Proteases and Glioma Angiogenesis

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Angiogenesis, the process by which new branches sprout from existing vessels, requires the degradation of the vascular basement membrane and remodeling of the ECM in order to allow endothelial cells to migrate and invade into the surrounding tissues. Serine, metallo, and cysteine proteinases are 3 types of a family of enzymes that proteolytically degrade various components of extracellular matrix. These proteases release various growth factors and also increase adhesive molecules and signaling pathway molecules upon their activation, which plays a significant role in angiogenesis. Downregulation of these molecules by antisense/siRNA or synthetic inhibitors decreases the levels of these molecules, inhibits the release of growth factors, and decreases the levels of various signaling pathway molecules, thereby leading to the inhibition of angiogenesis. Furthermore, MMPs degrade specific substrates and release angiogenic inhibitors which inhibit angiogenesis. Downregulation of 2 molecules, such as uPA and uPAR, uPAR and MMP-9, or Cathepsin B and MMP-9, are more effective to inhibit angiogenesis rather than downregulation of single molecules. However, careful testing of these combinations are most important because multiple effects of these combinations play a significant role in angiogenesis.

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A tumor's capacity to grow is linked to its capacity to recruit a supply of new blood vessels. When a tumor remains in a dormant state, the cellular proliferation rate is balanced by the apoptotic rate and it is unable to grow in size beyond a few millimeters in the absence of the acquired angiogenic phenotype. In order to do this, it sends out "signals" to already existing blood vessels to sprout new branch in a process called angiogenesis. In response to these chemical signals, endothelial cells divide and grow, breakdown their surrounding tissue barriers with proteases (namely cysteine, serine, and metalloproteinases) and migrate toward the tumor to form a connection to the body's blood supply. The mechanisms that lead the tumors to switch to the angiogenic phenotype are unknown. The development of new blood vessels follows a well-defined pattern beginning with an initial increased vascular permeability leading to extravasation of plasma, plasma proteins, and deposition of proangiogenic matrix proteins (41-43, 50). The process is complex and involves multiple steps and pathways with positive and negative signals (53, 54, 66). Endothelial cells then assemble themselves to form a central lumen, elaborate a new basement membrane, and eventually recruit pericytes and smooth muscle cells to surround the mature vessel. Thus, angiogenesis is a complex process involving a multitude of tightly controlled sequence of events that lead to the development of new vasculature. Loss of the tight control over this highly regulated process can have disastrous effects in developmental, pathological and physiological states. In neoplasia, the angiogenic balance is tipped in favor of new vessel growth (66).

Glioblastomas are among the most dramatically neovascularized neoplasms; progression of a glioma to its more malignant form of glioblastoma is usually associated with striking neovascularization, evidenced by vasoproliferation, altered endothelial cell cytology, and endothelial cell hyperplasia. In the case of astrocytoma, the low grade (WHO grade II) tumors show slight increases in vessel density, while high microvessel density and evidence of neo-angiogenesis is part of the histological definition of grade III and IV astrocytomas (82). In addition, glioblastomas (WHO grade IV astrocytomas) are characterized by two histological characteristics relevant to angiogenesis: glomeruloid vascular proliferation, a characteristic architecture of new vessels, and necrosis with pseudopalisading cells, a focus of hypoxia that stimulates angiogenesis (13). Endothelial proliferation is so frequently found within and adjacent to high-grade gliomas that it is one of the pathologic criteria for grading these tumors. The obvious conclusion is that high-grade gliomas must stimulate the proliferation of endothelial cells and generate new blood vessels to support their growth.

It is well known that in addition to cytokines and growth factors, the extracellular matrix (ECM) also plays a significant role in angiogenesis. Several number of distinct families of molecules have been identified that play a functional role in the angiogenic process including growth factors and their receptors, proteolytic enzymes, cell adhesion receptors, and extracellular matrix components (14, 23, 101, 143, 147). Vascular endothelial cells are capable of producing and subsequently interacting with a wide array of matrix molecules including laminin, collagen types, fibronectin and heparan sulfate proteoglycans (73, 163, 172, 174). Recent studies demonstrated that proteolytic remodeling of ECM proteins plays an important role in the regulation of angiogenesis (104, 125, 143). This proteolytic remodeling has been suggested to be mediated by a number of matrix degrading enzymes including serine, metallo and cysteine proteases (143, 218). In this review, we are mainly focused on the role of uPA, uPAR, MMP-9 and Cathepsin B in brain tumor angiogenesis.

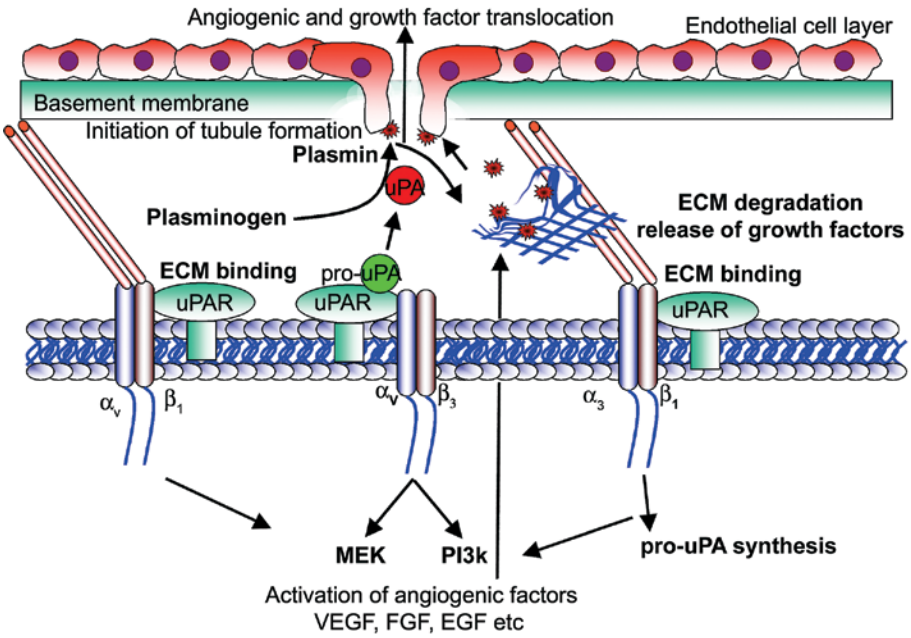

Figure 1. *Represents the involvement of the uPA-uPAR system in the progression of angiogenesis.* The binding of uPA and pro-uPA to uPAR at the invading front of the tumor cells initiates a cascade of events mediated by alpha and beta integrin heterodimers. At the cell surface, the interaction of uPAR with uPA initiates the activation of plasminogen to plasmin, a broad-spectrum protease that is involved in the degradation of extracellular matrix (ECM). The degradation of the ECM and the basement membrane by plasmin releases ECM bound growth factors that stimulate the growth of the tumor cells and induce the vascularization of the endothelial cells. The interaction of uPAR with ECM components such as vitronectin and fibronectin initiates a cascade of signaling events that involve MEK and PI3k, which in turn causes the upregulation of proangiogenic molecules like VEGF, FGF, EGF etc.

