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Laboratory Investigation

Proteases and the biology of glioma invasion

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Summary

Despite optimal clinical treatment, the prognosis for malignant gliomas remains poor. One of the primary reasons for treatment failure is not diffuse dissemination, but local invasion. Recently, there has been an increase in information regarding specific molecules that determine the aggressiveness and invasion potential of high-grade astrocytic tumors. In particular, expression of matrix metalloproteases in high-grade gliomas appears to correlate with tissue invasiveness. It is the purpose of the present review to describe the connection between alterations in growth-related genes, protease activity, and tumor biology, and how these connections may suggest potential novel therapeutic targets.

Abbreviations: bFGF – basic fibroblast growth factor; ECM – extracellular matrix; EGF – epidermal growth factor; EGFR – epidermal growth factor receptor; EGFRvIII – truncated constitutively active EGFR; FGFR-1 – fibroblast growth factor receptor-1; GBM – glioblastoma multiforme; MAPK – mitogen activated protein kinase; MMP – matrix metalloprotease; MT1-MMP – membrane-type 1 matrix metalloprotease; PDGF – platelet-derived growth factor; PKC – protein kinase C; PLC – phospholipase C; TBR – TGF-beta receptor; TGF-beta – transforming growth factor-beta; TIMP – tissue inhibitor of matrix metalloprotease.

Introduction

High-grade glial tumors (anaplastic astrocytoma and glioblastoma multiforme (GBM)) are the most frequent primary brain tumors in adults [1,2]. They usually occur sporadically without identifiable familial tendency or environmental risk factors. Commonly arising in the deep white matter of the cerebral hemispheres, they are often difficult to remove without damage to eloquent brain areas. Typically, they present with headache, seizures, mental status or personality changes, signs and symptoms of increased intracranial pressure, hemiparesis, or other neurologic deficit. Despite optimal current therapy, including surgery, radiation therapy, chemotherapy, and interstitial brachytherapy, high-grade gliomas are associated with a dismal prognosis [3]. Therefore, a more thorough understanding of the mechanisms of aggressive gliomas is essential to developing rational therapeutic strategies.

Local invasion: determinant of aggressiveness

In the first textbook devoted to brain tumors, Bramwell (1888) emphasized that the

> tendency to infiltrate the nervous structures is the most characteristic feature of the gliomatous tumour. The tumour tissue is never limited by a capsule, and it is impossible to say, without microscopical examination, where the tumour tissue ceases and the normal brain begins [4].

This characteristic, local tissue invasion, is the crucial cytologic attribute that distinguishes high-grade gliomas and makes efforts at resection often futile [5]. Furthermore, the intrinsic motility of glioma cells may be increased in high-grade tumors [6]. Morbidity and mortality from high-grade gliomas is directly related to their ability to invade and infiltrate surrounding tissue [1]. In careful studies, microscopic evidence of

malignant cells can be found well beyond the gross radiographic margins of the tumor [7]. Following surgical debulking or radiation therapy, such microscopic tumor foci lead to eventual local recurrence, usually within 2 cm of the original lesion [1,8,9]. In addition, stereotactic biopsy studies show scattered distribution of malignant cells [10]. Thus, the poor prognosis of high-grade gliomas is critically dependent on glioma invasion.

Biology of glioma invasion

Tumor invasion consists of several discrete steps, including tumor cell interaction with extracellular matrix (ECM) ligands, hydrolytic destruction of the matrix by release of proteolytic enzymes, and subsequent migration of the tumor cells through the area of destruction. Of all these steps, recent studies indicate that the ability of tumor cells to digest the ECM by secretion of proteolytic enzymes best correlates with the propensity for tissue invasiveness. Indeed, immunohistochemical analysis of the border zone between glioma and brain (glial *limitans externa*) has shown it to contain interstitial collagen, fibronectin, laminin, and type IV collagen [11]. The invasion of most primary human brain tumors is thought to be accomplished at least in part by elevated levels of proteases that breach connective tissue barriers, cause vascular remodeling and destruction of normal brain tissue.

