

Vascular endothelial growth factor in astrogloma stem cell biology and response to therapy

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Abstract

Malignant astroglomas are among the most aggressive, highly vascular and infiltrating tumours bearing a dismal prognosis, mainly due to their resistance to current radiation treatment and chemotherapy. Efforts to identify and target the mechanisms that underlie astrogloma resistance have recently focused on candidate cancer stem cells, their biological properties, interplay with their local microenvironment or ‘niche’ and their role in tumour progression and recurrence. Both paracrine and autocrine regulation of astrogloma cell behaviour by locally produced cytokines such as the vascular endothelial growth factor (VEGF) are emerging as key factors that determine astrogloma cell fate. Here, we review these recent rapid advances in astrogloma research, with emphasis on the significance of VEGF in astrogloma stem-like cell biology. Furthermore, we highlight the unique DNA damage checkpoint properties of the CD133-marker-positive astrogloma stem-like cells, discuss their potential involvement in astrogloma radioresistance and consider the implications of this new knowledge for designing combinatorial, more efficient therapeutic strategies.

Keywords: astrogloma • cancer stem-like cells • ‘vascular niche’ • radioresistance

Introduction

Considering their poor prognosis and resistance to chemotherapy and radiotherapy, brain tumours in general, and high-grade astroglomas in particular, are among the most devastating types of human

cancer. At the cell population level, brain malignancies are typically heterogeneous lesions composed of cells with diverse morphologies that express a variety of neural lineage markers. Prognosis and

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response to treatment differ significantly not only among histologically different brain tumours, but also among those that appear similar in terms of morphology and patterns of phenotypic markers. This notorious heterogeneity of brain tumours likely facilitates development of treatment-resistant clones, a process that is further fuelled by the ability of astroglia cells to shift between quiescent and proliferating states [1]. Given such unusual plasticity and the frightening rate of treatment failure, it is now commonly believed that treatment outcome and hence patients' survival can only improve if the biology of the brain cancer cell populations is better understood.

Classification of adult brain tumours is based on the World Health Organization (WHO) classification of nervous system tumours [2]. The WHO grading of astrocytomas establishes a malignancy scale based on histologic features of the tumour. The histological grades are as follows: WHO grade I includes lesions with low proliferative potential, a frequently discrete nature, and the possibility of cure following surgical resection alone. WHO grade II lesions are generally infiltrating and display low mitotic activity but recur. Some lower-grade lesions tend to progress to higher grades of malignancy. WHO grade III includes lesions with histological evidence of malignancy, generally in the form of mitotic activity, clearly expressed infiltrative capabilities, and anaplasia. WHO grade IV lesions are mitotically active, necrosis-prone and commonly associated with a rapid pre-operative and post-operative evolution of disease. The most malignant types of astroglomas are anaplastic astrocytoma (grade III astrocytoma) and glioblastoma multiforme (GBM, grade IV astrocytoma). In this article, we commonly use the general term astroglomas for simplicity, yet our discussion mainly concerns the grade III and particularly grade IV tumours.

One of the key features of astroglomas, affecting their biological and clinical behaviour, is dense vascularization. Since the introduction of the concept of an 'angiogenic switch' as a pivotal component of tumour growth and metastasis by Folkman *et al.* [3], multiple therapies targeting the molecular regulators of this mechanism have been tested with variable clinical efficacy. Despite such mixed initial results, anti-angiogenic therapy is regarded as a promising treatment strategy, and various pre-clinical experimental approaches targeting neovascularization turned out to be effective *in vivo*. For example, one study showed that systemic therapy with a mono-

clonal antibody against vascular endothelial growth factor receptor 2 (VEGFR2) inhibited tumour growth in mice by some 80% [4]. The ligand that operates through VEGFRs to promote tumour angiogenesis is VEGF, a versatile regulatory cytokine that is aberrantly up-regulated in a wide range of tumour types and acts as a potent modulator of tumour growth and metastasis in numerous pre-clinical tumour models [5]. Particularly relevant to this review, several pieces of evidence indicate prognostic significance of VEGF in high-grade astroglomas [6–8]. In this article, we highlight the emerging role(s) of VEGF signalling in astrogloma biology, including the possible links with DNA damage checkpoints in response to radiation treatment. Furthermore, we postulate and discuss a hypothetical function of autocrine VEGF signalling in regulation of the so-called brain tumour-derived cancer stem-like cells (BTCSC) and their intriguing resistance to therapy.

Adult brain – a dynamic structure with active stem cells?

