Cytostatic Agents in the Management of Malignant Gliomas
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The basic scientific studies of the angiogenic and migratory capacity of malignant brain tumors provide new areas for potential therapeutic strategies.

Background: Cytotoxic therapy for malignant gliomas is limited by poor delivery and drug resistance, and local therapy is ineffective in managing migratory cells. However, recent developments in malignant glioma therapy involve trials of cytostatic rather than conventional cytotoxic agents.

Methods: The biology of the brain extracellular matrix, tumor invasion, and angiogenesis are reviewed, and the cytostatic agents that inhibit matrix metalloproteinases, angiogenesis, cell proliferation, and signal transduction are discussed, as well as studies of the angiogenic and migratory capacity of malignant brain tumors.

Results: Two specific and interrelated areas, anti-invasion (migration) and anti-angiogenesis, are potential areas to develop new treatment strategies. Tumor invasion and angiogenesis are important components of the spread and biologic effects of malignant gliomas. Several proteinase inhibitors are in clinical trial, as well as anti-angiogenic agents and signal transduction cascade inhibitors.

Conclusions: Biologic control of brain tumor cell populations may offer a new management approach to add to currently available management options for malignant brain tumors.

Outcomes in Malignant Gliomas

The prognosis for patients with malignant gliomas has not significantly changed in recent years. Despite debulking surgery, radiation, and cytotoxic chemotherapy, median survival has changed little and is still measured in weeks. In the United States in 1995, these tumors affected 17,200 patients and caused 13,300 deaths, for a case-mortality ratio of 77%. Brain tumors constitute the No. 2 cause of cancer deaths in patients under 15 years of age, the No. 3 cause for adult men, and the No. 4 cause for women aged 15 to 34 years. In the 35- to 54-year age-group, brain tumors remain the No. 4 cause of cancer deaths in men.¹ These statistics bear out the presumption that brain tumors are highly fatal and often strike patients in their most productive years. The incidence of glioblastoma is also rising, especially in older adults, the poorest prognostic group.² Due to the lack of significant progress with conventional cytoreductive approaches, novel therapies and approaches to therapy are well warranted.

Cytotoxic vs Cytostatic Therapy

The concept of cytostatic agents being used to restrain tumor progression (rather than induce cytotoxic cytoreduction) has recently emerged.³ This concept questions the current therapeutic model in cancer management derived from microbiology, in which cancer cells are considered to be different from the host and these differences are exploited therapeutically. Continuing the analogy to infection, conventional wisdom has purported that unless cells are killed and totally eliminated, they will overwhelm the host.

A regulatory model has recently been proposed in which cancer can be viewed as a dynamic maladaptive process that originates within the host, is constantly in evolution, and is potentially reversible.⁴ This model is consistent with the molecular genetic understanding of cancer processes such as clonal evolution that has been demonstrated in gliomas.⁵ One implication of such a model is that by reimposing biological control on a cell population or a malignant phenotype, functional control of a tumor may be gained without requiring complete tumor elimination. Management of these malignant phenotypes, then, constitutes a novel avenue for therapeutic research. Anti-invasion/anti-angiogenic therapy represents one such strategy in malignant gliomas and relies on a molecular understanding of these phenotypes.

Invasion in Human Glial Tumors at Onset and at Clinical Recurrence

Gliomas in general and more highly anaplastic gliomas in particular infiltrate and spread great distances in the brain. The regional infiltration during tumor progression has been shown most strikingly in the whole-mount studies of Scherer⁶ and Burger et al⁷ in which glioblastoma cells appear to arise within a bed of better-differentiated tumor. In histological sections, most glioblastomas contain a central area of necrosis surrounded by a highly cellular rim of tumor and a peripheral zone of infiltrating cells. Infiltration of tumors cells along white matter tracts, around nerve cells, along blood vessels, and beneath the pia (secondary structures of Scherer⁸,⁹) is responsible for local and widespread recurrence and clinical tumor progression. Angiogenesis, the proliferation of neovasculature, is also a pathologic hallmark of malignant gliomas. The recruitment and proliferation of new vessels, which typically do not form an intact blood–brain barrier, result in the pattern of contrast enhancement seen in magnetic resonance imaging (Figure).
Biology of Tumor Invasion and Angiogenesis

The invasive nature of glioma cells and the accompanying neovascularature is perhaps the key feature in their persistence beyond therapeutic margins and is the primary reason for tumor recurrence and malignant progression. Both invading glioma cells and neovascular endothelial cells must pass through the brain extracellular matrix (ECM), a process that involves three major interrelated steps: (1) adhesion/disadhesion, (2) enzymatic degradation of the components of the parenchymal matrix, and (3) locomotion through the parenchymal barrier.10-12

Adhesion and Disadhesion

Coordinated adhesion and proteolysis of adhesive contacts occurs in many normal and pathologic processes, including trophoblast implantation, wound healing, tumor cell invasion, and angiogenesis. Proteinase/matrix interactions are presumed to regulate process extension by invadopodia and endothelial cells as modification of local matrix interactions permits process extension and cell locomotion. The relation of ECM adhesion and signaling with regard to tumor cell invasion is being pursued in our laboratory as well as many others institutes.