Proteases and tissue invasion

What are the proteases involved in ECM digestion? CNS tissue contains three major groups of proteases and their inhibitors: (1) *matrix metalloproteases* (MMPs) and tissue inhibitors of MMPs (TIMPs); (2) *serine proteases*, including urokinase, tissue plasminogen activator (tPA) and plasminogen activator inhibitors (PAIs); and (3) *cysteine proteases*, recently implicated in apoptosis (programmed cell death). Of these groups, by far the most is known about the role of MMPs in tumor invasion. Interestingly, however, recent reports correlate the level of expression of cathepsins (cysteine proteases) with clinical invasiveness in gliomas [12] and meningiomas [13].

Matrix metalloproteases

MMPs are a multigene family of Zn^{+2}-dependent enzymes that degrade a variety of ECM molecules such as proteoglycans, glycoproteins, and types of collagen [14] (Table 1). To date, well over 20 MMPs have been identified [15]. MMPs are divided into several groups by substrate specificity (collagenases, stromelysins, gelatinases, and membrane-type MMPs); however, all contain Zn^{+2} and require Ca^{+2} for proteolytic activity (hence the name metalloprotease). Similar to pancreatic enzymes or coagulation factors, MMPs are secreted in latent precursor (zymogen) form containing an amino-terminal propeptide sequence removed upon activation.

Table 1. Matrix metalloproteases

MMP #	Common name
1	Collagenase 1
	Fibroblast collagenase
	Interstitial collagenase
2	Gelatinase A
	72-kDa gelatinase
3	Stromelysin 1
4	Not used
5	Not used
6	Not used
7	Matrilysin
8	Collagenase 2
	Neutrophil collagenase
9	Gelatinase B
	92-kDa gelatinase
10	Stromelysin 2
11	Stromelysin 3
12	Macrophage elastase
13	Collagenase 3
	Rat osteoblast collagenase
14	MT1-MMP
15	MT2-MMP
16	MT3-MMP
17	MT4-MMP
18	Collagenase 4 (*Xenopus*)
19	No trivial name
20	Enamelysin
21	XMMP (*Xenopus*)
22	CMMP (chicken)
23	No trivial name

The normal functions of MMPs involve processes requiring degradation of the ECM, including wound healing, bone growth and remodeling, angiogenesis, macrophage infiltration, and axonal growth cone extension [16,17] (Table 2). In 1980, Liotta et al. [18] were the first to demonstrate elevated expression of MMPs in melanoma cells with metastatic/invasive potential. Since then, this central role of proteases in tumor invasion has been amply demonstrated [19–23].

Table 2. Physiologic and pathologic roles of matrix metalloproteases

Physiologic roles	Pathologic roles
Wound healing	Tumor invasion
Bone growth and remodeling	Tumor angiogenesis
Angiogenesis	Tumor metastasis
Macrophage and neutrophil function	Rheumatoid arthritis
Embryogenesis	Gastric ulcer
Axonal growth cone extension	

Of central importance for the biology of glial tumors in particular is the extent to which MMP expression reflects histologic and clinical invasiveness. Indeed, immunohistochemical studies have shown that high-grade human gliomas (GBMs and anaplastic astrocytomas) express MMPs, whereas non-invasive low-grade astrocytomas and normal brain do not [24]. In addition, abundant evidence indicates that in high-grade gliomas proteolytic activity appears to be strongly correlated with destructive and invasive properties *in vitro* and *in vivo* [25–31]. The member of the MMP family emerging as most central in glioma invasiveness is MMP-2 (72 kDa-type IV collagenase). *In vitro* studies reveal that inhibitors of MMP-2 block glioma invasion, whereas increasing MMP-2 activity increases glioma invasiveness [32,33]. Recently, *ex vivo* studies as well demonstrate increased MMP-2 activity in resected glioblastoma specimens compared to normal brain or low-grade glioma [34].

