, which can enhance the stimulating effect of SF/HGF on tumor cell migration.[41] Mahabir et al. found that the expression levels of mesenchymal markers were increased in clinically recurrent malignant glioma. These markers included those related to EMT, such as vimentin, fibronectin, α-SMA, collagen, and matrix-metalloproteinase (MMP), and those related to the mesenchymal subtype based on the TCGA study, such as CD44 and YKL-40. In addition, they identified Snail as the master regulator of the radiation-induced transition possibly through the phosphorylation of GSK-3β and extracellular signal regulated kinase (ERK)1/2, resulting in the promotion of migration and invasion.[42] Wnt/β-catenin signaling plays important roles in maintaining the stemness of cancer stem cells, and it is an important regulator that promotes cellular invasiveness through regulation of EMT in many neoplasms including glioma.[43] Dong et al. examined the pro-invasive effects of irradiation on U87 cells and demonstrated a pivotal role for the Wnt/β-catenin pathway in radiation-induced invasion of GBM cells.[44] VEGF is a negative regulator of EMT. Lu et al. demonstrated that VEGF enhanced recruitment of the protein tyrosine phosphatase 1B (PTP1B) to a MET/VEGFR2 heterocomplex, thereby suppressing HGF-dependent MET phosphorylation and tumor cell migration.[45] Consequently, VEGF blockade restores and increases MET activity in GBM cells in a hypoxia-independent manner, while inducing EMT. This finding is compatible with clinical evidence that anti-VEGF therapy induces glioma invasion. Also, they showed it was a possible therapeutic strategy. Inhibition of MET in the GBM mouse models blocks mesenchymal transition and invasion provoked by VEGF ablation, resulting in a substantial survival benefit.

II. Proneural–mesenchymal shift

Radiotherapy has been found to induce a phenotypic shift away from a proneural expression pattern toward a mesenchymal one in malignant gliomas. Analysis of paired specimens from primary and recurrent tumors has indicated that there is a shift from a proneural to mesenchymal phenotype at the time of tumor recurrence.[7] Halliday et al. showed a proneural to mesenchymal shift after radiation using an in vivo glioma model.[46] In their study, besides changes in regulators of the radiation response such as p53 and E2F, targets for Stat3 and CEBPB were up-regulated by radiation. Also, IL-15, LIF, and IL-7 (activators of Stat3 via gp130/JAK) are all among the most up-regulated transcripts following radiation. These data suggest that cytokine-mediated activation of the JAK/STAT pathway may drive the proneural to mesenchymal shift. In another study, microglia-derived TNFα was reported to induce a mesenchymal phenotype in a subset of proneural GBM neurospheres through activation of NF-κB.[47] The NF-κB and JAK/STAT pathways are known mechanisms of EMT. While it is not known whether the proneural to mesenchymal shift is equal to EMT, both shifts lead to increased invasive behavior of the glioma cells. At the very least, it seems that there is crosstalk between two phenomena.

III. Migration/proliferation dichotomy (Go or Grow mechanism)

At the macroscopic level, the progression speed of a GBM tumor is determined by two key factors: the cell proliferation rate and the cell migration speed. Although uncontrolled proliferation and extensive cell migration are two of the main characteristics of malignant glioma growth, proliferation, and migration appear to be mutually exclusive phenotypes at the single cell level. Experiments with cultured glioma cells have shown a relationship between migratory and proliferative behavior. Berens et al. observed that tumor cells harvested from the vital core of a GBM

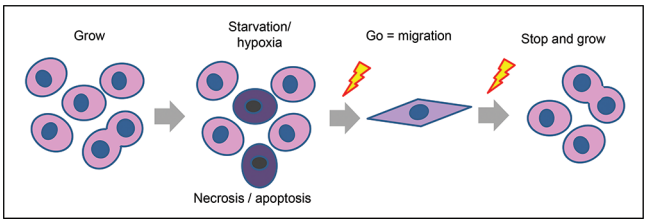

Fig. 3 Schematic illustration of migration/proliferation dichotomy (Go-or-Grow mechanism) theory. According to the theory, glioma cells are in one of two categories: proliferative or migratory. They grow when oxygen and nutrients are rich. They start migrating when under metabolic stress (starvation/hypoxia). They switch phenotypes depending on the environment.