UROKINASE-TYPE PLASMINOGEN ACTIVATOR AND ITS RECEPTOR

uPA is secreted by the cells as a 411 amino acid single chain zymogen prourokinase (pro-uPA), also known as single chain urokinase plasminogen activator (ScuPA). The C-terminal 253 residues comprise the serine protease domain having the usual catalytic triad of His, Ser, Asp. The N-terminal region or amino terminal fragment (ATF) is comprised of a 40 amino acid region, containing three disulfide bonds called the growth factor domain (GFD) joined with a larger triple disulfide kringle. Activation of 2 chain urokinase (uPA, tcuPA) requires enzymatic cleavage at the Lys 158-Ile 159 bond leaving A and B chains joined via an S-S bond (C148-C279). Activation can be catalyzed by plasmin or kallikrein, by tumor–associated trypsin or cathepsins (B, L) by mast cell tryptase or by HuTSP-1 (17, 184). Cleavage at Glu 143-Leu 144 by MMP-3 or MMP-7 and lysis by plasmin or slow autolysis by uPA at Lys 135-Lys 136 can truncate the A-chain, releasing the ATF and forming low molecular weight (LMW) uPA, essentially a pure catalytic domain. The binding of uPA to its cell receptor, uPAR is directed by the GFD (112). Signal transducing activities by uPA or its isolated ATF have been reported (134, 155). The phosphorylation of uPA has also been reported as an unusual modification for an extracellular enzyme (67).

The receptor for the urokinase-type plasminogen activator (uPAR), also called CD87, is related to members of the human CD59 and the mouse Ly-6 family (208). The uPAR gene has been mapped to chromosome 19q13.2 and is composed of 7 exons, separated by 6 introns, and occupies about 21.23 kb (204). The expression of the uPAR gene can be regulated via the Sp1, AP-2, NFκB, and AP-1 transcription factors by specific motifs contained within the first 188 bp upstream of the transcriptional start site (138, 205). The mRNA for uPAR also contains another specific sequence within the coding region (nucleotides 195–246) that can destabilize mRNA upon binding to an unidentified protein (176). Human uPAR is encoded as a 1.4-kb mRNA transcript (164). Primer extension experiments have demonstrated that the transcription start site is located 50 bp upstream of the translation start site (ATG) of the human uPAR gene (204).

The uPAR is synthesized as a 313-amino acid polypeptide that is folded through disulfide bonding into 3 homologous repeats known as domains 1, 2, and 3. A heavily glycosylated protein, uPAR contains 5 potential glycosylation sites. Its hydrophobic carboxy-terminal domain is processed during biosynthesis and substituted by a glycosylphosphatidylinositol (GPI) anchor that targets the receptor to the outer leaflet of the plasma membrane bilayer. At the cell surface, uPAR exists in both a 3-domain form that is capable of binding uPA and a 2-domain form (devoid of domain 1) that does not bind uPA (35). Formation of the uPAR-uPA complex with high affinity seems to require that residues located at equivalent positions in uPAR domains 1 and 3 be in close physical proximity to each other (146). Pro-uPA, di-isopropyl fluorophosphate (DFP)-inactivated uPA, uPA, and the amino-terminal fragment of uPA all bind with the same affinity to uPAR. The interaction of uPA with its receptor exhibits some species specificity; human uPA does not bind to murine uPAR, and murine uPA does not recognize human uPAR. The amino acids Asn22, Asn27, His29, and Trp30 in human uPA have been identified as being key determinants in the species-specific binding of uPA to uPAR (154). Four regions within the uPAR sequence have been found to bind directly to uPA: 2 distinct regions in uPAR domain 1 containing amino acids 13–20 and amino acids 74–84, and two regions in the putative loop 3 of domains 2 and 3, uPAR(154–176) and uPAR(247–276) (102).

Independent of catalytic activity, uPAR is also involved in cell signaling, interactions with integrins, cell motility, adhesion, invasion and angiogenesis. (Figure 1). uPAR plays a role in the migration of a variety of cell types and evidence is accumulating that uPAR-dependent migration is mediated through integrins. Several signaling pathways, including MEK-ERK (mitogen-activated protein kinase–extracellular signal-regulated kinase) and Jak-STAT (Janus kinase-signal transducer and activator of transcription), have been implicated in uPAR-mediated cell migration and adhesion. These effects, which involve cytoskeletal components and cytosolic and transmembrane kinases, were shown to be

associated with uPAR interactions with various molecules such as vitronectin, several members of the integrin family, caveolin and G protein-coupled receptors (12, 161). Antisense-mediated downregulation of uPAR in a human glioma cell line altered cell morphology, cell diffusion and cytoskeletal organization and upregulated α3β1 integrin expression (28). uPAR is now known to interact directly with ECM components including vitronectin (VN) on various cell types (79, 203). Association of uPAR with VN is enhanced by ligand occupancy of uPAR (79, 202) cells and the intact receptor is required for the VN–uPAR interaction to occur (71).

Several growth factors (bFGF, VEGF, HGF) sequestered by the ECM (in addition to TGF-β) can also be activated or released from binding sites as part of this cascade and feedback into the uPA system, resulting in an increase in uPA and uPAR expression (109, 142, 144). The addition of basic fibroblast growth factor (bFGF) or transforming growth factor-α (TGF-α) increased the invasiveness of human gliomas cell lines as well as uPA and uPAR mRNA levels. The growth factor-induced invasiveness can be reversed with uPAR-specific antibodies (124). In a collagen lattice assay, TGF-β1 induced collagen lattice contraction of T98G glioma cells via enhanced expression of the subunit α2-integrin expression in a concentration-dependent manner. An anti-α2 antibody, P1-E6, and antisense phosphorthioate oligonucleotides against α2 integrin have been shown to inhibit these effects of TGF-β1 (117). TGF-β1 and TGF-β2 promote migration, induce αv and β3 integrin mRNA expression, and enhance the cell surface expression of αvβ3 in a concentration-dependent manner (145).

Gliomas, the most common of the primary brain tumors, extensively invade the surrounding normal brain tissue and are extremely refractory to therapy. Proteolytic degradation of the ECM (166) is thought to be the initial step in the process of glioma tumor cell invasion and neovascularization. The roles of uPA and its receptor in this process have attracted a great deal of research attention. An increase in uPA activity has been found in the more malignant astrocytoma cell lines in vitro (120, 122) and in malignant human brain tumors in vivo (94, 211). In the latter situation, the presence of high uPA levels was associated with poor prognosis (11, 72). The invasive capability of tumor cells is facilitated by the expression of uPAR on the tumor cell surface (106). uPAR-mediated pericellular proteolysis or the interaction of uPAR with adhesion molecules results in variable adhesion or detachment events and is pivotal for the control of cell migration and invasion.

We found that the expression of uPAR in human glioblastoma cell lines could contribute to their invasive capability (58, 122). uPAR expression was significantly higher in anaplastic astrocytoma and glioblastoma cells than in normal brain tissues or in low-grade gliomas, and uPAR tended to be found in the greatest amounts at the leading edges of the tumors (58, 213). In U251MG glioblastoma cells, uPAR was found to be localized at the cell–ECM focal contacts, together with the αvβ3 integrin (58). Other studies indicated that LRP was overexpressed in malignant astrocytomas, especially in glioblastomas, and that the increased expression of LRP seemed to correlate with the expression of uPAR and the malignancy of the astrocytomas (210). The addition of βFGF or TGF-α to human glioma cells produced increases in cellular mRNA levels of uPAR and uPA, and the addition of anti-uPAR monoclonal antibodies to these cells significantly inhibited their βFGF- or TGF-α-induced invasiveness (124). We also found that uPAR promoter activity was upregulated in high-grade glioblastoma cell lines, a finding that suggests that increased transcription is likely to be the mechanism underlying the increase in uPAR expression in high-grade gliomas (10).