Unlike the early-stage embryonic brain harbouring large numbers of neural stem and progenitor cells, the adult brain is mainly composed of highly differentiated and specialized cell types, including neurons, glia (oligodendrocytes, astrocytes, microglia, ependymal cells), vascular endothelium and meningeal cells [9]. Over the past century, the brain has traditionally been viewed as static with respect to its very limited turnover and regenerative capacity. However, it has recently become clear that neurogenesis continues into adult life in restricted germinal zones [10–13]. A small percentage of quiescent cells present in the adult hippocampal dentate gyrus (sub-granular zone, SGZ), sub-ventricular zone of the lateral ventricles (SVZ) and olfactory bulbs are undifferentiated and multipotent neural stem cells (NSC) capable of self-renewal [14–15]. These stem cells divide to generate rapidly cycling transit-amplifying cells with a limited proliferation and differentiation potential. The transit-amplifying cells give rise to restricted progenitor cells undergoing terminal differentiation [16].

In the 1970s, an idea of specific anatomical locations termed 'niche' was first suggested on the basis of transplantation studies of haematopoietic progenitors. It has been hypothesized that other types of somatic stem cells reside in analogous 'niches' [17]. In the adult mammalian brain, we are just beginning

to identify the cellular and molecular features that characterize the neurogenic 'niches' in the SVZ and SGZ, and the mechanisms by which the full range of adult NSC development is regulated. NSCs are grown *in vitro* either as neurospheres in suspension or as an adherent monolayer [18]. Some of the cells within the neurospheres proliferate as multipotent, self-renewing NSCs upon stimulation with either epidermal growth factor (EGF) or basic fibroblast growth factor (bFGF) [18]. The NSCs residing in the adult human brain are attractive candidates for isolation, *in vitro* expansion and autologous transplantation to replace neurons lost to neurodegenerative diseases, stroke and traumatic brain injury [19].

Cancer stem-like cells in brain tumours

Recently, a small population of cancer stem-like cells (CSCs) has been identified in adult and paediatric brain tumours, as well as in established cell lines [20–24]. These cells express a number of NSC markers including CD133, a cell surface protein which is extensively exploited for enrichment of stem-like cells using fluorescence activated cell sorting (FACS) and/or magnetic bead separation, thereby providing cells to be examined in diverse applications [25–26]. The human CD133 antigen, also known as AC133, was originally identified as a marker of haematopoietic stem cells, however its function has remained unclear [27]. A characteristic feature of the CD133 protein is its rapid down-regulation during cell differentiation [28–29], which makes it a cell surface marker uniquely suited for the identification and isolation of stem-like and progenitor cells. Besides expressing the CD133 antigen, CSCs express mRNAs for several additional recognized NSC markers, including *bmi-1*, *Sox2* and *musashi-1* [21].

Inspired by identification of cancer stem cells from leukaemia and breast cancer [29–31], BTCSCs were reportedly successfully cultured according to the following criteria: (i) Expression of NSC markers CD133 and nestin; (ii) Generation of spheres morphologically indistinguishable from neurospheres; (iii) Self-renewal and proliferation and (iv) Production of differentiated progeny *in vitro* or recapitulation of the parental tumour mass growth when implanted into

immunodeficient animals [20–22, 24, 32–33]. As few as 100 CD133-positive human cells were sufficient to form brain tumours as xenografts in NOD-SCID mice [22]. Importantly, the CD133-positive BTCSCs also exhibit high resistance to current chemotherapy and radiotherapy, in contrast to their CD133-negative counterparts [33–34], an intriguing difference discussed in more detail later in this article. Among the unresolved questions of BTCSCs' biology is also whether, or to what extent, do the CD133-positive BTCSCs require interaction with the CD133-negative bulk sub-population of cells. Another largely open issue concerns the emerging role of the local microenvironment in supporting the maintenance of these candidate CSCs within tumour mass. As discussed below, identification of CD133-positive BTCSCs and attempts to understand their biology provide powerful new tools and approaches to better understand tumourigenesis in the CNS, a research area likely to become crucial in developing novel therapies based on BTCSCs as a target.