Proteolysis

Proteolysis of brain ECM has been suggested by the observation of overexpression of all major proteinase classes, including matrix metalloproteinase (MMP),13 cysteine proteinase (CP),14-17 serine/threonine proteinase (SP),18,19 and aspartic proteinase (CD). Few functional studies have been done to substantiate these observational studies.

Locomotion

Tumor cell locomotion involves a coordinated set of cellular responses; morphologic polarization (receptor asymmetry for integrin/cytoskeletal contacts), membrane extension (invadopodia), cell-substratum attachments, contractile force/traction, and release of focal attachments.20 Cathepsin B has been localized to focal regions in breast cancer and glioma cells in contact with the ECM,21 and the proteolysis of matrix components can be seen beneath such focal contacts, which can be inhibited by cathepsin B inhibitors. Inhibitors of cathepsin B inhibit melanoma cell motility induced by autocrine motility factor (AMF)22 in melanoma cells and in response to glioma-conditioned media (T.M., unpublished observations, 1997).

Molecular Mechanisms of Angiogenesis

Angiogenesis, the formation of new blood vessels, occurs in a variety of normal and pathologic conditions.23 In physiologic states, such as embryonic development and wound healing, neovascularization is a strictly regulated balance of expression of stimulatory and inhibitory angiogenesis factors.24 The disruption of this finely tuned regulatory pathway and the formation of a pathologic capillary network occur in a variety of disease states, including cancer, diabetic retinopathy, hemangiomata, and vasculitides.25 Tumor neovascularization begins with the sprouting of new capillary buds from an existing vessel in response to direct or indirect angiogenic stimuli. The angiogenesis response occurs as a result of proteinase secretion and basement membrane remodeling, endothelial cell proliferation, and endothelial cell migration to form capillary sprouts and neovascular lumina.26 The parallels between tumor cell invasion and endothelial cells in angiogenesis are striking. For example, the role of the lysosomal proteinase cathepsin B (CB) in the process of angiogenesis has been shown in invasive prostate cancer by immunoelectron microscopy and in situ hybridization.27 CB was demonstrated in proliferative neoendothelial cells in the invading zone. Our own work in immunohistochemistry has demonstrated CB expression, not only in tumor cells, including the infiltrating margin, but also in neovascular endothelial cells.28

Invasion and Angiogenesis: Proteinases, Inhibitors, and Malignancy

The proteinases that participate in malignant progression are numerous. Among the proteinases implicated in the progression of animal and human tumors are members of the four classes of endopeptidases: (1) matrix metalloproteinases such as stromelysin and gelatinases A and B, (2) serine proteinases such as urokinase, (3) aspartic proteinases such as cathepsin D, and (4) cysteine proteinases such as cathepsin B. There is an increasing awareness of the role played by cell surface proteinases in the malignant phenotype, due in part to the activation of other matrix metalloproteinases at the cell surface by the recently discovered membrane-associated matrix metalloproteinases.

Proteinases may affect infiltrative capacity of tumor cells in several ways. First, proteinases are capable of degrading ECM and basement membrane (BM) components, which act as barriers to tumor infiltration and metastasis. Limited degradation of the ECM, upon which cells also migrate, divide, and differentiate, allows movement of tumor cells through perivascular channels and white matter (myelin) tracts of the brain. Expression of ECM components is largely limited to the perivascularure of the brain. Production of several ECM components is altered in intracranial tumors. As the complement of proteinases in both intracranial and extracranial tumors is similar, it is possible that the unique BMs ECMs of the tumor perivascularure prevent formation of brain tumor metastases to tissues outside the central nervous system (CNS). The mechanisms by which MMPs and uPA degrade ECM and BM surrounding arteries and veins of injured brain have been described.29

In addition to opening migratory pathways, proteinases can alter cell adhesion properties regulated through several classes of cell surface receptors. These receptors, including cadherins, CD-44, integrins, and receptors for fibronectin, laminin, and vitronectin, negatively regulate cell motility and growth through cell-cell and cell-matrix
interactions. Thus, proteolytic degradation of receptors and/or ECM components could release tumor cells from these constraints. Proteolysis of cell–matrix interactions is tightly controlled by tumor cells that must maintain a substratum upon which to move at their leading edge while detaching from that same support at their trailing edge. This regulation may be accomplished through increased production of proteinases at the leading edge of the tumor where they are in an ideal location to down-regulate proteolytic activity. As described below, the increased expression of several inhibitors has been positively correlated with increased infiltrative capacity of several tumors. Although contradictory at first glance, up-regulation of inhibitors maintains the balance between proteolysis and inhibition. This balance is required for the cyclic attachment of tumor cells to the ECM, followed by focal dissolution of ECM components and substrate-binding cell surface receptors and release from the ECM. Inhibitors not only protect tumor cells from degradation during this process, but also ensure focal degradation of the ECM. Proteinases and inhibitors are known to be secreted from both tumor and host cells and to be stored in the ECM. Growth factors are also trapped in the ECM and may also be released upon its degradation.

