Specific Interference of Urokinase-type Plasminogen Activator Receptor and Matrix Metalloproteinase-9 Gene Expression Induced by Double-stranded RNA Results in Decreased Invasion, Tumor Growth, and Angiogenesis in Gliomas*

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We have previously demonstrated the effectiveness of adenovirus-mediated expression of antisense urokinase-type plasminogen activator receptor (uPAR) and matrix metalloproteinase-9 (MMP-9) in inhibiting tumor invasion in vitro and ex vivo. However, the therapeutic effect of the adenovirus-mediated antisense approach was shown to be transient and required potentially toxic, high viral doses. In contrast, RNA interference (RNAi)-mediated gene targeting may be superior to the traditional antisense approach, because the target mRNA is completely degraded and the molar ratio of siRNA required to degrade the target mRNA is very low. Here, we have examined the RNAi-mediated target RNA degradation of uPAR and MMP-9 in human glioma cell lines. Using RNAi directed toward uPAR and MMP-9, we achieved specific inhibition of uPAR and MMP-9. This bicistronic construct (pUM) inhibited the formation of capillary-like structures in both in vitro and in vivo models of angiogenesis. We demonstrated that blocking the expression of these genes results in significant inhibition of glioma tumor invasion in Matrigel and spheroid invasion assay models. RNAi for uPAR and MMP-9 inhibited cell proliferation, and significantly reduced the levels of phosphorylated forms of MAPK, ERK, and AKT signaling pathway molecules when compared with parental and empty vector/scrambled vector-transfected SNB19 cells. Furthermore, using RNAi to simultaneously target two proteases resulted in total regression of pre-established intracerebral tumor growth. Our results provide evidence that the use of hairpin siRNA expression vectors for uPAR and MMP-9 may provide an effective tool for cancer therapy.

RNA interference (RNAi) is a sequence-specific, post-transcriptional gene silencing mechanism, which is triggered by double-stranded RNA and causes the degradation of mRNA homologous in sequence to the double-stranded RNA (1–3). This is an ancient and ubiquitous antiviral system used by organisms to maintain the integrity of the genome, to defend cells against viral infection, and to regulate expression of cellular genes (4). RNAi depends upon the formation of double-strand RNA (double-stranded RNA) whose antisense strand is complementary to the transcript of a targeted gene. Recently, it has been shown that sequence-specific inhibition RNAi can also be induced in mammalian cells (4, 5). In one implementation of RNAi, selective degradation of target mRNAs in mammalian cells is achieved by transfection with double-stranded, short interfering RNAs (siRNAs), leading to rapid and efficient degradation of the target (4). These siRNAs were shown to avoid the well documented nonspecific effects triggered by longer double-stranded RNAs in mammalian cells.

Glioblastoma multiforme is a highly malignant primary central nervous system neoplasm, which is extremely refractory to therapy. One property that makes glioblastoma resistant to treatment is the tendency of the tumor cells to invade normal brain tissue (6). Invasiveness is thus considered to be a major determinant of the malignant behavior of human gliomas. The ability of tumor cells to migrate into extracellular matrix in vitro is known to be mediated by the cooperative expression of a family of adhesion receptors called integrins and cell surface proteinases such as uPA and MMPs. The serine protease uPA and its receptor (uPAR) are produced by several tumor cells and strongly implicated in tumor progression (7). Several reports related to mRNA expression, enzyme kinetics, receptor binding, and cell invasion assays have demonstrated the key role of uPAR in the process of tumor cell invasion and metastasis (8). We have previously shown that uPAR mRNA is localized in astrocytoma cells and the endothelial cells within brain tumor tissue. Expression of uPAR in the invading astrocytoma cells appears to have a critical role in the invasive behavior of glioblastoma (9). Stable transfection of a human glioblastoma

uPAR, urokinase-type plasminogen activator receptor; CMV, cytomegalovirus; PBS, phosphate-buffered saline; FITC, fluorescein 5-isothiocyanate; GFP, green fluorescent protein; MMP, matrix metalloproteinase; MAPK, mitogen-activated protein kinase; EV, empty vector; SV, scrambled vector; H&E, hematoxylin & eosin; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate-buffered saline; pUM, plasmid siRNA vector for uPAR and MMP-9; pMMP-9, plasmid siRNA vector for MMP-9; pGFP, plasmid siRNA for GFP; puPAR, plasmid siRNA vector for uPAR.

