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Adenovirus-mediated expression of antisense MMP-9 in glioma cells inhibits tumor growth and invasion

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Matrix metalloproteinase 9 (MMP-9) is known to play a major role in cell migration and invasion in both physiological and pathological processes. Our previous work has shown that increased MMP-9 levels are associated with human glioma tumor progression. In this study, we evaluated the ability of an adenovirus containing a 528 bp cDNA sequence in antisense orientation to the 5′ end of the human MMP-9 gene (Ad-MMP-9AS) to inhibit the invasiveness and migratory capacity of the human glioblastoma cell line SBN19 in *in vitro* and *in vivo* models. Infection of glioma cells with Ad-MMP-9AS reduced MMP-9 enzyme activity by approximately 90% compared with mock- or Ad-CMV-infected cells. Migration and invasion of glioblastoma cells infected with Ad-MMP-9AS were significantly inhibited relative to Ad-CMV-infected controls in spheroid and Matrigel assays. Intracranial injections of SNB19 cells infected with Ad-MMP-9AS did not produce tumors in nude mice. However, injecting the Ad-MMP-9AS construct into subcutaneous U87MG tumors in nude mice caused regression of tumor growth. These results support the theory that adenoviral-mediated delivery of the MMP-9 gene in the antisense orientation has therapeutic potential for treating gliomas.
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Introduction

Cancer cell invasion is a complex process that requires coordinated interactions between adhesive proteins and pericellular proteolysis. It is known that serine proteinases, such as urokinase and matrix metalloproteinases (MMPs) are essential for cell invasion. MMPs are a family of secretory Zn dependent endopeptidase with a diverse hydrolytic spectrum of extracellular proteins. MMP-9 is known to degrade basement membranes, which normally separate the epithelial from the stromal compartment (Stetler-Stevenson *et al*., 1993). It is believed that the finely regulated temporal and spatial MMP activity on the surface of both tumor and endothelial cells is essential for the aggressiveness of these cells in the process of tumor metastasis and invasion. Thus the control of matrix proteolysis has long been proposed as a rational therapeutic antitumor strategy (Brown, 1999; Liotta *et al*., 1991; Yip *et al*., 1999).

The human MMP gene family contains 24 identified members in four major groups – the collagenases, gelatinases, stromelysins, and MT-MMPs (Shingleton *et al*., 1996). The collagenases (MMP-1, MMP-8, and MMP-13) cleave triple helical collagen into two fragments; gelatinases (MMP-2 and MMP-9) degrade denatured collagen; and stromelysins (MMP-3, MMP-7, MMP-10 and MMP-11) degrade a broad range of molecules, including aggrecan, fibronectin, laminin and procollagens. MT-MMPs (MMP-14, MMP-15, MMP-16, and MMP-17) are similar to other MMPs except for the addition of a transmembrane domain and a cytoplasmic tail at the C terminus; and MT-MMPs are also expressed in many carcinomas (Seiki, 1999). The activities of the MMPs are regulated by gene expression (Eberhardt *et al*., 2000; Sato and Seiki, 1993), proenzyme activation (Crabbe *et al*., 1994; Imai *et al*., 1995) and inhibition of the active enzymes by their specific tissue inhibitors (Gomez *et al*., 1997).

Levels of MMP-2, MMP-9, and MT1-MMP are reportedly elevated in various cancers (Kahari and Saarialho-Kere, 1999). For example, MMP-9 was detected in 68% of primary breast carcinomas, either in the stromal compartment or adjacent to tumor cells (Jones *et al*., 1999). A significant correlation between the expression of MMP-9 and tumor grade has also been reported in bladder cancer (Davies *et al*., 1993b). Furthermore, elevated serum level of MMP-9 was shown to correlate with spontaneous metastasis in rat mammary tumor models (Nakajima *et al*., 1993). Inhibition of MMP-9 expression and/or activity resulted in reduction of tumor invasion and metastasis in animal studies (Davies *et al*., 1993a; Garbisa *et al*., 1987; Lozonschi *et al*., 1999). Mice deficient in MMP-9 were viable but exhibited an abnormal pattern of skeletal growth plate vascularization (Vu *et al*., 1998). Cultured growth plate cells from MMP-9-null mice showed a delayed release of some angiogenic activators, suggesting that MMP-9 may play a positive role in regulating angiogenesis.