Immunohistochemical studies of proteinases in both gliomas and extracranial tumors have indicated that they may also play a role in angiogenesis. Further, because the BM of new arterioles and veins is incomplete, tumor cells may be able to migrate through this partial barrier and metastasize to distant regions of the CNS and occasionally to extracranial sites. Several proteinases and proteinase inhibitors have been implicated in these processes leading to tumor progression and infiltration, as already noted. The putative role(s) of individual proteinases and inhibitors in intracranial tumor cell infiltration are discussed in more detail below.

Matrix Metalloproteinases and Inhibitors

MMPs are metal-dependent endopeptidases that may be divided into two classes: those that are secreted (as inactive zymogens) and the newly described membrane-type MMP (MT-MMP), which is associated with the cell surface via a transmembrane domain near the carboxy-terminus. Only one study has addressed the expression of MT-MMP in brain tumors. Results of Northern blot, reverse transcriptase-polymerase chain reaction, and immunohistochemical analyses in this study indicated that MT-MMP mRNA and protein are expressed in astrocytoma cells but not in normal brain tissue. Furthermore, expression of MT-MMP was shown to be positively correlated with gelatinase A expression during malignant progression of gliomas. Interestingly, in both of these studies, using immunohistochemistry MT-MMP protein was localized to tumor cell surfaces. MT-MMP has been shown to activate pro-gelatinase A in the absence of tissue inhibitor of metalloproteinases (TIMP)-2. As discussed below, gelatinase A is expressed in several human brain tumors and tumor cell lines. Amberger et al also described a membrane-bound MMP purified from rat C6 glioblastoma cells. Homology of this proteinase and MT-MMP has not been determined. When O-phenanthroline (an inhibitor for MMPs) or a synthetic substrate selective for MMPs was added to C6 cultures, spreading on myelin plates was inhibited. This may indicate a role for MT-MMPs in rat glioblastoma cell migration or invasion.

Abe and colleagues demonstrated a correlation between increasing gelatinase A expression at the mRNA level and glioma cell-line invasion as measured with Matrigel barrier invasion assays. In this study, nine cell lines demonstrating variable abilities to invade Matrigel were examined for gelatinase A expression by Northern blotting. Those cell lines most active in the invasion assay also contained the highest amount of gelatinase A mRNA. Gelatinase A mRNA production has also been detected in glioma cell lines by Costello et al. Expression of matrilysin and stromelysin message in glioma cell lines has been shown to be highly variable and does not seem to correlate with invasive capacity. Similarly, the expression of interstitial collagenase mRNA seems to vary according to the glioma line examined. Expression of gelatinase B and stromelysin-2 has not been examined in intracranial tumor cell lines at the mRNA level.

At the level of protein activity, several investigators have detected gelatinase A in conditioned media of intracranial human tumor cell lines and rat BTSC glioblastoma cells. Levels of gelatinase A mRNA production correlate with protein activity and expression. Gelatinase A activity as measured by zymography was highest in those cell lines that were most invasive as measured by the Matrigel invasion assay. Both the zymogen and active forms of gelatinase A are secreted by CNS tumor cell lines. Although such results may argue for a role for gelatinase A in intracranial infiltration, the ECM of the brain does not resemble the makeup of Matrigel. Thus, these studies, like those in extracranial tumors, may imply that gelatinase A may be involved in intracranial infiltration but do not provide direct evidence of such a phenomenon. As with mRNA expression, the levels of protein activity and expression have not been determined for gelatinase B, interstitial collagenase, or stromelysin-2 in vitro. Likewise, the expression of matrilysin and stromelysin protein expression and activity has yet to be undertaken in intracranial tumor cell lines.

The major inhibitor of gelatinase A is TIMP-2, which prevents degradation of solubilized collagen by gelatinase A purified from the rat glioma cell line BTSC. Lund-Johnsen and coworkers have shown that gelatinase A purified from BTSC glioma cell-conditioned media is capable of destruction of fetal rat brain aggregates in a manner similar to that observed for normal rat brain spheroids confronted with BTSC spheroids. Such results suggest a direct role for gelatinase A in intracranial tumor cell infiltration. These results also uphold the circumstantial evidence of increased expression of gelatinase A correlating with increased infiltrative capacity. Although gelatinase B is also expressed by BTSC cells, its correlation with infiltrative capacity was not further studied by these two groups. The roles of interstitial collagenase, matrilysin, stromelysin, and stromelysin-2 in intracranial tumor cell progression remain unclear in vitro.

In clinical material, many researchers have used Northern blotting to examine mRNA expression of MMPs in intracranial tumor specimens. Nakano and colleagues examined nonmatched normal and glioma tissue specimens for expression of several MMPs, including interstitial collagenase, gelatinases A and B, matrilysin, and stromelysin. Interstitial collagenase and stromelysin were not expressed in any samples. Such results may indicate that these MMPs are not directly involved in infiltration of normal CNS tissue by any of these tumor types. Expression of gelatinases A and B is increased in parallel with glioma tumor progression. Thus, these MMPs may play a role in glioma cell infiltration as glioblastoma multiforme (GBM) is the most aggressive of the tumor types followed by anaplastic astrocytoma (AA) and astrocytoma. Although not consistent among all samples, matrilysin mRNA levels were also increased in GBM in comparison to lower-grade tumor and normal specimens. These results indicate that matrilysin may play only a minor role in infiltration of the ECM by brain tumor cells.