Selective inhibition of the uPA-uPAR interaction is considered a feasible approach for the treatment of malignant brain tumor.

Several in vivo studies have indicated that modulation of uPAR expression or blockade of uPAR with catalytically inactive uPA fragments leads to reductions in tumor growth and tumor angiogenesis and an increase in tumor dormancy in a variety of tumor types (194). Strategies that target uPA or its receptor with the aim of disrupting the interaction between the two or the ligand-independent actions of uPAR include antisense technology, monoclonal antibodies, cytotoxic antibiotics and synthetic inhibitors of uPA.

A reduction in uPAR expression in response to antisense techniques inhibited tumor cell invasiveness, prevented metastasis and reversed the invasive phenotype (33, 90). We have shown that transfection of the human glioblastoma cell line SNB19 with antisense uPAR-expressing plasmid DNA significantly alters the invasive properties of glioblastoma cells in vitro (119). Furthermore, glioblastoma cells in which uPAR expression was down regulated with antisense-uPAR-cDNA or transfection with an adenovirus (Ad) expressing antisense uPAR gene did not form tumors after their intracerebral injection in nude mice (59, 118). In addition, injection of the Ad-uPAR construct into previously established subcutaneous U87MG tumors in nude mice caused tumor regression (118). These findings support the therapeutic potential of targeting the uPA–uPAR system for glioma treatment. The antisense-mediated downregulation of uPAR expression in glioblastoma cells has been shown to increase the expression of the α3β1 integrin, but not that of α5β1 in vitro (28). Reducing uPAR expression in human glioma cells leads to changes in cell morphology, decreased cell diffusion and cytoskeletal disorganization, indicating that the coordinated expression of uPAR and integrins may be involved in glioma cell spreading (28). Ligation of uPAR by uPA directly induces brain tumor cell migration and markedly enhances invasion in a manner independent of uPA-mediated proteolysis and in concert with ECM degradation (106). Other studies have reported that high molecular weight serum protein complexes promote both uPAR–focal adhesion co-localization and cell migration in glioma cells (68). Glioblastoma cells stably transfected with antisense-uPAR undergo apoptosis when injected intracerebrally in nude mice or when grown on tissue culture plates coated with the extracellular matrix components fibronectin or vitronectin. Increase in apoptotic cell death in vitro was associated with the increased expression of the apoptotic protein BAX (80). These transfected cells also demonstrated losses in mitochondrial transmembrane potential with the corresponding release of cytochrome c from the mitochondria and subsequent activation of caspase-9 relative to the behavior of the parental cells (217). Finally, these antisense-uPAR-transfected glioblastoma cells exhibited higher lev-

els of the tumor necrosis factor-α-related apoptosis-inducing ligand (TRAIL) receptors DR4 and DR5 than did parental cells, thereby rendering the transfectants susceptible to apoptotic stimuli (92). These findings indicate that the downregulation of uPAR expression in human glioblastoma cells inhibits the invasiveness of those cells and enhances their susceptibility to apoptotic stimuli. mRNA levels of both uPA and uPAR genes are increased in high grade gliomas, possibly suggesting increased gene transcription (10).

The effects of uPA and uPAR on tumor cell invasion may also involve the tumor suppressor gene *CDKN2A*—encoding 2 tumor suppressors, INK4A and ARF—which is frequently inactivated in gliomas. Recent reports indicate that restoration of the INK4A protein inhibits αvβ3 integrin-mediated signaling of vitronectin (49). Our recent work demonstrated that infecting malignant glioblastoma cells with this Ad-uPAR-INK4A vector in the presence of vitronectin resulted in decreased αvβ3 integrin expression and integrin-mediated biological effects, including reductions in adhesion, migration, proliferation, and survival (3). These results support the concept that down regulation of the expression of the integrin αvβ3 and integrin-mediated signaling in glioma cells through the adenovirus-mediated transfer of antisense-uPAR and sense-INK4A genes may have therapeutic potential for treating human gliomas (2). Recent studies have also demonstrated that the diphtheria toxin (DT) amino-terminal (AT) fragment of the urokinase-type plasminogen activator gene caused a statistically significant regression of glial tumors and was highly potent and selective in killing uPAR-expressing glioblastoma cell lines (199). Rustamzadeh et al, (170) demonstrated that DTAT (diphtheria toxin amino-terminal fragment) caused the regression of small subcutaneous uPAR-expressing tumors with minimal toxicity to critical organs. Further, in vivo DTAT13 (diphtheria toxin amino-terminal fragment and IL-13 molecule) caused the regression of small tumors when administered at 10 μg/day given on a 5-dose schedule every other day (192). Orthotopically implanted U87MG cells in mice with single species-specific or combination of uPA ligands resulted in significant decreases in tumor size and increased survival rate (18). Adenovirus-mediated downregulation of bicistronic constructs of uPA and uPAR expression inhibited glioma cell migration, invasion and angiogenesis in vitro and inhibition of tumor formation ex vivo (61).

In particular, the uPA system seems to be important for the migration and invasion of endothelial cells during angiogenesis. Angiogenesis involves several processes, including proteolysis and remodeling of the basement membrane, endothelial cell activation, proliferation, migration and tissue infiltration from preexisting blood vessels. In vitro experiments have shown the importance of proangiogenic factors, such as βFGF and VEGF, in inducing endothelial cells to migrate (139, 162). These same growth factors are released by the proteolytic action of the uPA system in cancer. A peptide derived from the connecting peptide region of uPA inhibits the migration of microvascular endothelial cells and GBM tumor progression in combination with an agent that inhibits endothelial cell proliferation (cisplatin [CDDP]) (116). In analyzing vascularization of tissue in uPA-deficient mice, the possibility of uPA may only be necessary solely for vessel development when endothelial cells are invading fibrin (143). It has been demonstrated that soluble uPA induces neovascular growth in the avascular rabbit cornea and dose dependently promotes growth, chemotoxins and matrix invasion of cultured endothelial cells (113). uPA antisense-transfected stable cells showed markedly reduced levels of invasion and reduced tumor formation in nude mice compared to control group (120). Interaction of uPA with its receptors appears to be mandatory for the angiogenic effect of uPA since anti-uPA and anti-uPAR monoclonal antibodies blocked the proangiogenic effects of uPA at the endothelial cell level. Impairment of uPAR availability by monoclonal antibodies and by antisense oligonucleotides against uPAR-mRNA inhibited endothelial cell proliferation, chemotoxins and chemoinvasion (52).