Nervous system tumors are one of the leading causes of cancer related death. The diffusely infiltrative nature of glioblastoma multiforme is one of the major obstacles to its successful surgical control. The specific mechanisms facilitating the invasive behavior of brain cancer remains obscure; however, the interactions between cancer cells and the surrounding normal cells and ECM are thought to be key aspects in tumor cell invasion. The ECM in the blood vessel basement membrane of the normal brain and in the perivascular locations of brain tumors includes collagen types I, III, IV, V, VI, VII, and VIII, laminin, and fibronectin (Gomez *et al*., 1997). We and others (Chintala *et al*., 1996a,b; Kahari and Saarialho-Kere, 1999; Gomez *et al*., 1997; Koochekpour *et al*., 1995) have reported that various protein components of the ECM play significant roles in the migration and invasion of glioma cells. We have also demonstrated higher levels of ECM components in glioblastomas than in low-grade glioma or normal brain tissue (Chintala *et al*., 1996b). Moreover, the metalloproteinases that degrade various ECM components are present in increasingly high amounts in increasingly malignant forms of human glioma tissues and cultured glioma cells (Forsyth *et al*., 1998; Yamamoto *et al*., 1996; Rao *et al*., 1993). We have recently shown that stable transfection of human glioblastoma cell line SNB19 with a plasmid containing antisense MMP-9 cDNA alters the invasive properties of these cells, both *in vitro* and *in vivo* (Kondraganti *et al*., 2000).

For patients with malignant glioma, present treatment options mostly consist of surgery and radiation. Recent advances have made it possible to consider using gene transfer techniques for the treatment of cancer. Adenoviral vectors have been widely used to transfer genes because of their unique ability to accomplish efficient gene delivery in various tissues. In the present study, we constructed a replication-deficient recombinant adenovirus (Ad-MMP-9AS) containing a 528 bp antisense expression segment for human MMP-9 and used this vector to infect glioblastoma cells in culture and tumors *in situ*. Our goal was to down-regulate the overexpression of MMP-9 in glioblastoma cells and tumors, thereby inhibiting the invasion of tumor cells into surrounding normal tissue and hence the development of tumor growth.

Results

Infection with Ad-MMP-9AS decreased MMP-9 enzyme activity and protein expression in SNB19 glioblastoma cells

We constructed a replication-deficient recombinant Ad (Ad-MMP-9AS) with a 1.8 kb mini expression cassette that expresses a truncated 528 bp antisense message to the 5′ end of the MMP-9 gene. The MMP-9 expression cassette was cloned into the E1-deleted region of the adenovirus genome, driven by a CMV promoter with a bovine growth hormone polyadenylation signal. Ad-MMP-9AS infection significantly decreased MMP-9

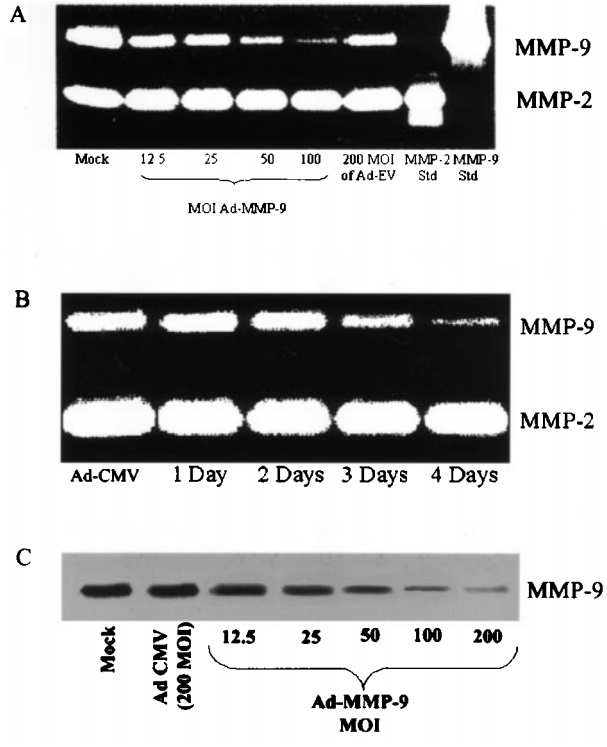

Figure 1 Infection with Ad-MMP-9AS decreased MMP-9 enzymatic activity and protein in SNB19 glioblastoma cells in a dose and time dependent manner. (**A**) Conditioned medium containing equal amounts of protein (20 μg) extracted from the conditioned medium from mock-infected, vector-infected (Ad-CMV), and Ad-MMP-9AS-infected SNB19 cell cultures was mixed with Laemmli sample buffer and run on 10% SDS–PAGE gels containing 0.1% gelatin (gelatin zymography). (**B**) SNB19 cells were infected with 100 MOI of Ad-MMP-9AS for various periods and enzyme activity was measured by gelatin zymography as described above. (**C**) Western blot analysis of MMP-9 protein expression in conditioned medium in mock, Ad-CMV and Ad-MMP-9AS infected SNB19 cells. Western analysis was performed using antibody against MMP-9

enzyme activity as determined by gelatin Zymography when compared to mock or Ad-CMV infected SNB19 cells (Figure 1A). Quantification of MMP-9 enzyme activity showed a significant ($P<0.001$) decrease in cells that had been infected with Ad-MMP-9AS at more than 25 MOI; this decrease reached 90% in cells infected with 100 MOI (relative to the enzyme activity in the control cells). The extent of decrease in MMP-9 enzyme activity levels also increased with time (Figure 1B). Quantification of MMP-9 enzyme activity over time showed a significant ($P<0.001$) decrease in Ad-MMP-9AS infected SNB19 cells after 3 days; this decrease reached above 90% in cells infected with Ad-MMP-9AS after 4 days. A dose and time dependent decrease in MMP-9 enzyme activity over 4 days was also observed in Ad-MMP-9-infected U87MG cells, another human glioblastoma cell line (data not shown). Figure 1C shows the adenovirus mediated decrease in MMP-9 protein expression as determined by Western blotting using anti MMP-9 antibody suggesting that the decrease in enzyme activity is due to decrease in MMP-9 protein expression.

