

Downregulation of uPA, uPAR and MMP-9 using small, interfering, hairpin RNA (siRNA) inhibits glioma cell invasion, angiogenesis and tumor growth

CHRISTOPHER S. GONDI¹

SAJANI S. LAKKA¹

DZUNG H. DINH²

WILLIAM C. OLIVERO²

MEENA GUJRATI³

AND

JASTI S. RAO^{1,2}

The diffuse, extensive infiltration of malignant gliomas into the surrounding normal brain is believed to rely on modification of the proteolysis of extracellular matrix components. Our previous results clearly demonstrate that uPA, uPAR and MMP-9 concentrations increase significantly during tumor progression and that tumor growth can be inhibited with antisense stable clones of these molecules. Because antisense-mediated gene silencing does not completely inhibit the translation of target mRNA and high concentrations of antisense molecules are required to achieve gene silencing, we used the RNAi approach to silence uPA, uPAR and MMP-9 in this study. We examined a cytomegalovirus (CMV) promoter-driven DNA-template approach to induce hairpin RNA (hpRNA)-triggered RNAi to inhibit uPA, uPAR and MMP-9 gene expression with a single construct. uPAR protein levels and enzymatic activity of uPA and MMP-9 were found to significantly decrease in cells transfected with a plasmid expressing hairpin siRNA for uPAR, uPA and MMP-9. pUM-transfected SNB19 cells significantly decreased uPA, uPAR and MMP-9 expression compared to mock and EV/SV-transfected cells, determined by immunohistochemical analysis. Furthermore, the effect of the single constructs for these molecules was a specific inhibition of their respective protein levels, as demonstrated by immunohistochemical analysis. After transfection with a plasmid vector expressing dsRNA for uPA, uPAR and MMP-9, glioma-cell invasion was retarded compared with mock and EV/SV-treated groups, demonstrated by Matrigel-invasion assay and spheroid-invasion assay. Downregulation of uPA, uPAR and MMP-9 using RNAi inhibited angiogenesis in an in vitro (co-culture) model. Direct intratumoral injections of plasmid DNA expressing hpRNA for uPA, uPAR and MMP-9 significantly regressed pre-established intracranial tumors in nude mice. In addition, cells treated with RNAi for uPAR, uPA and MMP-9 showed reduced pERK levels compared with parental and EV/SV-treated SNB19 cells. Our results support the therapeutic potential of RNAi as a method for gene therapy in treating gliomas.

Keywords: gliomas, invasion, RNA interference, angiogenesis

INTRODUCTION

Tumor progression involves modulation of tumor-cell adhesion during migration and degradation of the extracellular matrix (ECM) during invasion. An intricate balance of proteases, their activators and their inhibitors regulates both these processes during tumor invasion. Three classes of ECM-degrading proteinases are the serine proteinases, metallo-proteases and cysteine proteinases. A large body of evidence assigns a key role in tumor progression and invasion to uPA by virtue of its ability to initiate a cascade of proteases that can degrade most matrix and basement membrane components and interfere with cell–cell and cell–matrix interactions (Andreasen *et al.*, 1997; Carroll *et al.*, 1999). uPA, bound to its cell surface receptor uPAR, is the principal participant of ECM degradation, as demonstrated by a several-fold increase in

plasminogen activation (Alonso *et al.*, 1996; Ellis *et al.*, 1991). uPA also activates several growth factors after degradation of ECM components (Naldini *et al.*, 1995; Werb *et al.*, 1996). The absence of uPA negatively affects the progression of chemically induced neoplasms in mice (Shapiro *et al.*, 1996). The inoculation of metastatic Lewis lung carcinoma cells into plasminogen-deficient mice resulted in the formation of either smaller or less hemorrhagic tumors than those observed in control, wild-type mice (Bugge *et al.*, 1997). Regulation of either uPA or uPAR is associated with increased aggressiveness in diverse tumor types (Blasi, 1999). In murine tumor models, either decreased expression of uPAR or administration of uPAR antagonists markedly inhibits the metastatic ability of cancer cells (Crowley *et al.*, 1993), primary tumor growth (Min *et al.*, 1996) and downregulation of uPAR, which leads to dormancy of carcinoma cells *in vivo* (Yu *et al.*, 1997). Targeted deletion

of the gene encoding uPAR in mice significantly reduces the uPA-mediated activation of plasminogen in peritoneal macrophages (Dewerchin *et al.*, 1996), indicating that the principal role of uPAR is to localize plasminogen activation to the cell surface (Ellis *et al.*, 1992). MMPs are also implicated in cancer invasion through ECM degradation and the processing of a range of molecules, including growth factors and cytokines. These processes are involved in promoting all aspects of tumor growth, such as cell proliferation, adhesion and dispersion, migration, differentiation, angiogenesis, apoptosis and host-defense evasion (Egeblad and Werb, 2002).

Malignant gliomas are characterized by rapid cell proliferation, a high level of invasiveness into the surrounding brain and increased vascularity. In humans, diffuse astrocytomas, glioblastoma multiforme in particular, invade the brain preferentially along white matter fiber tracts (Giese and Westphal, 1996; Pedersen *et al.*, 1995), resulting in rapid infiltrative growth that prevents successful surgical resection. This invasive behavior seems to depend in part on several proteolytic enzymes. Our previous studies and those of others have reported significantly increased levels of uPA and uPAR in human glioblastoma tissue samples compared to low-grade and normal brain tissue samples (Gladson *et al.*, 1995; Yamamoto *et al.*, 1994a; Yamamoto *et al.*, 1994b). Downregulation of uPAR expression using antisense and gene-therapy approaches has resulted in increased survival in animal models. The antisense, stable, uPAR glioblastoma clones result in an inability of the cells to generate tumors when transplanted into nude mice (Go *et al.*, 1997) and reduced invasiveness in *in vitro* models (Mohanam *et al.*, 1997). The infection of glioblastoma cells with antisense uPAR adenoviral vectors in cultures resulted in tumor growth *in vivo* (Mohan *et al.*, 1999). Adenovirus-mediated downregulation of bicistronic constructs of uPA and uPAR expression inhibited glioma cell migration, invasion and tumor-induced capillary formation (Gondi *et al.*, 2003). Antisense stable clones for uPA significantly reduced invasiveness and tumor formation in *in vitro* and *in vivo* models (Mohanam *et al.*, 2001). In another study, stably transfected glioma cells expressing the amino terminal fragment (ATF) domain (residues 1–46) of uPA, which binds uPAR, did not form tumors in nude mice (Mohanam *et al.*, 2002). Several studies report significantly increased levels of MMP-9 during the progression of human gliomas (Forsyth *et al.*, 1999; Raithatha *et al.*, 2000; Rao *et al.*, 1993; Rao *et al.*, 1996).

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 (Kondraganti *et al.*, 2000). Intracranial injections of glioblastoma cells (SNB19) infected with an adenovirus that expresses antisense MMP-9 did not produce tumors in nude mice (Lakka *et al.*, 2002a). A bicistronic Ad-construct with antisense uPAR and MMP-9 had more effect with regard to inhibition of invasion, angiogenesis and tumor growth *in vivo* (Lakka *et al.*, 2003). Our recent studies demonstrate that a siRNA bicistronic construct for cathepsin B and MMP-9 completely repressed pre-established intracranial tumors (Lakka *et al.*, 2004).