Angiogenesis in astroglomas

Angiogenesis is a highly regulated process essential not only in early embryogenesis but also during tissue growth and repair, female reproductive cycle and diverse pathologies, such as inflammation or tumour development and progression [35–36]. Localized breakdown of extracellular matrix (ECM) precedes proliferation, migration and tissue infiltration of endothelial cells. In time these cells re-model back into capillary structures, and a new ECM is deposited [37]. Highly malignant astroglomas exhibit striking angiogenesis and markedly increased expression of VEGF (Fig. 1). VEGF expression strongly correlates with tumour aggressiveness, metastatic potential, a short time to relapse and, consequently, it commonly indicates poor prognosis in patients with cancer [6, 38–43]. Recent work [44] identified the BTCSCs to be a key source of angiogenic factors in brain cancer, suggesting that anti-angiogenic therapy targeting these stem-like tumour cell sub-populations might improve the therapy outcome. Exuberant angiogenesis is a key event in astrogloma progression [45–47]. Understanding angiogenesis and its relation to tumour growth and resistance to therapy is of considerable interest, particularly because diffusely infiltrating

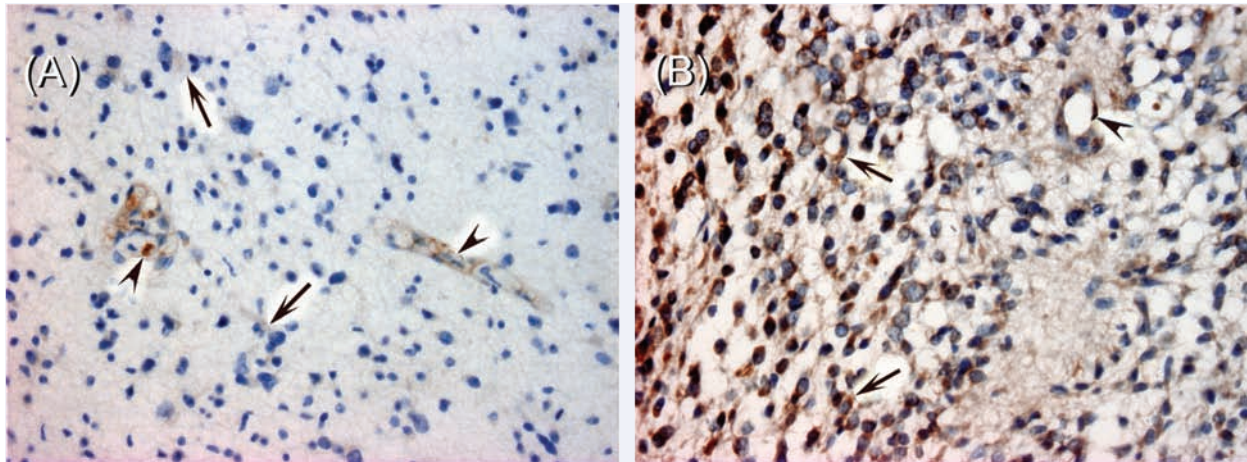


Fig. 1 Immunohistochemical analysis of VEGF expression in a low and high-grade astroglioma. Arrows indicate very weak diffuse VEGF staining in the cytoplasm of astroglial elements of a low grade astroglioma WHO grade II (A), in contrast to strong cytoplasmic positivity in astroglial elements in a high-grade astroglioma specimen WHO grade IV (B). Both tumour grades exhibit certain level of cytoplasmic positivity in endothelial elements (arrowheads) (Original magnification 200x).

astrogliomas are mostly refractory to current surgical and adjuvant treatments [48].

Neovascularization in brain tumours correlates markedly with their enhanced aggressiveness, degree of malignancy and poor clinical prognosis and inversely with the post-operative survival time of patients [49–51]. Newly formed tumour blood vessels possess an ineffective blood–brain barrier that contributes to the pathogenesis of tumour-associated oedema [52]. The characteristic vascularity of astrogliomas has led to a hypothesis that the formation of new blood vessels is crucial to tumour growth [53]. Astroglioma cells have angiogenic activities, in that they promote capillary morphogenesis and endothelial proliferation *in vitro* [54].

As mentioned above, the pathologic features that distinguish GBM from lower grade astrogliomas are the presence of necrosis with pseudopalisades and a distinct form of angiogenesis, microvascular hyperplasia [54–56]. These palisades are in part caused by vessel regression [57] and increased tumour cell proliferation [56, 58]. Analysis of the shapes and sizes of pseudopalisades suggests that these structures evolve and enlarge overtime, giving rise to a gradually expanding coagulative necrosis [59]. Such features inspired formulation of a vaso-occlusive and pro-thrombotic model of pseudopalisade formation in GBM [60]. This emerging concept postulates hypoxic

tumour cells migrating away from the central hypoxia that arises after a vascular insult. Hypoxia resulting from such conditions is then thought to induce new blood vessels that supply the tumour with necessary metabolites [61]. VEGF is highly expressed in pseudopalisading tumour cells adjacent to necrotic zones and hyperplastic vessels in astroglioma. This phenomenon reflects an elevated transcriptional activity of hypoxia-inducible factors 1 and 2 (HIF-1 and -2) [62–64]. Secretion of VEGF, in turn, causes endothelial cell proliferation and angiogenesis followed by microvascular hyperplasia and formation of glomeruloid bodies in GBM and other tumours [65–66].