Ad-MMP-9AS infection inhibited the migration of SNB19 cells

We examined the migrating capacity of the spheroids of SNB19 cells that had been infected with mock Ad-CMV and Ad-MMP-9AS. Figure 2A shows that there was significant migration of cells from the spheroids infected with mock and Ad-CMV in 72 h. However, there was very little migration of cells in Ad-MMP-9AS infected spheroids. Figure 2B shows a significant reduction ($P<0.001$) in the migration of cells from spheroids infected with Ad-MMP-9AS when compared to mock and Ad-CMV.

Ad-MMP-9AS infection inhibited the invasion of SNB19 cells through matrigel

SNB19 cells infected with Ad-MMP-9AS invaded Matrigel-coated filters to a much lesser extent than did the mock-infected or Ad-CMV-infected cells (Figure 3A). Quantitative analysis of the number of cells by 3-(4,5-dimethylthiazol-2-2,5-diphenyl-tetrazolium bromide) assay (Konduri *et al*., 2000) showed that 45% of the mock-infected cells and 39% of the Ad-CMV-infected cells but only 14% of the Ad-MMP-9AS-infected SNB19 cells invaded to the lower side of the membrane at 48 h ($P<0.001$) (Figure 3B).

Ad-MMP-9AS infection inhibited invasion of SNB19 cells into fetal rat brain aggregates

In the spheroid coculture experiments, fetal rat brain aggregates stained with the fluorescent dye DiO were confronted with spheroids of parental, vector-infected, or antisense Ad-MMP-9AS-infected cells that had been stained with DiI fluorescent dye. The tumor spheroids merged with the rat brain aggregates within 24 h. At 72 h, the tumor spheroids consisting of parental cells or vector control cells had progressively invaded the fetal rat brain aggregates (Figure 4A), producing a corresponding decrease in brain aggregate volume (Figure 4B). In contrast, Ad-MMP-9AS infection greatly inhibited the invasion of SNB19 cells into rat brain aggregates. Quantitative analysis of the rat brain aggregates remaining after 72 h of coculture revealed only 10–15% remaining in the parental and vector-infected cocultures compared with 85–90% remaining in Ad-MMP-9AS infected cells (Figure 4B) ($P<0.001$).

Ad-MMP-9AS infection inhibited tumor formation and caused regression of established tumors in mice

Having demonstrated that Ad-MMP-9AS infection decreased migration and invasion in SNB19 cells *in vitro* we tested the effect of this Ad-MMP-9AS in an *in vivo* model. As shown in Figure 4C, SNB19 cell infected with 100 MOI of Ad-CMV and then injected intracerebrally into nude mice (10 mice per group) developed tumors in nude mice by 4 weeks. But all the 10 mice that were injected with Ad-MMP-9. As

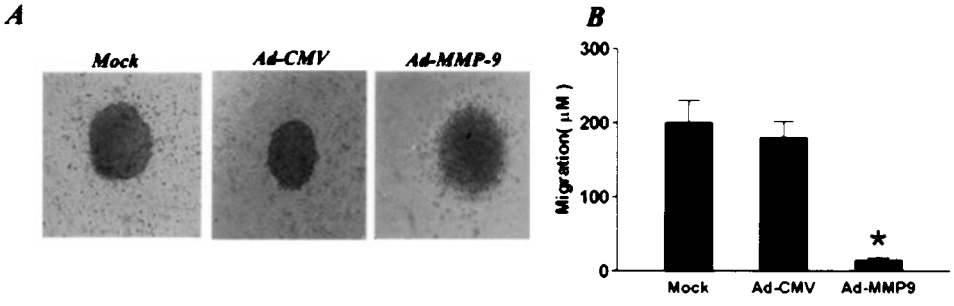

Figure 2 Migration of SNB19 cells infected with Ad-MMP-9AS or Ad-CMV. SNB19 cells (3×10^6 cells) were suspended in DMEM and seeded onto 0.5% agar-coated plates and cultured until spheroids were formed. Spheroids of 100–200 μm in diameter were selected and infected with 5×10^8 PFU of Ad-CMV or Ad-MMP-9AS. After 3 days, single glioma spheroids were placed in the center of a vitronectin-coated well in 96-well plate and cultured at 37°C for 48 h. At the end of the migration assay, spheroids were fixed and stained with Hema-3 stain and photographed. (**A**) The migration of cells from the spheroid was measured using a microscope calibrated with a stage and ocular micrometer. (**B**) The data shown are mean $\pm$ s.d. values of four independent experiments from each group (*$P<0.001$)