Together, these studies indicate the biological significance of uPAR, uPA and MMP-9 in glioma invasion and tumor growth. The stability/specificity of the delivery approach of

Ad-vector and antisense technology to intracellular target RNA seems to be a crucial limiting factor in exerting an inhibitory effect on the targeted molecule. The siRNA duplex was significantly more stable in cells than the cognate, single-stranded sense of anti-sense RNA with transcription under the control of identical promoter in each case (Miyagishi and Taira, 2002). RNAi was shown to be superior to antisense because only a few molecules of dsRNA per cell can trigger gene silencing (Zamore, 2001). The activity of siRNA is high, even at low concentrations, and without apparent toxicity. In a recent study, siRNA was quantitatively more efficient than antisense oligonucleotides at suppressing cotransfected GFP expression *in vitro* and *in vivo* (Bertrand *et al.*, 2002).

Therefore, RNAi might provide a more powerful strategy for the regression of pre-established intracranial tumor growth and inhibition of invasion/angiogenesis through a nucleic acid drug or a gene therapy approach. We show here that the siRNA-mediated suppression of uPAR, uPA and MMP-9 results in significant regression of pre-established intracranial tumor growth as well as inhibition of invasion and angiogenesis. Our results demonstrate that a single vector capable of expressing siRNA for three genes can block expression of the targeted proteases/receptor and indicate a potentially useful method for developing highly specific, siRNA-based, gene-silencing therapeutics for the treatment of glioma.

OBJECTIVES

The present study was conducted to determine the effectiveness of downregulating multiple targets in human glioma cells to control invasion, growth and angiogenesis. We hypothesized that the simultaneous RNAi-mediated targeting of uPAR, uPA and MMP-9, using a single CMV promoter, would retard tumor-cell invasion, growth and angiogenesis, and that the effects would be synergistic rather than additive. The study was conducted in three stages. Stage 1 dealt with the development of plasmid-based, CMV-driven, RNAi vectors to target uPAR, uPA and MMP-9 either singly or together, and the determination of their ability to effectively downregulate the target molecules. Stage 2 dealt with the *in vitro* behavior of SNB 19 human glioblastoma cell with respect to invasion and angiogenesis. Stage 3 dealt with the *in vivo* ability of the RNAi vectors to retard the growth of pre-established intracranial tumors in nude mice.

METHODS

Constructing a vector expressing siRNA for uPAR, uPA and MMP-9

pcDNA3 was used for the construction of a vector expressing siRNA for uPAR, uPA and MMP-9 downstream of the cytomegalovirus (CMV) promoter. The uPAR sequence from +77 to +98 was used as the target sequence and for convenience a self-complementary oligo was used. The uPAR sequence 21 bases long with a 9 base loop region with BamHI sites incorporated at the ends (5'gacacctacagcagtgaggagcattata-tataataatcgctctccactgctgtag3') was used. The oligo was self-annealed in 6× SSC using standard protocols and ligated into the BamHI site of a pcDNA3 vector plasmid. Similarly, uPA complementary sequence from +346 to +367 (5'agcttgagagcctgctggcgccatataatggcgccagcaggctctca3') with HindIII

sites incorporated at the ends was ligated into the HindIII site and MMP-9 +360 to +381 (5'aattcaagtggcaccaccacaacaataataattgttggtggtgctcacttg3') was ligated into the EcoRI site of the vector containing the siRNA sequence for uPAR and uPA. This finally resulted in a siRNA expression plasmid for uPAR, uPA and MMP-9 designated pU₂M (Fig. 1). Single siRNA expression vectors for uPAR (puPAR), uPA (puPA) and MMP-9 (pMMP-9) were also constructed. The orientation of the insert in either the single or tricistronic construct was not crucial because the oligos were self-complementary and had bilateral symmetry. BGH poly A terminator served as a stop signal for RNA synthesis for all four constructs.

Cell culture and transfection

The SNB19 (or SNB19 GFP) cell line, established from a human, high-grade glioma, was used for this study. Cells were grown in Dulbecco's modified Eagle medium/F12 media (1:1, v:v) supplemented with 10% fetal calf serum in a humidified atmosphere containing 5% CO₂ at 37°C. SNB19 and SNB19 GFP cells were transfected with plasmid constructs empty vector (EV), scrambled vector (SV), puPAR, puPA, pMMP-9 and pU₂M using lipofectamine (Life Technologies) according to the manufacturer's instructions.

Western blot analysis

SNB19 cells were transfected with EV/SV, puPAR, puPA, pMMP-9 and pU₂M. After 48 hours, cells were collected and total cell lysates prepared in extraction buffer containing 0.1 M Tris (pH 7.5), 1% Triton-X114, 10 mM EDTA, aprotinin and phenylmethylsulfonyl fluoride as described previously (Mohan *et al.*, 1999). The extracts were incubated at 37°C for 5 minutes and centrifuged to separate the lower (detergent) phase that contains mainly hydrophobic membrane proteins including the glycosylphosphatidylinositol-anchored uPAR. Subsequently, 20 µg of protein from these samples were separated under non-reducing conditions by 12% SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell). The membranes were probed with polyclonal antibodies against uPAR (#399 American Diagnostics) and secondary antibodies (anti-rabbit-horseradish peroxidase) as required, and developed according to enhanced chemiluminescence protocol (Amersham). The membranes were stripped and probed with monoclonal antibodies for GAPDH as described above to verify that similar amounts of protein was loaded in each lane.

Gelatin zymography

The enzymatic activity and molecular weight of MMPs was determined by gelatin zymography in the conditioned medium of SNB19 cells transfected with EV/SV, puPAR, puPA, pMMP-9 and pU₂M, as described previously (Lakka *et al.*, 2003). To prepare conditioned medium, cells were washed with serum-free medium and resupplied with fresh, serum-free medium. After 24 hours, the conditioned medium was harvested, centrifuged to remove cellular debris and protein concentrations determined. Equal amounts of protein were electrophoresed under non-reducing conditions through 8% SDS-PAGE containing 0.1% gelatin. Gels were washed in 2.5% Triton X-100 and incubated overnight in Tris-CaCl₂ buffer. The gels were then stained with 0.1% amido black for 1 hour

and destained in 20% methanol and 10% acetic acid. The clear bands represented gelatinase activity.

Fibrin zymography

The enzymatic activity and molecular weight of electrophoretically separated forms of uPA were determined in conditioned medium of SNB19 cells transfected with EV/SV puPAR, puPA, pMMP-9 and pU₂M by SDS-PAGE as described previously (Mohanam *et al.*, 2001). Briefly the SDS-PAGE gel contains acrylamide to which purified plasminogen and fibrinogen were substrates before polymerization. After polymerization, equal amounts of proteins in the samples were electrophoresed and the gel was washed and stained as described previously (Mohanam *et al.*, 2002).