Regulation of metalloprotease activity

Regulation of MMP expression is crucial in determining the overall level of protease activity and hence the propensity toward tissue invasion. Regulation of MMPs is accomplished at three levels. First, since MMPs are released from cells in latent precursor (zymogen) forms, they must be activated *in vivo* by other proteases. MMPs are activated by either the urokinase/plasminogen/plasmin system or by other membrane-bound MMPs [14,23]. MMP-2, the protease most prominently implicated in glioma invasiveness, has been shown to be activated not by the soluble urokinase/plasminogen/plasmin system but rather by an integral plasma membrane-bound protease termed MT1-MMP (membrane-type 1 MMP) [20,35]. MT1-MMP is also upregulated in human gliomas [31], and serves to concentrate the activation of MMP-2 activity at the cell surface, presumably to facilitate ECM digestion and thus invasion locally at the tumor margin.

Interestingly, the most common pattern of spread of malignant glioma cells is along the path of the deep white matter tracts, in particular the corpus callosum, producing the so-called 'butterfly glioma' [36,37]. How these cells can do this at all is of interest since CNS myelin contains proteins inhibitory to migration of most cell types. However, two recent studies shed light on this issue. First, white matter microglia have been shown to express high levels of MT1-MMP [38], and perhaps could activate MMPs secreted by invading glioma cells thus providing a permissive substrate for infiltration [23]. Second, C6 glioma cells and human glioblastoma cells themselves express MT1-MMP, and this appears to be required for migration through CNS white matter [39]. In support of this contention is the observation from the same study that transfection of naïve rat 3T3 fibroblasts with MT1-MMP bestows the ability to migrate on the (previously) nonpermissive myelin substrate and invade adult rat optic nerve explants [39].

The second mode of regulation of MMPs is transcriptional activation or repression. Most MMPs are not constitutively expressed but are modulated at the mRNA level by growth factors, cytokines, and oncogene expression (see below). For example, bFGF induces collagenase mRNA in endothelial cells [40], whereas transforming growth factor (TGF)-β1 inhibits [41] and EGF induces [42] stromelysin in fibroblasts.

The third mechanism of regulation of MMP activity is post-translational interactions with naturally-occurring TIMPs (TIMP-1 through TIMP-4) [43]. These proteins bind to MMPs and abrogate their proteolytic activity [14,44]. Thus, the net invasiveness of any tumor is thought to rely on the protease/antiprotease balance of tissue activity rather than absolute levels of protease gene expression. Indeed, as expected, potent anti-invasive activities of TIMPs have been amply demonstrated in multiple *in vitro* cancer models [45]. Transfection of cells and glioma cell lines with TIMPs greatly reduces local tumor invasiveness *in vitro* [46,47]. In concert, *ex vivo* data from glial tumor specimens show that TIMP levels correlate negatively with invasiveness (lowest for glioblastomas, higher for lower-grade gliomas and normal brain) [24,48]. In other words, just those molecules (TIMPs) expected to limit invasiveness by neutralizing MMPs are underexpressed in high-grade glial tumors. Whether and how TIMPs are coordinately regulated in glial tumors along with MMPs remains to be investigated.

Genetic alterations in growth-related genes

A prevalent current theory of neoplasia derived from studies of colon carcinoma holds that high-grade tumors result from step-wise and progressive loss of tumor suppressor genes (such as P53) and/or mutation-induced activation of important growth-related proto-oncogenes. These genetic changes together result in the promotion of growth and/or prevention of cell death in the tumor, and the acquisition of characteristics of malignancy, such as tissue invasiveness, the ability to metastasize, and resistance to antineoplastic chemotherapy. Similarly, alteration of growth-related genes in gliomas lead to increased tumor growth and invasiveness [49,50]. As summarized below, the alterations in these genes may lead to increased tissue invasiveness of gliomas via alteration of protease activity.

Tumor suppressor genes

Inactivation of tumor suppressor genes has been found to underlie many types of human cancer, including malignant glial tumors. P53 is the most common tumor suppressor gene implicated in human tumors. This gene encodes a 53-kDa protein product that is a transcription factor known to influence progression through the cell cycle, especially via inhibition of cyclin-dependent kinases (CDKs) [51,52]. Inactivation of P53 results in loss of growth control and subsequent neoplasia. The tumor suppressor genes NF1 and RB1 have also been associated with glioblastomas [49]. Interestingly, however, in contrast to the case with proteases, P53 mutations are common in low-grade glial tumors as well as in glioblastomas [53], although the number of P53 and other tumor suppressor gene mutations may differ between *de novo* GBMs and GBMs arising from prior low-grade tumors [54]. Thus, mutation of P53 may be involved in early stages of astroglial neoplasia but not be sufficient alone for the progression to high-grade tumors. From this, one might expect that the prognostic value of P53 is limited, and indeed the presence of a p53 gene mutation does not predict survival in glioma patients [55,56]. In contrast, there is some evidence that mutations in the tumor suppressor gene RB1 (retinoblastoma gene) may be involved in a subset of glioblastomas [57,58]. Also, loss of more recently described tumor suppressor genes such as PTEN on chromosome 10 may contribute to oncogenesis following deletion of a variety of chromosomal loci in high-grade glial tumors [59,60].