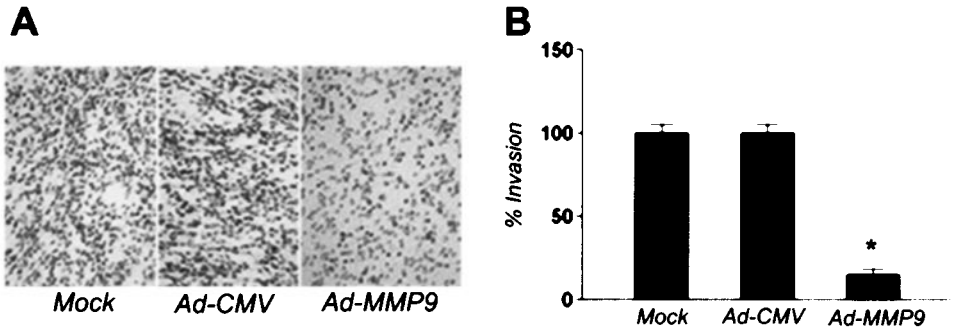

Figure 3 Ad-MMP-9AS infection inhibits invasion of SNB19 cells. SNB19 cells were infected with 100 MOI of Ad-CMV or 100 MOI of Ad-MMP-9AS. Four days later, 1×10^6 cells were allowed to migrate for 24 h through transwell inserts (8 μm pores) coated with Matrigel. The cells that invaded through the Matrigel-coated inserts were stained, counted and photographed under a light microscope at $20 \times$ magnification (**A**) and invasion was quantified (**B**) as described in Materials and methods. Data shown are the average values from four separate experiments from each group ($\pm$s.d.; $^*P < 0.001$)

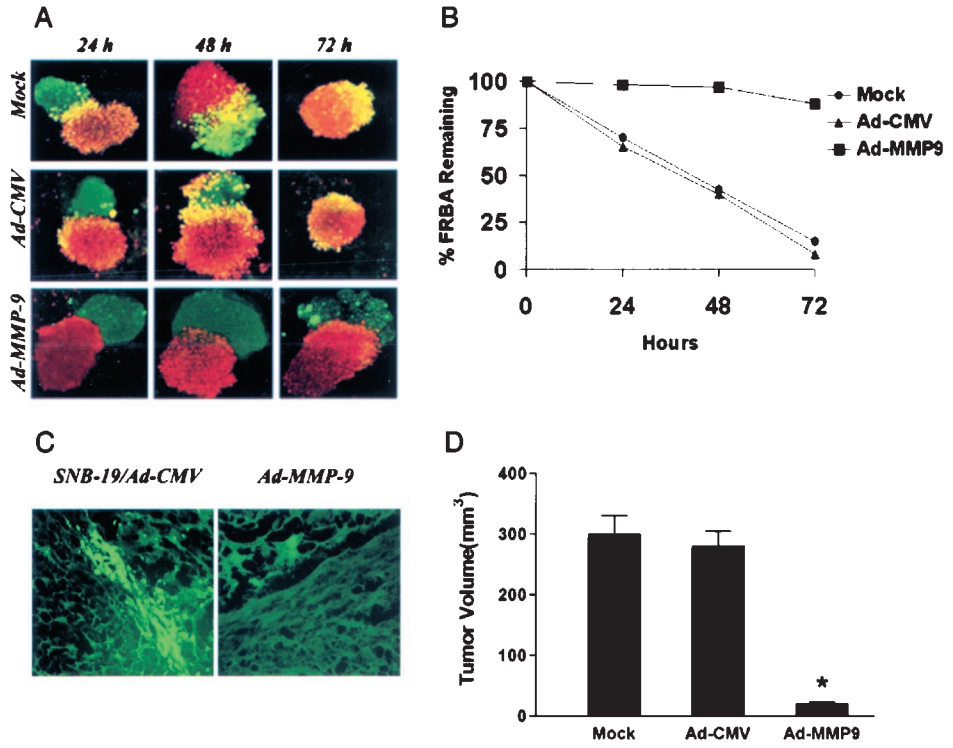

Figure 4 SNB19 cells (3×10^6 cells) were suspended in DMEM and seeded onto 0.5% agar-coated plates and cultured until spheroids formed. Tumor spheroids (red fluorescence) 100–200 μm in diameter were selected, infected with 5×10^8 PFU of Ad-CMV or Ad-MMP-9AS, and co-cultured with fetal rat brain aggregates. After 3 days, progressive destruction of fetal rat brain aggregates (green fluorescence) and invasion of SNB19 cells was observed upon confocal laser scanning microscopy (**A**). Quantification of remaining fetal rat brain aggregates by SNB19 spheroids infected with Ad-CMV or Ad-MMP-9AS vectors as described in Materials and methods (**B**). Data shown are the mean $\pm$ s.d. values from four separate experiments for each group ($^*P < 0.001$). (**C**) Tumor growth inhibition of SNB19 GFP cells infected with Ad-MMP-9AS. SNB19 glioblastoma cells, but not SNB19 cells infected with antisense Ad-MMP-9AS, formed intracerebral tumors in nude mice. Cells were infected with Ad-MMP-9AS (100 MOI), Ad-CMV (100 MOI), or phosphate-buffered saline for 4 days, trypsinized, counted and then inoculated intracerebrally (1×10^6 cells in 10 μl of PBS) into nude mice (10 mice in each group). The animals were examined for tumor formation over a 5–6 week period, and tumor sizes were estimated on tumor sections from GFP expressing cells (**C**). Semi quantitation of tumor volume in mock/Ad-CMV and Ad-MMP-9AS infected SNB19 cells 4 weeks after intracranial injection of these cells as described in Materials and methods (**D**). Data shown are the $\pm$s.d. values from 10 animals from each group ($^*P < 0.001$)

