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Angiogenesis and Apoptosis in Glioma: Two Arenas for Promising New Therapies

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Abstract New therapies for gliomas are urgently needed in view of the very marginal increase in patient survival that has been achieved over the past two decades, which is only somewhat mitigated by improvements in quality of life. Two relatively recent fields of research that hold out great promise in this area, are angiogenesis and apoptosis. Depriving growing tumors of the blood supply they need, or tipping the balance in the cancer cell towards cell death, both provide conceptually elegant approaches to therapy, with the hope of great efficacy and little toxicity. However, attempts at successfully translating exciting laboratory findings to the clinic have been slowed by the complexity of the underlying biology. In this article we examine some of the issues that have impeded progress, and examine the potential role that integrins may play as targets, with a role in both angiogenesis and apoptosis. J. Cell. Biochem. 96: 16–24, 2005.

Key words: glioma; astrocytoma; angiogenesis; apoptosis; invasion; translation

The development of molecularly based therapies for cancer in general, and glioma in particular, holds the promise for an effective means of combating the disease. Two fields that are particularly important in this regard are angiogenesis, where the hope is that interfering in the tumor's blood supply will not only remove existing lesions but also prevent their recurrence, and apoptosis which would produce, particularly, potent therapies if one could selectively turn on the machinery of cellular self-destruction in the cancer. Here, we briefly introduce these fields in the context of brain cancer with emphasis on advances and their translation to therapy.

GLIOMA ANGIOGENESIS: MOLECULAR BASIS

The rationale for targeting angiogenesis is now widely appreciated, resting on the insights that the new formation of blood vessels is relatively restricted to tumors in the adult, that removal of a good blood supply will limit the growth of clinically significant tumors, and that the endothelium may be a less difficult target than the cancer [Folkman, 1972]. Gliomas are angiogenic: even low-grade astrocytomas (WHO grade II) show increased vessel density, while

Abbreviations used: Ang-1/2, angiopoietin-1/2; APAF-1, apoptosis activating factor-1; BH, bcl-2 homology domain; CAI, carboxyamidotriazole; CARD, caspase activation and recruitment domain; DD, death domain; DED, death effector domain; DISC, death inducing signaling complex; DR, death receptor; ECM, extracellular matrix; FADD, fas-associated protein with death domain; FAK, focal adhesion kinase; HIF-1, hypoxia inducible factor-1; PI3K, phosphatidyl inositol 3-kinase; TIMP, tissue inhibitor of metalloproteinase; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis inducing ligand; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; WHO, World Health Organization.

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the histological definition of grade III and IV astrocytomas includes high microvessel density and evidence of neo-angiogenesis, as well as evidence of abnormal and glomeruloid blood vessel formation [Kleihues et al., 2000; Brat and Van Meir, 2001; Brat et al., 2003]. Therefore, despite a lack of concrete advances in the clinic, angiogenesis remains a compelling target, and the rapid advances in understanding of its molecular underpinnings continues to suggest that a successful intervention is not long off.

Newly formed blood vessels in gliomas are abnormal at several levels, including structure and the absence of the blood brain barrier [Plate and Risau, 1995], and so glioma angiogenesis has to be understood in its own right, and not only in terms of its normal counterpart. One major driving force in this process is signaling by vascular endothelial growth factor (VEGF), which is typically overexpressed in higher-grade tumors [Plate and Risau, 1995; Schmidt et al., 1999], and the reduction of which can significantly reduce the angiogenic potential of the glioma cell lines in xenografts [Cheng et al., 1996; Sonoda et al., 2003]. Not all forms of VEGF are equivalent in their actions, however, and a significant level of complexity arises from the existence different VEGF genes and different isoforms produced by a single gene [see, e.g., Cheng et al., 1997]. The receptors for the VEGF signaling pathway, such as the high-affinity VEGF receptors VEGFR-1/Flt-1 and VEGFR-2/Flk-1, show upregulation in the endothelium associated with gliomas, but how is this achieved is not clear [Plate et al., 1992; Plate and Risau, 1995]. Another signaling pathway that is increasingly appreciated as a major factor in glioma angiogenesis is that initiated by the angiopoietins, Ang-1 and -2, which also work through tyrosine kinase receptors, in this case predominantly Tie-2 [Yancopoulos et al., 2000; Hu et al., 2003]. The Tie-2 receptor is restricted to endothelial cells and stimulation with Ang-1 leads to activation, while Ang-2 leads to inhibition, but the simple model suggests, where Ang-1, which comes from cells other than the endothelium, acts as an angiogenic signal, while Ang-2, which is made predominantly by endothelial cells, acts to provide feedback does not hold up, because some forms of Ang-1 are inhibitory and because other signaling networks are engaged [Yancopoulos et al., 2000; Zadeh and Guha, 2003]. The observation that Ang-1, Ang-2, and Tie-2 all increase in expression levels with increasing grade of astrocytoma, and this is paralleled by increased levels of Tie-2 activation in glioblastoma, and interfering in its signaling negatively impacts growth of gliomas [Ding et al., 2001] means that it is a potentially valuable target for therapy development.