At the protein level, immunohistochemical studies have shown that interstitial collagenase levels are increased in parallel with malignant progression of brain tumors including GBM, AA, and astrocytoma. Increased levels of interstitial collagenase protein were not found in meningioma or neurinoma. While correlative, these results suggest that although expression of message for interstitial collagenase is below detectable limits of the assay used by Nakano et al., posttranscriptional controls may modulate protein production. Such results might also lead to the hypothesis that interstitial collagenase plays a major role in glioma infiltration. Measurement of interstitial collagenase activity has not been reported for intracranial tumor specimens, although they would provide evidence to prove the involvement of interstitial collagenase in the infiltrative capacity of CNS tumors. Levels of stromelysin protein detected in GBM, AA, and astrocytoma do correlate with the Northern blot analyses cited above. Expression of stromelysin protein did not correlate with formation of meningiomas or neurinomas. This proteinase might not be expected to play a major role in infiltration by any CNS tumors examined; studies have yet to be performed that examine stromelysin activity in intracranial tumors and normal brain specimens.

Interestingly, significant increases in gelatinase A protein are not found consistently in gliomas, although gelatinase A mRNA expression increases in correlation with progression of gliomas. This lack of agreement between mRNA and protein studies may reflect the use of nonmatched normal and tumor specimens in each of these studies as levels of proteinase expression may well be expected to be dependent on the individual. In support of gelatinase A having a role in intracranial infiltration, expression of protein has been detected in glial tumor cells by zymography and has been localized immunohistochemically to tumor cells, to endothelial cells of...
vascular structures surrounding the tumor mass, and to the tumor microvascular endothelium. These results may indicate that gelatinase A is important to infiltration of glioma and formation of neovessels in some but not all patients. Further studies of matched normal and tumor tissues might clarify the regulation and role of this protease in progression of gliomatous tumors. Expression of gelatinase A in meningioma and neurinoma has been shown to be increased as compared to nonmatched normal specimens in some cases, but the data are also inconsistent. No conclusions can be drawn concerning the role of gelatinase A in these intracranial tumors.

Among the MMPs, gelatinase B protein and activity as measured by zymography is most consistently associated with progression of gliomas. Protein expression3,41 can also be seen to correlate well with mRNA levels37 in gliomas and normal tissues, suggesting that this MMP plays an integral role in infiltration of normal brain by glioma cells. Gelatinase B may also modulate formation of meningioma foci as increases in gelatinase B protein and activity have been indicated in zymographic analyses of nonmatched meningioma and normal brain specimens by two laboratories.13,41 In addition, gelatinase B protein is highly expressed in neurinoma specimens as compared to nonmatched normal tissues.42 To our knowledge, levels of matrilysin protein and activity have not been determined in brain tumor specimens.

In studies of extracranial tumors, TIMPs and inhibitors of metalloproteinases (IMPs) may aid in controlled degradation of the ECM.30,42 These inhibitors have, in fact, been used to substantiate a role for MMPs as a class in tumor cell invasion in vitro studies of extracranial tumor cell lines,43-45 TIMP-1 strongly inhibits interstitial collagenase, pro- and active gelatinase B, and active streptolysin. Gelatinase A is inhibited by TIMP-1 to a lesser degree but is strongly inhibited by TIMP-2/IMP-2 at a 2:1 inhibitor-to-proenzyme ratio or 1:1 inhibitor-to-active enzyme ratio.46 A biological function has yet to be ascribed to IMP-1 and IMP-3.

**Effects of Cell-Cell and Cell-Matrix Interactions on TIMPs and MMPs**

The expression, secretion, and localization of TIMPs and MMPs comprise a complex process believed to be regulated in part by cell-cell and cell-matrix interactions. In an immunohistochemical study of high-grade bladder tumors, Grignon and colleagues47 have shown that TIMP-2 protein is expressed in stromal cells only in areas where BM has been extensively degraded and that gelatinase A and B proteins are overexpressed by actively invading tumor cells (ie, at the invading edge). Arguably, either collagen or tumor cells themselves signal stromal cells to express TIMP-2 in this system. TIMP-2 mRNA, however, was not localized in this study, and thus the protein may have been produced in and secreted from tumor cells only to localize elsewhere. Additional studies of bladder cancers have indicated that gelatinase A mRNA is expressed primarily in stromal cells surrounding the tumor mass,48 whereas the protein is localized to tumor cells. Gelatinase A and B are more strongly expressed in all tumor cells, particularly at the leading edge of the tumor. Interestingly, all areas of vascular proliferation in tumor specimens exhibit strong staining for uPA mRNA, indicating a possible role for the serine proteinase in angiogenesis. Expression of uPA mRNA (measured in unmatched normal specimens, astrocytoma, AA, and GBM) also positively correlates with increasing tumor grade of gliomas.70 In situ hybridization corroborated the Northern results; uPA mRNA