The delayed angiogenesis was seen recently in plasminogen-deficient mice in an in vitro model of aortic rings embedded in a collagen gel (38) and an in vivo model of malignant keratinocyte transplantation (6). It has been reported that loss of either uPA or plasminogen was shown to reduce tumor growth of T241 fibrosarcoma (34, 63) and carcinoma (19). A comparative study of control and plasminogen-deficient mice using the pymT mouse mammary tumor model (20, 171) demonstrated no difference in tumor growth. Although angiogenic endothelial cells require uPA and plasmin to degrade ECM components and migrate, this proteolysis needs to be controlled by physiological inhibitors. The importance of balanced proteolysis in the process of angiogenesis might provide an explanation for the dual role of proteinases and inhibitors in angiogenesis.

uPA can be inactivated irreversibly by reaction with any one of several protein inhibitors including PAI-1 (which is abundant within the ECM), PAI-2 (which is induced in lymphocytes and macrophages), PAI-3 (protein C inhibitor), α2-macroglobulin and protease nexin (also found in ECM). Paradoxically, high tumor levels of the uPA inhibitor plasminogen activator inhibitor-I (PAI-I) are associated with a poor prognosis, and as such, high levels of PAI-1 have been correlated with poor prognosis in cancer patients (127). PAI-1 mRNA is abundantly expressed in glioblastomas and anaplastic astrocytomas but weakly expressed in low-grade astrocytomas or normal brain, suggesting that high expression of PAI-1 is associated with the malignant progression of astrocytic tumors. The distribution of PAI-1 mRNA expression has been found to be particularly abundant around areas of vascular proliferation and in remnant tumor cells surrounding necrotic foci and could be possibly associated with intratumoral necrosis in glioblastomas (212). Localization of PAI-1 in proliferating vessels of intracranial tumors (glioblastoma multiforme, 5 anaplastic gliomas, 4 malignant meningiomas, and 9 metastatic tumors) suggests that PAI-1 may be involved in angiogenesis (89). In addition to recruiting, focusing, and enhancing the activation of plasminogen by uPA locally on cell surfaces, uPAR also participates in the regulation of this activity through internalization of the uPA-PAI complexes. Ternary complexes of uPAR-uPA-PAI are rapidly endocytosed through recognition and uptake by the lipoprotein receptor-related protein (LRP, α2-macroglobulin receptor). The uPA and PAI components are degraded while uPAR is recycled to the cell surface (159). As demonstrated by RT-PCR analysis, LRP mRNA was frequently expressed in glioblastomas and anaplastic astrocyto-

mas as compared with low-grade astrocytomas and was well-correlated with uPAR expression. Altered LRP expression might contribute to the stimulation of cell-surface proteolytic activity that, in turn, facilitates the invasiveness of glioblastomas in vivo (210). PAI-1 shares a mutually exclusive binding site for VN (in the somatomedin B domain) with uPAR and is capable of displacing uPAR from VN (37). Andreasen et al reported that PAI-1, independent of its role as a proteinase inhibitor, inhibits cell migration by competing for vitronectin binding to integrins, while the interference of PAI-1 with binding of vitronectin to the urokinase receptor may play a secondary role (5). The effect of adenoviral-mediated transfer of the PAI-1 gene in regulating the in vitro invasiveness of D54MG glioma cells into Matrigel and into fetal rat brain aggregates was studied (69). The results show that PAI-1 overexpression can inhibit glioma cell motility and invasion through extracellular matrix (ECM) components, such as laminin and collagen, but does not inhibit tumor cell invasion in a 3-dimensional invasion assay that simulates normal brain tissue with varying ECM and interstitial composition. The different results obtained in the 2 invasion assays reflect the complex biological effects of the uPA/PAI-1 system.

Elevated levels of PAI-1 have also been reported in primary tumors of advanced neuroblastomas (188). Furthermore, PAI-1 inhibited endothelial tube formation on Matrigel in the presence of vitronectin, but had a stimulatory effect in the presence of fibronectin suggesting that PAI-1 acts as a positive switch for angiogenesis by promoting endothelial cell migration away from their vitronectin-containing perivascular space toward fibronectin-rich tumor tissue (75). PAI-I seems to be multifunctional as it is expressed by multiple cell types and has multiple molecular interactions.

uPAR has also been associated with several members of the β1, β2, and β3-integrin families, indicating the strong involvement of uPAR in cell adhesion and migration as well as supporting the concept that integrins may function as signal transducers for plasminogen activation. The coexistence of uPAR with specific integrins at different cellular locations is a prerequisite for the regulation of integrin function by the uPAR (200). In leukocytes, uPAR interacts with various β2 integrins, and consequently the inhibition or removal of uPAR will result in integrin dysfunction. In an in vivo study, the β2 integrin-dependent recruitment of leukocytes to the inflamed peritoneum of uPAR-deficient mice was significantly reduced relative to that in wild-type animals (111), indicating that both β2 integrin-mediated leukocyte–endothelial cell interactions and their recruitment to inflamed areas require the presence of uPAR. The association between uPAR and the β2 integrins have been clearly shown to initiate a variety of direct and indirect regulatory mechanisms for both adhesive and proteolytic processes (149).

The uPAR forms "cis"-interactions with integrins as an associated protein and thereby transduces proliferative or migratory signals to cells upon binding of uPA. Binding of suPAR to α4β1 and αvβ3 is blocked by known soluble ligands and by mutated integrins that inhibit ligand binding, suggesting that uPAR is an integrin ligand in addition to being an integrin-associated protein (191). In addition, GPI-anchored uPAR on the cell surface specifically binds to integrins on contiguous cells, suggesting that uPAR–integrin interactions may mediate cell-to-cell interactions ("trans"-interactions) (191). In nonhematopoietic cells that do not express β2 integrins, the uPAR seems to interact with β1 and β3 integrins (209). uPAR specifically associates with certain members of the β1 and β3 integrin families on adherent tumor cells, and ECM components induce specific integrin–uPAR associations to enable directional proteolysis by which tumor cells can migrate and invade (137, 149, 209). In recent experiments, a peptide homologous to integrin sequences suspected of being a uPAR binding site was identified and shown to disrupt uPAR-integrin interactions (178). Several studies point to the possibility that the level of caveolin influences the outcome of uPAR-integrin interactions (206). However, results obtained with the tumorigenic carcinoma cells HEp3, which do not contain caveolin, indicate that the density with which uPAR is expressed and the association of uPAR with respective integrins determines the state of integrin activation (4).

Several studies revealed that both in vivo and in vitro findings strongly suggest that α1β1, α2β2, α5β1, α6β1, αvβ3 and αvβ5 play a significant role in angiogenesis (40, 70, 110, 136, 169). A significant body of experimental evidence has demonstrated that αvβ3 antagonists can inhibit angiogenesis during development, wound healing, retinal neovascularization and in growing tumors (15, 16, 32, 65). Recently, it has been shown that specific cyclic peptides EMD 121974, 85189 and 66203 selectively inhibit αvβ3 and α5β5 in vitro angiogenesis induced by angiogenic cytokines in three-dimensional fibrin or collagen gels (133).

The αvβ3 integrin receptor is closely associated with tumor growth and spread, acting as a marker of invasiveness in malignant melanomas and in Grade III and IV glioblastomas, and plays an essential role in neovascularization (15, 57). Glioblastomas express αvβ3 prominently at the invasive borders, as do angiogenic endothelial cells in tumors (7, 48). Our own results have shown that vitronectin is highly expressed in glioma cells and integrin ligation with vitronectin inhibits apoptosis (197). Binding of cell surface integrins to ECM stimulates a variety of intracellular signaling pathways which enhances cell survival pathways (186). Our work demonstrated that infecting malignant glioblastoma cells with Ad-uPAR-p16 vectors in the presence of vitronectin resulted in decreased αvβ3 integrin expression and integrin-mediated biological effects including reductions in adhesion, migration, proliferation and survival (3). Our recent studies in combination of several siRNA constructs significantly reduced the levels of integrins which in turn reduced angiogenesis in both in vitro and in vivo models (personal communication).