Overexpression of VEGF by tumour cells frequently occurs not only in response to hypoxia [62, 67], but also upon loss of function of certain tumour suppressor genes [68–69] and oncogene activation [70]. Under hypoxic conditions, transcriptional regulation of VEGF is dominated by HIF-1 that, together with its target genes, plays a key role in astroglioma-induced angiogenesis [71–72]. Chronic oncogenic stimuli, such as activated Ras and PI3K pathways appear to enhance HIF-1 α expression and likely contribute to GBM progression [60, 73]. The proto-oncogene ETS-1 and the transcription factor STAT3 are also capable of VEGF induction and/or activation. ETS-1 activates genes for VEGF receptors, matrix metalloprotease proteins (MMPs) and urokinase plasminogen activator

(uPA) [74], all features that can promote angiogenesis and tumour progression.

In addition, several cytokines and growth factors involved in astrogloma pathogenesis, including TGF- β , EGF, PDGF-B, bFGF, also up-regulate VEGF [48]. Genetic alterations common in astroglomas, such as mutational activation of the epidermal growth factor receptor (EGFR) and loss-of-function mutations of the PTEN tumour suppressor, lead to enhanced VEGF expression and increased angiogenic activity [75–76]. Astroglomas frequently overexpress EGFR, and its truncated mutant isoform EGFRvIII has been implicated in relapse and poor prognosis [77]. Enhanced EGFR signalling can up-regulate VEGF production in brain cancer, while blockage of EGFR inhibits secretion of VEGF and other angiogenic factors [78]. Overall, this accumulating evidence supports the notion that VEGF plays a central role in the molecular pathogenesis of astroglomas and strongly affects the biological behaviour of these tumours.

VEGF and the ‘stem cell niche’

The importance of microvasculature and proper local microenvironment (‘niche’) including a plethora of regulatory cytokines and growth factors for the maintenance of ‘stemness’ of the normal NSCs is known [79]. Related critical issues for astroglomas have been whether the BTCSCs also depend on their niche and, if yes, whether the mutual interdependencies of the niche microenvironment and the stem cells may differ between normal NSCs and BTCSCs. If such differences exist, the specific features of the BTCSC-niche interplay might offer potential targets for novel therapeutic interventions.

There is little doubt that the nature of the niche microenvironment may represent an important factor in the behaviour of normal NSCs and CSCs in terms of their stem cell self-renewal and cell-fate decisions. The microenvironment composed of the ECM, stem and progenitor cells, as well as mature, differentiated cells secreting a range of growth factors seems to be important in such processes. Balanced microenvironmental levels of various mitogens, including bFGF, EGF and sonic hedgehog (Ssh), support the propagation of adult NSC in culture and appear to perform similar functions *in vivo* [80–83]. Histological and *ex vivo* cell culture studies of mouse tissues suggest

that NSC lie within a ‘vascular niche’ in which endothelial cells regulate stem cell self-renewal [84–87]. Growth factors, such as VEGF, bFGF, PDGF and EGF represent the mitogenic and trophic factors that regulate neurogenesis, while exerting direct neurotrophic and neuroprotective activities [88]. bFGF together with EGF are crucial growth factors necessary for NSC, as well as for CSC proliferation and maintenance of their self-renewal properties *in vitro* [22, 89–90]. For example, VEGF stimulates neurogenesis both in mouse brain cultures *in vitro* and in neuroproliferative regions (Sub-Granular Zone and Sub-Ventricular Zone) of the non-ischaemic mouse brain *in vivo* [91]. Naturally coupled to astrocytes through astrocytic end-feet, endothelial cells are also important components of the ‘niche’ structure and closely co-operate with astrocytes to regulate adult neurogenesis [84–85]. BTCSCs also interact with endothelial cells that secrete factors supporting the maintenance of these cells in stem-like cell state [92].

While current evidence supports the idea of VEGF as a regulator of neuronal cell fate and a key ingredient of such stem-cell niche in both, normal brain and astrogloma scenarios, one possibly important difference may reflect the plausible role of the VEGF as an autocrine factor in astroglomas, as opposed to only paracrine role under the normal niche conditions. Tumours known to overexpress VEGF have strong angiogenic potential which, in turn, could explain the documented importance of VEGF in astrogloma progression. Since VEGF and both VEGFRs are co-expressed in tumour cells in the majority of nascent primary GBM lesions (Fig. 2), an autocrine role of the VEGF in GBM might significantly contribute to tumour growth and invasivity [93–95]. On the other hand, in contrast to VEGFs well-established paracrine effects on endothelial cells, less is known about such potential autocrine role of VEGF in glioblastoma. While some authors [96] reported that exogenous VEGF not only stimulates endothelial cells, but also directly enhances astrogloma cell motility, invasion and proliferation (Fig. 3A), others published virtually opposite effects [97] and this issue remains controversial.