Immunohistochemical analysis

SNB19 cells (1×10⁴ well⁻¹) were seeded in 8-well chamber slides, incubated for 24 hours and transfected with EV/SV, puPAR, puPA, pMMP-9 and pU₂M. After 72 hours, cells were fixed in 3.7% formaldehyde and incubated with 1% bovine serum albumin in PBS at room temperature for 1 hour for blocking. The slides were then incubated with PBS and 1% bovine serum albumin, with mouse IgG anti-uPAR (R&D Systems), mouse IgG anti-uPA (Calbiochem), or rabbit IgG anti-MMP-9 (Biomedica) at a concentration of 1:500 and incubated at room temperature for 1 hour as described previously (Lakka *et al.*, 2004). The slides were subsequently washed three times with PBS to remove excess primary antibody and then incubated with either anti-mouse HRP conjugate or anti-rabbit HRP conjugate IgG (1:500 dilution) for 1 hour at room temperature. The slides were washed three times, DAB peroxidase substrate (Sigma #D-4168) was added, covered with glass cover slips, and observed in a bright-field microscope.

Matrigel invasion assay

Invasion of glioma cells *in vitro* was measured by the invasion of cells through Matrigel-coated (Collaborative Research Inc.) transwell inserts (Costar). Briefly, transwell inserts with 8 µm pores were coated with a final concentration of 1 mg ml⁻¹ of Matrigel, SNB19 cells transfected with EV/SV, puPAR, puPA, pMMP-9 and pU₂M or parental cells alone were trypsinized and 200 µl aliquots of cell suspension (1×10⁶ cells ml⁻¹) were added in triplicate wells. After a 24-hour incubation, cells that passed through the filter into the lower wells were quantified as described earlier (Mohan *et al.*, 1999; Mohanam *et al.*, 1997) and expressed as a percentage of the sum of cells in the upper and lower wells. Cells on the lower side of the membrane were fixed, stained with Hema-3 and photographed.

In vitro angiogenic assay

SNB19 cells (2×10⁴ well⁻¹) were seeded in 8-well chamber slides and transfected with EV/SV, puPA, puPAR, pMMP-9 and pU₂M or with parental cells above. After a 24-hour incubation period, the conditioned media was removed and added to a monolayer of 4×10⁴ human dermal endothelial cells in 8-well chamber slides, and the human dermal endothelial cells were allowed to grow for 72 hours. Cells were then fixed in 3.7% formaldehyde, blocked with 2% bovine serum albumin and the endothelial cells were incubated with factor VIII primary

antibody (DAKO Corp.). The cells were then washed with PBS and incubated with a FITC-conjugated secondary antibody for 1 hour. The slides were washed and the formation of capillary-like structures was observed using a fluorescent microscope. Similar slides were H&E stained to visualize network formation. *Image Pro* software was used to quantify angiogenesis. The degree of angiogenesis was measured by the following method: number of branch points and the total number of branches per point were counted, with the product indicating the degree of angiogenesis.

***In vitro* spheroid invasion assay**

Multicellular SNB19 spheroids were cultured in 6-well ultra low attachment plates. Briefly, 3×10^6 cells were suspended in 10 ml of medium, seeded onto the plates and cultured until spheroids formed. Spheroids of 100–200 μm diameter were selected and transfected with EV/SV, puPAR, puPA, pMMP-9 and pU₂M or with parental spheroids alone. Three days after infection, tumor spheroids were stained with the fluorescent dye DiI and confronted with fetal rat brain aggregates stained with DiO. The progressive destruction of fetal rat brain aggregates and invasion of SNB19 spheroids were observed by confocal laser scanning microscopy and photographed as described previously (Lakka *et al.*, 2003; Lakka *et al.*, 2004). The remaining volume of the rat brain aggregates at 24, 48 and 72 hours by these glioma spheroids with these constructs were quantified using image analysis software as described previously (Gondi *et al.*, 2003; Gondi *et al.*, 2004; Lakka *et al.*, 2003).

Intracranial tumor growth inhibition

For the intracerebral tumor model, 2×10^6 SNB19 GFP cells were injected intracerebrally into nude mice. Tumors were allowed to grow for 10 days. At this time, animals were randomized into seven groups and EV/SV, puPAR, puPA, pMMP-9 and pU₂M (150 μg of each construct were injected into the brain using Alzet mini pumps at the rate of $0.25 \mu\text{l h}^{-1}$ (six mice in each group). Five weeks after tumor inoculation, mice were sacrificed by cardiac perfusion with 3.5% formaldehyde in PBS. Their brains were removed and placed in 4% paraformaldehyde for 24 hours, paraffin embedded and sectioned. The sections were screened for GFP fluorescence to examine tumor growth under a fluorescent microscope. The sections were reviewed blindly and scored semiquantitatively for tumor size. The average tumor area in each section was used to calculate tumor volume and compared between controls and treated groups.

RESULTS

Effect siRNA constructs on uPAR protein, and uPA and MMP-9 enzymatic activity in SNB19 glioblastoma cells

To simultaneously inhibit three endogenous genes with hairpin siRNA, we constructed a vector (pU₂M) expressing siRNA for uPAR (77–98 bases of human uPAR in RNA), uPA (346–367 bases of human uPA in RNA) and MMP-9 (360–381 bases of human MMP-9 in RNA) under the control of the CMV promoter (Fig. 1). Western blot analysis was performed to examine the effect of empty vector/scrambled vector

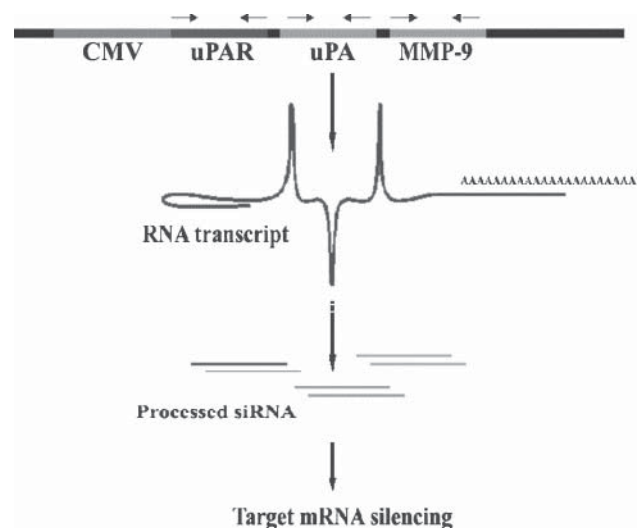


Fig. 1. Schematic of the possible formation of hpRNA molecules from a single, CMV-driven, tri-inverted repeat construct for uPAR, uPA and MMP-9. The powerful CMV viral promoter drives the formation of an RNA molecule that possesses self-complementary inverted repeats for uPA, uPAR and MMP-9.

