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Heterogeneity Maintenance in Glioblastoma: a social network

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Abstract

Glioblastoma multiforme (GBM), the most common intracranial tumor in adults, is characterized by extensive heterogeneity at the cellular and molecular levels. This insidious feature arises inevitably in almost all cancers and has great significance for the general outcome of the malignancy because it confounds our understanding of the disease and also intrinsically contributes to the tumor's aggressiveness and poses an obstacle to the design of effective therapies. The classical view that heterogeneity arises as the result of a tumor's "genetic chaos", as well as the more contemporary cancer stem cell (CSC) hypothesis tend to identify a single cell population as the therapeutic target: the prevailing clone over time in the first case and the CSC in the latter. However, there is growing evidence that the different tumor cell populations may not be simple bystanders. Rather, they can establish a complex network of interactions between each other and with the tumor microenvironment that eventually strengthens tumor growth and increases chances to escape therapy. These differing but complementary ideas about the origin and maintenance of tumor heterogeneity and its importance in GBM will be reviewed here.

Introduction

As instructed by sociology and population genetics, diversity confers an evolutionary advantage to humans and other organism communities, while the lack of diversity is synonymous with vulnerability. Likewise, tumor cells can be considered as members of a society where the presence of distinct phenotypes is the key for adaptation to fluctuations in their environment that can be both intrinsic to tumor progression and extrinsically induced by radio- or chemo-therapy. Tumors are almost never composed of a single homogeneous population, but rather by a heterogeneous ensemble of cells that differ in many biological features, such as morphology, proliferation rate, invasive behavior, metastatic potential, and drug resistance.

For example, an early study of the mouse B16 melanoma model (1) demonstrated that different clones derived from a parental tumor had quite different metastatic capabilities when injected into syngeneic mice. Consistent with this, a variety of different clones isolated from a single GBM tumor displayed a wide range of sensitivity to chemotherapeutics (2). These two features, widespread metastasis and drug resistance, are the most common causes

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of treatment failure, and therefore constitute among the most urgent challenges for cancer research and therapy.

In this review, we focus on different aspects of heterogeneity, with particular attention to how the intratumoral and tumor-microenvironment interactions operate in GBM to maintain the heterogeneous tumor composition and to promote tumor growth. We also discuss different theories on the origins of heterogeneity and its relevance in drug treatment. A better understanding of how heterogeneity is maintained, and the molecules responsible in promoting and maintaining this complex tumor network environment, should help drive the design of more effective therapies against this highly malignant brain tumor.

Glioblastoma multiforme: a heterogeneous disease

GBM is the most common and malignant type of brain tumor in adults, and is characterized by diffuse infiltration throughout the brain parenchyma, robust angiogenesis, intense resistance to apoptosis, necrogenesis, and genomic instability (3). The moniker – 'multiforme' – derives from the first histopathological descriptions of its varied morphological features and the presence of heterogeneous cell populations within a single tumor, where lesions with a high degree of cellular and nuclear polymorphism and numerous giant cells coexist with areas of high cellular uniformity (4).

A molecular basis of heterogeneity in gliomas was evidenced by early studies that found markedly different karyotypes among cells freshly isolated from clinical specimens (5) or even within an established cell line (6), and variable expression of antigenic markers (6). Consequences of this heterogeneity were reflected in the *in vitro* phenotype of those cells: in their morphologies, growth rates, and, most importantly, their drug responses (2). Similarly, analysis by CGH of several microdissected regions of individual GBM tumors demonstrated the presence of area-specific chromosomal aberrations in addition to other aberrations common to the whole tumor (7). Other studies (8) demonstrated intratumoral cytogenetic heterogeneity that is correlated with DNA aneuploidy and caused by the high genetic instability characteristic of GBM. Also mutation of p53, which is known as one of the earliest and more common events in gliomagenesis, is usually detected only in a subset of tumor cells, even in low-grade gliomas, suggesting that p53 mutation is not an initiating event in these tumors (9). Another example of a gene heterogeneously expressed in GBM is O6-methylguanine–DNA methyltransferase (MGMT), which is detected in distinct areas of positive tumor cells surrounded by negative cells (10). The principal mechanism for MGMT silencing is promoter hypermethylation and silencing of MGMT in GBM has been correlated with a better response to alkylating agents such as temozolomide.