This paper is available online at http://www.jbc.org/
Regression of Glioma Growth by uPAR and MMP-9 siRNA

MMPs enhance tumor cell invasion by degrading extracellular matrix proteins, activating signal transduction cascades that promote motility (17), and activating growth factors, such as transforming growth factor β, that are implicated in glioblastoma multiforme motility. Recent studies focusing on the mechanisms of glioblastoma multiforme invasion suggest that the family of matrix metalloproteases plays a critical role in this process. In particular, expression of the gelatinases MMP-2 and MMP-9 closely correlate with the invasive and metastatic potentials of various cancers, including gliomas (18, 19). We have previously shown that MMP-9 levels were highly correlated with the histological grade of glioma malignancy (20). MMP-9 has also been shown to be important in endothelial cell morphogenesis and capillary formation in glial/endothelial co-cultures in vitro. Furthermore, antisense oligonucleotides that blocked MMP-9 gene expression in SNB19 glioblastoma cells inhibited tumor formation in nude mice, thereby providing evidence that MMP-9 expression facilitates glioma invasion in vivo (21, 22).

Taken together, these findings indicate the biological significance of uPAR and MMP-9 in glioma invasion and tumor growth. Our previous studies using adenovirus-mediated antisense delivery of these proteins have shown successful treatment of brain tumors in animal models when the tumor cells were treated ex vivo (12, 22). The delivery and approach of antisense nucleotides to intracellular target RNA seems to be a crucial limiting factor in exerting its inhibitory effect on the targeted molecule. Conversely, the activity of siRNA is high even at low concentrations and efficiently causes down-regulation of target genes. siRNA, which are 21- to 23-nucleotide RNA duplexes with a 2-nucleotide overhang, are considered to be much more resistant to nucleases. The siRNA duplex was significantly more stable in cells than the cognate single-stranded sense or antisense RNA, with transcription under the control of the identical promoter in each case (23). Therefore, RNAi may provide a more powerful strategy for the inhibition of tumor growth and angiogenesis through a nucleic acid drug or a gene therapy approach. We show here that the siRNA-induced suppression of uPAR and MMP-9 results in significant inhibition of glioma tumor growth and angiogenesis. Our results demonstrate that a single vector capable of expressing small interfering RNA for two genes can block expression of the targeted proteases and suggest a potentially useful method for the development of highly specific, siRNA-based gene-silencing therapeutics in gliomas.

**EXPERIMENTAL PROCEDURES**

**Construction of a Vector Expressing siRNA for uPAR and MMP-9**—pcDNA 3 was used for the construction of a vector expressing siRNA for both uPAR and MMP-9 downstream of the cytomegalovirus (CMV) promoter (Scheme 1). The uPAR sequence from +77 to +98 was used as the target sequence, and for convenience a self-complimentary oligonucleotide was used. The uPAR sequence 21 bases in length with a 35-bp separation was digoxigenin-labeled (Roche Applied Science) as per the manufacturer’s instructions.

**Determination of Processing of Long RNA with Hairpin Loops to siRNA**—Total RNA was isolated from pGFP, EV (empty vector), SV (scrambled vector), puPAR, pMMP-9, and pUM transfected cells after 72 h. The isolated RNA was fractionated on a 2% agarose gel after which small RNA molecules (<100 bases) were extracted from the gel as per standard protocols. 21-bp DNA sense oligonucleotides used for the construction of the plasmid vectors were digoxigenin-labeled (Roche Applied Science) as per the manufacturer’s instructions (see pGFP, puPAR, and pMMP-9 in Table I). For each microgram of fractionated RNA, 1 ng of appropriately labeled probe was added in 6× SSC. The mixture was heated to 80 °C and allowed to slowly cool to room temperature. The resulting DNA/RNA hybrid solution was run on a non-denaturing 15% polyacrylamide gel and electroblotted onto a nylon membrane. The membrane was then processed to visualize the digoxigenin-labeled probe as per the manufacturer’s instructions.

**Cell Culture and Transfection Conditions**—For this study, we used the established human glioblastoma cell line SNB19. Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 1% glucose, 100 μg/ml streptomycin, 100 units/ml penicillin, and 10% fetal bovine serum (pH 7.2–7.4) in a humidified atmosphere containing 5%
CO₂ at 37 °C. Cells were sub-cultured every 3–5 days. Cell transfection was performed with the plasmid (1 μg/ml medium) using Lipofectamine (Invitrogen). The complexes of plasmids and Lipofectamine were pre-pared as described previously (24) using 10% SDS-PAGE analyses containing gelatin (0.5 mg/ml). Gels were stained with Amido Black (Sigma Aldrich), and gelatinase activity was visualized as areas of clear bands in blue. MMP-2 activity was quantified as arbitrary units compared with controls.

**Gelatin Zymography—** Conditioned media were collected from cells transfected with either U6 or CMV promoter vector EV/SV, puPAR, pMMP-9, and pUM and centrifuged to remove cellular debris. Twenty micrograms of the resulting samples was assayed for gelatinase activity as described previously (24) using 10% SDS-PAGE analyses containing gelatin (0.5 mg/ml). Gels were stained with Amido Black (Sigma Aldrich), and gelatinase activity was visualized as areas of clear bands in blue. MMP-2 activity was quantified as arbitrary units compared with controls.