MATRIX METALLOPROTEINASES

Matrix metalloproteinases (MMPs) constitute a family of zinc-containing enzymes with more than 20 members identified to date. They are divided into 4 groups such as interstitial collagenases, stromelysins, gelatinases and membrane type metalloproteinases (MT-MMPs) based on protein structure (165). Unlike MMPs, MT-MMPs are membrane-bound, and studies have shown that they are activated either intracellularly or on the cell surface (215). Another group of proteolytic metalloproteases is the ADAMs family, which consists of at least 33 different enzymes (151). More recently, a new group of metalloproteases called AD-

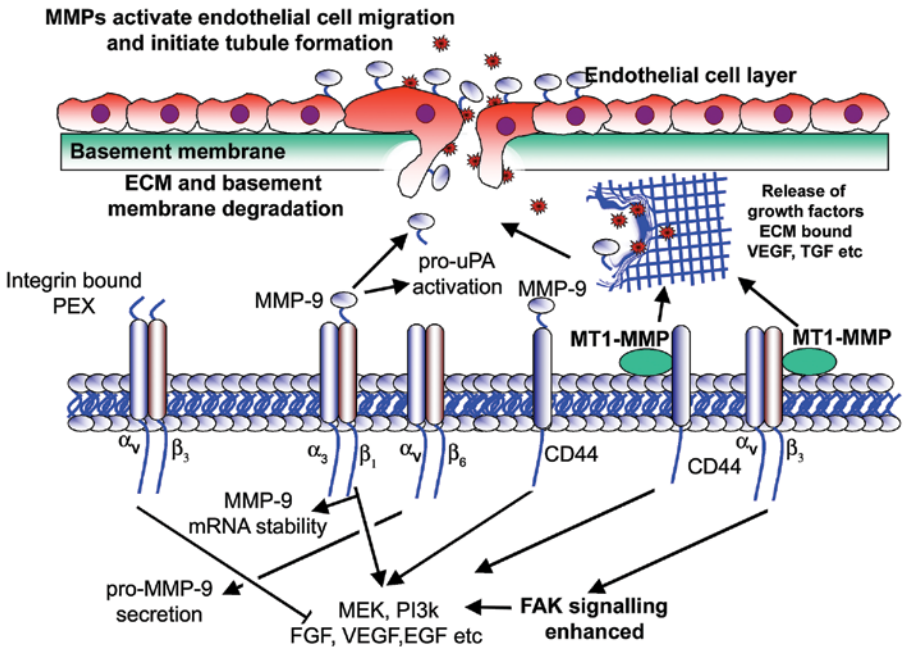

Figure 2. *Representation of the involvement of MMPs in angiogenesis.* MMP-9 is an important component of tumor cells involved in invasion and metastasis. MMP–9 is secreted as pro-forms and activated by plasmin, cathepsins or active MMPs. The degradation of the ECM by MMPs releases ECM bound growth factors, which stimulate the growth and the vascularization of the endothelial cells towards the tumor cells. The binding of MMP-9 to its receptor CD44 and the binding of MMP-9 to integrin heterodimer initiate a cascade of cellular events involving MEK and PI3k, and the activation of these kinases initiate the production of proangiogenic factors such as VEGF, FGF, EGF etc.

AMTs has been identified. Like the ADAMs, these enzymes contain a disintegrin and metalloprotease domain. However, instead of a transmembrane domain, the ADAMs express one or several thrombospondin 1-like repeats (190). Although their physiological functions are not yet completely understood, nineteen different members have been identified (21).

MMPs are secreted as pro-MMPs and then activated following sequential cleavage steps (128, 175). Removal of the signal peptide and propeptide domains or a change in configuration activates the enzymes. MMP expression and proteolytic activity are tightly regulated at three stages: gene transcription, proenzyme activation and activity of natural inhibitors (tissue inhibitors of metalloproteinase known as TIMPs). The balance between production, activation and inhibition prevents excessive proteolysis or inhibition. Several factors like cytokines, growth factors, phorbol esters, cell-cell and cell-matrix interactions are thought to control MMP expression (207). Most MMPs are secreted as inactive zymogens, which may be proteolytically activated by different proteinases such as other MMPs, plasmin, trypsin, chymotrypsin and cathepsins. Several cell types produce MMPs in the CNS by microglia, astrocytes, oligodendrocytes and neurons (62, 107, 198). MMP-2 and MMP-9 are secreted by microglia and astrocytes as active forms (126).

Among the MMPs, the gelatinase MMP-9 have been strongly implicated in glioma invasion and angiogenesis (Figure 2). Significantly increased levels of MMP-9 are expressed in human glioma cell lines and human glioma tissue specimens with the degree of expression correlating with tumor grade (55, 56, 156-158). There is a strong correlation between the expression of MMP-9 and the ability of glioma cells to invade matrigel in vitro (1, 86). Furthermore, glioblastoma cells injected intracranially into nude mice resulted in significantly increased levels of MMP-9 during the progression of tumors (173). It has been reported that MMP-9 was strongly expressed in blood vessels at proliferating margins (130) as well as in the tumor cells (156).

We have previously shown that MMP-9 production is induced by cytoskeletal changes involving protein kinase C activation mediated by NFκB (29). TNFα induces MMP-9 transcription (via NFκB and AP-1 sites) and secretion by vascular smooth muscle cells (123). Another study demonstrated that vanadate and phenyl arsenic oxide inhibited migration and invasion of glioma cells by their effects on the cytoskeleton and inhibition of MMP-9 expression (27). On the other hand, MMP-9 knockout mice exhibit abnormal skeletal growth plate angiogenesis and delayed ossification, which is eventually compensated to produce a normal appearing skeleton (201). TGF-β1 stimulated the expression of matrylisin in two human glioma cell lines and activated MMP-9 in C6 rat glioma cells (131). The mitogen-activated kinase/extracellular signal-regulated kinase (MEK/ERK) signaling pathway is essential for MMP-9 upregulation in astrocytes after PMA (PKC induction and TNF-α [cytokine] stimulation). It has been reported that SNB19 cells transfected with dominant negative JNK, MEKK and ERK1 expression vectors decreased MMP-9 expression as well as promoter activity (99). The mutated-ERK stable SNB19 cells showed decreased levels of MMP-9 and less invasiveness as compared to parental and vector-transfected stable clones (98).

MMPs mediate cell-surface-receptor cleavage and release, cytokine and chemokine activation and inactivation, and the release of apoptotic ligands (45). These cellular processes are all involved in promoting aspects of tumor growth, such as cell proliferation, adhesion and dispersion, migration, differentiation, angiogenesis, apoptosis and host defense evasion. β1 integrin is highly expressed in invasive gliomas, and in vitro invasion of β1-integrin expressing glioma cells can be blocked with the exogenous application of specific antisera (167). We have previously observed that stimulation of intracellular β1 integrin can increase the activation of MMP-2 and MMP-9 and the invasiveness of glioma cells in vitro (30).