The serine proteinases plasmin, tPA, and uPA have been implicated in the malignant progression of breast,53 colorectal,54 lung,55 prostate,27 and intracranial tumors, and tPA and uPA are secreted as inactive precursors that may bind to ECM components. The specific cell surface receptor for uPA (uPAR) is approximately 45 to 60 kDa and has been isolated from both a human lung carcinoma cell line38 and transformed U937 cells.57 uPAR is bound to the cell surface via a glycosyl-phosphotidyl insitol moiety that replaces a portion of the COOH-terminus of the protein during maturation in the endoplasmic reticulum. An extensive review of the functional properties of uPAR has been written.59,60 Although known to bind to the cell surface, no specific receptors have been identified for plasminogen or tPA; both have been shown to bind to fibrin during clot dissolution, and such binding increases the activity of tPA.59,60

Pro-uPA may be activated by plasmin,61 plasminogen,62 and CB.63 Both tPA and uPA can activate plasminogen to active plasmin61 and are inhibited by the plasminogen activator inhibitors 1 and 2 (PAI-1 and PAI-2). When either free or bound to uPAR, uPA can be inactivated by inhibitors. Upon inactivation at the cell surface, tPA and uPA are internalized and degraded. Plasmin, however, cannot be inactivated or internalized once bound to the cell surface. In addition to degrading specific ECM components, plasmin has been shown to activate latent collagenase,64,65 whereas uPA has been demonstrated to activate gelatinase A.66,67 Thus, proteinases of this class may facilitate brain tumor cell infiltration both directly and indirectly.

To our knowledge, expression of uPA at the mRNA level has not been examined in human or rat intracranial tumor cell lines. Mohanam et al18 have shown uPA to be expressed in human glioblastoma cell lines U251, UWR1, UWR2, and UWR3. Slightly less uPA mRNA was expressed in UWR1 cells than in the other lines examined; uPAR mRNA expression was highly variable among these same cell lines.

Bykowski et al36 demonstrated that tPA (capable of inducing fibrinolysis) is secreted from the rat tumor cell line RT4-71-1. BTSC rat glioblastoma cells, which infiltrate both in vitro and in vivo, have been shown to secrete tPA that activates plasminogen on zymograms.38 However, in a study of human GBM primary cultures, tPA was not produced by tumor cells as demonstrated by an enzyme-binding assay, expression of uPAR was variable among the cell lines. The expression of uPAR protein correlates well with the variable expression of uPAR mRNA in these same cell lines, as noted previously.

Although active tPA has been demonstrated to be secreted by BTSC cells, to our knowledge, no studies have correlated in vitro activity of tPA with invasive potential. Reith and Ruckdeschel38 have shown that uPA secreted by the glioma cell line BTSC is active and can activate not only plasminogen but also progelatinase A secreted by these cells. Active plasminogen (ie, plasmin) and gelatinase A38,40 both are capable of degrading ECM components; thus, this cascade may enhance ability of tumor cells to infiltrate host tissue. Increased expression of uPAR mRNA and protein has also been shown to correlate positively with the ability of human glioblastoma cell lines to invade Matrigel.18 Anti-uPAR antibodies block the invasion of Matrigel by all cell lines examined. Together, these studies and data concerning activity of uPAR-bound uPA suggest that, in vivo, uPAR may be an important mediator of uPA activity necessary for intracranial tumor cell infiltration.

Northern blots indicate that increased expression of uPA mRNA correlates with malignant progression of intracranial neoplasms.68-70 Using in situ hybridization, uPA was weakly expressed in endothelial cells of normal brain tissue and tumor cells of astrocytoma specimens. Staining of GBM samples showed that mRNA was strongly expressed in all tumor cells, particularly at the leading edge of the tumor. Interestingly, all areas of vascular proliferation in tumor specimens exhibit strong staining for uPA mRNA, indicating a possible role for the serine proteinase in angiogenesis. Expression of uPAR mRNA (measured in unmatched normal specimens, astrocytoma, AA, and GBM) also positively correlates with increasing tumor grade of gliomas.70 In situ hybridization corroborated the Northern results; uPAR mRNA
By immunohistochemistry, tPA is detectable in endothelial cells of stromal vessels and in neoplastic cells of low-grade tumors but not in high-grade tumor cells. Immunocytochemical staining for tPA in astroblastoma, ependymoma, choroid plexus papilloma, ganglioglioma, meningioma, oligodendroglioma and multiple grades of gliomas has indicated that expression of this proteinase is restricted to vascular endothelial cells. This is particularly true for those that are hyperplastic, and thus are indicative of a possible role for tPA in angiogenesis. Conversely, tPA activity and antigen in tumor tissue decrease with increasing tumor grade, suggesting that tPA may not play a role in infiltration of high-grade tumor cells. Also, tPA production is inversely correlated with the amount of necrosis in GBM specimen. As tPA is responsible for maintenance of vascular patency, decreases in tPA production or increases in PAI-1 production in GBM (see below) may be responsible for the formation of necrotic lesions found in this tumor type.