Despite our effort to understand the stem cell niche is in its infancy, particularly with regard to the BTCSC niche and its potential aberrant features, recent studies in this area are encouraging and support a crucial role of a perivascular niche for brain tumour stem-like cells [92, 98]. The emerging significance of this concept for development of new

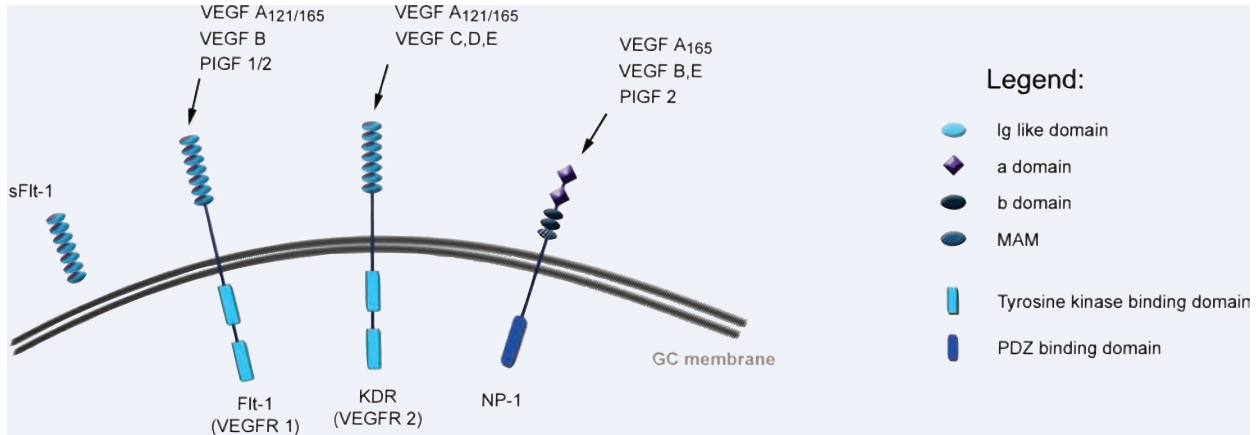


Fig. 2 Schematic representation of membrane VEGF receptors expressed by astroglial tumour cells. Known receptor types are indicated and identified ligands/ligand isoforms are listed directly in the figure above. Cell membrane (GC membrane) receptors of the VEGF tyrosine kinase receptor family consist of seven Ig-like domains, transmembrane region and an intracellular tyrosine kinase binding domain interrupted by a kinase-insert sequence. The aberrant sFlt-1 receptor form lacking one Ig-like domain as a result of VEGFR1 truncation is also shown. The neuropilin receptor acts as a co-receptor for VEGFR2, enhancing binding and biological activity of VEGF₁₆₅. Ligand binding through electrostatic interactions with specific sequences of sulphation within the HS chains leads to receptor dimerization and tyrosine domain phosphorylation, thereby activating the corresponding signalling pathway.