Inactivation of known tumor suppressor genes may directly regulate metalloprotease expression and/or activity and thus directly influence tumor invasiveness. For example, mutations in the TGF-beta receptor (TBR)-II gene, one of the three TBRs, in various carcinomas suggest that these molecular alterations are responsible for altered matrix metabolism (in tumor and stromal cells) [61]. Indeed, P53 has been shown to directly downregulate MMP-1 expression [62]. Similarly, reintroduction of wild-type P53 in human melanoma cell lines reduces tissue invasiveness in association with reduced levels of secreted MMP-2 [63].

Cyclins

Cyclins, which trigger downstream signaling cascades by activating CDKs, are integrally involved in cell growth and differentiation. Cyclin D1 in particular is known to regulate the G1-S cell-cycle phase transition and has been implicated in tumorigenesis [64,65]. Furthermore, cyclin D1 is amplified or over-expressed in a variety of tumors and is associated with a greater risk of relapse [52,66].

MMPs may be part of the effector mechanism for cyclin regulation of tumor progression and invasiveness. For example, a recent study shows that overexpression of cyclin D1 increases MMP activity and resultant cell motility and invasiveness [67]. Therefore, increased expression of growth-related genes such as cyclin D1 may not be simply a proliferative tumor signal but may also directly elaborate proteases to increase tissue invasiveness. Further studies are necessary to delineate the alterations in cyclin activity during glial tumorigenesis.

Growth factors and growth factor receptors

Mutations in many growth factors and their receptors have been discovered in malignant gliomas. The co-expression of growth factors and their cognate receptors by the same tumor allows for autocrine stimulation of growth. For example, gliomas are known to concurrently express the angiogenic factor and astroglial mitogen basic fibroblast growth factor (bFGF) and its receptor FGFR-1 [68–70]. Similarly, gliomas have been shown to co-express platelet-derived growth factor (PDGF) and its receptor [71–73].

However, the most common and best-described alteration appears to be amplification of the receptor for epidermal growth factor (EGFR) in a high proportion

of malignant gliomas, occurring in approximately 40% of GBM tumors but in very few low-grade glial tumors [74–76]. In addition, some GBMs express a truncated receptor that is constitutively phosphorylated (termed EGFRvIII) [77,78]. This constitutively active receptor has been associated with increased activation of downstream metabolic signaling pathways (e.g. ras-MAP kinase cascade) and with increased growth potential.

The prognostic relevance of EGFR gene amplification or mutation in human glioblastoma has been controversial [49]. However, a recent study examined the molecular phenotype of EGF receptors and downstream signaling transduction pathways in a group of astrocytic neoplasms [79]. In this study, specimens from 15 tumors (12 GBMs, 2 gliosarcomas, and 1 low-grade glioma) and control tissue were analysed for EGFR phenotype and ras/MAPK activation. The authors found higher levels of activated ras and MAP kinase in GBM specimens compared to low-grade glioma or normal brain. In addition, they demonstrated a correlation between expression of the constitutively active form of the EGF receptor (EGFRvIII) and shorter life expectancy (4 months for EGFRvIII-positive tumors and 11 months for EGFRvIII-negative tumors). Such evidence suggests that molecular classification of glial tumors may help prognosticate outcome as well as target molecular therapy.