We have demonstrated that glioblastoma cells expressing antisense MMP-9 exhibited decreased migration and invasion in vitro and did not form tumors when injected intracranially in nude mice (86). Intracranial injections of glioblastoma cells (SNB19) infected with an adenovirus expressing antisense MMP-9 did not produce tumors in nude mice (100). A bicistronic Ad-construct with antisense uPAR and MMP-9 had more effect in regard to inhibition of invasion, angiogenesis and tumor growth in vivo (96). Our recent studies demon-

strated that siRNA bicistronic construct for cathepsin B and MMP-9 completely repressed pre-established intracranial tumors (97). Stable transfection of PTEN (Phosphatase and tensin homologue) reduced MMP-9 secretion caused by hyaluronic acid-induced phosphorylation of focal adhesion kinase and ERK1/ERK2 signaling (140). Glioblastomas with EGFR variant amplification demonstrated the highest levels of MMP-9 (31). Both signal transduction pathways, MAPK/ERK and PI3K pathways, are responsible for mediating increased MMP-9 levels following EGFR activation and appear to promote the association of pro-MMP-9 with the cell surface (31, 46). Our recent studies with bicistronic constructs for uPAR and MMP-9 and for MMP-9 and cathepsin B constructs demonstrated regression in pre-established tumor growth of glioblastoma cell lines in vivo (95, 97).

Exogenous MMP-9 has been shown to enhance endothelial cell growth in vitro (148). Our own studies demonstrated that addition of recombinant MMP-9 (100 ng) increased capillary formation, but excess recombinant MMP-9 (200 ng) inhibited capillary formation in co-cultures in vitro (25). Another study showed that radiation differentially affects the production of MMP-9, MMP-9 and TIMP-1 during glial-endothelial morphogenesis and suggests mechanisms by which microvessels in the CNS respond to radiation (132). A recent study from our group showed that the addition of conditioned medium from glioblastoma cells to endothelial cells treated with antisense Ad-MMP-9 vectors or co-cultures of GBM cell lines with MMP-9 reduced endothelial cells, resulting in reduced capillary-like tube formation and demonstrating the key role of MMP-9 in endothelial cell network organization (77). Using the MMP-9 knockout mice model, it was demonstrated that MMP-9 expression was required for the angiogenic switch, and also that exogenous addition of MMP-9 caused increased VEGF levels (9). It has been reported that forced overexpression of MMP-9 in human breast cancer MCF-7 cells resulted in increased tumor angiogenesis due to increased levels of VEGF (115). In other studies, implantation of tumor cells into either MMP-2 or MMP-9 knockout mice have demonstrated reduced tumor-induced angiogenesis compared to implantation into wild-type mice (26, 76). Further, addition of exogenous active MMP-9 or MMP-9 induced the release of VEGF in ovarian carcinoma cells, suggesting that tumor cell-expressed MMPs can act in an autocrine manner to induce secretion of angiogenic factors (8).

MMPs have been shown to generate endogenous angiogenesis inhibitors by proteolytic cleavage of plasminogen (MMP-2, 3, 7, 9 and 12) which generates angiostatin (39, 135, 141), and MMP-3, 9, 12, 13 and 20 might be involved in the generation of endostatin, a C-terminal fragment of basement membrane collagen type XVIII (51). Another inhibitor, tumstatin, is also released by MMP-9 and inhibits endothelial cell proliferation and promotes apoptosis via signaling through integrin αvβ3 in an RGD-independent manner (64, 187). Thrombospondins at low concentrations induced MMP-9 secretion, tube formation and invasive activity by endothelial cells, but higher concentrations of TSP-1 induced high concentrations of MMP-9 and reduced tube formation and invasion (153). Further, several synthetic inhibitors have shown antiangiogenic activity and reduced the levels of MMPs (105, 108, 129, 193). Our recent studies demonstrated that siRNA bicistronic constructs for cathepsin B and MMP-9 or uPAR and MMP-9 inhibited angiogenesis in both in vitro and in vivo models (95, 97) .

CATHEPSINS

Cathepsins are a group of cysteine proteases predominantly located in the lysozymes. There are 11 cysteine cathepsins present in the human genome (B, C, F, L, K, V, S, X/Z, H, W and O), each with different expression patterns, levels and specificities, all of which contribute to their varying physiological roles. While some are completely specific, others such as cathepsins B, L, and H are very abundant and most are highly active but differently stable at neutral pH—collectively making these proteases potentially harmful if transposed outside of their normal endosomal/lysosomal localization (196). Cathepsins are implicated in tumor angiogenesis, apoptosis and inflammatory and immune responses (195) via their involvement either directly in the degradation of ECM components, such as laminin, fibronectin, and collagen, or through the modulation of protease-sensitive regulatory networks involving other proteases (ie, uPA) as well as non-proteases (ie, annexin II) found at the cellular surface of cancer cells (84, 168, 168).

CATHEPSIN B

Cathepsin B is a lysosomal cysteine proteinase of the papain family of enzymes (81). Proteolytic processing of procathepsin B results in a 31- to 33-kDa single chain. Further processing results in a 26-kDa glycosylated double chain form and a 24-kDa unglycosylated form. Cathepsin B is regulated transcriptionally, post-transcriptionally and post-translationally. Means of regulation include increased transcription, different transcription start sites, the presence of different splice variants of cathepsin B mRNA in individual tissues, and post-translational modification of the protein by proteolytic processing, glycosylation, trafficking and inhibition (181).

Both the human and murine cathepsin B genes have a promoter containing a GC-rich region and numerous Sp1 sites, resembling a housekeeping gene promoter (152). It has been reported that in the U87 glioblastoma cell line Sp1 and Sp3 could activate cathepsin B transcription, and that the regulation of cathepsin B transcription by Sp1 and Sp1-related factors is mediated through multiple GC boxes (214). In addition, cathepsin B promoter activity and the number of Sp1 complexes are much higher in glioblastoma cell lines than in anaplastic astrocytoma or low-grade glioma cell lines (87). Cathepsin B has also been suggested to mediate dissemination of cancer cells by degrading major ECM components (183)—interestingly, it has also been reported recently that integrins play a significant role in the activation of cathepsin B (85)—or by activating other proteases that are capable of degrading the matrix (83). Membrane-associated cathepsin B may also be important for the conversion of pro-uPA to uPA (83). Cathepsin B has been shown to play an active role in the initiation of the proteolytic cascade involving uPA, plasminogen and plasmin that activates latent TGF-β (182).

The proteolytic activity of MMPs depends on their activation, as they are secreted from tumor cells or host cells as latent precursors. All 3 latent MMPs (MMP-2, MMP-3 and MMP-9) can be activated by plasmin, which is, itself, derived from its