rapidly grew to large colonies in soft agar, whereas cells that were plated from regions of invaded brain developed smaller colonies.[48] On the other hand, when they were tested for migration in vitro, cells derived from invaded brain had higher motility rates compared with cells from solid tumor. These results suggest that invasive glioma cells are more likely migratory than proliferative, i.e., cells proliferate only when they do not move. This phenomenon is known as the migration/proliferation dichotomy or 'Go or Grow' mechanism (Fig. 3).[29,49,50] A phenotypic shift from one to the other is known. Hatzikirou et al. proposed that the transition to invasive tumor phenotypes can be explained in the context of the microscopic 'Go or Grow' mechanism (migration/proliferation dichotomy) when studying a hypoxic environment of a growing tumor with the help of a simple growth model, a lattice-gas cellular automaton.[29] On the other hand, reverse phenotypic shift from invasive to proliferative may occur. Undetectable invasive glioma cells using conventional neuroimaging techniques become detectable when they generate a recurrent, satellite lesion either adjacent to or far from the resection margin. This means migratory tumor cells, in fact, do become proliferative at some point in their biology.[50]

IV. Angiogenesis-invasion shift

In rapidly growing tumors such as GBM, where oxygen and glucose may fluctuate, cells must engage adaptive strategies to survive periods of hypoxic and metabolic stress. Glioma cells ensure an adequate oxygen and glucose supply through increased angiogenesis or migration. For example, anti-angiogenic therapy paradoxically enhances tumor progression by promoting an invasive phenotype that allows the tumor to escape angiogenesis inhibition. Two independent phenomena, angiogenesis and invasion, link together and they are mutually exclusive in phenotypic expression of each cell. Bikfalvi identified a molecular mechanism in tumor cells that allows the switch from an angiogenic to invasive program.[51] Their results indicate that anti-angiogenesis treatment in the experimental glioma model drives expression of critical genes which relate to disease aggressiveness in GBM patients. We searched for key factors regulating angiogenesis and invasion of malignant gliomas using our novel animal models.[3,52,53] We have established two sibling glioma subclones, J3T-1 and J3T-2, showing different invasive and angiogenic phenotypes. One showed angiogenesis-dependent cell growth and no single-cell invasion, and the other showed massive single-cell invasion without angiogenesis. First, we demonstrated that annexin A2 is expressed at higher levels in J3T-1 than J3T-2 cells by proteomic analysis.[52] Next, the function of annexin A2 in relation to angiogenesis and invasion was investigated using these models by silencing annexin A2 in J3T-1 (J3T-1shA) cells or by overexpressing annexin A2 in J3T-2 (J3T-2A) cells.[53] Histopathologic analysis of animal brain tumors revealed that J3T-1 and J3T-2A tumors displayed marked angiogenesis and tumor cooption along the neovasculature, whereas J3T-2 and J3T-1shA tumors exhibited diffuse, infiltrative invasion without angiogenesis. Immunohistochemical analysis of human GBM samples confirmed higher expression of annexin A2 in tumor cells clustered around neovasculatures, but not in diffusely invasive tumor cells. According to our results, annexin A2 is one of the key factors regulating angiogenesis and invasion of malignant gliomas (Fig. 4).

Conclusion

In general, glioma cells are in one of two phenotypic categories: higher proliferative activity with angiogenesis, or higher migratory activity with attenuated proliferative ability. Further, they switch phenotypic status to survive depending on the situation. To date, a multidimensional approach has been employed to clarify the mechanisms of phenotypic shifts of glioma. Various molecular and signaling pathways are involved in phenotypic shifts of glioma, possibly with crosstalk between them. Further studies are necessary to fully elucidate these mechanisms, which may provide new insights into targeted therapeutic strategies to treat gliomas.

