Selective Suppression of Matrix Metalloproteinase-9 in Human Glioblastoma Cells by Antisense Gene Transfer Impairs Glioblastoma Cell Invasion


Abstract

Increased expression of matrix metalloproteinases (MMPs) has been associated with human glioblastoma tumor progression. In this study, we sought to down-regulate MMP-9 expression by stably transfecting a high-grade glioblastoma cell line with a plasmid vector capable of expressing an antisense transcript complementary to a 528-bp segment at the 5' end of human MMP-9 cDNA. Stable transfectants were obtained through selection with G418. Of the clones transfected with vector, sense, and antisense constructs, Northern blotting, Western blotting, and gelatin zymography showed that MMP-9 expression was significantly reduced only in the antisense-transfected cells. A Matrigel invasion assay revealed marked reductions in invasiveness for the antisense clones relative to the parental, vector, and sense clones. Cocultures of tumor spheroids and fetal rat brain aggregates showed that MMP-9 expression was significantly reduced only in the antisense-transfected stable clones. Intradural injection of antisense stable transfectants in nude mice produced no tumors or very small tumors, but intracerebral injection of parental or vector clones did produce tumors. These results suggest that MMP-9 expression is essential for the invasiveness of glioblastoma cells.

Introduction

Local invasive growth is a key feature of primary malignant brain tumors. Malignant brain tumor cells cause massive tissue destruction at the border between tumor and normal brain tissue (1). The most common and malignant brain tumor, glioblastoma multiforme, is characterized by the presence of necrosis, vascular proliferation, and aggressive invasion into surrounding normal brain tissue (2). Glioblastoma multiforme spreads along nerve fiber tracts and frequently penetrates beyond the glial membrane limits externa, leading to leptomeningeal dissemination of the tumor. The diffuse infiltrative nature of glioblastoma multiforme is one of the major obstacles to its successful surgical control.

The specific mechanisms facilitating the invasive behaviors of brain cancers remain obscure; however, the interactions between cancer cells and the surrounding normal cells and ECM are thought to be key aspects in tumor cell invasion (3). To invade and spread through surrounding normal tissue, tumor cells must degrade multiple elements of the ECM, including fibronectin, laminin, and type IV collagen (4). Several different proteases, including serine proteases, MMPs, and cysteine proteases, are thought to potentiate the invasiveness and metastatic ability of a variety of malignant tumors (5).

MMPs are neutral proteinases encoded by a multigene family. These proteinases mediate ECM degradation and tissue turnover during physiological and pathological processes (6). These enzymes have been grouped into four broad categories on the basis of their substrate specificity: (a) interstitial collagenases (MMP-1, MMP-8, and MMP-13) that degrade fibrillar collagens; (b) type IV collagenases (MMP-2 and MMP-9) that degrade basement membrane collagens, gelatin, and elastin; (c) stromelysins (MMP-3, MMP-10, and MMP-11) that degrade proteoglycans, fibronectin, laminin, gelatin, and the globular portions of type IV collagen; and (d) MT-MMPs (MT1-MMP or MMP-16, MMP-15, MMP-16, and MMP-17) that contain a unique transmembrane domain in their COOH terminus that localizes these MMPs at the cell surface (7). All of these enzymes are secreted in latent forms and are activated by cleavage of NH2-terminal propeptides at a conserved sequence. This cleavage can be mediated by proteases such as plasmin, trypsin, kallikreins, and cathepsins and by organomercury compounds (7). The activities of the MMPs are regulated by gene expression (8), proteolytic activation, and inhibition of the reactive enzymes by their specific tissue inhibitors (TIMPs; Ref. 9). Levels of MMP-2, MMP-9, and MT1-MMP are reportedly elevated in various cancers (10, 11). Evidence that MMPs are involved in invasion and angiogenesis in gliomas comes from observations that MMP-2, MMP-9, and MT1-MMP have been found in several glioma cell lines and surgical specimens (12–17), that MMP-2 or MMP-9 protein is localized to the tumor neovascularure (16), and that the extent of MMP overexpression correlates with glioma grade (16, 17).