**In Vitro Angiogenic Assay—** SNB19 cells (2 × 10⁴) were seeded in 96-well round-bottomed plates (Fisher) coated with vitronectin (50 μg/ml) and incubated for 24 h. The medium was removed and replaced with 100 μl of serum-containing medium at 37 °C, and the cells were allowed to migrate through Matrigel-coated Transwell inserts (8-μm pores) for 24 h. The amount of bone wax. A 1.5- to 2-cm superficial incision was made horizontally along the edge of the dorsal air sac, and the air sac was opened. With the help of forceps the chambers were placed underneath the skin and sutured carefully. After 10 days the animals was anesthetized with ketamine/xylazine and sacrificed by intracardial perfusion with saline (10 ml) followed by 10 ml of 10% formalin/0.1 M phosphate solution and followed by 0.001% FITC solution in PBS. The brains were removed and the implanted chambers were removed from the subcutaneous air sac. The skin fold covering the chambers was photographed under visible light and for FITC fluorescence. The number of blood vessels within the chamber in the area of the air sac fascia was counted, and their lengths were measured.

**Matrigel Invasion Assay—** The effect of RNAi was determined by two complement Boyden chambers (Costar, Cambridge, MA) and basement membrane Matrigel invasion assay as described previously (22). Briefly, the 8-μm pore polycarbonate filters were coated with basement membrane Matrigel (50 μg/filter) (Collaborative Research, Inc., Boston, MA). SNB19 cells were then transfected with EV/SV, puPAR, pMMP-9, and pUM. Three days later, cells were trypsinized, counted, and 1 × 10⁶ cells were suspended in 100–150 μl of sterile PBS and injected into the chamber through the opening of the “O” ring. The opening was sealed by a small amount of bone wax. A 1.5- to 2-cm superficial incision was made horizontally along the edge of the dorsal air sac, and the air sac was opened. With the help of forceps the chambers were placed underneath the skin and sutured carefully. After 10 days the animals was anesthetized with ketamine/xylazine and sacrificed by intracardial perfusion with saline (10 ml) followed by 10 ml of 10% formalin/0.1 M phosphate solution and followed by 0.001% FITC solution in PBS. The brains were removed and the implanted chambers were removed from the subcutaneous air sac. The skin fold covering the chambers was photographed under visible light and for FITC fluorescence. The number of blood vessels within the chamber in the area of the air sac fascia was counted, and their lengths were measured.

**Spheroid Invasion Assay—** SNB19 cells (3 × 10⁵ cells) were suspended in Dulbecco’s modified Eagle’s medium and seeded onto 0.5% agar-coated plates and cultured until spheroids formed. Tumor spheroids (red fluorescence) 100–200 μm in diameter were selected and then transfected with EV/SV, puPAR, puPA, and pUM. After 48 h, cells were co-cultured with fetal rat brain aggregates. Progressive destruction of fetal rat brain aggregates (green fluorescence) and invasion of SNB19 cells was observed for 72 h using confocal laser scanning microscopy. The remaining volumes of the brain aggregates or tumor spheroids were quantitated using image analysis software as described previously (22).

**Animal Experiments—** SNB19 GFP glioblastoma cells grown in serum-containing culture medium were washed with Hank’s balanced buffer and centrifuged at 1500 rpm for 5 min. Cell pellets (2 × 10⁶ cells/mice) were re-suspended in 10 μl of PBS and injected intracerebally into nude mice.

All experiments were performed in compliance with institutional guidelines set by the Institutional Animal Care Use Committee that approves experiments at the University of Illinois College of Medicine at Peoria. Tumors were allowed to grow for 10 days. At this time, animals were randomized into several groups and mock (PBS), empty vector (150 μg), puPAR vector (150 μg), pMMP-9 vector (150 μg), and pUM vector (150 μg) were injected into the brain using Alzet mini...
FIG. 1. To determine whether long hairpin (hp) RNA are processed to siRNA, molecules were transfected in SNB19 cells with control/EV, SV, puPAR, pMMP-9, and pUM. Cells were also transfected with an unrelated construct targeting GFP in non-GFP cells to determine the processing of appropriate siRNA molecules. Small RNA molecules fractionated on a 2% agarose gel (see “Experimental Procedures”) were allowed to hybridize with appropriate digoxigenin-labeled sense oligonucleotide in the presence of 6× SSC. The resulting hybrid solution was run on a 15% polyacrylamide gel and electroblotted onto a nylon membrane. The membrane was processed to visualize the 21-bp DNA:RNA hybrid as per the manufacturer’s instructions as shown in A. The probes used are represented as numbers (see Table I): 1, puPAR; 2, pMMP-9; and 3, sGFP. B shows reverse transcription-PCR of SNB19 cells transfected with CMV vector constructs control/EV (lane a), SV (lane b), puPAR (lane c), pMMP-9 (lane d), and pUM (lane e). Reverse transcription-PCR was performed as per standard protocols; briefly, 72 h after transfection, total RNA was isolated as per standard protocols, and first strand cDNA was synthesized using a cDNA synthesis kit (Invitrogen). The PCR reaction was set up using the first strand cDNA as template for uPAR and MMP-9; PCR for GAPDH was also set up to serve as loading control (see Table I).