infected cells developed very small or undetectable tumors (Figure 4C). Quantitation of tumor size showed a significant reduction ($P < 0.001$) in Ad-MMP-9AS infected cells compared to mock and Ad-CMV infected cells (Figure D). This represented a 30-fold reduction in the tumor volume compared to control virus treated cells. In a separate experiment, 5×10^6 U87MG glioblastoma cells were injected subcutaneously in nude

mice. After the tumors reached 4–5 mm in diameter (8–10 days), they were injected every second day with a total of four doses of Ad-CMV or Ad-MMP-9AS (5×10^8 PFU). All 10 mice injected with the Ad-MMP-9AS vector showed regression of tumors beginning on the fifth day after the second injection and continuing for 21 days at which about 80% inhibition was seen relative to the Ad-CMV injected tumors (Figure 5). By 25 days, inhibition was about 90% relative to subcutaneous tumors that had been injected with Ad-CMV (Figure 5).

Discussion

The etiology of brain cancer, like other malignancies, involves a complex interplay between epigenetic and genetic changes occurring during the natural history of tumor growth and development. Genetic changes accumulate in the form of mutations, activation of proto-oncogenes, or the loss of tumor suppressor genes that could promote tumor progression, invasion, and metastasis; this process provides a rational basis for targeting one or more critical genetic defects in cancer cells by using gene-mediated therapy approaches. A variety of different vectors and delivery techniques are being studied for gene transfer within the central nervous system (Parks and Bramson, 1999). Substantial work over the past decade has shown that MMPs play a pivotal role in the process of malignant progression and that inhibition of MMP-9 expression and/or activity resulted in reduction of tumor invasion and metastasis in animal studies (Davies *et al*., 1993a; Garbisa *et al*., 1987; Lozonschi *et al*., 1999). In the present study, we demonstrated that adenoviral-mediated transfer of an antisense-MMP-9 gene sequence can inhibit tumor growth in gliomas.

Malignant astrocytomas (glioblastomas) are the most invasive of brain tumors and frequently demonstrate extensive infiltration of surrounding tissues. Indirect evidence that MMPs are involved in invasion and angiogenesis in gliomas comes from observations that

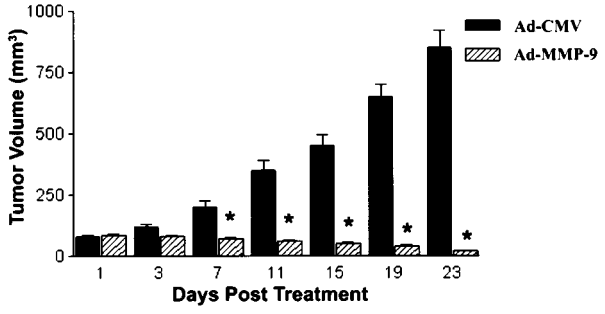

Figure 5 Growth inhibition of pre-established tumors by the intratumoral injection of Ad-MMP-9AS. 5×10^6 U87 cells in 100 μl of phosphate-buffered saline were injected in nude mice. After 8–10 days, the resultant 4–5 mm subcutaneous tumors were injected intratumorally with Ad-CMV (vector control), or Ad-MMP-9AS (5×10^8 PFU) in a 100-μl volume. A total of four injections were given, one every other day, and the tumor size was measured with calipers. Tumor volumes are shown as means $\pm$ s.d. ($^*P < 0.001$)