One signature example of a molecular marker that is not uniformly expressed in GBMs is the common truncated EGFR mutant known as ΔEGFR (also known as EGFRvIII or EGFRde2-7), which results in a ligand-independent constitutively active receptor with potent tumorigenic activity (11). The occurrence of this mutation is typically associated in GBM tumor masses with the amplification and over-expression of wild-type EGFR (wtEGFR) (12). Paradoxically, despite ΔEGFR's potent ability to enhance tumorigenicity (which is not shared by the wtEGFR), its expression is typically observed only in a subpopulation of cells and almost never in the entirety of the tumor. For example, in one study specific ΔEGFR immunostaining of human GBM samples demonstrated that ΔEGFR-positive cells were scattered diffusely or showed geographical distribution within the tumors and only 1/20 cases showed homogeneous staining (13). This disconnect between oncogenic potential and the frequencies and proportions of amplified ΔEGFR and wtEGFR in high-grade gliomas might arise if mutant EGFR-expressing cells occur later in tumor progression and never have the time to become homogeneous in these rapidly fatal tumors. However,

our recent data support an alternative, although not mutually exclusive, possibility that the minority of cells that express ΔEGFR not only enhance their own intrinsic tumorigenic abilities, but also actively potentiate the proliferation of the neighboring majority of tumor cells expressing amplified wtEGFR (14). Interestingly in this regard, non-uniform patterns of expression in GBM have also been demonstrated for other growth factor receptors (15–16), angiogenic factors (17), adhesion molecules (18), suggesting in some cases the existence of functional subdomains within tumor masses.

Alternative theories to explain the origin of heterogeneity: clonal evolution vs cancer stem cell

Cancer has long been considered to be an evolutionary process where natural selection occurs and better adapted clones survive and are responsible for the tumor growth. In this light, tumor heterogeneity is thought to arise from clonal evolution, where even though most non-hereditary tumors have a monoclonal initiation, the expansion and acquisition of mutations during tumor progression promotes genetic variability and an increase in tumor heterogeneity. Under selective pressures, such as chemo- or radio-therapy, clones that have acquired resistant properties survive (Figure 1A) (19). More recently, a different idea has been proposed to explain tumor heterogeneity: the cancer stem cell (CSC) model (Figure 1B). This theory postulates a hierarchical organization where a tumor arises from and is maintained by a small subpopulation of CSCs, which are also inherently responsible for the drug resistance and tumor relapse observed after treatment. These cells are able to self-renew and to give rise to phenotypically diverse non-tumorigenic daughter cells with limited division properties that can differentiate and that compose the bulk of the tumor. CSCs have been identified in several types of cancer, including GBM. Initially, the expression of the surface marker CD133 (prominin-1) appeared to be a robust marker of brain tumor stem cells, and cells lacking this marker where considered to lack tumorigenic potential (20). However, numerous studies have since demonstrated that this marker does not consistently distinguish between tumorigenic and non-tumorigenic glioma cells and both CD133+ and CD133− are able to form tumors upon transplantation into immunocompromised mice (21–22).

Although the models of clonal evolution and CSC have often been considered to be mutually exclusive, they need not and might actually be complementary. In fact upon appropriate interrogation, most cancers in which CSCs have been identified, present intra-clonal heterogeneity suggesting that they evolve through a divergent process of clonal evolution (23). Whether heterogeneity initiates and arises from clonal evolution or from CSCs, the mechanisms by which it is maintained over time also need to be considered and understood. For example, an alternative mechanism of tumor progression and heterogeneity maintenance called inter-clonal cooperativity that takes into account cancer cell-cancer cell interaction has been proposed (24) (Figure 1C). This model suggests that some clones within a tumor acquire oncogenic mutations that result in a pro-oncogenic microenviromental phenotype characterized by the production of factors that confer an advantage to other nearby clones. One interesting concept derived from this theory is that even a small minority of phenotypically distinct cells can have a profound impact on the behavior of the rest of the population, for example, by inducing metastasis, conferring an enhanced growth rate, or even promoting the growth of cells that are not *per se* tumorigenic. Indeed, our experiments with Ink4/Arf null astrocytes over-expressing wtEGFR, which are not tumorigenic upon intracranial injection into nude mice and are prompted for tumor growth when co-injected with Ink4/Arf null ΔEGFR over-expressing astrocytes, support the possibility that inter-clonal cooperativity exists in GBM (Figure 1D) (14).

Mouse models

While most *in vivo* studies are based on the use of xenografts composed of a single established cell line and many useful data illuminating the biology of GBM have been obtained from them, these approaches do not recapitulate the characteristic heterogeneity of the native tumor. Attempts to overcome this issue and obtain *in vivo* models that more closely resemble the human tumors have been done by generating genetically engineered mouse models. Indeed, the tumors arising in these mice reproduce some histopathological features of various grades of gliomas, depending on the original genetic background (for a review, see Huse & Holland (25)). Moreover, some of these tumors acquire additional mutations and molecular alterations that target pathways typically perturbed in gliomas, thus exhibiting a relatively complex heterogeneous composition (26).