Another area in which therapeutic targets are being identified is the regulatory machinery that tells the cell to make the angiogenic factors that were just discussed. The most important stimulus here is hypoxia, a state that leads to the activation of the hypoxia inducible factor one (HIF-1), a transcriptional regulator consisting of a constitutively expressed β and a tightly regulated α subunit. Under normoxic conditions, HIF-1α is efficiently degraded, but is stabilized by reduction in oxygen levels and then increases the expression of VEGF and VEGFR-1/Flt-1 [reviewed in Brat et al., 2003]. Disruption of HIF-1 ability to activate transcription has a profound effect on the rate of glioma growth in mice [Kung et al., 2000]. Strategies targeting HIF-1 include the use of existing compounds, such as geldanamycin, which promotes HIF-1α degradation [Mabjeesh et al., 2002] or the discovery of new drugs [Rapisarda et al., 2002], and these have been recently reviewed [Powis and Kirkpatrick, 2004]. An alternative approach is to use HIF-1 response elements to target gene therapy vector expression to the tumor [Post and Van Meir, 2001; Post et al., 2004].

GLIOMA ANGIOGENESIS: TRANSLATION

Several practical issues have complicated translation of therapies aimed at exploiting glioma angiogenesis. Some of these issues are common to all cancers, such as the redundancy in the signaling cascades that control the formation of the tumor vasculature, which makes the elimination of a single signal of questionable value in the long term. Combination therapies are the obvious conceptual answer, but pose challenges when the multiple agents are clinically unproven and need to first clear clinical trial hurdles in their own right. Other issues are either unique to gliomas or particularly pronounced in this family of tumors, and these include the diffuse nature of the disease and profound intra-tumoral heterogeneity, which means that not all tumors are homogeneously angiogenic. In addition, the simple assessment

of the disease burden on an ongoing basis in clinical practice is problematic. One should also not underestimate the difficulties of delivering therapeutic agents to a heterogeneous and diffuse target site, without prior knowledge of the optimized dose or schedule and without validated tools to easily determine therapeutic impact.