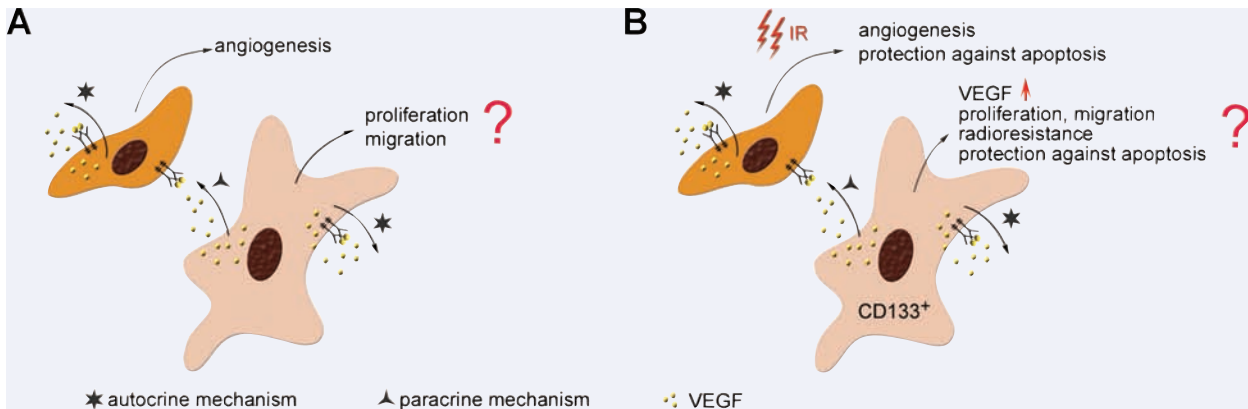


Fig. 3 Autocrine and paracrine effects of VEGF signalling in astroglomas. **(A)** VEGF secreted by tumour endothelial cells (orange) induces angiogenesis in an autocrine manner, while VEGF secreted by astroglial tumour cells (pink) stimulates tumour angiogenesis in a paracrine manner. As reports on other biological effects of the autocrine VEGF function in astroglial tumour cells are currently contradictory, this aspect remains to be clarified by further studies. **(B)** Hypothetical distinct effects of VEGF on the CD133-positive (VEGF secreting and radioresistant [34,44]) versus CD133-negative (radiosensitive) astroglial cell population. IR-induced VEGF secretion by the CD133-positive astroglial cells (cancer stem-like cells) could enhance tumour angiogenesis and protection of endothelial cells against apoptosis. Furthermore, IR-induced VEGF could modulate proliferation of the CD133-positive cells, thereby regulating their sensitivity to IR. IR-induced VEGF, massively secreted by the CD133-positive astroglial cell sub-population, might also help to protect these cells from apoptosis and increase their migration in an autocrine manner.

astroglioma treatment strategies is discussed in the ultimate section of this article.

Resistance to DNA-damaging therapy in astroglomas

Given the severity of the health problem posed by astroglomas to the society, embarrassingly little is known about the biological basis and molecular mechanisms that allow brain tumours to survive treatment and recur. Apart from surgery and some newly tested targeted therapies, the major modalities presently used in the clinic to treat astroglomas are DNA damaging treatments by ionizing radiation (IR) and chemotherapy. In this section, we briefly highlight how the cancer stem cell concept and recent advances in understanding the cellular machinery that responds to DNA damage are beginning to converge to shed more light on the notoriously difficult issue of the treatment resistance. Although chemotherapeutics, particularly DNA alkylating drugs, such as temozolomide are currently widely used in combination with traditional radiotherapy to treat astroglomas, the molecular basis of sensitivity versus resistance to such drugs is relatively well understood [99–100], and it will not be further discussed here. Rather, we focus our discussion on IR and cellular responses to the most toxic IR-induced lesions, the DNA double strand breaks (DSBs).

In response to DSB-causing insults such as IR, human cells activate their DNA damage response machinery, a sophisticated network of signalling and effector pathways that co-ordinate cell cycle checkpoints with DNA repair and cell death mechanisms [101–102]. Relevant for our discussion on cancer cells, DSBs can be generated not only through external genotoxic insults, but also from events within the cell itself, for example, due to metabolic reactive oxygen species, or errors during DNA replication. The latter insult, often referred to as replication stress, is commonly caused by various activated oncogenes and loss of some tumour suppressors, leading to constitutively activated DNA damage checkpoint signalling in tumours [102–104]. Such constitutive activation of the DNA damage checkpoints is particularly apparent in borderline pre-malignant and early malignant lesions, resulting in enhanced apoptosis or

induction of senescence as an inducible biological barrier against tumour progression [102–107]. Despite the existence of such physiological barrier response has been demonstrated for multiple types of human solid tumours, particularly carcinomas and melanomas, it is largely absent in testicular germ cell tumours [108], and its relevance for astroglomas remains to be explored. What is important for our discussion about sensitivity versus resistance to DNA-damaging therapies is the fact that during their progression, many malignant tumours, at least partially, disable the activated checkpoint barrier through mutations or epigenetic changes in relevant genes, such as p53 [102, 109]. Alternatively, malignant cells might progress in the face of constitutive DNA damage by enhancing the efficiency of their DNA repair pathways, in either case altering the overall sensitivity towards potential subsequent therapy by DNA damaging modalities, such as IR.

At the molecular level, the key element of the cellular DSB response is activation of the apical signalling kinase ATM (and also the ATR and DNA-PK kinases), which rapidly phosphorylate a wide range of substrates including the effector kinases Chk2 and Chk1 [110]. Chk2 and Chk1 become activated upon their phosphorylation by ATM and ATR, respectively, and further propagate the DNA damage alert signal to diverse effectors such as the tumour suppressor p53, or other checkpoint and DNA repair proteins. The severity of the DNA damage, the effectiveness of the activated cell cycle checkpoint and DNA repair mechanisms, as well as other parameters including the protective signals from the microenvironment, then jointly affect the final outcome and cell fate of the irradiated cell. In cancer cells, such a cell-fate decision may be widely variable due to the heterogeneity of tumour cell populations, and it may also reflect alterations in the DNA damage machinery acquired during cancer progression (see above).