Although suitable for the study of tumor-stroma interactions and for preclinical testing of anticancer therapies, these models are intrinsically limited by the dependence on their original genetic background and, most importantly, do not account for an essential, though often underrated, aspect of tumor biology: the interactions between genetically different cancer cells. The recapitulation of heterogeneity using engineered cell lines, mixed in known proportions, serves as an alternative approach not only to study these interactions, but also to decipher whether heterogeneity is simply the result of stochastic events, destined to disappear with time by clonal selection, or is actively maintained. We have used this approach and discovered an unexpected interaction between cells over-expressing wtEGFR and ΔEGFR, that is mediated by secreted factors that might suggest a new therapeutic strategy in which tumor cells and the signals between them are conjointly targeted (14).

Cell-to-cell interactions

Tumors are increasingly appreciated as not being composed only of a mass of malignant cells, but to also include a proportion of host cells and tissues that either infiltrate the tumor attracted by tumor secreted molecules such as cytokines, chemokines, growth factors, or that are engulfed during its uncontrolled growth. This contribution of normal tissue, which can represent a high percentage of the tumor bulk, together with a variable amount of tissue immediately surrounding the tumor constitute what is usually referred to as the tumor microenvironment. The cells in this compartment are profoundly affected by the tumor and their presence is not as simple bystanders, but as actively influencing the biology of the tumor. The importance of the microenvironment in tumor initiation and progression is strongly indicated by the selective patterns of metastatic spread and through intravenous injection experiments, where even the most aggressive cancer cells that spread throughout the organism, form tumors only where they find a permissive microenvironment (27). In brain tumors the microenvironment is composed of microglia, macrophages, astrocytes, oligodendrocytes, neurons, glial and neuronal progenitors, extracellular matrix, pericytes and endothelial cells. This complex structure renders a network where mutual interactions between neoplastic and non-neoplastic cells produce a local milieu that favors tumor cell growth, invasiveness, cell death/therapy resistance and immune escape (Figure 1C).

One particularly illustrative example of interaction between glioma cells and different host cells is provided by prostaglandins. GBMs have long been known to have altered arachidonic acid metabolism and to secrete high levels of prostaglandins and thromboxanes, and some of these compounds contribute to their immunosuppressive capacity (28). On the other hand, it has been demonstrated that Prostaglandin E2, though being a potent immunosuppressor, can induce the up-regulation of IL-6 in microglia, which is a promoter of glioma cell proliferation (14, 29). Thus, by acting on different targets, GBM-produced factors can elicit multiple effects, like maintaining a favorable inflammatory environment,

while at the same time inhibiting its adverse effects. Similarly, CD133+ glioma cancer-initiating cells potently inhibit T-cell proliferation and the production of pro-inflammatory cytokines, as well as inducing regulatory T-cells and triggering T-cell apoptosis (30).

GBM cells preferentially invade along myelinated axons, vascular basement membranes or the subependyma, suggesting that some microenvironmental features might be involved in this invasion (31). Another example of the interactions between glioma cells and their microenvironment is provided by the demonstration that brain tumor CSCs closely interact with endothelial cells resulting in the maintenance of the stem cell-like state (32). The increase of endothelial cells or blood vessels in orthotopic brain tumor xenografts, resulted in the expansion of the self-renewing population and accelerated tumor growth. Conversely, the depletion of blood vessels using erlotinib, that through EGFR inhibition downregulates VEGF, or bevacizumab, which neutralizes VEGF directly, reduced tumor growth and decreased the number of self-renewing cells.

As mentioned above, tumors can be considered from an ecological perspective, where the different cancer cells represent the individuals or species and the rest is the environment. These components create an ecosystem within which interactions exist not only between cancer cells and their environment, but also between different cancer clones and cooperate to create what can be considered a social network. In this social network, different types of interactions occur. Tumor clones can compete between themselves for oxygen, nutrients or space, resulting in the prevalence of the stronger through natural selection. For example, in some Bcr-Abl-driven leukemias there is an up-regulation of an iron transporter molecule, 24p3, by Bcr-Abl cells that results in the exhaustion of iron leading to apoptosis of wildtype cells (33). Tumor clones can also cooperate positively to help each other (mutualism) or to just benefit from one clone (commensalism). In mammary carcinoma it has been demonstrated that the co-existence of two different cells populations, “E-cells” and “M-cells”, is necessary for the secretion of high levels of collagen-degrading matrix metalloproteases and thus the promotion of metastasis (34). While this type of interaction between different tumor clones has been studied in different tumor types, such as breast cancers or leukemia, no studies to date have analyzed the interaction between common genetic alterations present in different cells in GBM, despite the number of reports of its heterogeneous nature. We recently provided the first direct evidence of this type of interaction in GBM between clones over-expressing ΔEGFR and clones over-expressing wtEGFR (Figure 1D). The expression of ΔEGFR in glioma cell lines results in a strong induction and secretion of soluble factors, such as IL-6 and/or LIF, that potently act on wtEGFR-expressing cells through two mechanisms, directly by binding to their receptor (IL-6R or LIF-R) in complex with their common subunit gp130 and activating the STAT3 pathway, and indirectly through trans-activation of EGFR by gp130 interaction, together resulting in increased tumor growth and cell survival (14). The implications of these findings might go beyond the simple paracrine stimulation between different clones. It has been reported that LIF can maintain self-renewal of glioma stem cells and increase their oncogenic properties (35), raising the possibility that ΔEGFR could enhance tumor growth by expanding the cancer-stem cell population through secretion of this cytokine. This model complements the unidirectionality of both clonal evolution and CSC models, rendering a bidirectional model where different tumor clones interact between themselves and with the CSC compartment and the selected phenotypes are those that give advantage to the community, rather than to the single cells. Further studies are needed to determine the relevance of these interclonal cooperative interactions that drive GBM malignant progression.