In this study, we down-regulated MMP-9 by transfecting a human glioma cell line with an antisense vector capable of expressing an antisense transcript complementary to a 528-bp segment at the 5' end of human MMP-9 cDNA. Cells transfected with this antisense MMP-9 showed inhibition of MMP-9 at the mRNA, protein expression, and enzymatic activity levels as well as subsequent reduction of invasiveness in vitro and in vivo.

Materials and Methods

Materials. DMEM/F-12 medium was obtained from Life Technologies (Gaithersburg, MD). PMA was obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Culture. SNB19, an established human glioma cell line, was routinely grown in high-glucose DMEM/F-12 medium (1:1) supplemented with 10% fetal bovine serum, 20 μg/ml HEPES, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO2 at 37°C. Preliminary experiments revealed that at 50 ng/ml, PMA had a maximal stimulatory effect on MMP-9 expression without affecting cell viability (as assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay).

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4 The abbreviations used are: ECM, extracellular matrix; MMP, matrix metalloproteinase; MT-MMP, membrane-type MMP; TIMP, tissue inhibitors of metalloproteinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PMA, phorbol myristate acetate.

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Preparation of MMP-9 Constructs. A 528-bp cDNA fragment of MMP-9 was amplified by PCR with synthetic primers and subcloned into the pcDNA3 vector (Invitrogen, Carlsbad, CA) between the HindIII and XhoI polycloning sites in the sense and antisense orientations. The following primers were used: (a) for sense MMP-9, the forward primer was 5'-AATAAGCTTACAC- CTTGCTCACCACATTAG-3', and the reverse primer was 5'-AATCTC- GAGAATCTGAGGATCAGGATGC-3'; and (b) for antisense MMP-9, the forward primer was 5'-AATTCGGAGACACTCTGCTC- CACCATGAG-3', and the reverse primer was 5'-AATAAGCTTAACTGGAT- GAGGATTCGTCGTC-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 (18).

Transfection of SNB19 Cells. SNB19 cells were transfected with the MMP-9 constructs in sense and antisense orientations by using LipofectAMINE (Life Technologies) according to a previously described procedure (19). Stable transfectants were selected by growing cells in 800 μg/mL G418; the cells that survived were then expanded in the absence of G418 for additional studies. Stable transfectants were screened on the basis of MMP-9 expression in the presence of 50 μg/mL PMA.

Northern Blot Analysis. Total cellular RNA was extracted from confluent cultures as described previously (19). Aliquots of 10 μg of RNA were separated by electrophoresis on 1.2% agarose-formaldehyde gels, transferred and exposed to X-ray film. The filters were hybridized at 65°C with a MMP-9 cDNA probe labeled with [32P]dCTP by random primer labeling (16). The filters were washed in SSC (3 M sodium chloride, 0.3 M sodium citrate) and 0.1% SDS for 20 min at room temperature and then washed in 0.5 X SSC, 0.1% SDS for 15 min at 65°C and exposed to X-ray film at −70°C. The membranes were then stripped and rehybridized with GAPDH cDNA to verify equal loading in all of the lanes.

Gelatin Zymography. MMP-2 and MMP-9 expression was analyzed on SDS polyacrylamide gels impregnated with 0.1% gelatin (w/v) and 10% polyacrylamide (w/v) as described previously (14, 16). Cells were treated with or without PMA for 6–8 h, and then the culture medium was replaced with 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 CaCl2, 150 mM NaCl, and 0.05% NaN3. 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 radioimmunoprecipitation assay buffer (1% NP40, 20 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.1 mg/mL aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Twenty-μg aliquots of cell lysate from each treatment were then 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). Immunoreactive bands were visualized for MMP-9 using a horseradish peroxidase-conjugated antimouse IgG secondary antibody and enhanced chemiluminescence reagents and exposed to X-ray film.