Despite these obstacles, therapies of various classes, based on what aspect of the angiogenesis cascade they target, are being actively tested in the clinic because they have demonstrated significant efficacy in animal models of angiogenesis and, usually, tumor growth [Kerbel and Folkman, 2002]. These include the so-called "true" angiogenesis inhibitors that target new vessel formation by inhibiting pro-angiogenic factors and their receptors, and show their therapeutic effect typically in days to weeks. They include angiostatin [O'Reilly et al., 1994, 1996], endostatin [O'Reilly et al., 1997], and the cyclic pentapeptide, EMD121974. This agent, which blocks the $\alpha_v ß_3$ and $\alpha_v ß_5$ integrins, has been shown to prolong survival in an animal model of glioma [Chatterjee et al., 2000], and is currently in Phase 2 clinical trial for recurrent glioblastoma. Vascular targeting agents, on the other hand, which act to destroy pre-existing tumor vasculature, exert their therapeutic effect within hours, with acute endothelial cell death, vessel thrombosis, hypoxia, and, often, necrosis. They include anti-tubulin agents, combretastatin A4 prodrug (CA4DP) and ZD6126, flavonoids DMXAA squalamine [for review see Siemann et al., 2002]. Finally, non-selective anti-angiogenic agents, which may act via their anti-proliferative, anti-invasive, or cytotoxic effect on multiple cell types, including endothelial cells, exert their effects over prolonged periods, depending on dosing. Dosing of these biologically non-specific agents may produce relative specificity to endothelial cells, as has been seen, for example, with certain types of low-dose chemotherapy including cyclophosphamide, 5-FU, methotrexate, paclitaxel, and vinblastine [Kerbel and Folkman, 2002]. Matrix metalloprotease inhibitors, such as BMS275291, captopril, col-3, marimastat, neovastat, prinomastat, and solimastat, also affect angiogenesis indirectly. Anti-cytokine agents thalidomide and its derivatives are another group of well-known angiogenesis inhibitors [D'Amato et al., 1994]. Cell locomotion inhibitors IFN-2-α and ion-flux inhibitors such as carboxyamidotriazole (CAI) have also been studied pre-clinically [Jacobs et al., 1997] with positive result, but with negative results in clinical trial (TM, unpublished results). In summary, despite strong rationale and promising pre-clinical results for all agents tested, clinical experience has not yet realized this promise. However, these early clinical trials with angiogenesis inhibitors have provided insights that can be used to refine drug development efforts and have underlined the need for new tools to assess clinical performance in patients which are not as simple, uniform, and tractable as animal models. This is a particular pertinent in gliomas, where it is widely acknowledged that measurement of patient response is difficult, and as it represents a significant bottleneck bears further investigation.

GLIOMA APOPTOSIS

There have been tremendous advances in the understanding of apoptosis, the most common form of programed cell death, particularly, in the identification and cataloging of involved molecules and understanding function and pathways [Lowe and Lin, 2000; Bogler and Weller, 2002]. Two categories of apoptotic pathways commonly serve as a framework for discussion. The "extrinsic" pathways start with ligand-mediated activation of transmembrane death receptors, which recruit and activate caspases through intermediary adaptor proteins. Death receptors, such as the prototypical CD95/Fas/APO-1, are activated by trimerization following ligand binding, in this case CD95L. The receptors then bind adapter molecules, such as Fas-associated protein with death domain (FADD) via a homophilic interaction mediated by death domains (DD), which in turn recruit regulatory pro-caspases, such as caspase-8, via another homophilic interaction, involving death effector domains (DED). This aggregation is called the death-inducing signaling complex (DISC) and its formation triggers the conversion of pro-caspases into active enzymes by proteolytic cleavage of the pro-domain, eventually leading to proteolytic activation of effector caspases, paradigmatically caspase-3 [Ashkenazi and Dixit, 1998]. These in turn then dismantle the cell by proteolytic cleavage of structural proteins [Thornberry and Lazebnik, 1998]. The "intrinsic" pathways

are triggered by intracellular events, and are regulated by the bcl-2 family of proteins, which has over 20 members that share protein–protein interaction motifs, called bcl-2 homology (BH) domains. BH domains mediate the complex homodimerization and heterodimerization, which are the basis of their activity [Adams and Cory, 1998]. Some members are anti-apoptotic, such as the oncoprotein Bcl-2, while others are pro-apoptotic, such as the p53-induced gene bax. Their main mechanism of activity appears to be regulation of pore formation in the mitochondrial membrane, which results in the release of cytochrome c and so the assembly of the apoptosome complex together with the apoptosis-activating factor (APAF)-1 protein. These proteins in turn recruit and activate the regulatory caspase-9, via a caspase activation and recruitment domain (CARD)-mediated interaction [Thornberry and Lazebnik, 1998; Gross et al., 1999]. Activation of effector caspases and dismantling of the cell ensues. An additional regulatory component comes from members of the bcl-2 family that have only the BH3 domain, and are important sensors of cellular damage and initiators of apoptosis by an intrinsic pathway that is independent of the nucleus [Huang and Strasser, 2000]. While the apparent dichotomy of the extrinsic and intrinsic pathway as described suggests that they function independently, this is actually rarely the case. The majority of cells, so called type II cells, require amplification via a mitochondrial loop to trigger apoptosis from an extrinsic signal and this applies to gliomas [Glaser et al., 2001]. There are some type I cells that are strong DISC generators which produce enough caspase activity at the DISC to undergo apoptosis [Scaffidi et al., 1998].