MMP-2, MMP-9, and MT1-MMP are present at elevated levels in several glioma cell lines and surgical specimens (Rao *et al*., 1993, 1996; Forsyth *et al*., 1998; Yamamoto *et al*., 1996; Nakada *et al*., 1999). The regulation of the expression and activity of MMP-9 is more complex than that of most other MMPs (Dubois *et al*., 1999). MMP-9 is not produced constitutively by most cells (Collier *et al*., 1988), but its activity is induced by different stimuli depending on the cell type (Houde *et al*., 1993), thereby providing a means of increasing its activity in response to specific pathophysiological events. Glioblastomas express greater amounts of MMPs than do either low-grade gliomas or normal brain *in vivo* (Rao *et al*., 1993; Forsyth *et al*., 1998; Yamamoto *et al*., 1996), and the activity of MMP-9 in particular in surgical specimens was proportional to the grade of the glioma (Rao *et al*., 1996). In the present study, we constructed a replication-defective recombinant adenovirus containing a 528 bp antisense gene to human MMP-9 and used it to effectively down-regulate MMP-9 enzymatic activity in glioma cells. The decrease in MMP-9 activity resulting from Ad-MMP-9AS infection inhibited the invasiveness of SNB19 glioblastoma cells by 90% in Matrigel and spheroid models. However we did not see any inhibitory effect ($\sim$10%) of Ad-MMP-9AS compared to mock and Ad-CMV in a proliferation assay (data not shown). In the present study we did not study the expression of MMP-9 gene transcript following infection with Ad-MMP-9AS. In our earlier work with adenoviral mediated transfer of uPAR antisense gene, although the uPAR protein levels and *in vitro* translation studies with ^{35}S-labeling of uPAR decreased, there was no significant decrease in target mRNA (Mohan *et al*., 1999). Studies on the co-localization of antisense RNAs with the target mRNA suggest that there is difference in RNA susceptibility. The RNA that is transcribed from the a chromosomal location (cis) is targeted by antisense RNA but is not targeted by the antisense RNA transcribed by the extra chromosomal units like adenovirus (Arndt and Rank, 1997). We then assessed the *in vivo* effect of Ad-MMP-9AS using two nude mice models. SNB19 cells infected with Ad-MMP-9AS formed very small tumors or undetectable upon intracerebral injection in nude mice. Intratumoral injection of this virus in the subcutaneous model efficiently inhibited tumor growth. We believe that the data from the nude mice indicated that the suppression of tumor growth is because of reduced invasion/angiogenesis in Ad-MMP-9AS infected cells compared to the parental and Ad-CMV controls. Previous studies with uPAR antisense colonies has shown that these cells remained dormant after injection into nude mice (Aguirre-Ghiso *et al*., 2001) and analysis of brain sections indicated that these cells have undergone apoptosis (Go *et al*., 1997; Kin *et al*., 2000). We are investigating the fate of the Ad-MMP-9AS infected cells in nude mice at present. Taken together, these findings indicate that the efficient down-regulation of MMP-9 by adenoviral-mediated delivery of an antisense gene has an antitumor effect *in vivo*.

MMP-9 expression and activation involve multiple steps. We showed previously that inhibition of MAP kinases through the use of dominant-negative c-*jun*, ERK-1, ERK-2, or MEKK or specific inhibitors like PD98059 markedly inhibited the expression of MMP-9 and the invasive potential of gliomas (Lakka *et al*., 2000). At present, inhibition of the function of MMPs in the extracellular matrix is being actively pursued for anticancer therapy. Several reports have implicated the plasmin system in MMP-9 activation. Activation of the type IV collagenases has been shown to occur in a dose- and time-dependent manner in the presence of physiological levels of plasminogen and uPA. The addition of antihuman uPA IgG delayed the migration of human bronchial epithelial cells in a dose-dependent manner, indicating that uPA activity is essential for the migration of these cells; this treatment also resulted in a decrease in the amount of activated MMP-9 (Chambers and Matrisian, 1997). In a rat sarcoma model, a ribozyme directed against MMP-9 mRNA was used to show that MMP-9 expression is involved in metastasis but not in tumor growth (Hua and Muschel, 1996).

Synthetic MMP inhibitors have been reported to inhibit tumor progression and angiogenesis in a variety of *in vivo* models (Rasmussen and McCann, 1997; Shalinsky *et al*., 1999). The synthetic inhibitors batimastat and marimastat effectively reduced glioma invasion in Matrigel-coated transwell assays and co-cultures of tumor spheroids with fetal rat brain aggregates, although higher concentrations were required in co-culture systems (Tonn *et al*., 1999). AG3340, another synthetic inhibitor, inhibits the growth of the U87 glioma cell line implanted subcutaneously in SCID-NOD mice (Price *et al*., 1999). These compounds inhibit the *in vitro* activities of many MMPs, and most are synthetic analogues that use hydroxamic acid as the zinc-binding group (Scatena, 2000). Several of these hydroxamate-based inhibitors have demonstrated a dose limiting arthralgia/myalgia in clinical trials (Brown, 2000), and it has been suggested that this dose limiting side effect may result from the inhibition of MMP-1. Clinical toxicity may also result from inhibiting the proteolytic processing of other cell surface molecules, such as L-selectin, IL-1-R11, and IL-6R (Baxter *et al*., 2000). Another approach to down-regulate these molecules is by using specific antisense oligonucleotides. A potential advantage of antisense strategies is that, they are selective for a specific type of MMP, thereby potentially resulting in fewer systemic toxic effects. Previous work from our laboratory has shown that successful transfection of SNB19 cells with an antisense-MMP-9 construct led to reduced invasiveness in Matrigel and spheroid assays, indicating that MMP-9 is required for invasiveness, and antisense MMP-9-transfected clones did not form tumors in nude mice (Kondraganti *et al*., 2000).