Recent work on human glioblastomas now indicates that the CD133-positive stem-like cells may have an enhanced checkpoint response to radiation, and this may contribute to their selective survival and radioresistance [34]. After irradiation, the CD133-positive fraction of human GBM cultures and xenografts was enriched up to fivefold compared with CD133-negative cells. This was not attributable to induction of CD133 expression in CD133-negative tumour cells, but to lower rates of apoptosis among the CD133-positive subset. Analysis of the checkpoint

machinery pointed to an augmented activation of the Chk2 and Chk1 kinases, faster repair of the IR-induced DSBs, and overall better survival and ability to recur among the CD133-enriched BTCSCs [34]. Furthermore, the authors used a chemical inhibitor to block the activities of the checkpoint kinases Chk1 and Chk2 after IR, and this treatment radiosensitized the CD133-positive subset more than the CD133-negative astrogloma cells from the same tumour. Collectively, these intriguing results identify a possible mechanism that contributes to radioresistance of BTCSCs and thereby of the tumour, and suggest that targeting the DNA damage checkpoints may be worth considering as an option in the GBM radiotherapy scenario. Whether this mechanism may be in any way linked with the effects of VEGF or other cytokines in the BTCSC niche, and how to exploit these new insights into therapy resistance for improved treatment strategies in the future, is discussed in the following section.

VEGF and DNA damage response: implications for astrogloma therapy

In response to IR, VEGF secretion by GBM cell lines was highly increased in a radiation dose-dependent manner [94, 111]. Among all the cell lines tested, the U87MG is highly radioresistant and expresses the highest IR-induced VEGF levels [111], a correlation that inspired a hypothesis about a potential involvement of IR-enhanced VEGF secretion in radioresistance. Based on observations of decreased GBM cell proliferation in response to exogenous VEGF [97], it was proposed that expression of VEGF would at the same time induce growth of new blood vessels ensuring a better supply of oxygen and nutrients and reduce GBM cell proliferation resulting in decreased sensitivity to IR. Since proliferating cells are more sensitive to irradiation than quiescent cells [112] and since VEGF protects tumour blood vessels from irradiation-mediated toxicity [94], this suggests a possible mechanism through which GBM cells can escape the consequences of radiation treatment.

After irradiation, the VEGF gene promoter becomes stimulated *via* multiple mitogen-activated protein kinase (MAPK) dependent pathways in both cultured

normal human astrocytes and GBM cell lines [113]. Since HIF-1 was not overexpressed under such conditions, hypoxia doesn't seem to be involved in this mechanism [113]. Given the radioresistance of BTCSCs discussed in the previous section [34], could these data jointly indicate a connection between radiation-induced VEGF secretion, increased angiogenesis and selective survival of the CD133-positive BTCSCs? Could IR-induced VEGF secretion by BTCSCs selectively regulate their own migration and/or proliferation in an autocrine manner while protecting them from IR-induced apoptosis (Fig. 3B), possibly through some so-far unidentified link with DNA damage checkpoint signalling or repair? Despite speculative at present, this idea seems indirectly supported by recent analysis of the so-called bystander effects of radiation [114]. This study showed that conditioned media from irradiated human glioblastoma cells contained factors including cytokines, whose membrane-mediated signalling to non-irradiated cells resulted in activation of the checkpoint kinase ATR and cellular DNA damage response without direct exposure of such bystander cells to radiation. These results imply that secreted cytokines may be capable of inducing DNA damage checkpoints, and that such bystander response differs in GBM cells compared with normal astrocytes [114], suggesting that these effects might be exploited through therapeutic targeting.

The arguments discussed so far point to critical roles of VEGF and BTCSC niche, as well as the status of the DNA damage response machinery, both of which show unique features in the treatment-resistant astrogloma stem-like cells. These accumulating results identify both VEGF-mediated and DNA damage signalling cascades as promising targets for treatment of astroglomas, a notion that is supported also by recent successful attempts to experimentally target either VEGF signalling alone [4, 115], or combine such vascular niche-targeting treatment with DNA-damaging chemotherapy (see below). Formation of multiple 'vascular BTCSC niches', each possibly capable of giving rise to a recurrent tumour, may strongly facilitate tumour growth and invasion [116–118]. If BTCSC are true tumour-initiating cells, then drugs selectively killing these cells could prove highly effective treatments for astroglomas. Encouraging are recent data providing proof of principle that selective targeting of CSCs is possible, at least in some types of malignancies [34, 44, 119].