(EV/SV), puPAR, puPA, MMP-9 and pU₂M transfection on uPAR protein concentrations in SNB19 cells. The uPAR protein band was present in SNB19 cells transfected with EV/SV, puPA and pMMP-9, whereas it was reduced significantly in puPAR- and pU₂M-treated cells (Fig. 2A). The effect of the tricistronic construct (pU₂M) was greater than the puPAR (Fig. 2A). The levels of GAPDH determined that equal quantities of protein were loaded in the gel (Fig. 2A). Fibrin zymography was performed to examine the effect of EV/SV, puPAR, puPA, pMMP-9 and pU₂M-treated SNB 19 cells on uPA enzymatic activity. Gelatin zymography was performed to determine the effect of these constructs on the levels of MMP-9 in SNB19 cells. MMP-9 levels were significantly reduced in SNB19 cells treated with puPAR, pMMP-9 and pU₂M compared to parental, EV/SV- and puPA-treated cells (Fig. 2B). Interestingly, MMP-2 levels were also downregulated in pU₂M-treated cells. Care was taken to load equal quantity of proteins. (Fig. 2B). The uPA enzymatic activity (MR 55 000) was reduced significantly in puPA- and pU₂M-treated cells compared with the parental, EV/SV-, puPAR- and pMMP-9-treated groups (Fig. 2C)

The effect of the tricistronic construct was more pronounced than that of the single siRNA constructs for these molecules. Determined by immunohistochemical analysis, puPAR, puPA, pMMP-9 and pU₂M transfection decreased uPAR, uPA and MMP-9 concentrations in SNB19 cells. Figure 2D shows the protein levels of uPAR, uPA and MMP-9 in parental, EV/SV-, puPAR-, puPA-, pMMP-9- and pU₂M-transfected cells using specific antibodies for uPAR, uPA and MMP-9. The respective intensities of uPAR, uPA and MMP-9 were very high in parental cells and in cells transfected with EV/SV. By contrast, uPAR intensity decreased in SNB19 cells transfected with puPAR and pU₂M. puPA and pU₂M transfection significantly decreased the intensity of uPA protein compared with parental, EV/SV-, puPAR- and pMMP-9-transfected cells. Further, MMP-9 protein concentration decreased significantly in pMMP-9- and pU₂M-transfected cells compared with parental, EV/SV, puPA- and puPAR-transfected cells. These results demonstrate that the effect of

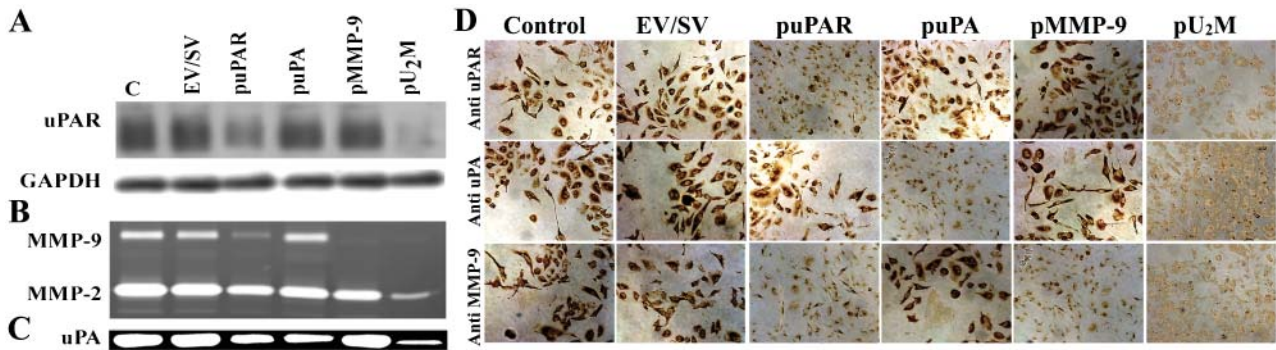


Fig. 2. Western blotting, fibrin/gelatin zymography and immunohistochemical analysis of uPA and MMP-9. SNB19 cells were either mock transfected or transfected with an empty vector/scrambled vector (EV/SV) and vectors encoding siRNA uPAR (puPAR), uPA (puPA), MMP-9 (pMMP-9) and a combination of the three together (pU₂M). After a 3-day-incubation period, total cell lysates were prepared in extraction buffer and 50 µg of protein from these samples were separated by 12% non-reducing SDS-PAGE and immunoblotted with anti-uPAR antibody (A). GAPDH was immunoprobed simultaneously as a loading control. Conditioned medium was collected from these samples (20 µg) and gelatin and fibrin zymography performed to detect MMP-9 (B) and uPA activity (C). (D) SNB19 cells (1×10^4) were seeded onto Lab-Tek II chamber slides and either mock transfected or transfected with EV/SV and vectors encoding siRNA puPA, puPAR and pMMP-9 either singly or together (pU₂M). After 72 hours, cells were fixed, washed for 1 hour with blocking buffer and stained for uPAR, uPA and MMP-9 expression using specific antibodies for uPA, uPAR and MMP-9.

the single constructs is molecule-specific and that the effect of the tricistronic construct is much more pronounced than that the single constructs alone.

puPAR, puPA, pMMP-9 and pU₂M inhibit tumor-induced capillary network formation

The growth of a glial tumor depends on the induction of new capillary blood vessels that are necessary to support the developing tumor mass. We used a co-culture system in which microvascular endothelial cells were induced by conditioned media from glial cells to form capillary-like structures to examine the effect of RNAi-mediated suppression of uPAR, uPA and MMP-9. We performed immunohistochemical analysis using factor VIII antigen to evaluate tumor-induced vessel formation in an *in vitro* co-culture system and performed H&E staining. Endothelial cells form capillary-like structures in the presence of conditioned media from SNB19 parental and EV/SV-transfected cells (Fig. 3A). By contrast, transfection of SNB19 cells with vectors expressing siRNA for uPA, uPAR and MMP-9 either individually or in combination partially or completely inhibited tumor-induced microvessel formation (Fig. 3A). New branch points and/or an increase in the number of branches were not detected in pU₂M-transfected cells compared with EV/SV-treated cells (Fig. 3A). Furthermore, compared with EV/SV-treated cells, the formation of capillary-like structures was inhibited by ~55% in puPAR-treated cultures, ~36% in puPA-treated cultures and ~60% in pMMP-9-treated cultures (Fig. 3B).

puPAR, puPA, pMMP-9 and pU₂M inhibit invasion in SNB19 cells

Proteolytic degradation of ECM components is crucial for tumor-cell invasion. To evaluate the impact of siRNA-mediated inhibition of uPAR, uPA and MMP-9 on glioma invasiveness, we utilized two models. In the first model, we compared the invasive ability of SNB19 cells transfected with puPAR, puPA, pMMP-9 and pU₂M to those infected with the EV/SV vector. SNB19 cells transfected with EV/SV and parental cells demonstrated extensive invasion through

Matrigel-coated transwell inserts, as indicated by the intense staining of cells. By contrast, puPAR-, puPA-, pMMP-9- and pU₂M-transfected cultures were markedly less invasive through the reconstituted basement membrane, as indicated by the staining intensity compared with the controls (Fig. 3C). Quantification confirmed that transfection with puPAR, puPA, pMMP-9 and pU₂M vectors reduced invasion by SNB19 cells to 9%, 40%, 15% and 2%, respectively, of that of parental and EV/SV transfected controls (Fig. 3D). Inhibition of invasion was higher in cells transfected with the tricistronic construct compared to single constructs alone.