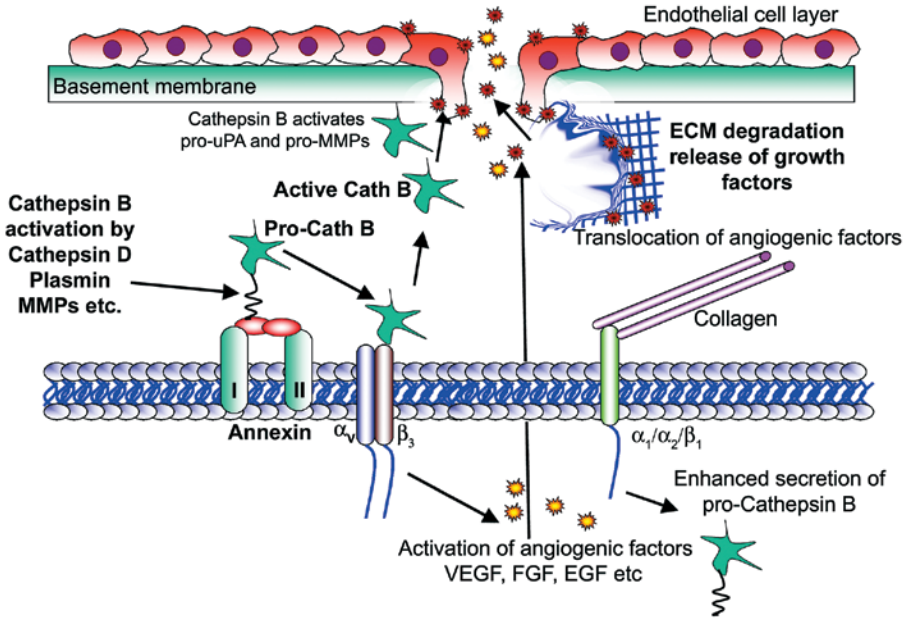

Figure 3. *Represents the involvement of cathepsin B in the initiation of angiogenesis.* Cathepsin B is a lysosomal protease and is secreted from vesicles; extracellular pro-cathepsin B is known to be bound to annexins and is activated by other proteases such as cathepsin D, MMPs and plasmin. Active cathepsin B is either bound to cell surface integrins heterodimer, which may or may not be associated with uPAR, or a free cathepsin B actively degrading the ECM. The degradation of the ECM, as in all proteases, releases ECM bound growth and angiogenic factors, which initiate the endothelial cells to vascularize and migrate towards the tumor cells. The binding of ECM components such as collagen to α or β subunits of integrins is known to enhance the synthesis of pro-cathepsin B, and hence behaves as a feedback loop inducing the production of cathepsin B as long as collagen molecules are present and associated with the cell surface integrins. The interaction of cathepsin B with integrin heterodimer is also known to initiate the activation of proangiogenic factors (VEGF, FGF, and EGF etc.). Cathepsin B also activates pro-collagenases and pro-uPA to their active forms.

latent precursor by a cascade involving cathepsin B and uPA (Figure 3). Therefore, in addition to indirectly activating MMPs via the plasminogen/activator/plasmin cascade, cathepsin B may also directly activate MMPs (eg, collagenase-1 and stromelysin-1) (44).

In human colon carcinoma, increased cathepsin B activity has been found to coexist with increased MMP-2 activity, implying that cathepsin B is crucial to the proteolytic cascade in tumors(47). Cathepsin B is an important mediator of tissue degradation in tumor invasion as it directly participates in tissue destruction. It also enhances the activity of the MMPs by destroying their inhibitors—it inactivates TIMP-1 and TIMP-2 in human articular chondrocytes (91)—even in the absence of mechanisms capable of MMP up regulation, and so acts as an angiogenic stimulator. Cathepsin B is responsible for shifting the balance between MMPs and TIMPs through inactivation of the latter (91).

Cathepsin B has been the most investigated of the lysosomal enzymes and several studies indicated that the activity of cathepsin B is an order of magnitude higher in glioma tissue than in matched normal brain tissue, and these levels also strongly correlated with clinical invasion as assessed by MRI (160) Cathepsin B promotes tumor progression by direct proteolytic activity of the ECM components or by activating other proteases (83). Our previous work has demonstrated that cathepsin B activity and 25- to 26-kDa mature enzyme proteins were the highest in glioblastoma, lower in anaplastic astrocytomas and the lowest in low-grade gliomas and normal brain tissue (180).

The involvement of cathepsins in regulation of angiogenesis reveals yet another distinct role for cathepsins in tumor progression (78). Increased abundance and intensity of cathepsin B staining was observed in 79 glioma tissue samples as compared with 5 normal brain tissue samples (114). This study also demonstrated intense cathepsin B staining at the tumor margin and in endothelial proliferation in high grade tumors, especially in the region of tumor infiltration into adjacent normal brain, suggesting that cathepsin B is functionally significant in the process of tumor invasion and angiogenesis during glioma progression.

In biopsies of human gliomas, cathepsin B levels correlated with pathological evidence of invasion into normal brain tissue and with survival rate of patients (185). Significantly higher expression of cathepsin B correlating with clinical symptoms was demonstrated in studies involving 100 primary brain tumor patients, including 73 malignant and 27 benign tumors of different histologies. This study also demonstrated that intense cathepsin B staining in endothelial cells was a significant prognostic marker for glioblastoma multiforme (36).

Previous studies have demonstrated that cathepsins play a role in apoptosis of cultured cells (74, 177). The antiapoptotic activity of cathepsin B was reported using cysteine cathepsin inhibitor Z-Phe-Gly-NHO-Bz, which induces rapid apoptotic death in glioblastoma cells (219). Cathepsins B and D are involved in the H_2O_2-induced apoptosis in cultured astrocytes; cathepsin D acts as a death-inducing factor upstream of caspase-3 and caspase-independent apoptosis is regulated antagonistically by cathepsins B and D (189). Prolonged inhibition of the lysosomal proteolytic pathway is incompatible with cell survival, leading to apoptosis of neuroblastoma cells. Treatment with E-64 or CA074Me (2 specific inhibitors of cathepsin B) or with pepstatin A (a specific inhibitor of cathepsin D) was cytotoxic for 2 neuroblastoma cell lines having differing degrees of malignancy. Since inhibition of either cathepsin B or D affected cell viability, it was hypothesized that in cultured neuroblastoma cells, cathepsin B and cathepsin D are sequentially involved in the degradation of proapoptotic factors and that the inhibition of either enzyme leads to accumulation of substrates that eventually impact on the caspase cascade (24).

Cysteine protease inhibitors (CPI) of the cystatin family are important endogenous proteins, which protect extracellular structures from abnormal proteolysis and from cysteine proteases. Inhibitors capable of blocking both intra and extracellular fractions of cathepsin B were the most effective in reducing the invasive potential of tumor cells (150). They form tight, equimolar non-covalent complexes with the

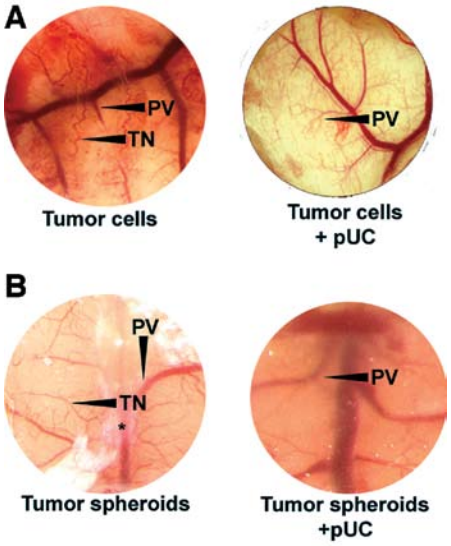

Figure 4. A. *Demonstrates the induction of angiogenesis in SNB19 glioma cells and the inhibition of angiogenesis in SNB19 cells infected with pCU vector.* SNB19 glioma cells and SNB19 cells infected with pCU vector were placed in a cylindrical chamber with a diameter of 14 mm and a height of 2 mm. The largest areas of the disc were made of 0.45 μm Millipore filter discs, which prevent direct cell contact with the animal tissue. These discs were placed in a dorsal sac of nude mice and sutured as per standard protocols control discs containing PBS or serum-free media were also used. Ten days following implantation the discs were removed and the corresponding skin fold in contact with the disc was photographed to visualize the development of tumor-induced neo-vasculature (TN) when compared to pre-existing vasculature (PV). **B.** Shows the induction/inhibition of angiogenesis by cranial window model. Nude mice were anesthetized and a circular opening was made by cutting the cranium and skin with an approximate diameter of 8 mm. Pre-existing vasculatures (PV) were identified and SNB19 glioma spheroids or SNB19 glioma spheroids infected with pCU vector were placed on prominent branching points on the brain surface. A circular glass cover slip (10-mm diameter) was placed on the circular opening and glued onto the cranium bone with surgical glue (cyano-acrylate). The glass cover slip served as a window to monitor the progression of the development of tumor-induced neo-vasculature (TN). (* indicates the position of glioma spheroid)