In addition to these apoptosis pathways there are other signaling networks that have a major impact on cell death, including the survival signals emanating from the PI3 kinase-Akt pathway. In malignant glioma, amplification of the epidermal growth factor receptor gene and mutation or deletion of the *PTEN* tumor suppressor gene, both of which activate the PI3K/Akt pathway, occur in 40%–50% and 30%–40% of glioblastomas, respectively [Smith et al., 2001]. Stimulation of tyrosine kinase growth factor receptors, including the epidermal growth factor receptor, promotes cell survival including that of glioma cells by activation of Akt [Wick et al., 1999]. It is likely that this deregulation of a pro-survival signal is a component of the intrinsic drug resistance of the tumor, reflected in generally poor results with chemotherapy [Weller et al., 1998; DeAngelis, 2001]. Therefore, inhibition of the PI3K/Akt survival pathway should decrease drug resistance and sensitize tumor cells to pro-apoptotic chemo and radiation therapy [Shingu et al., 2003].

In glioma, the relationship between spontaneous rates of apoptosis and tumor grade or treatment response are not straightforward. The dominant form of cell death in glioblastoma is necrosis rather than apoptosis, and this histological feature is part of the definition of this tumor type [Kleihues et al., 2000]. Analysis of glioma apoptosis by histological criteria or by use of the TUNEL assay has revealed the apoptosis rates vary greatly between different tumors of the same type, and within a tumor. Nevertheless, a positive correlation between apoptosis rate and tumor grade has been described in astrocytomas, and in some carefully controlled studies, a higher rate of spontaneous apoptosis predicted longer survival, but this was not the case in the majority of studies published to date [reviewed in Bogler and Weller, 2002]. Nevertheless, the possibility that triggering apoptosis in glioma cells by means of targeted therapies that alter the molecular balance in the favor of cell death affords strong hope for a breakthrough.

GLIOMA APOPTOSIS: TRANSLATION

Perhaps the simplest therapy would be to trigger an extrinsic pathway via the natural ligand, and most interest in this arena is currently focused on the Apo2 ligand (Apo2L)/TNF-related apoptosis-inducing ligand (TRAIL) and its death signaling receptors, death receptors (DR) 4 and 5, also known as TRAIL-R1 and TRAIL-R2. This is because there is a greater likelihood that this molecule will make a clinical contribution, as TRAIL does not induce the severe toxicities caused by CD95L [Ashkenazi et al., 1999], while remaining effective as a non-modified ligand, for example, in the treatment of human glioma xenograft in nude mice, in the absence of any neurotoxicity [Roth et al., 1999; Fulda et al., 2002]. Tumor cells, including those from gliomas, preferentially express the agonistic receptors, however, they also make TRAIL itself, suggesting that the presence of

ligand and receptor at the cell surface is not enough to result in significant levels of apoptosis in tumors, and allowing that an inhibitor of this pathway is also overexpressed, and may serve as a novel therapeutic target in its own right [Rieger et al., 1999]. An alternative hypothesis would be that there is not enough TRAIL in brain tumors to trigger apoptosis, which is supported by the observation that delivery of exogenous TRAIL kills glioma cells in culture and in xenografts [Roth et al., 1999].