We further examined the effect of puPAR, puPA, pMMP-9 and pU₂M vectors using a spheroid invasion assay. A significant, potential advantage of using glioma spheroids is that tumor cells grown in three-dimensional cultures exhibit properties that more closely resemble those of tumors *in vivo*. In the spheroid co-culture system, control spheroids and spheroids transfected with the EV/SV vector progressively invaded fetal rat-brain aggregates whereas spheroids transfected with puPAR, puPA, pMMP-9 and pU₂M demonstrated partial to almost complete inhibition of invasion (Fig. 4A). Quantification revealed that glioma spheroids invaded the fetal rat brain aggregates by 30% within 1 day, 55% within 2 days and 95% by 3 days, at which time the tumor spheroid and brain aggregates had combined into single entity (Fig. 4B). A similar trend was observed with glioma spheroids transfected with the EV/SV vector. By contrast, tumor spheroids transfected with the pU₂M vector did not invade fetal rat brain aggregates. By 3 days, the rat brain aggregates were invaded by approximately 96%, 95%, 45%, 25% and 15% in the parental, EV/SV-, puPA-, pMMP-9- and puPAR-transfected co-cultures, and by 1% in the pU₂M-transfected co-cultures (Fig. 4B). These results provide strong evidence that pU₂M strongly inhibits glioma invasion in both *in vitro* models.

pU₂M completely regresses intracranial tumor growth

Here, we determined whether the downregulation of uPAR, uPA and MMP-9 levels using either single or tricistronic constructs causes regression of pre-established intracranial

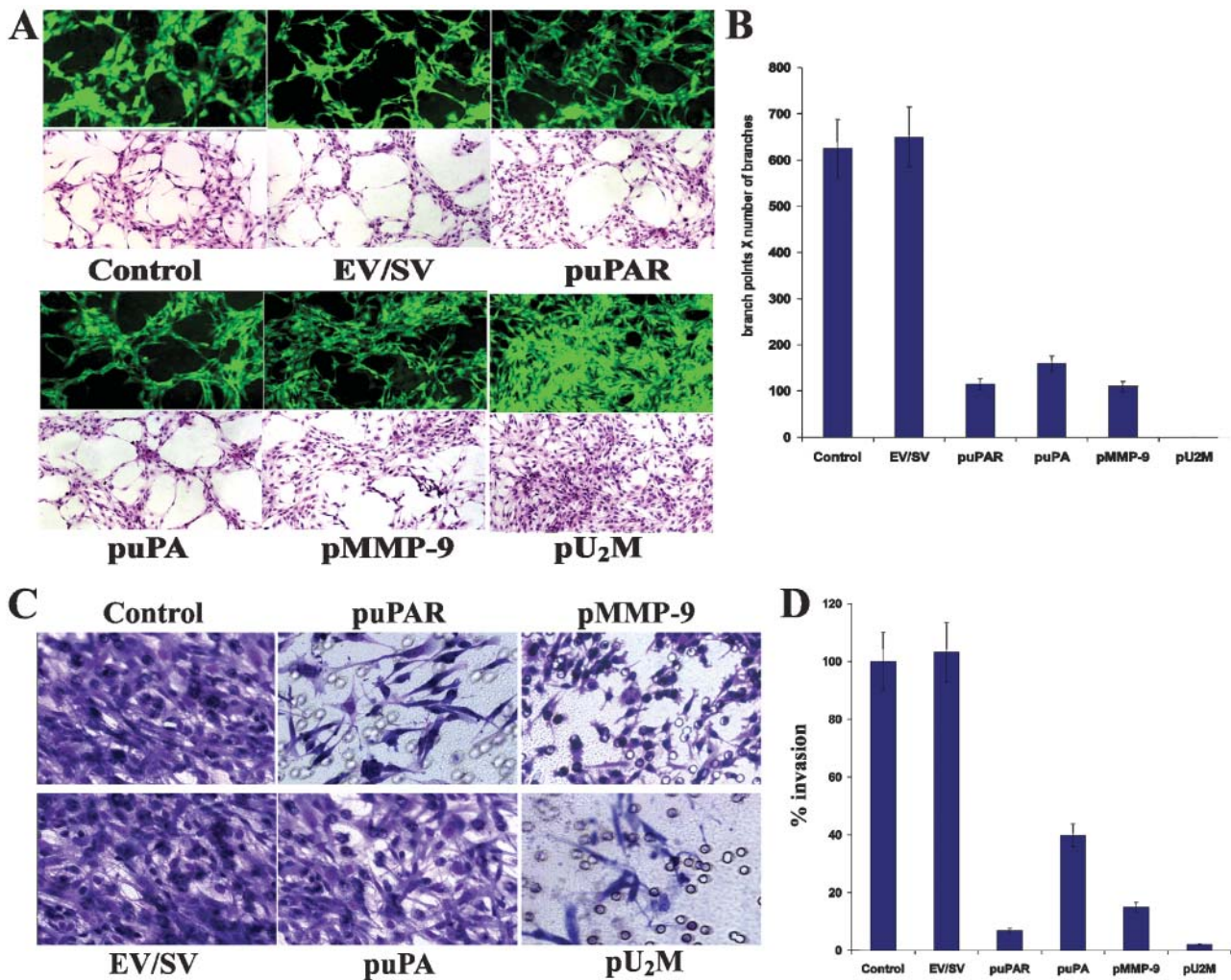


Fig. 3. Inhibition of glioma angiogenesis and invasion by siRNA constructs. SNB19 cells (2×10^4) were seeded onto 8-well-chamber slides and transfected with EV/SV and vectors encoding siRNA uPAR (puPAR), uPA (puPA), MMP-9 (pMMP-9) and a combination of three together (pU₂M). After a 24-hour-incubation period, the medium was removed, cells were co-cultured with either 4×10^4 human endothelial cells or 4×10^4 endothelial cells alone and were grown in the presence of conditioned media. After 72 hours endothelial cells were stained for factor VIII antigen in the co-cultures (green fluorescence). Cells grown in the preserved conditioned media were H&E stained and examined under either a fluorescent microscope or a bright field microscope (A). (B) Quantification of angiogenesis in co-cultures infected with EV/SV, puPAR, puPA, pMMP-9 and pU₂M vectors. Values are mean \pm SD of four experiments. SNB19 cells were trypsinized 72 hours after transfection with EV/SV, puPAR, puPA, pMMP-9 and pU₂M, washed with PBS and resuspended in serum-free medium. (C) Invasion assays were carried out in a 12-well transwell unit on polycarbonate filters with 8- μ m pores coated with matrigel (0.7 mg ml^{-1}). After a 24-hour-incubation period, the cells that had passed through the filter into the lower wells were stained, counted and photographed under a bright-field microscope. (D) The invasion was quantified as described Methods (D). Values are mean \pm SD of four experiments.

tumor growth in nude mice. All animals in the control and EV/SV-treated groups had intact cerebral tumors that were characterized by strong GFP fluorescence (Fig. 4C) whereas brain sections of mice treated with puPAR, puPA and pMMP-9 had small tumors, illustrated by minimal GFP fluorescence. Notably, GFP fluorescence was not detected in brain sections of mice treated with pU₂M (Fig. 4C). Further quantification of these sections (scored by a neuropathologist blinded to treatment conditions) revealed no difference in tumor size between the parental and EV/SV treated groups and significant regression of pre-established intracranial tumor growth in the groups treated with puPAR, puPA and pMMP-9 (80%, 55% and 68% respectively) compared to control groups (Fig. 4D). However, complete regression of pre-established intracranial tumor growth was revealed in the pU₂M treated group. These results demonstrate that RNAi-mediated suppression

of uPAR, uPA and MMP-9 using a tricistronic construct completely eradicated malignant glioma growth in nude mice.