Plasmid-based CMV Promoter-driven 21-bp Inverted Repeats Are Processed to siRNA—To determine whether the CMV promoter-driven transcript is processed correctly to siRNA, we transfected SNB19 cells with control/EV, SV, puPAR, pMMP-9, and pUM. Cells were also transfected with an unrelated construct targeting GFP in non-GFP cells to determine the processing of the RNA transcript to siRNA and to confirm the fact that the obtained results are not merely degradation products of the target gene. Non-GFP SNB19 cells transfected with pgFP resulted in the processing of the RNA transcript to siRNA (Fig. 1A). Similarly, cells transfected with puPAR, pMMP-9, and pUM resulted in the processing of the RNA transcript to the appropriate siRNA. EV-transfected cells did not produce any siRNA-like fragment targeting uPAR or MMP-9; indicating that the siRNA fragment seen is processed from the inverted repeat loops incorporated in the construct. SV-transfected cells also did not produce any siRNA-like fragment targeting uPAR or MMP-9; SV consisted of an imperfect inverted repeat sequence with no homology to any known gene. When probed with a 21-bp sense oligonucleotide for SV, no 21-bp DNA was observed. However, RNA hybrid was seen, indicating that this construct did not process to siRNA-like fragments (data not shown). SNB19 cells transfected with pUM caused the down-regulation of both uPAR and MMP-9 mRNA. To determine whether the plasmid construct containing inverted 21-bp sequence homologous to uPAR and MMP-9 would induce RNAi, we transfected SNB19 cells with control/EV, SV, puPAR, pMMP-9, and pUM. Total RNA was isolated from the transfected cells, and the first strand cDNA was synthesized using a cDNA synthesis kit (Invitrogen). The cDNA was then subjected to PCR as per standard protocols. Using specific primers for uPAR, MMP-9, and GAPDH (Table I) in cells transfected with control/EV and SV, there was no reduction in the levels of uPAR or MMP-9; whereas cells transfected with puPAR showed significantly reduced levels of uPAR and mRNA and the levels of MMP-9 mRNA were not changed. In cells transfected with pMMP-9, significant reduction in the levels of MMP-9 mRNA was observed, whereas the levels of uPAR mRNA were not changed indicating the specificity of the vectors to target molecules. Cells transfected with pUM showed a decrease in both uPAR and MMP-9 mRNA levels. GAPDH levels did not change (Fig. 1B).

Inhibition of MMP Activity and uPAR Protein Levels by RNA Interference—We transfected SNB19 cells with either U6 or CMV promoter-based vectors, control, empty vector (EV), scrambled vector (SV), puPAR, pMMP-9, and pUM. uPAR levels were determined in cell lysates by Western blotting, and MMP-9 activity was determined in conditioned media by gelatin zymography. SNB19 cells transfected with pUM vectors (CMV or U6 promoter-based) decreased the amount of uPAR protein expression when compared with controls, EV- or SV-transfected cells as determined by Western blotting (Fig. 2A). GAPDH protein levels were also determined in the same blot to serve as loading control. Conditioned media from pUM-transfected SNB19 cells (CMV or U6 promoter-based) showed decreased levels of MMP-9 activity when compared with control or EV- or SV-transfected cells (Fig. 2A). MMP-9 activity levels also showed a decrease in activity (89–97% for CMV-based vectors and 45–64% for U6-based vectors) when compared with controls (Fig. 2B). GAPDH levels did not show any change and served as internal loading control. Quantitative analysis of uPAR protein levels by densitometry revealed a decrease in uPAR protein expression (87–93% decrease for CMV-based vectors and 47–62% decrease for U6-based vectors) when compared with controls (Fig. 2B). MMP-9 activity levels also showed a decrease in activity (89–97% for CMV-based vectors and 45–64% for U6-based vectors) when compared with controls (Fig. 2B). SNB19 cells transfected with puPAR (CMV or U6) or pMMP-9 (CMV or U6) inhibited uPAR or MMP-9 expression similar to the bicistronic constructs. There was no change in the levels of MMP-2 enzymatic activity in SNB19 cells after transfection with these constructs (Fig. 2, A and B). The down-regulation of the target molecules was more pronounced in the bicistronic constructs when compared with single constructs alone, whether CMV-based or U6-based. Further experiments were conducted using only the CMV-based vectors due to their greater efficiency when compared with the U6 promoter.