Leaving aside global triggers of response to cellular damage, such as P53, which are beyond the scope of this review, most attention on the intrinsic side of apoptosis has focused on the bcl-2-related proteins, and in the context of neuro-oncology to the founder, bcl-2, and those members discovered soon after it, such as the pro-apoptotic protein bax. Much like the analysis of apoptosis rates itself, no consistent and simple correlation between tumor type and grade and bcl-2 expression level has been described, although some studies have shown that increased expression of anti-apoptotic family members was a negative prognostic marker in astrocytoma III and recurrent glioblastoma [reviewed in Bogler and Weller, 2002]. In cultured cells, it has been shown for gliomas, as for many other tumor types, that bcl-2 can protect from a variety of therapeutics including chemotherapies and radiation [Weller et al., 1995]. No small molecule inhibitors of bcl-2 are yet showing clinical promise, so approaches to downregulating the expression have been predominant and have showed some pre-clinical results in glioma [Julien et al., 2000].

The other approach to exploiting the bcl-2 family of proteins is to try and increase the activity of the pro-apoptotic members. Again analysis of the expression of these molecules has not yet revealed a simple pattern. However, levels of bax protein correlated with sensitivity of pediatric glioma cells to chemotherapy [McPake et al., 1998], and in vivo, reduced expression was associated with recurrence in glioblastoma [Strik et al., 1999], and with shorter times to progression in oligodendroglioma [Deininger et al., 1999]. Increased bax expression correlates with treatment sensitivity and increasing its levels induces apoptosis and sensitizes cells to other treatments [Shinoura et al., 1999; Vogelbaum et al., 1999]. Increased emphasis is now being placed on the BH3-only proteins, as they act as another starting point for the initiation of apoptosis and can be highly effective against gliomas [Naumann et al., 2003].

Integrins: Targets to Integrate Apoptosis and Angiogenesis Therapies

A third characteristic of glioma, besides resistance to apoptosis and very active angiogenesis, which makes them hard to treat is invasiveness. The parallels between tumor cell invasion and endothelial cells migration in the course of angiogenesis are well appreciated. However, a link between invasion and apoptosis is also coming to light, suggesting that there are molecular nodes important in all these aspects of tumor biology. Even lower grade gliomas, with the exception of pediatric pilocytic astrocytomas (WHO Grade I), do not have clean margins and residual, invading cells likely represent a major source of tumor recurrence. Understanding the state of these invading cells is important as they, rather than the bulk of the tumor, are the common target of radiation and chemotherapy that follows resection of most or all the tumor at the gross level. Examination of the gene expression profile of migrating glioma cells indicates that such cells are less prone to programed cell death than less motile counterparts [Mariani et al., 2001], suggesting an intriguing link between the two. This is substantiated by the report of a molecular link, via CAS/Crk, between the promotion of migration and the suppression of apoptosis, in the context of cellular invasion of a matrix: CAS/Crk signaling through Rac (a small GTPase) led to activation of invasion genes and survival genes in the cells; parallel recruitment of ERK-activated pathways led to myosin light chain kinase activity resulting in cell motility [Cho and Klemke, 2000]. This mechanism may extend to other cell types as well, of course, as suggested by the observation that cervical cancer cells, via expression of the anti-apoptosis clone 11 gene, are activated to invade along with a marked suppression of apoptosis [Kim et al., 2000]. Melanoma cells whose invasion activity is arrested via overexpression of TIMP are also rendered more susceptible to undergo apoptosis [Ahonen et al., 1998]. Direct manipulation of the propensity to undergo apoptosis can also alter migration, as demonstrated by the transfection of human glioma cells with the survival factor, bcl-2, which led to accelerated migration and more successful invasion into brain aggregates

[Wick et al., 1998]. Therefore, it appears that in addition to an inverse link between motility and cell division [Wild-Bode et al., 2001; Joy et al., 2003] that there may be a similar inverse relationship between invasion and apoptosis.

Cells, of course, exist in a context, and the extracellular matrix, which has long been seen as an active element in invasion and angiogenesis, is now being recognized as a cell survival factor [Meredith et al., 1993]. Therefore, another potential anti-apoptotic influence on invading glioma cells is interaction with the normal brain matrix, rather than the abnormal matrix in the bulk of the tumor. Survival signals in the ECM are potent modulators of tumor cell behavior, negating p53-mediated apoptosis [Ilic et al., 1998], and conferring heightened drug resistance [Sethi et al., 1999]. With the elucidation of the receptors mediating cellular interactions with the ECM, more detailed understanding of the role of physical adhesion to a substrate and the consequent signaling pathways engaged by the integrins has emerged [Meredith et al., 1996; Aplin and Juliano, 1999; Parise et al., 2000]. Therefore, strategies that interfere with blood vessel formation might at the same time render glioma cells more prone to undergo apoptosis.