Inhibition of ERK1/2 phosphorylation

To better understand the effect of siRNA-mediated down-regulation of uPAR, uPA and MMP-9 on signaling pathways, we assayed for total and phosphorylated levels of ERK1/2, which are involved directly in tumor-cell survival, migration and proliferation. Western blots showed that there was no significant difference in total ERK1/2 concentrations in control and EV/SV-transfected cells compared with puPAR-, puPA-, pMMP-9- and pU₂M-transfected cells (Fig. 5). However, the concentration of phospho-ERK1/2 was reduced significantly in SNB19 cells transfected with the pU₂M vector compared with the control, EV/SV-, puPAR-, puPA- and pMMP-9-

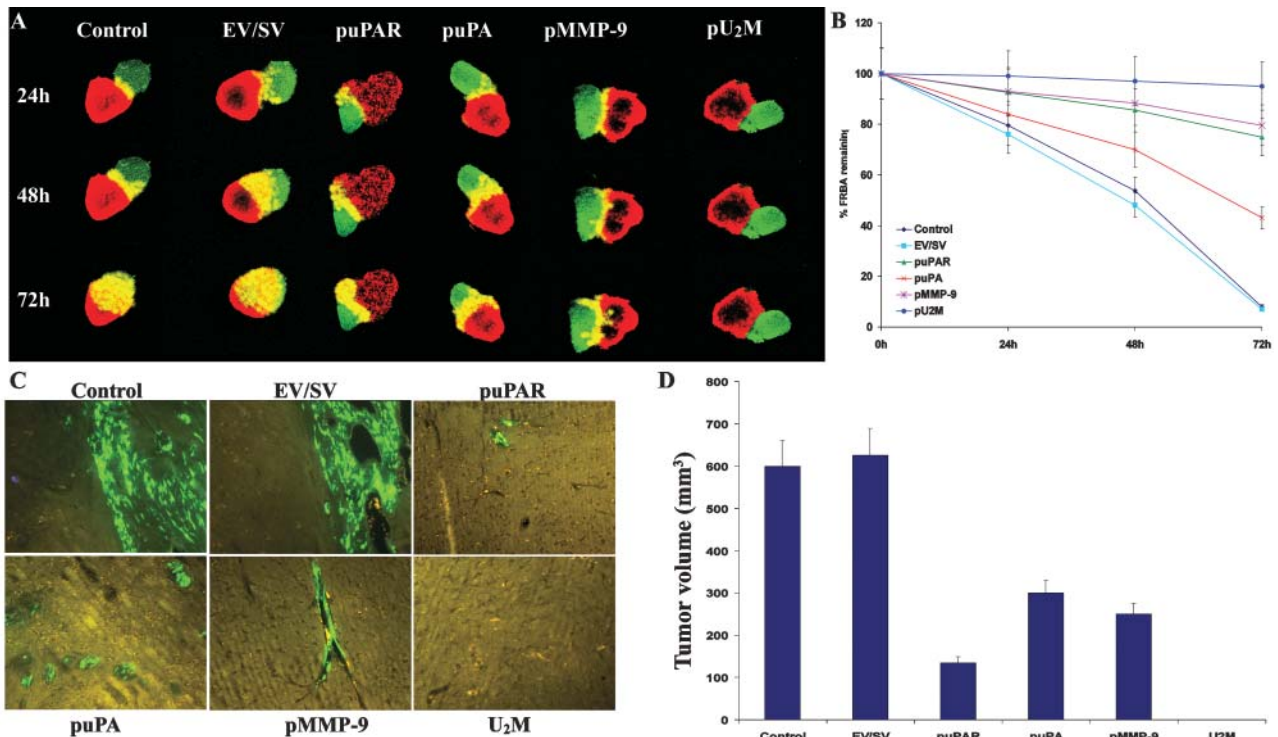


Fig. 4. Inhibition and regression of invasiveness and tumor growth by siRNA in spheroid and intracranial assays. (A) Invasiveness of glioma spheroids was measured by co-culturing glioma spheroids with fetal rat brain aggregates. Spheroids of SNB19 cells were transfected with EV/SV, puPA, puPAR, pMMP-9 and pU₂M and stained with DiI and co-cultured with DiO-stained fetal rat brain aggregates. Serial, 1- μ m-thick sections were obtained from the surface through the center of the co-cultures with a confocal laser scanning microscope at the indicated time points. (B) The remaining volume of the rat brain aggregate transfected with EV/SV, puPA, puPAR, pMMP-9 and pU₂M was measured as described in Methods. The values are mean \pm SD of three experiments. (C,D) RNA-mediated regression of pre-established tumor growth. SNB19 GFP cells in suspension (2×10^6 in 10 μ l serum-free medium) were injected intracranially. One week later, the mice were injected with either EV/SV or siRNA-expressing vectors (puPAR, puPA, pMMP-9 and pU₂M) using an Alzet mini osmotic pump (constructs diluted to $1.5 \mu\text{g ml}^{-1}$ in PBS and injected at $0.25 \mu\text{g hour}^{-1}$, with six mice in each group). After a 5-week follow-up period, mice were sacrificed, their brains removed, paraffin embedded and sectioned. Sections were observed under fluorescence microscopy for GFP-expressing cells. (D) Semi-quantification of tumor volume in control, EV/SV, puPAR, puPA, pMMP-9 and pU₂M-treated groups was assessed after 5 weeks. Data are shown mean \pm SD of six animals from each group.

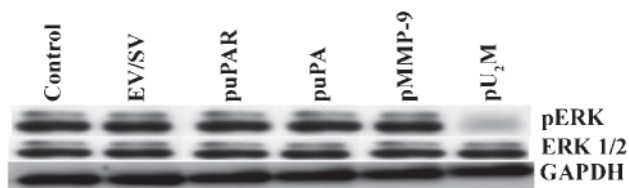


Fig. 5. RNAi-mediated downregulation of uPAR, uPA and MMP-9 reduces phosphorylation of ERK. Western blot analysis of total and phosphorylated ERK (pERK) protein after transfection of glioblastoma cells with EV/SV, puPAR, puPA, pMMP-9 and pU₂M constructs. GAPDH levels served as loading control.

transfected SNB19 cells. Notably, there was no effect on the levels phospho-ERK in SNB19 cells transfected with any of the single constructs. GAPDH levels indicated that equal quantities of protein were loaded in the gel (Fig. 5).