Interestingly, as for cyclins, effects of growth factors on tissue invasion may be directly related to their modulation of MMP expression. For example, EGFR expression in non-small-cell lung cancer [80] and head and neck squamous cancers [81] strongly correlates with increased MMP-9 activity and poor clinical outcome. bFGF is known to upregulate collagenase in endothelial cells [40] and EGF induces stromelysin in fibroblasts [42]. These effects may be at least partially via growth factor-mediated stimulation of protein kinase C (PKC), a phospholipid-dependent serine–threonine kinase that is a central intermediate in signal transduction pathways from the cell surface to the nucleus. PKC activity is high in high-grade gliomas [82], and inhibition of PKC via chemical inhibitors or antisense oligonucleotides dramatically reduces the invasiveness of malignant gliomas *in vitro* [83] and *in vivo* [84]. Similarly, PKC inhibition with calphostin C reduces and PKC activation with phorbol esters increases MMP-2 activity in concert with invasiveness *in vitro* [33]. In recent work, Yong and colleagues [23] have suggested that the mechanism by which PKC activation leads to increased MMP-2 activity is via transcriptional activation of MT1-MMP (the primary activator of MMP-2). Through these mechanisms, there appears to be a direct link between upregulation of growth-related genes and downstream expression of increased matrix-degrading protease activity.

Angiogenesis and protease expression

Vascular remodeling and growth of new vessels (angiogenesis) is a critical component of tissue invasion and tumor progression. Tumor vasculature often exhibits distinct expression of cell surface proteins, including integrins, growth factor receptors, and MMPs. Thus, in addition to the role for proteases described above in tissue invasiveness and their potential connection to tumor suppressor genes, cyclins, and growth factors, proteases may also be specifically involved in tumor angiogenesis.

Indeed, MMP-2 (gelatinase A) is known to be upregulated in GBM, and especially in endothelial cells of blood vessels, suggesting a role in the marked neovascularization that is a hallmark of glioblastomas [34]. Consistent with this possibility, MMP-2-deficient mice demonstrate reduced angiogenesis and tumor progression originating from implanted melanoma or lung carcinoma cells [85].

From a therapeutic perspective, selective upregulation of molecules in tumor endothelium provides a window for molecular discrimination between normal and tumor tissue, and this approach is actively being pursued. For example, TIMPs may act to inhibit tumor angiogenesis [43]. The precise interactions between MMPs and tumor angiogenic factors such as VEGF and their regulation remain to be better defined. However, tantalizing evidence exists for such a connection. For example, MMP-9 is known to release extracellular VEGF to accomplish the ‘angiogenic switch’ in pancreatic islet cell carcinogenesis [86].

Prognostic factors and therapeutic targets

The constellation of cellular alterations in glial cells that leads eventually to a highly-invasive glial tumor has been extensively studied. As described above, a variety of mutations in growth-related genes (oncogenes) and tumor suppressor genes have been discovered [49]. Accumulation of these mutations putatively leads to cellular alterations in signal transduction pathways involving molecules such as PKC that have as

their downstream targets protease activities that mediate tumor invasiveness. It is clear that rate of proliferation of tumor cells alone does not account for the 'malignancy' of a glial tumor; rather, the invasiveness of high-grade glial tumors is at least as insidious and is directly related to local recurrence and eventual treatment failure.

Therefore, the most clinically relevant downstream effect of genetic alterations in glial tumorigenesis may be the elaboration of molecules supporting invasiveness. Recent advances in understanding the signal transduction pathways and effectors of tumor invasion have not only provided potential molecular prognostic factors for individual tumors but also suggested critical steps that provide therapeutic targets for modification and inhibition of the cascade of tumor invasion.

Growth factor signaling mechanisms have been targeted in pre-clinical and early clinical trials of novel therapeutic agents. For example, radiolabeled (^{125}I) monoclonal antibodies to the EGF receptor have entered Phase II clinical trials [87]. In another approach, immunotoxins directed at novel EGF receptors expressed selectively in glioblastomas have shown activity in an animal model of neoplastic meningitis [88]. In addition, antisense oligonucleotides to EGF RNA inhibit growth of C6 glioma cells *in vitro* and *in vivo* [89]. Protein kinase C, a second messenger target of EGF, is also a promising target of anticancer therapy [90]. In a small subset of glioma patients, chronic high-dose oral tamoxifen, known to inhibit PKC [91], provided some improvement [92,93]. In addition, inhibition of phospholipase C (PLC)-γ, a cytosolic enzyme that is an important downstream effector of EGFR activation, abrogates glioma invasion in animal models [94]. It is possible that determining the specific growth factors and growth factor receptors expressed by a particular tumor (molecular phenotyping) would aid in more specific therapy in the future.