Inhibition of Cell Proliferation by siRNA for uPAR and MMP-9—We used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay to assess the effect of the siRNA vectors (EV/SV, puPAR, pMMP-9, and pUM) on proliferation of cells cultured on vitronectin-coated microplates. After 3 days of infection, the puPAR, the puPAR, pMMP-9, and pUM vector-infected SNB19 cells showed a decrease in proliferation rela-
tive to that of parental and EV/SV-transfected SNB19 cells (Fig. 3). pUM vector effect was much higher in SNB19 proliferation compared with the single siRNA constructs (puPAR and pMMP-9). There was no difference in proliferation between parental and EV/SV-transfected SNB19 cells.

RNA Interference Inhibited uPAR and MMP-9 Immunofluorescence and Tumor-induced Angiogenesis—SNB19 cells transfected with puPAR and pMMP-9 caused the down-regulation of uPAR and MMP-9 protein levels as determined by immunocytochemistry. Cells transfected with pUM caused the down-regulation of both uPAR and MMP-9 protein levels as determined by immunocytochemistry (Fig. 4A). To determine the effect of the combined construct expressing siRNA for both uPAR and MMP-9, we transfected SNB19 cells with puPAR, pMMP-9, and pUM; cells were also transfected with EV and SV, which served as controls. From the results, it was clear that cells transfected with puPAR alone showed a down-regulation of uPAR protein levels. Cells transfected with pMMP-9 showed a down-regulation of MMP-9 alone, whereas cells transfected with pUM caused a down-regulation of uPAR and MMP-9 protein levels, indicating that the dual construct was as efficient, if not more, at down-regulating the target protein levels. To test if siRNA for uPAR and MMP-9 could also inhibit tumor-induced capillary formation, we co-cultured transfected and untransfected SNB19 glioma cells with human endothelial cells. Immunohistochemical analysis was performed using factor VIII antigen to evaluate tumor-induced vessel formation in an in vitro co-culture system and H&E staining of these co-cultures after transfection with EV/SV, puPAR, pMMP-9, and pUM. Fig. 4B shows that endothelial cells cultured with SNB19 cells formed distinct capillary-like networks in mock- and empty vector-transfected cultures within 24–48 h. In contrast, pUM-transfected SNB19 cells did not induce capillary-like network formation in endothelial cells. Quantification of the branch points and number of branches were significantly reduced in pUM-transfected co-cultures compared with parental and empty/scrambled vector-transfected co-culture (Fig. 4C). Furthermore, the effect was <50% in puPAR and pMMP-9 vector and <50% in puPA and pMMP vector-transfected co-culture, when compared with the parental, EV/SV-treated group in relation to capillary-like structure formation. To con-

**TABLE I**

| Primers used for RT-PCR | uPAR | GAPDH |
|-------------------------|------|-------|
| uPAR                    | CATGCAGTGTAAGACCCAACGGGGA 75–98 mRNA (gi 8050814) | CGGAGTCGCAACGGATTTGGTCTAT 93–116 (gi 7669491) |
| MMP-9                   | AATAGGTGACAGCCCGGCCAGAGT 328–305 mRNA (gi 8050814) | AGGCCCTTCCATGTTGTTAGAACAGC 376–399 (gi 7669491) |
| GAPDH                   | TGGGCACTCGCTGAGCTGACGAGCT 1554–1537 (gi 4826835) | GAGCTTGTTCACCCGGTTGCTGTG 1989–2008 (gi 4826835) |
|                         | CTACGCGGTCGGAGAGCCGATT     | CAAGTGGCACCCACCCACCAAACAA |
| Probes used             | sGFP (3)                    | sMMP-9 (2)            |
| suPAR (1)               | CTACAGCAGTGGAGAGCGATT       | CTACGCGGTCGGAGAGCCGATT |
| sMMP-9 (2)              | CAAGTGGCACCCACCCACCAAACAA | CAAGTGGCACCCACCCACCAAACAA |