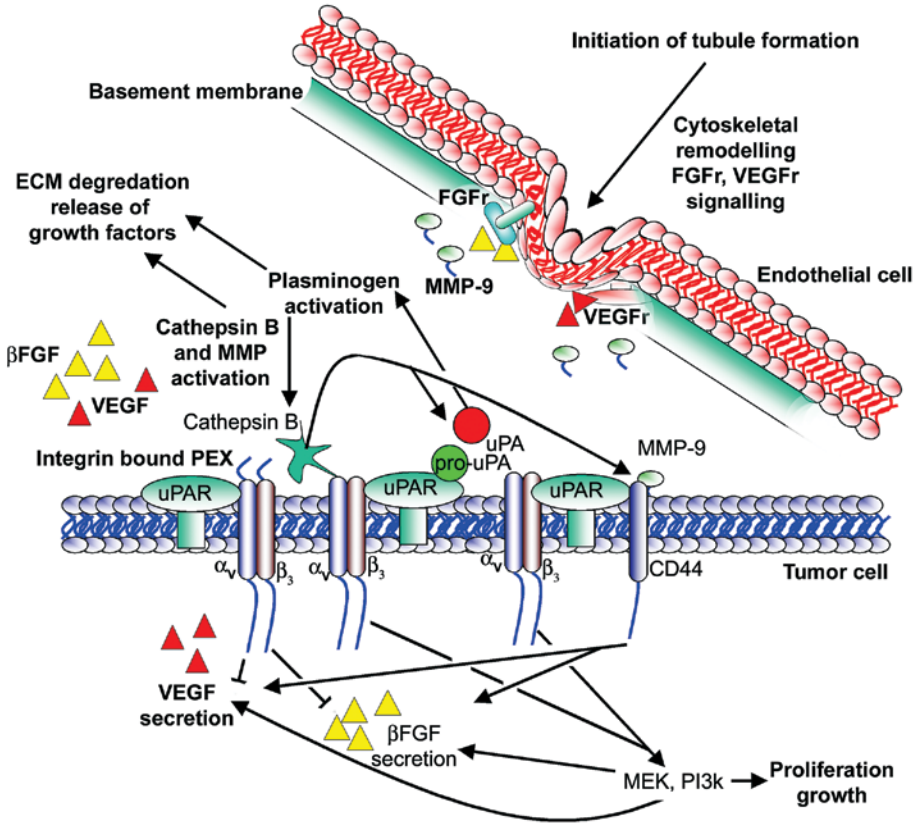

Figure 5. *Schematic representation of the involvement of proteases in the initiation of angiogenesis.* The initiation of angiogenesis is a well-orchestrated cascade of events that involves the combination of proteases, growth factors and angiogenic factors. uPAR appears to play a central role in the initiation of angiogenesis. The activation of plasminogen to plasmin activates other proteases such as cathepsin B and MMPs. Together these proteases degrade the ECM, releasing ECM bound growth factors and enabling the tumor cells to proliferate. The released angiogenic factors such as VEGF, FGF and EGF bind to their corresponding receptors on the cell surface of endothelial cells, which initiate cytoskeletal remodelling and migration towards the tumor cells. The activation of integrin and transmembrane molecules such as CD44 by proteases on the tumor cells initiate the proliferative cascade involving MEL and PI3k, which in turn initiates the synthesis of proangiogenic factors VEGF and FGF. The complex interplay of the proteases with the ECM causes the release of proangiogenic factors and induces the vascularization of the endothelial cells.

cysteine proteases to protect cells from the harmful effects of continuous proteolysis. We have previously reported high cysteine protease inhibitory activity in glioma cells and culture medium. Low levels of cysteine protease inhibitory activity were observed in high-grade gliomas (anaplastic and glioblastoma) as compared to low-grade and benign tumors. Similarly, CP inhibitory activity was high in benign and atypical meningiomas as compared to invasive meningiomas (179).

Three types of CPI inhibitors have been identified: stefins (Mr ~11 kDa), cystatins (Mr ~13 kDa) and kininogens (Mr ~60 and 100 kDa) (22). The major intracellular or endogenous cysteine proteinase inhibitors are stefins A and B. Cystatin C is an extracellular molecule that is synthesized within the human brain including choroid plexus, cerebral and cerebellar neurons and astrocytes (103). We have demonstrated that cystatin C levels were significantly higher in low-grade and anaplastic astrocytoma cell lines as compared to glioblastoma cells (88).

The synthetic cathepsin B inhibitor K11017 blocked glioblastoma migration and substantially reduced glioma cell invasion in a Matrigel assay and reduced the infiltration of glioma spheroids into normal brain aggregates (36). An in vitro invasion assay has demonstrated that the invasiveness of glioblastoma cells was significantly impaired by cysteine protease inhibitor E-64 (87) and by intracellular and extracellular inhibition of cathepsin B by its selective inhibitors, Ca074Me and Ca074, respectively (93). Glioblastoma cells expressing antisense cathepsin B cDNA exhibited significant reductions in cathepsin B mRNA and protein and invasiveness in Matrigel and spheroid models in vitro. Intracerebral injection of SNB19 stable antisense transfectants resulted in reduced tumor formation in nude mice (121).

Blockade of cathepsin B expression in human glioblastoma cells is also associated with suppression of angiogenesis. Antisense cathepsin B stable clones demonstrated a marked reduction in capillary network formation by endothelial cell in vitro and mi-

crovasculature development in vivo dorsal air sac assay as compared to vector-transfected and parental controls (216). Stable transfection of cystatin C gene in the sense orientation reduced the invasiveness of glioblastoma cell line in vitro and did not form tumors in nude mice in vivo (88). The bicistronic antisense construct for uPAR and cathepsin B (Ad-uPAR-Cath B) infected cells revealed a marked reduction in migration, invasion, angiogenesis and tumor growth in both in vitro and in vivo models (60). Furthermore, RNAi of uPAR and cathepsin B reduces glioma cell invasion and angiogenesis in both in vitro and in vivo models and regressed pre-established tumor growth (Figure 4). We also demonstrated that these constructs inhibited cell proliferation and reduced the levels of pERK and pFAK. Our recent studies demonstrated that bicistronic siRNA construct against cathepsin B and MMP-9 regressed completely pre-established intracerebral tumors in nude mice and also marked reduction in the migration, invasion and angiogenesis (97).

These studies clearly demonstrate the interaction and significant role of these proteases and uPAR in angiogenesis (Figure 5).

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