Matrix-degrading proteases appear to be an extremely promising target for therapy [23]. Whatever the upstream mechanism of neoplasia (e.g. EGFR amplification, P53 mutation, cyclin alterations), proteases may constitute a 'final common pathway' for tumor invasion [23]. Alterations in oncogenes, growth factors, second messenger molecules (e.g. PKC), and cyclins act in concert to modulate protease activity. Increasing evidence suggests that virtually all tumors utilize proteases to invade the ECM. Furthermore, knowledge of the regulation of protease activity has led to synthetic inhibitors of specific proteases, which may specifically target therapy toward individual proteases and individual tumors. For example, since MMP-2 appears to be the MMP most strongly implicated in glioma invasiveness, specific MMP-2 inhibitors that do not affect other MMPs would perhaps better target glioma invasion and be better tolerated than broad-spectrum MMP inhibitors. An alternative target would be MT1-MMP, which activates MMP-2 and by itself can confer invasiveness to glioma cells *in vitro* [39].

Indeed, *in vivo* studies have already demonstrated efficacy of MMP inhibitors at inhibiting tumor invasion [95,96]. BB-94 (batimastat), a synthetic broad-spectrum MMP inhibitor, reduces invasiveness of a variety of tumors in animal models [97,98]. However, its poor oral bioavailability led to the synthesis of marimastat [99], which is in early clinical trials. Both of these molecules are synthetic peptides that mimic the structure of collagen and form a complex with the zinc ion at the active site of the MMPs. In a recent study, both BB-94 (batimastat) and BB-2516 (marimastat) demonstrated efficacy at inhibiting glioma invasion *in vitro* [100]. AG3340, a newly-developed potent MMP inhibitor, has shown strong antitumor properties *in vitro* and *in vivo* in a variety of tumor models [101]. Once-daily injections of AG3340 markedly inhibited U87 glioma growth in nude mice [102]. Histologic evidence of invasion was also reduced in AG3340-treated tumors. In this study, AG3340 decreased tumor size by 78% compared to vehicle-treated control mice; most dramatically, treated mice survived an average of 71 days compared to 31 days for controls [102].

Of course, the normal functions of proteases in the brain and body must be taken into account when considering protease inhibition as a therapeutic intervention. It is of note, however, that inhibitors of angiotensin-converting enzyme (ACE), a metalloprotease, have been well-tolerated for many years in the treatment of hypertension and congestive heart failure, with minimal long-term side effects. This establishes a clinical precedent for nontoxic systemic delivery of a specific metalloprotease inhibitor.

Conclusion

Recent studies have delineated some of the molecular factors involved in invasiveness and aggressiveness of glial tumors. These include tumor suppressor gene mutations, alterations in cyclins, mutations in growth factors and their receptors, and increased matrix-degrading protease activity. At minimum, therapies aimed at these targets could be seen as adjuncts to

the traditional modalities of resective surgery, cytotoxic chemotherapy, radiation therapy, and interstitial brachytherapy. One serious issue is raised by a recent study that demonstrated that sublethal radiation may lead to an increase in the MMP expression and invasiveness of glioma cells [103]. Assessment of the status of growth factor and growth factor receptor gene expression and protease activity may aid in prognostication of tumor invasion and clinical outcome. The level of protease activity may constitute a 'final common pathway' for tumor invasiveness and as such may be a particularly appropriate target for anti-invasive therapies. Successful limitation of tumor spread by anti-invasive agents at particular stages of glial tumorigenesis could help to convert an infiltrative tumor into a local tumor and thus convert a lethal to a chronic disease, or at least restore efficacy to focal tumor therapies. To the extent that invasiveness constitutes the single most destructive characteristic of malignant gliomas leading to poor clinical outcomes, it is likely that the development of potent and specific anti-invasive agents will be an invaluable addition to the treatment armamentarium.

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