At present, adenovirus is still an attractive vector to deliver anti-invasive and/or antiangiogenic gene products in cancer because of its high infectivity *in vivo*, which allows direct vector injection in clinic. In our study intratumoral delivery of Ad-MMP-9AS suppressed the growth in nude mice. We did not see the tumor growth up to approximately 10–12 weeks. Although the described protective effect of the Ad-MMP-9AS therapy in study appears to be transient since adenoviral delivery of genes is known to last for only 4–6 weeks, it did emphasize the potential of such a protocol as a further advance in cancer gene therapy. However, the substantial clinical benefit and further extension of this strategy should certainly await the development of vectors allowing long term expression of genes at circulating therapeutic levels and /or the use of vectors with altered tropism to permit gene transfer into specific cell types. Together with our previous results we have demonstrated the anticancer efficiency of delivering antisense MMP-9 by adenoviral vector. Since MMP-9 is not only tightly associated with invasion/metastasis but also was shown to mediate angiogenesis and is considered to be prioritized therapeutic target (Folkman, 1999), gene therapy targeting this molecule is not restricted to cancer because its potential clinical use should include any angiogenic-relevant diseases such as rheumatoid arthritis, retinopathy and vascular stenosis (Folkman, 1999; Baker *et al*., 1998).

Materials and methods

Materials

DMEM/F12 medium was obtained from Fisher Scientific (Pittsburgh, PA, USA). PMA was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Matrigel was obtained from Collaborative Research, Inc. (Boston, MA, USA), and transwell plates were from Costar (Cambridge, MA, USA).

Construction of the Ad-MMP-9AS vector

A 528 bp cDNA fragment of MMP-9 was amplified by PCR with synthetic primers and subcloned into the pcDNA3 vector (Invitrogen, Carlsbad, CA, USA) between the *Hind*III and *Xho*I polycloning sites in the antisense orientation (pcDNA-MMP-9-AS). The forward primer was 5′-AATCTCGAGAGACACCTCTGCCCTCACCATGAG-3′; the reverse primer was 5′-AATAAGCTTAACTGGATGACGATGTCTGCGTCC-3′ (enzyme recognition sites are underlined). Sequence analysis of the 528-bp internal sequence verified its 100% homology with the published sequence of MMP-9 cDNA (Wilhelm *et al*., 1989). This mini expression cassette containing the 528 bp DNA fragment of the 5′ end of MMP-9 gene, with the CMV promoter and the polyadenylation signal of bovine growth hormone, was extracted from the pcDNA-MMP-AS clone by digestion with *Pvu*II. The resulting 3.5 kb DNA fragment containing the 528 bp antisense expression cassette for MMP-9 was purified from agarose gels and partially digested with *Nru*I to yield a 1.7 kb DNA fragment, which was blunt end-ligated into the *Eco*RV site of the adeno-shuttle vector pAdΔE1sp1A. The sequence of the resulting Ad-MMP-9AS clone was confirmed, and the Ad-MMP-9AS construct was cotransfected with the 40.5 kb pJM17 vector into human embryonic kidney 293 cells to isolate recombinant adenovirus (Graham and Prevec, 1991). The recombinant virus was purified by ultracentrifugation in

cesium chloride step gradients (Graham and Prevec, 1991). The viral DNA was also sequenced to confirm the configuration of the expression cassette. The purified viral preparations were verified to be free of wild-type Ad by PCR with E1-specific primers.

Cell lines and infection conditions

SNB19, an established human glioblastoma cell line, was grown in DMEM/F12 (1:1, v/v) supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% CO_2 at 37°C. Viral stocks were suitably diluted in serum-free medium to obtain the desired MOI or PFU and added to cell monolayers of tumor cell spheroids (1 ml per 60 mm dish or 3 ml per 100 mm dish) and incubated at 37°C for 30 min. The necessary amount of culture medium with 10% fetal bovine serum was then added, and the cells were incubated for the desired times.

Gelatin zymography

MMP-9 expression was analysed on SDS polyacrylamide gels impregnated with 0.1% gelatin (w/v) and 10% polyacrylamide (w/v) as described previously (Rao *et al*., 1993; Forsyth *et al*., 1998; Yamamoto *et al*., 1996). Cells were treated with or without PMA for 6–8 h, after which the culture medium was replaced with fresh serum-free medium. The conditioned medium was collected 48 h later, and medium containing equal amounts of protein (20 μg) was mixed with sample buffer before electrophoresis. The gels were run at a constant current and then washed twice for 30 min in 50 mM Tris-HCl, pH 7.5, plus 2.5% Triton X-100; the gels were then incubated overnight at 37°C in 50 mM Tris-HCl (pH 7.6), 10 mM $CaCl_2$, 150 mM NaCl and 0.05% NaN_3. The gels were stained with Coomassie brilliant blue R-250 and then destained.