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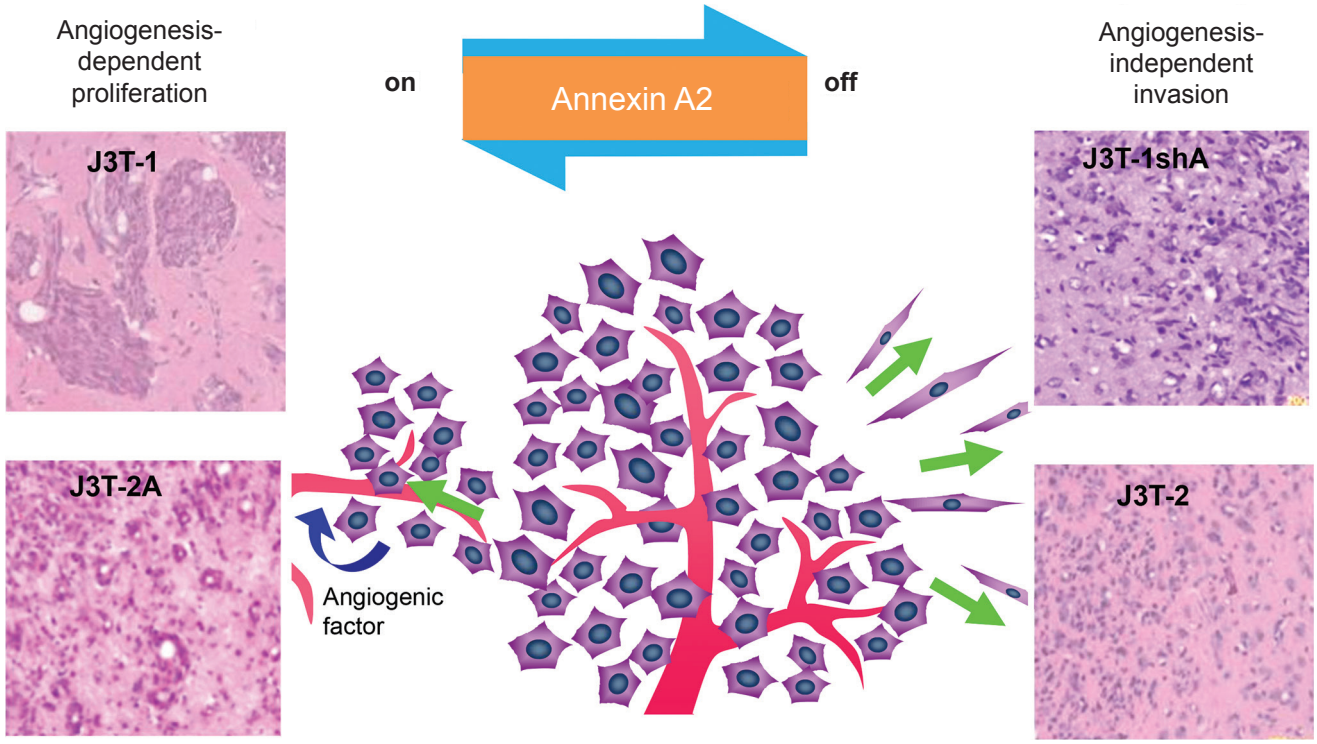

Fig. 4 We functionally analyzed annexin A2 in our animal glioma models. J3T-1 cells originally show remarkable angiogenesis and proliferation around neovasculatures, whereas J3T-2 cells show broad single cell infiltration without angiogenesis. Transduction (J3T-2A) or suppression (J3T-1shA) of annexin A2 resulted in reversal of phenotype. Therefore, annexin A2 is a key factor that switches the phenotype of the glioma cell.

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Conflicts of Interest Disclosure

The authors declare that there are no conflicts of interest with regard to this manuscript. All authors have registered online Self-reported COI Disclosure Statement Forms through the website for JNS members.

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