Relevance of tumor heterogeneity to therapy

GBM intratumoral heterogeneity is a hallmark feature that may contribute to its poor response to targeted therapy. According to the clonal evolution model, the therapeutic strategy should be directed to achieve the maximal cell kill, while proponents of the CSC model suggest that therapy should be directed against CSCs, since these are the cells with unlimited proliferation capacity. CSCs have been implicated as responsible for tumor relapse after therapy due to their intrinsic resistance to chemo- and radio-therapy (36–37). The presence of different tumor clones might be responsible for therapy failure not only because of the pre-existence of resistant clones within the tumor, as suggested by the clonal evolution model, but also by facilitating the survival of nonresistant cells through positive interaction with other tumor cells or the microenvironment. One of the factors responsible for this failure might be IL-6. We demonstrated in our heterogeneity model that ΔEGFR cells secrete elevated levels of IL-6 promoting wtEGFR cells growth, but, as other studies suggest, IL-6 can also confer a drug resistant phenotype. In Burkitt's lymphoma, IL-6 and Timp-1 are released in the thymus in response to DNA damage creating a chemo-resistant niche that promotes lymphoma cell survival and serves as a reservoir for tumor relapse (38). Similarly, in lung cancer, Yao et al found up-regulation of IL-6 expression as a mechanism to unleash lung cancer cells from their addiction to mutant EGFR (39). The stimulation of lung cancer cells with recombinant IL-6 was sufficient to decrease sensitivity to the EGFR tyrosine kinase inhibitor, erlotinib.

Future directions

GBM is a heterogeneous disease among tumors and numerous studies have shown that cells within an individual glioma differ in their morphology, genetics and biological behavior. However, little attention has been given to understanding the role of this heterogeneity in therapeutic resistance and few studies have attempted to decipher how heterogeneity in GBM is maintained. We have shown that heterogeneity can be actively maintained through inter-clonal cooperativity. We showed that Ink4/Arf null astrocytes over-expressing wtEGFR are not tumorigenic upon transplantation into nude mice, however, the presence of ΔEGFR expressing astrocytes provided a positive microenvironment where wtEGFR astrocytes are able to survive and proliferate, thus generating and maintaining a heterogeneous tumor. It is important to decipher the nature of interactions between different tumor clones, since ablating this intercellular communication might represent a new tool in our arsenal to treat these highly malignant tumors.

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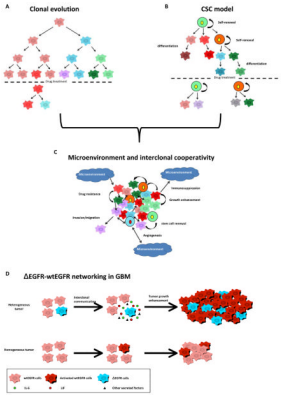

Figure 1. Heterogeneity origins and maintenance

According to clonal evolution (A), heterogeneity is caused by the acquisition of mutations and expansion. Upon a selective pressure, such as drug treatment, more adapted clones survive and, through consequent mutations, give rise again to a heterogeneous population. The CSC model (B) postulates that only CSCs are able to divide indefinitely, giving rise to cells that will differentiate heterogeneously. CSCs can also acquire mutations and generate a heterogeneous CSC population. CSCs are considered to be drug resistant, such that surviving cells serve as a reservoir for tumor relapse. Whether heterogeneity arises by clonal evolution, CSCs or CSCs undergoing clonal evolution, its maintenance requires interactions between CSCs/tumor cells and their microenvironment and between different CSCs/tumor cell clones (C). Those interactions can drive an increase in tumor growth, drug resistance, immune suppression, angiogenesis, invasion, or even CSC renewal. (D) ΔEGFR cells secreted factors, such as IL-6 and LIF, which through a paracrine mechanism activate wtEGFR cells, resulting in a significant tumor growth enhancement as compared to a wtEGFR homogeneous tumor.