CONCLUSIONS

- Plasmid-based, CMV promoter-driven hpRNA targeting uPAR, uPA and MMP-9, either singly or simultaneously, induces RNAi in the SNB19 human glioma cell line.
- The simultaneous, RNAi-mediated downregulation of uPAR, uPA and MMP-9 in SNB19 human glioma cells caused:

- (1) Inhibition of invasion and angiogenesis *in vitro*.
- (2) Regression of pre-established intracranial tumors in nude mice *in vivo*.
- (3) Reduction in the phosphorylation of ERK 1 and 2 signaling molecules.

DISCUSSION

In mammals, dsRNA larger than 30 nt activates an antiviral defense mechanism that includes the production of interferons and results in the non-specific degradation of RNA and a general shutdown of the host protein-synthesis machinery (Williams, 1997). To circumvent this major defense response, small, 21 bp dsRNA molecules were used, so that the dsRNAs were sufficiently large to induce gene-specific suppression but small enough to evade the antiviral defense mechanism (Caplen *et al.*, 2001; Elbashir *et al.*, 2001). The potential application for RNAi in mammals is the simultaneous inhibition of multiple genes in somatic cells. Earlier observations have demonstrated that inhibition of a synthetic reporter by a transfected hairpin siRNA vector is constant, even in the presence of another hairpin siRNA vector. This demonstrates that there is no significant competition between co-transfected hairpin siRNA vectors. In the present study, we demonstrated that plasmid-based RNA interference has the potential to inhibit

uPA, uPAR and MMP-9 target molecules. Figure 2 showed that there are significantly decreased levels of uPAR protein, and uPA and MMP-9 enzymatic activity in glioma cells transfected with pU₂M vector compared to mock and empty vector. We also demonstrated the significant reduction in the levels of these molecules by immunohistochemistry using specific antibodies for these molecules in puPAR, puPA, pMMP-9 and pU₂M-transfected cells. It has been reported that treatment of a human T98G glioblastoma cell line with uPA antisense phosphorothioate oligonucleotides resulted in greater decreases of uPA mRNA expression than sense vectors (Engelhard *et al.*, 1996). Our own results demonstrate that uPA and uPAR protein and mRNA levels were decreased significantly by antisense uPA and antisense uPAR clones (Mohanam *et al.*, 1997; Mohanam *et al.*, 2002). In a rat sarcoma model, MMP-9 expression was decreased with ribozyme directed against MMP-9 mRNAi. (Hua and Muschel, 1996). The reduced levels of MMP-9 in stable MMP-9 antisense clones and SNB19 cells transfected with antisense MMP-9 Ad-constructs were also determined (Kondraganti *et al.*, 2000; Lakka *et al.*, 2002b). Earlier studies showed that RNAi can be transmitted from cell to cell, and to the next generation, and might also be amplified by an RNA-dependent RNA polymerase (Carmell *et al.*, 2003; Shuey *et al.*, 2002).

Our results show that conditioned medium from a glioblastoma cell line transfected with pU₂M inhibited the capillary-like structures compared with mock or empty vector (Fig. 3A,B). This indicates that the angiogenic signal necessary for the induction of angiogenesis was not present in pU₂M-transfected cells. As demonstrated by the absence of angiogenic induction in pU₂M-transfected SNB19 glioma cells, downregulation of uPA, uPAR and MMP-9 by hpRNA caused the downregulation of angiogenic factors. This accords with our previous work in which antisense-mediated downregulation of uPAR and MMP-9 retarded angiogenesis (Lakka *et al.*, 2003). Other studies have shown that VEGF induces sustained cellular permeability in capillary endothelial cells that is mediated by activation of the uPA/uPAR system, which substantiates the involvement of uPAR in angiogenesis (Behzadian *et al.*, 2003). This also indicates possible cross-talk between uPA/uPAR and VEGF. It was shown that the absence of uPA or tissue type plasminogen activator (tPA) significantly decreased the development of experimental choroidal neovascularization compared with either wild-type or uPAR-deficient mice (uPA^{-/-}) (Li *et al.*, 1998). It has been reported that primary tumor growth is reduced significantly in uPA^{-/-} and plasminogen activator inhibitor-1-deficient (PAI-1^{-/-}) mice relative to wild-type mice. Moreover, tumors in uPA^{-/-} and PAI-1^{-/-} mice have lower proliferative and higher apoptotic indices, and different neovascular morphology compared with wild-type mice (Gutierrez *et al.*, 2000).

Previous studies have demonstrated that several peptides that have been shown to inhibit uPA binding by bacteriophage display inhibit angiogenesis and primary tumor growth in syngenic mice (Gutierrez *et al.*, 2000).

MMPs also regulate tumor angiogenesis and might be required for the 'angiogenic switch' that occurs during tumor neovascularization (Bergers *et al.*, 2000). Two transgenic models of tumor progression, the K14-HPV16 [G] skin cancer model (Coussens *et al.*, 2000) and the RIP1-Tag2 [G] model (Bergers *et al.*, 2000), demonstrate that MMP-9 plays a significant role in angiogenesis. We used a co-culture system in which glioma and endothelial cells were cultured together to

evaluate endothelial-tube formation. In this model, we showed that capillary-like structure formation was decreased in the presence of anti-MMP-9 antibodies, TIMP-1, a synthetic inhibitor batimastat (BB-94) and the protein kinase C inhibitor calphostin because of reduced activity of MMP-9 (Chandrasekar *et al.*, 2000). Other studies have demonstrated that the inhibition of the ERK1/ERK 2 pathway with PD98059, a specific inhibitor of MEK1, abrogated basic fibroblast growth factor-mediated tube formation by mouse endothelial cells (Tanaka *et al.*, 1999).

Diffuse single-cell invasion, which occurs in all glial tumors regardless of histological grade, is defined as a translocation of neoplastic cells through host cellular and ECM barriers. Malignant gliomas express higher levels of uPA, uPAR and MMP-9 compared with low grade and normal brain tissue (Forsyth *et al.*, 1999; Raithatha *et al.*, 2000; Rao *et al.*, 1993; Rao *et al.*, 1996). Inhibitors and antibodies of uPA inhibit the invasiveness of tumor cells into ECM, and amniotic and chick chorioallantoic membranes (Alonso *et al.*, 1998; Holst-Hansen *et al.*, 1996; Jarrard *et al.*, 1995; Min *et al.*, 1996). Moreover, uPA antibodies blocked metastasis of Hep3 human carcinoma cells in chick embryos and inhibited the local invasiveness of subcutaneous Hep3 tumors in nude mice (Ossowski *et al.*, 1991). Our previous results showed that antisense, stable clones of uPA, uPAR and MMP-9 were much less invasive in *in vitro* models (Go *et al.*, 1997; Kondraganti *et al.*, 2000; Mohanam *et al.*, 1997; Mohanam *et al.*, 2001). We also showed that clones stably expressing an aminoterminal fragment of uPA (ATF-uPA) were markedly less invasive in *in vitro* models compared to controls (Mohanam *et al.*, 2002). CD44 has been demonstrated to serve as a docking molecule to retain MMP-9 proteolytic activity at the cell surface (Yu *et al.*, 1996; Yu and Stamenkovic, 2000) and cleavage of MMP-9 from CD44 resulted in impairment of migration (Kajita *et al.*, 2001). Localization of MMP-9 to the cell surface mediated the activation of latent transforming growth factor β and promotes tumor cell invasion and angiogenesis (Yu *et al.*, 2000).