**Fig. 2.** Western blot analysis for uPAR and gelatin zymography for MMP-9. SNB19 cells were transfected with U6- or CMV-based vectors mock, an empty/scrambled vector, and a vector encoding single or bicistronic siRNA for uPAR and MMP-9 (puPAR, pMMP-9, and pUM). A, Western blot analysis of uPAR protein expression in cell lysates from SNB19 cells transfected with EV/SV, puPAR, pMMP-9, and pUM. Western blot analysis was performed using an antibody specific for uPAR. GAPDH was simultaneously immunodetected to verify the loading of similar amounts of cell lysates. MMP-9 activity was determined in conditioned media (20 μg) of from EV/SV-, puPAR-, pMMP-9-, and pUM-transfected cells by gelatin zymography. Quantification of uPAR protein, MMP-2, and MMP-9 enzymatic activities was obtained by scanning the autoradiograms with a densitometer. B, data are shown as mean values ± S.D. of four different experiments from each group (**, control protein expressions normalized to each other; *, CMV promoter-driven down-regulation of target protein expression compared with controls; p < 0.001).

**Fig. 3.** RNAi-mediated down-regulation of uPAR and MMP-9 reduces SNB19 glioma cell proliferation. Briefly 5 × 10⁴ SNB19 cells transfected with CMV-based EV/SV, puPAR, pMMP-9, and pUM were seeded in VN-coated 96-well microplates under serum-free conditions. The number of viable cells was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay. Shown are the mean ± S.D. values from four separate experiments.
firm the in vitro co-culture experiments, we examined whether the pUM vector can inhibit tumor angiogenesis in vivo as assessed by the dorsal window model. Implantation of a chamber containing parental EV-transfected SNB19 cells resulted in microvessel development with curved, thin structures, and many tiny bleeding spots. In contrast, implantation of SNB19 cells transfected with the pUM vector did not result in the development of any additional microvessels (Fig. 4D).

**siRNA for uPAR and MMP-9 Inhibits Invasion of SNB19 Cells**—Because siRNA expression inhibited uPAR and MMP-9, we assessed its ability to inhibit cell invasion. SNB19 cells transfected with EV/SV, puPAR, pMMP-9, and the pUM vector were allowed to invade through Matrigel-coated filters. Fig. 5A illustrates that the staining of pUM-transfected SNB19 cells was significantly less than that of the parental- and EV/SV-transfected cells. Quantitative analysis of cells showed that only 8% of pUM-transfected cells invaded compared with parental- and EV/SV-transfected cells (Fig. 5B). Furthermore, quantitative analysis of invasion of SNB19 cells transfected with puPAR and pMMP-9 vector invaded 25 and 50% as compared with parental and EV/SV-transfected SNB19 cells (Fig. 5, A and B). RNAi also inhibited the invasion of SNB19 cells in a three-dimensional spheroid invasion model. Fig. 5C demonstrates that glioma spheroids, transfected with mock and empty/scrambled vector, attached to rat brain aggregates and progressively invaded the aggregates. However, co-cultures with pUM-transfected glioma spheroids failed to attach to rat brain aggregates and did not invade. Quantitative analysis indicated
that only 2–4% of the fetal rat brain aggregates remained in the parental and EV/SV-transfected spheroids, whereas 90–95% of the fetal rat brain aggregates remained in the pUM-transfected spheroids (Fig. 5D). At 72 h, the rat brain aggregates revealed ~25 and 45% of invasion in the puPAR- and pMMP-9-transfected co-cultures. Taken together, these findings provide strong evidence that RNAi-mediated silencing of uPAR and MMP-9 greatly inhibits glioma cell invasion in both in vitro models compared with single siRNA constructs for uPAR and MMP-9. These results showed that single siRNA constructs for uPAR was more effective than single siRNA construct for MMP-9.

Therapeutic Effect of siRNA for uPAR and MMP-9—To evaluate the effectiveness of RNAi-mediated interference of uPAR and MMP-9 gene expression in tumor progression, the pUM vector was injected in tumor-bearing mice using a stereotactic pump. To facilitate the detection of invasive tumor cells, we first transfected human glioblastoma cells (SNB19) with the cDNA for green fluorescent protein (SNB19-GFP). Microscopic examination of brain sections revealed that control animals receiving PBS or empty vector (EV) alone developed significant tumor growth after a 5-week follow-up period as visualized by GFP fluorescence and H&E staining of similar sections. In contrast, we could not detect any tumor growth or GFP fluo-
rescence or H&E staining in animals receiving the pUM vector under the same conditions (Fig. 6, A and B). Quantification of H&E-stained brain sections or GFP sections by a neuropathologist who was blinded as to treatment revealed no difference in tumor size between the control and empty vector-treated groups; however, total regression of tumors was revealed in the pUM vector-treated group (Fig. 6C). In the case of single siRNA-treated constructs for uPAR and MMP-9, pre-established intracranial tumor growth was inhibited 70 and 40%, respectively. These results demonstrated that RNAi-mediated suppression of uPAR and MMP-9 dramatically inhibited pre-established intracranial tumor growth.