Increased uPA antigen expression and activity parallel tumor grade in comparisons of unmatched normal brain, glioma, and GBM. When measured through immunohistochemistry, uPA is expressed at low or undetectable levels in astrocytoma, normal brain, and endothelial cells but is highly expressed in tumor cells and endothelial cells in AA and GBM specimens. In this study, protein expression corresponded to mRNA expression. Yamamoto and coworkers used fibrin zymography to demonstrate that the uPA expressed in these unmatched normal and tumor tissue specimens is active and that uPA activity has been correlated with increased grade of intracranial tumors. From the results presented above, it may be argued that this activity is primarily representative of uPA expression in tumor cells, although tPA activity derived from vascular endothelial cells cannot be ruled out. In another study by Yamamoto et al., uPAR protein expression was measured by radioactive ligand-binding assay and showed a positive correlation with increased tumor grade. As in the in vitro analyses, the in vivo studies imply that expression of uPA/uPAR and plasmin may be important to infiltration of intracranial tumors.

Keohane et al. demonstrated that differentiation of GBM explants correlates with increased expression of PAI-1 and α2-antiplasmin (an inhibitor of plasmin). The amount of α1-antitrypsin (capable of inhibiting PA) antigen has been associated with increased PA activity in acoustic neuroma, adult neuroblastoma, ependymoma, glioma, intracranial lymphoma, meningioma, and sarcomatous glioblastoma. In vitro experiments on HT1080 fibrosarcoma cells and normal fibroblasts indicate that expression of both PAI-1 and uPA is higher in the malignant cell line. Active uPA has been localized to extracellular sites of cell-cell contact and focal, stria-like areas of cell-substratum (fibronectin) contact. PAI-1 antigen has been localized to surface of HT1080 cells in contact with the substratum and appears as a somewhat homogenous layer. There was no co-localization of uPA and PAI-1 in HT1080 cells. The identity of PAI-1 was confirmed by immunoblotting, immunoprecipitation, and its binding to purified uPA. The extracellular localization of uPA in tumor cell lines suggests that it may be involved in the degradation of ECM at discrete focal sites. These results and those of Sawaya et al. suggest that PAI-1 localized to the cell surface may mediate protection of tumor cells.

Cysteine Proteinases and Inhibitors

To our knowledge, CB is the only cysteine proteinase that has been correlated with the malignant progression of intracranial tumors. CB expression and activity are regulated at the transcriptional, posttranscriptional, processing, trafficking, and inhibitor levels. Changes in regulation occurring during brain tumor progression may account for the reported increases in CB mRNA, protein, and activity, and the altered localization of CB in tumor tissue specimens and glioma cell lines. Our laboratory is currently examining the role of CB in brain tumor cell infiltration and changes in regulation that have been correlated with tumor progression.

CB is distributed perinervously in normal cell lines derived from breast (ie, MCF-10A) and in the GBM cell line U251MGp. Of the glioma cell lines examined, U251MGp has the lowest mRNA, protein, and activity for CB. In U87, HF66, and HF140 cell lines, CB is distributed both perinervously and peripherally. CB staining in HF140 and HF66 is also observed in cell processes. Notably, tumor processes (eg, focal adhesions) stain for CB in HF140 cells. U251MGp cells implanted stereotactically into the right caudate putamen of CR-NU nude rats have been stained for CB. Normal brain does not stain for CB. Nests of infiltrating tumor cells as well as the main tumor mass overexpress CB antigen, indicating that CB may be important to the infiltration process. Also, CB has been found to be secreted from human glioma cell lines. CB is associated with the basal surface of U87 cells grown on laminin in areas of substratum degradation. Increases in CB protein and activity have been demonstrated in glioma cell lines. Increased production of CB mRNA, protein, and activity, as well as altered distribution of this proteinase, particularly to focal adhesions, implicates CB in infiltrative processes of many human tumors.

Increases in expression of CB protein have been positively correlated with glioma progression and infiltrative capacity. Western blotting and ELISAs have been used to demonstrate increased antigen levels in AA and GBM. Immunohistochemical staining has also indicated that an increase in CB antigen occurs in high-grade gliomas. In GBM, tumor cells expressing CB can be seen in blood vessels. As noted previously, intracranial tumors invade along these perivascular channels. Thus, this observation suggests a role for CB in glioma tumor cell invasion. CB has also been detected by immunohistochemistry in cells of proliferative vascular endothelial cells in glioma, suggesting that CB is involved in angiogenesis in these tumors.