In this context, integrins are one of the most promising molecules to consider, and in fact are being actively targeted for therapy already. One important mediator of the interaction between cell and matrix is integrin $\alpha_v ß_3$, a receptor for a wide variety of ECM ligands with an exposed RGD sequence, including vitronectin, fibronectin, fibrinogen, thrombospondin, proteolyzed collagen, von Willebrand factor, and osteopontin. In adults, integrin $\alpha_v ß_3$ has a rather limited tissue distribution, as it is not typically expressed on normal endothelial cells. Glioblastomas, however, express $\alpha_v ß_3$ prominently at the invasive borders, as do angiogenic endothelial cells in tumors [Enenstein and Kramer, 1994; Bello et al., 2001], suggesting that it is a key mediator of angiogenesis, invasion, and growth. Furthermore, ligand is also available. For example, it has been shown that vitronectin is highly expressed on glioma cells and integrin ligation with vitronectin inhibits apoptosis [Uhm et al., 1999]. Binding of cell surface integrins to the ECM stimulates a variety of intracellular signaling pathways including upregulation of both PI3K and ras-Akt signaling, which enhances cell survival pathways [Stupack and Cheresh, 2002]. Osteopontin expression in normal tissue, for example, appears to allow integrin ligation (via FAK) and enhanced migration [Gladson, 1999]. Integrin $\alpha_v ß_3$ ligation via vitronectin increases bcl-2 and inhibits bax [Uhm et al., 1999]. Therefore, these ligand interactions of $\alpha_v ß_3$ in migrating glioma cells contribute to the decreased susceptibility to apoptosis in response to chemotherapy and radiation, in addition to promoting invasion and playing a role in angiogenesis [Joy et al., 2003]. This suggests that therapeutics being developed as anti-angiogenic compounds, might also be evaluated for other effects on the tumor cells directly. At the present anti-neoangiogenic aspects of their function have been most actively investigated.

Disruption of $\alpha_v ß_3$ ligation with antibody (LM609) or cyclic peptide cRGDFV (Cilengitide, EMD121974) antagonists of $\alpha_v ß_3$ disrupts blood vessel formation in the chick chorioallantoic membrane (CAM) assay [Drake et al., 1995], without detectably influencing the preexisting blood vessels [Brooks et al., 1994a,b; Eliceiri and Cheresh, 1999]. Recent studies suggest that $\alpha_v ß_3$ can bind the matrix metalloproteinase-2 (MMP-2) in a non-RGD-dependent manner where it serves to localize the active form of the enzyme on the surface of endothelial cells [Brooks et al., 1996], enabling them to degrade and remodel the ECM during their invasion. Integrin $\alpha_v ß_3$ antagonists have been used in glioma efficacy studies, demonstrating evidence of glioma and endothelial cell apoptosis and prolonged survival in orthotopic models [Chatterjee et al., 2000]. Furthermore, $\alpha_v ß_3$ integrin antagonists are in active clinical use, either using humanized monoclonal antibodies (Vitaxin/Medi522) or the RGD-containing cyclic pentapeptide Cilengitide (EMD121974) [Smith, 2003; Tucker, 2003], currently in clinical trial for recurrent malignant glioma in the NABTT CNS Clinical Trials Consortium.

In summary, the molecular pathways controlling apoptosis and angiogenesis offer many opportunities for the development of targeted therapies, with the promise of effectiveness and little toxicity, while some are already reaching the clinic the complexity, and the extent of the signaling networks that control these processes suggest that there is more to come. Particularly, intriguing is the possibilities that some of the molecules under investigation have important roles in both areas, such as the integrins appear

to have along with the added bonus of functions in invasion. This could mean a single targeted therapy that reduces angiogenesis, increases apoptosis, and stops cells from invading.

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