Western blotting

Western blotting for MMP-9 was performed by lysing the cells with radio-immunoprecipitation assay buffer (1% Nonidet P40, 20 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.1 mg/ml aprotinin, and 1 mM phenylmethyl sulfonyl fluoride). Then 20 μg aliquots of cell lysate from each treatment were loaded onto a 10% resolving SDS–PAGE gel with a 4.5% stacking gel. Samples were electrophoresed and then electroblotted onto a nitrocellulose membrane. Western blot analysis was performed with a 1 : 2000 dilution of anti-MMP-9 antibody (Oncogene Science, Cambridge, MA, USA). Immunoreactive bands were visualized for MMP-9 by using a horseradish peroxidase-conjugated anti-mouse IgG secondary antibody and enhanced chemiluminescence reagents and exposed to X-ray film.

Migration of cells from spheroids

Migration of cells from spheroids was assessed as described previously (Adachi *et al*., 2001), with modifications. Spheroids of SNB19 cells were prepared by suspending 2×10^6 cells in DMEM, seeding them on 100 mm tissue culture plates coated with 0.75% agar, and culturing them until aggregates were formed. Spheroids measuring approximately 100–200 μm in diameter (about 4×10^4 cells per spheroid) were selected and infected with adenovirus vectors at 100 MOI. Three days after infection, a single glioma spheroid was placed in the center of each well in vitronectin-coated 96-well microplates and 200 μl of serum-free medium was added to each well. Spheroids were cultured at 37°C for 48 h, after which the spheroids were fixed and stained with Hema-3 and migration from the spheroids was assessed by light microscopy and photographed. The migration of cells from spheroid to monolayers was measured using a microscope calibrated with a stage and ocular micrometer.

Matrigel invasion assay

Invasiveness was assessed by the ability of parental SNB19 cells, control cells (those mock-infected with phosphate-buffered saline or infected with the Ad-CMV vector), or cells infected with antisense MMP-9 (Ad-MMP-9AS) to traverse a Matrigel-coated membrane as follows. Transwell inserts with 8 μm pores were coated with a final concentration of 0.7 mg/ml of Matrigel, and 500 μl of cell suspension (1×10^6 cells) were added in triplicate wells. After a 24 h incubation, the cells that passed through the filter into the lower wells were quantified as described elsewhere (Konduri *et al*., 2000) and expressed as a percentage of the sum of the cells in the upper and lower wells. Cells on the lower side of the membrane were fixed, stained with Hema-3 and photographed.

Spheroid invasion assay

Glioma invasiveness was assessed in a 3-dimensional model by using a previously described procedure (Go *et al*., 1997) in which glioma spheroids were co-cultured with fetal rat brain aggregates. Glioma spheroids were prepared as described above, stained with the fluorescent dye DiI and combined with fetal rat brain aggregates that had been stained with DiO. At different time intervals, serial 1 μm-thick sections were obtained from the surface through the center of the co-cultures with a confocal laser-scanning microscope. DiI and DiO fluorescence was detected by using an argon laser at 488 nm with a band-pass filter at 520–560 nm and a helium/neon laser at 543 nm with a long-pass filter at 590 nm respectively. The volumes of fetal brain aggregate or tumor spheroid that remained at 24, 48 and 72 h of co-cultures were quantified with the formula as described previously (Konduri *et al*., 2000).

Tumor implantation and regression experiments

For the intracerebral tumor experiments, cultured SNB19 GFP positive cells were infected with PBS (mock-infection condition), Ad-CMV, or Ad-MMP-9AS, cultured for 5 days, trypsinized, counted and intracerebrally inoculated into nude mice. Four weeks later, the mice were anesthetized and killed by intracardiac perfusion with phosphate-buffered saline followed by 4% paraformaldehyde in saline for *in situ* fixation of the tumors (Go *et al*., 1997). The brains were removed, placed in 6% paraformaldehyde, and allowed to stand at 4°C for 4 h, after which they were transferred to a solution of 0.5 M sucrose in phosphate-buffered saline and incubated overnight at 4°C. The following day, the brains were cut, embedded in microscopic slides, and frozen at −20°C. The frozen sections were screened for GFP fluorescence to examine tumor growth. All sections were reviewed by a neuropathologist, Dr M Gujrati, who was unaware of the treatment condition. The size of each tumor was scored semiquantitatively in terms of maximum cross-sectional diameter. The variation in the thickness between the sections in each group was less than 10%. For the subcutaneous tumor experiments, U87MG cells (5×10^6 cells) were injected subcutaneously into nude mice. After 8–10 days, when tumors reached 4–5 mm in diameter, they were

injected with Ad-CMV, or Ad-MMP-9AS (5×10^8 PFU) every other day for a total of four injections. Tumor size was measured every second day, and tumor volume was calculated from the formula $1/6\ \Pi\ (R\ max) \times (R\ min)^2$, where R max and R min are the maximum and minimum tumor radii respectively.

Abbreviations

ECM, extracellular matrix; MMP, matrix metalloproteinase; MT-MMP, membrane-type MMP; Ad, adenovirus; CMV, cytomegalovirus; MOI, multiplicities of infection; PFU, plaque-forming units; PMA, phorbol myristate acetate.

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