The relationship between CB expression and the ability of glioma cells to infiltrate surrounding tissue has been clarified by studies with synthetic CB inhibitors. Currently, two classes of CB inhibitors have been used in spheroid invasion assays (T.M., unpublished results, 1997). The lipophilic inhibitors P35033 and P35056 irreversibly bind CB specifically and are subsequently cleaved into a leaving group and a group that remains in the active site of the proteinase. The substituted vinyl sulfones K11017 and K11002 are nontoxic in cell cultures and animal models. Both classes of inhibitors, which are available in oral form, inhibit CB in animal models of diseases other than cancer. Also in animal models, the vinyl sulfones are unreactive to systemic thiol. Both classes of synthetic inhibitors significantly decrease the number of glioma cells infiltrating into normal brain aggregate in spheroid confrontation assays. These studies not only support the theory that CB is involved in brain tumor cell infiltration but also show that synthetic inhibitors, active when administered orally, may be useful therapeutically via reducing infiltration by gliomas. Given orally, these agents would be available to intracranial tumors as the blood-brain barrier is not intact in areas of tumor.

The use of proteinase inhibitors as therapeutic agents has recently been launched in a trial of the MMP inhibitor marimastat (British Biotech) in addition to an ongoing trial in pancreatic cancer. Currently, a randomized trial in newly diagnosed glioblastoma patients following their operation and radiation with this cytostatic agent seeks to prevent tumor infiltration and angiogenesis, thus prolonging the time to tumor progression. Other proteinase inhibitors for the MMPs and other proteinases are currently under development.

Other Therapeutic Approaches

Invasion and angiogenesis are the newest therapeutic targets to yield agents for phase I clinical trials. Three general strategies for anti-invasive/anti-angiogenic therapy have been proposed: (1) inhibition of the production of stimulatory factors by tumor cells, (2) blockade of invasive activity, and (3) interdiction of the signal directing the proliferative or invasive command. Growth factor antagonists, such as suramin, tyrphostins, and signal transduction inhibitors such as CAI (see below) and protein
kinase C (PKC) inhibitors are all in clinical development. Agents that block invasive activity may work at several biological or biochemical levels. Antibodies directed against vascular endothelial growth factor (VEGF) have been effective in xenograft models in inhibiting angiogenesis and resultant tumor growth. This strategy has not yet reached human trials. The hypothesis that inhibition of MMP activity may offer a novel and effective approach to inhibition of invasive potential has prompted the development of synthetic inhibitors of the matrix MMPs. BB-94, batimastat, a prototype agent, is a low-molecular-weight compound containing a peptide backbone that binds to the MMPs and a hydroxamic acid group that binds to the catalytically active zinc atom in the MMP. BB-94 has a broad spectrum of inhibition of MMPs and is effective at concentrations ranging from 3 nM for interstitial collagenase (MMP-1) to 20 nM for stromelysin (MMP-3). Intraperitoneal injection of BB-94 into ovarian cancer-bearing nude mice causes a decreased tumor burden associated with a dramatic increase in survival of the mice. Anti-invasive activity also has been observed in human colon cancers and in the inhibition of angiogenesis. Clinical trials are now ongoing using marimastat, an orally bioavailable analog, in a number of tumor types, including a phase III trial in newly diagnosed glioblastoma.

Several agents identified as inhibitors of angiogenesis are now under development or in clinical trial. AGM-1470 (TNP-470) is a synthetic analog of fumagillin, an angioinhibitory compound secreted by Aspergillus fumigates. AGM-1470 inhibits bFGF-stimulated proliferation and migration of endothelial cells in vitro and proliferation of tumor cells in vivo. In vivo studies on a variety of experimental tumors have shown significant inhibitory effects in both the number and dimension of tumor nodules including primary brain cancers. The specific mechanism of action of AGM-1470 has not been identified. Ongoing clinical trials have demonstrated no objective partial or complete responses; however, patients reportedly have had disease stabilization.

Thalidomide, a well-known teratogen, is a potent anti-angiogenic factor. Using the rabbit cornea micro-pocket assay, D’Amato et al. showed that orally administered thalidomide inhibited corneal neovascularization induced by bFGF. It requires hepatic metabolism for activation. Since thalidomide was originally developed as a sedative, it is already known to traverse the normal blood–brain barrier. Phase II clinical trials of thalidomide have been initiated in numerous solid tumors including glioblastoma multiforme.

Several agents that inhibit signaling events have anti-invasive or anti-angiogenic activity. An inhibitor of protein kinase C activity, saltingol, was shown to inhibit gastric cancer invasion in vitro. A phase I clinical trial has demonstrated that this agent can be administered without significant toxicity, suggesting that further clinical investigation is warranted. Another selective protein kinase C inhibitor, UCN-01, has recently entered clinical trial, and tamoxifen, an inhibitor of protein kinase C at high concentration, is under investigation in prostate cancer and in recurrent malignant gliomas. More specific PKC inhibitors, such as hypericin, are also under study. Tyrophostins, synthetic agents targeted against receptor tyrosine kinases, inhibit invasion and proliferation in a cytokine receptor-selective fashion. Narrow spectrum tyrophostins have been developed against EGF receptor, PDGF receptor, and more recently the VEGF receptors. Clinical trials of tyrophostins are pending.