There is no doubt that additional factors of astrogloma biology may hinder attempts to successfully introduce combined VEGF- and DNA damage checkpoint-targeting treatment strategies. For example, infiltration of tumour cells into surrounding brain contributes to the treatment-refractory nature of malignant astroglomas [120]. Moreover, the blood–brain barrier represents a significant obstacle, preventing the delivery of large-molecular-weight polar compounds to brain tumour cells. In addition, brain tissue is highly sensitive to cytotoxic treatments [121], and our own data show that inhibition of checkpoint kinases such as Chk1 causes endogenous DNA damage in proliferating human cells, thereby raising concerns as to the suitability of this approach for therapy [122]. With the exception of the modest activity associated with temozolomid, there is no standard chemotherapy available for patients with high-grade astroglomas, and resistance to chemotherapy is common [123].

On the optimistic side Bevacizumab (Avastin®), a recombinant, humanized monoclonal antibody targeting VEGF, has been recently approved for use in colorectal carcinoma based on significant survival benefit observed following its addition to fluorouracil-based chemotherapy [124]. Preliminary results from single-arm phase II study of Bevacizumab with Irinotecan (CPT-11), currently underway at the Preston Robert Tisch Brain Tumor Center at the Duke University Medical Center for patients with recurrent malignant astrogloma, indicate that the most effective therapy of GBM identified to date could be a combined treatment by Bevacizumab with a DNA-damaging drug such as CPT-11 [125].

Thus, despite possible numerous obstacles that must not be underestimated, we believe that the available evidence justifies attempts to identify a clinically feasible, effective combination of therapeutic approaches that would allow complementary targeting of the VEGF/niche, and the DNA damage checkpoint aspects of malignant astroglomas, with special emphasis on targeting the unique features of the candidate astrogloma stem cells (Fig. 4). In general terms, such a combined strategy may include available or future drugs or antibodies to inhibit VEGF and/or its receptor-mediated signalling, along with standard DNA damaging treatment modalities, such as IR and alkylating drugs, complemented by selective inhibitors of checkpoint signalling or DNA repair to counteract the mechanisms underlying astrogloma resistance to such treatments. In any

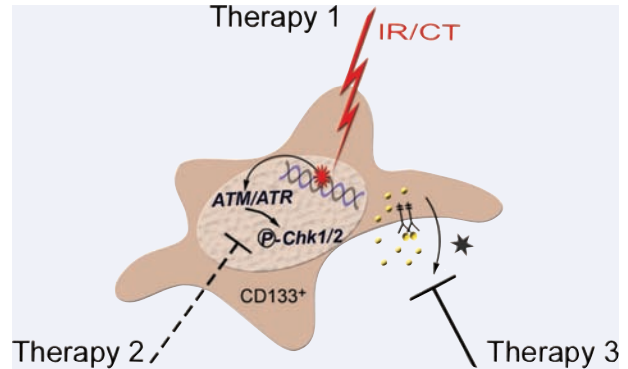


Fig. 4 Potential therapeutic approaches targeting ‘pathological vascular niche’ and DNA damage checkpoint as unique features of BTCSCs. Accumulating evidence suggests that the CD133-positive, candidate BTCSCs, harbour specific features that could be exploited in future combinatorial therapies of malignant astroglomas. Such potential treatment combination might include classical DNA-damaging modalities: ionizing radiation (IR), chemotherapy (CT) or both (Therapy 1), a concomitant blockade of DNA damage checkpoint signalling and/or DNA repair (Therapy 2), and inhibition of the cytokine-mediated paracrine/autocrine circuitry (asterisk) such as the VEGF effects (Therapy 3).

case, given the close relationship between tumour microenvironment, vascular architecture and tumour response to therapy, it seems logical to investigate the potential role of VEGF not only as an angiogenic factor stimulating formation of ‘pathological vascular niche’, but also as a potential regulator of BTCSC cell-fate. Perhaps in concert with acquired defects within the apoptotic machinery and/or selective regulation of DNA damage signalling/repair, VEGF and ‘vascular niche’ might help BTCSCs escape from the toxic effects of chemotherapy and radiotherapy by protecting them from DNA damage-induced apoptosis [126–129]. Better mechanistic understanding of these biological processes appears to be more plausible based on the recent advances in the field, and such research will hopefully help to improve the presently dismal prognosis of astrogloma patients.

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