siRNA against uPAR and MMP-9 Inhibits the Level of Phosphorylated ERK, MAPK, and AKT—These pathways play a major role in cell proliferation and survival. We performed Western blotting to compare the levels of total and phosphorylated forms of ERK, MAPK, and AKT by using antibodies specific for these molecules after transfection of SNB19 cells with EV/SV, puPAR, pMMP-9, and pUM. There was no significant difference in the amounts of total MAPK, ERK, and AKT by EV/SV, puPAR, pMMP-9, and pUM constructs (Fig. 7). However, levels of phosphorylated forms of MAPK, ERK, and AKT were decreased significantly by pUM compared with EV/SV-, puPAR-, and puPA-transfected SNB19 cells (Fig. 7). We demonstrated that the simultaneous blockade of uPAR and MMP-9 genes had an additive/synergistic effect on tumor re-

![RNAi-mediated regression of pre-established intracerebral tumor growth](image-url)
RNA interference (RNAi) is a conserved mechanism in which double-stranded, small, interfering RNAs (siRNAs) trigger a sequence-specific response mediated by a protein complex RNA-induced silencing complex where the homologues mRNA molecule is destroyed. However, unlike the antisense and triple helix approaches, RNAi results in the destruction of the target molecule. Earlier researchers have reported the destruction of target mRNA using the antisense approach (26), but the mechanism of antisense-mediated destruction is not fully understood. Gene silencing using chemically synthesized 19- to 22-bp siRNA has revolutionized functional genomics from worms to humans. In the present study we have demonstrated that plasmid vectors with 21-bp inverted repeat sequences homologous to uPAR and MMP-9, singly or in tandem, inserted downstream of the CMV promoter and terminated by a poly(A) signal sequence induces RNAi. In vitro and in vivo studies have demonstrated that the simultaneous down-regulation of uPAR and MMP-9 decreases tumor growth and angiogenesis in SNB19, the human glioma cell line. We also constructed vectors with U6 promoter pSilencer (Ambion, Austin TX). In contrast to earlier reports (3), we have used an RNA polymerase II promoter instead of RNA polymerase III where the inverted repeat sequences are terminated by a poly(A) signal sequence. We had performed initial experiments with U6 RNA polymerase III promoter and found that CMV-based constructs are more efficient than U6-based constructs (Western blot analysis, gelatin zymography, and reverse transcription-PCR). The Western blot analysis of SNB19 cells transfected with CMV- or U6-based vectors targeting uPAR and MMP-9, either singly or simultaneously, showed that CMV-based vectors had at least a 15- to 20-fold better down-regulation than U6-based vectors.

To determine whether the CMV promoter-based construct transcripts are efficiently processed to 21-bp siRNA-like molecules, we conducted solution hybridization experiments from fractionated RNA isolated from un-transfected SNB19 cells or cells transfected with EV, SV, puPAR, pMMP-9, and pUM. Cells were also transfected with unrelated constructs such as pGFP targeting non-GFP cells. From the results, it is clear that non-GFP SNB19 cells, when transfected with pGFP, did process the CMV promoter-driven construct transcript to siRNA when probed with sense GFP corresponding oligonucleotide (probe 3). Similarly, puPAR-transfected cellular fractionated RNA, when probed with sense uPAR corresponding oligonucleotide (probe 1), showed processing to siRNA for uPAR. Similar results were obtained with pMMP-9 (probe 2). SNB19 cells transfected with empty vector or scrambled vector did not show processing of either uPAR or MMP-9 siRNA, indicating that CMV-based plasmids with inverted repeats do process efficiently to siRNA-like molecules and induce RNAi. Inverse probing was also done where puPAR-transfected cellular RNA was probed for MMP-9 (probe 2) and MMP-9-transfected cellular RNA-probed for uPAR (probe 1). In both these cases, no hybridization was seen (data not shown).

It has been reported that both chemically synthetic and vector-based siRNA can successfully knock down specific gene expression in mammalian cells, including malignant cells (3). Vectors expressing short hairpin RNA for uPAR and MMP-9 (pUM) significantly inhibited the expression of uPAR and MMP-9 in glioma cells, as determined by Western blotting, gelatin zymography, and immunohistochemical analyses. Transfection of SNB19 cells with the pUM vector resulted in significantly inhibited tumor-cell invasion through Matrigel and fetal rat brain aggregates.