Signaling of several classes of molecules mediate interaction of tumor and endothelial cells with the ECM. Recently described fragments of ECM components have direct angioregulatory effect, including endostatin (collagen) and angiostatin (plasminogen). The role of these factors in brain tumors is under active investigation, and their development as clinical agents stems from their remarkable demonstrated effect in the serial regulation of animal tumors without demonstrated resistance or tachyphylaxis of angioinhibitory effects. Angioinhibitors such as thrombospondin bind to ECM, including to avbeta3 integrin, and agents inhibiting the matrix signaling of the avbeta3 integrin are in development.

**Carboxyamido-Triazole (CAI)**

The importance of calcium in normal cellular functions led to a screen of compounds that inhibited tumor cell migration in vitro and selected signal transduction pathways. Calcium is a vital component in many signal transduction cascades: as a ligand, as a second messenger, and as a tertiary messenger. Calcium regulates selected pathways involved in cell proliferation, cyto–skeletal rearrangement, migration, and invasion. Using a screen for inhibition of tumor cell migration and inhibition of calcium influx, we identified a novel synthetic agent, carboxyamido-triazole (CAI), as an inhibitor of tumor cell migration and angiogenesis.

CAI inhibits stimulated calcium influx, proliferation, and invasive potential of a variety of human tumor cell lines in the concentration range of 1 to 10 µM. A structure–activity relationship study indicated a statistically significant link between inhibition of calcium influx and calcium-mediated signaling pathways with tumor cell proliferation and metastatic potential. Exposure to CAI inhibits tumor cell growth in monolayer and in soft agar cultures and reduces the production of gelatinase A. All of the inhibitory effects of CAI against signaling, proliferation, and invasion are reversible; thus, CAI is a member of the new family of cytostatic agents. Oral administration of CAI to human melanoma or ovarian cancer-bearing nude mice resulted in greatly reduced tumor numbers, reduction in total tumor burden, a striking decrease in spontaneous metastasis compared with vehicle–treated control animals, and a reduction in tumor initiation. Plasma concentrations were in the range of 1 to 10 µg/mL (0.5–5 µM) in the experimental animals. An oral formulation has been developed for use in xenograft models and in human trials.

Recent studies have identified antitumor activity in vivo against human glioblastoma cell lines. Seven different cell lines of differing malignant aggressiveness were investigated for response to CAI. While a dichotomy was seen in the degree of responsiveness to CAI, invasion of all cell lines was inhibited. Inhibition of proliferation, gelatinase A secretion, adhesion to tissue culture plastic and type IV collagen substrata, and invasion in Matrigel barrier assays were demonstrated. The 50% inhibitory concentration for the antiproliferative and anti-invasive activities are in the range of 2 to 20 µM for most glioma cell lines. Preliminary work using CAI in a three-dimensional confrontation assay has demonstrated inhibition of glioma invasion (F.M., unpublished observations, 1997). Animal xenograft studies of human glioma are planned for pharmacokinetics and efficacy studies leading to clinical trials for glioma patients.

Angiogenesis is a critical component of aggressive primary brain tumors. CAI inhibits the proliferation and invasion associated with neovascularization. In the effective concentration ranges identified in the human tumor cell and brain cancer cell line studies, CAI inhibited umbilical vein endothelial cell proliferation, migration, adhesion, and tube formation on Matrigel. Administration of CAI in an in vivo assay, the chick chorioallantoic membrane assay, confirmed an anti-angiogenic effect with a marked inhibition of neovessel formation as well as a die-back in existing vessels. Further studies have focused on the calcium influx-sensitivity of endothelial cell spreading, a key component of migration and maintenance of vascular integrity. CAI has an immediate effect of inhibiting actin rearrangement and spreading of endothelial cells on basement membrane substrate.

Phase I clinical trials of orally administered CAI have demonstrated a safe therapeutic window for continuous daily administration. Disease stabilization has been observed in some heavily pretreated patients with colorectal cancer, pancreatic cancer, renal cell carcinoma, ovarian cancer, and breast cancer. The primary toxicities observed included gastrointestinal intolerance with the liquid and gelatin capsule formulations and a rare, reversible sensory peripheral neuropathy. Serious side effects that were reversible with drug discontinuation included retinal hyperemia and a concentration-dependent cerebellar ataxia possibly associated with cognitive dysfunction. Permanent sequelae were not observed in any of the affected patients. A patient with cerebellar ataxia and some cognitive dysfunction was successfully rechallenged at a 15% dose reduction (one dose level) without subsequent recurrence of toxicity. Phase II concentration-directed clinical trials are under development.
Conclusions

The recent advances in the field of tumor biology and molecular pathology have resulted in the identification of novel biologic targets, and agents that attack these targets are undergoing rapid development and preclinical testing. These agents by their nature are cytostatic rather than cytotoxic and have resulted in a shift in the treatment paradigm whereby biologic control of a tumor population may be reimposed rather than attempting to leverage the marginal therapeutic ratio of cytotoxics with resultant toxicity. Combination regimens, whereby biologic agents synergize with either radiation or chemotherapy to achieve maximal efficacy, may translate into improved results in the clinical population.

References


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