In the present study, almost complete inhibition of glioblastoma-cell invasion was observed in both Matrigel and spheroid-invasion assays (Fig. 3C,D and Fig. 4A,B). Thus, downregulation of uPA, uPAR and MMP-9 by RNAi significantly inhibit the ability of glioma cells to invade the surrounding ECM compared to approaches described earlier.

These results correlate well with the spheroid and Matrigel invasion assays, in that the pU₂M-transfected cells do not invade, even in the presence of brain tissue in an *in vitro* model. This might be because the cells have lost their ability to anchor to the ECM and are unable to invade the confronted brain tissue. Earlier studies showed that binding of uPA to uPAR in MCF-7 cells activates ERK 1 and ERK 2, which are required for cell motility (Nguyen *et al.*, 1998). Jo *et al.* (Jo *et al.*, 2003) have also shown that an alternate pathway links uPAR to ERK in the absence of the epidermal growth factor receptor (EGFR). However, this pathway is silenced by EGFR expression, which indicates the involvement of uPAR in cell motility. Physical association between uPAR and the integrin α V chain of the vitronectin receptor leads to functional interaction of these receptors, indicating that uPAR directs cytoskeletal rearrangements and cell migration by altering α V β 3 signaling specificity (Carriero *et al.*, 1999). Our findings confirm previous reports that both uPA and uPAR are required for cell migration in a Vitronectin environment (Busso *et al.*, 1994). Other studies have reported that high-molecular-weight serum-protein

complexes promote both uPAR-focal adhesion colocalization and cell migration of glioma cells (Hedberg *et al.*, 2000). Studies utilizing native MMP inhibitors, such as the TIMPs, have demonstrated that downregulation of both TIMP-1 and TIMP-2 contributed significantly to the invasive potential of gliomas (Lampert *et al.*, 1998; Mohanam *et al.*, 1995; Nakano *et al.*, 1995). Introduction of TIMP-1 cDNA into an invasive astrocytoma cell line reduced its invasive potential (Matsuzawa *et al.*, 1996). In addition, the synthetic MMP inhibitors batimastat, marimastat and AG3340 reduced glioma invasion *in vitro* (Tonn *et al.*, 1999). Stable transfection of phosphatase and tensin homologue (PTEN) reduced MMP-9 secretion caused by hyaluronic acid-induced phosphorylation of focal adhesion kinase and ERK1/ERK2 signaling (Park *et al.*, 2002). Glioblastomas with EGFR VIII amplification demonstrated the highest levels of MMP-9 (Choe *et al.*, 2002).

In earlier work (Lakka *et al.*, 2000) we have shown that transient transfection of SNB19 cells with either mt-ERK or mt-JNK repressed MMP-9 promoter activity, which indicated that interfering with either pathway might inhibit MMP-9 expression. Inhibition of ERK by MEK-specific inhibitors blocked MMP-9 expression in breast cancer cells (Yao *et al.*, 2001), and decreased MMP-9 production and attenuated the invasiveness *in vivo* in head and neck squamous carcinoma cells (Simon *et al.*, 1998). Our recent results showed that cells stably transfected with mt-ERK were less invasive and had significantly reduced levels of MMP-9 (Lakka *et al.*, 2002a). It has been reported that these two signaling pathways (MAPK and ERK1/2) are activated when uPA binds to uPAR (Konakova *et al.*, 1998; Tang *et al.*, 1998). Figure 5 shows that the pU₂M construct inhibited the phosphorylation of ERK1/2.

In the present study, we completely suppressed pre-established intracranial tumor growth during the 5-week follow-up period in nude mice injected with pU₂M vector compared to mock and empty vector/scrambled vector (Fig. 4C,D). These results *in vivo* correlate well with the spheroid-invasion assay. In contrast to our earlier experiments, in which uPAR-antisense cells were raised *ex vivo* before intracranial implantation (Go *et al.*, 1997), the pU₂M plasmid was delivered after tumor implantation.

Several studies using selective inhibitors of uPA and small, synthetic, cyclic, competitive uPA antagonists derived from the binding site of uPAR resulted in a significant reduction of tumor burden (Evans *et al.*, 1998; Sato *et al.*, 2002; Sturzebecher *et al.*, 1999). Recent studies have also shown that fusion of diphtheria toxin with ATF-uPA caused a significant regression of glial tumors and was highly potent and selective at killing uPAR-expressing glioblastoma cell lines (Vallera *et al.*, 2002). Adenovirus-mediated downregulation of uPA and uPAR (Gondi *et al.*, 2003), and cathepsin B and uPAR (Gondi *et al.*, 2004) constructs inhibited the migration and invasion of glioma cells, and inhibited *ex vivo* tumor growth compared with vector-treatment. Intracranial injections of SNB19 glioblastoma cells infected with an adenovirus expressing antisense MMP-9 did not produce tumors in nude mice (Lakka *et al.*, 2002b). A bicistronic Ad-construct with antisense uPAR and MMP-9 was more effective at inhibiting invasion, angiogenesis and tumor growth *in vivo* than a single construct (ad-uPAR) (Lakka *et al.*, 2003). Our recent studies demonstrated that a siRNA bicistronic construct for cathepsin B and MMP-9 completely repressed pre-established intracranial tumors (Lakka *et al.*, 2004). From our results, it can be concluded that downregulation of uPA, uPAR and MMP-9 is

achieved via a triple hpRNA that targets the corresponding mRNA molecules. Because no viral delivery methods were used, the question of viral toxicity does not arise (Alemany *et al.*, 1999). Thus, it can be concluded that RNAi is a powerful alternative to genetic tools such as antisense oligonucleotides and ribozyme technologies to reduce target-gene expression. Our results prove that downregulation of uPA, uPAR and MMP-9 by hpRNA decreases migration, invasion and angiogenesis in the SNB19 human glioma cell line. As such, the use of RNAi-based gene therapy has great potential for the treatment of gliomas and other metastatic tumors.

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AUTHORS' ADDRESSES

Program of Cancer Biology

- ¹ Department of Biomedical and Therapeutic Sciences
- ² Department of Neurosurgery
- ³ Department of Pathology

The University of Illinois College of Medicine
Peoria, IL

Correspondence should be addressed to:

Jasti S. Rao, PhD
Program of Cancer Biology
University of Illinois
College of Medicine at Peoria
One Illini Drive
Peoria, IL 61605
USA
phone: +1 309 671 3445
fax: 309-671-3442
email: jsrao@uic.edu