The production of pro-uPA and its subsequent activation to uPA via its interaction with uPAR is an important step in glioma cell invasion, because it is required for efficient activation of plasminogen to plasmin and extracellular matrix degradation (27). The ability of tumor cells to invade depends on multiple cellular activities such as mobility, adhesion, and cytoskeletal reorganization occurring in a temporally organized manner (28). uPAR influences integrin-dependent cell adhesion, migration, and proliferation, because these functions are blocked by uPAR antibodies (27). Similarly, uPAR-dependent migration of numerous cell types was blocked by anti-uPAR antibodies in, in vitro studies (27). The anti-uPAR antibody has been shown to completely block the induced invasion of U251glioma cells in invasion assays (29). In addition, the role of MMPs in promoting cell migration has often been associated with a direct cleavage of specific extracellular matrix components. CD44 has been demonstrated to serve as a docking molecule to retain MMP-9 proteolytic activity at the cell surface (30), and its cleavage from CD44 resulted in impairment of migration (31). The localization of MMP-9 to the cell surface mediates the activation of latent TGF-β and promotes tumor cell invasion and angiogenesis (32). Because siRNA for uPAR and MMP-9 are able to inhibit invasion of tumor cells in vitro, they may interfere with one or more of these activities in vivo as well.

In the present study we were able to completely suppress intracranial tumor growth during the 5-week follow-up period in nude mice injected with pUM vector. It has been reported that similar siRNA expression vectors can stably suppress certain genes (33). RNAi-mediated inhibition of uPAR and MMP-9 may inhibit tumor growth in several interdependent ways. We have previously reported that apoptosis measured by DNA fragmentation was higher in the brains of animals injected with the antisense uPAR stable clones than in the parental cell line (34). The antitumor effects observed in the intracranial tumor model could be due to induction of tumor-cell death.

Tumor cells depend on angiogenesis to survive and proliferate. RNAi-mediated inhibition of uPAR and MMP-9 significantly inhibited tumor-induced angiogenesis in our in vitro co-culture system. In this study, the anti-angiogenic effects of the pUM vector suppressed the ability of tumor cells to recruit blood vessels necessary for survival and directed anti-invasive effects onto the tumor cells themselves. The capacity of siRNA for uPAR to block tumor progression could also include the blocking of the anti-apoptotic and angiogenic effects of uPA. Similar observations were reported where an 8-mer uPA-derived peptide decreased tumor growth by blocking angiogenesis and promoting apoptosis of tumor cells in breast cancer (35). It has become increasingly evident that no single anti-angiogenic agent (including angiostatin and endostatin) used as monotherapy in preclinical models is able to reduce tumor burden once tumors have reached 100 mm (36). It was shown that the absence of placental growth factor, uPA, or tissue plasminogen activator significantly decreased the development of experimental choroidal neovascularization compared with wild type or uPAR-deficient mice (37). This effect was suggested to be partly due to a modulation of matrix metalloproteinase activity. Several reports have shown that the anti-tumor effect of many anti-angiogenic drugs and MMP inhibitors was stronger when treatment was started earlier (38), suggesting that MMPs may play critical roles in early aspects of cancer progression. Although a large number of studies have demonstrated the anti-angiogenic effect of synthetic MMP inhibitors, virtually all of these inhibitors lack specificity for a single MMP (39). For example, decreased vessel density and increased tumor cell apoptosis were observed in primary tumors and me-
Regression of Glioma Growth by uPAR and MMP-9 siRNA

Schematic representation of the plausible involvement of cell surface molecules like uPAR and CD44 at the invasion front of metastatic cells.

earlier reports we were successful in inhibiting glioma tumor growth using antisense RNA expressing plasmids but only in ex vivo studies (12, 22, 53). In the present study, RNAi-mediated inhibition of uPAR and MMP-9 completely suppressed pre-established glioma tumor growth in nude mice. Aoki et al. (54) have demonstrated that RNAi or RNAi-like effects were more potent than antisense effects in reducing target gene expression, also suggesting the potential applicability of RNAi. They have used a peptide vector comprising tumor-homing arginine-glycine-aspartic acid motif in a cyclic conformation, a DNA-binding oligonucleotide lysine, and histidyl residues to facilitate delivery into the cytosol (55), whereas this study demonstrated that the peptide vector could function as a carrier of siRNA. The actual mechanism by which small hairpin RNA causes silencing of target mRNA is not well understood; however, similarities can be drawn with work done on micro RNAs (56). Our results are based on the transfection of cells with plasmids expressing RNA molecules predicted to have structures similar to small nuclear RNA molecules (57) and are processed to siRNA or short hairpin RNA-like molecules. The actual mechanism of this processing would provide a better understanding of the RNAi machinery would aid in the development of an RNAi-based gene therapy approach for the treatment of gliomas and other metastatic tumors.

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Specific Interference of Urokinase-type Plasminogen Activator Receptor and Matrix Metalloproteinase-9 Gene Expression Induced by Double-stranded RNA Results in Decreased Invasion, Tumor Growth, and Angiogenesis in Gliomas

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