Matrigel Invasion Assay. Invasiveness of parental SNB19 cells and vector-, sense MMP-9-, and antisense MMP-9-transfected SNB19 clones was measured by the invasion of cells through Matrigel-coated transwell inserts (Becton Dickinson, Boston, MA). Briefly, transwell inserts with 8-μm pores were loaded on the Northern blots. Densitometric quantification of membranes with GAPDH showed that similar amounts of mRNA had been loaded on the Northern blots. For these experiments, parental cells and vector-transfected clones expressed the message for MMP-9 construct-transfected cells and antisense MMP-9 construct-transfected clones. Of these clones, the parental clones, the vector-transfected clones, and the sense-transfected clones all expressed MMP-9 mRNA in the presence of PMA (Fig. 1B). In contrast, none of the three antisense MMP-9 construct-transfected SNB19 cells expressed the message for MMP-9 in the presence of PMA. Probing the stripped membranes with GAPDH showed that similar amounts of mRNA had been loaded on the Northern blots. Densitometric quantification of MMP-9 mRNA was significantly higher (10–12-fold) in parental and vector controls and sense clones compared with antisense clones. After gelatin zymography confirmed that MMP-9 activity was reduced in the antisense clones, we characterized mRNA expression in parental clones, vector-transfected clones, sense MMP-9 construct-transfected clones, and antisense MMP-9 construct-transfected clones. After confirming by Northern blotting that MMP-9 mRNA was reduced in antisense-transfected clones, we determined MMP-9 protein levels in parental clones, vector-transfected clones, sense MMP-9 construct-transfected clones, and antisense MMP-9 construct-transfected clones by Western blotting using MMP-9-specific antibodies. Use of an anti-MMP-9 antibody revealed that MMP-9 protein was present in parental cells, vector-transfected cells, and sense MMP-9 construct-transfected cells treated with PMA (Fig. 1A).
agarose gels, blotted onto a nylon membrane, and hybridized with a 32P-labeled MMP-9 and transfected cells. Total RNA was isolated, and 10^6 treated with and without PMA.

0.1% gelatin. The zymographic results shown here were obtained after the cells were mixed with Laemmli sample buffer and run on 10% SDS-polyacrylamide gels containing 3C, but MMP-9 protein was not detected in antisense transfectants, regardless of PMA treatment (Fig. 1C).

Invasiveness of MMP-9 Stable Transfectants. Invasiveness of glioblastoma cells was measured in a Matrigel invasion assay and in a three-dimensional spheroid coculture assay. In the Matrigel invasion assay, parental SNB19 cells and vector-transfected and sense MMP-9 construct-transfected cells showed invasive ability, which could be induced slightly upon treatment with PMA (Fig. 2A). In contrast, the antisense MMP-9 construct-transfected clones were much less invasive, and PMA treatment did not augment their invasiveness (Fig. 2A). The percentage of invasion of parental, vector, and sense clones was much higher (45–48%) than that of antisense clones (14–16%; Fig. 2B). PMA treatment slightly increased the invasive behavior of parental, vector, sense (from 48% to 58%), and antisense clones (from 15% to 18%), and the effect was slightly higher in the parental, vector, and antisense clones (Fig. 2B). In spheroid coculture assays, fetal rat brain cell aggregates stained with a fluorescent dye, DiO, were confronted with spheroids of parental, vector, sense, and antisense MMP-9 clones that had been stained with Dil fluorescent dye. The tumor spheroids merged with the rat brain aggregates within 24 h. At 48 and 72 h, the tumor spheroids consisting of parental, vector, or sense clones (red fluorescence) had progressively invaded the rat brain aggregates (green fluorescence), producing a corresponding decrease in brain aggregate volume. On the other hand, the antisense MMP-9 spheroids failed to invade rat brain aggregates even at 72 h, although the two cell types did become attached to each other after 24 h (Fig. 3A). Quantitative analysis of the remaining fetal brain aggregates were 10–15% in parental, vector, and sense clones compared with 92–95% in antisense clones (Fig. 3B). The invasiveness of fetal rat brain aggregates was significantly decreased in antisense clones compared with parental, vector-, and sense construct-transfected clones (P < 0.001).

Intracranial Implantation. To assess the importance of the reduction in MMP-9 in vivo, cells were injected intracerebrally into nude mice. Five mice received an injection of parental cells, five mice received an injection of vector-transfected cells, five mice received an injection of sense-transfected cells, and five mice received an injection of antisense-transfected cells (for each clone); all injections contained 2 × 10^6 cells. Every mouse that received an injection of parental cells, vector-transfected cells, or sense MMP-9 construct-transfected cells developed tumors (Fig. 3C). In contrast, mice that received an injection of antisense-transfected cells showed no tumor formation or very small tumor formation at 4 weeks after the injection (Fig. 3C). The sections analyzed using H&E staining and blindly reviewed by a neuropathologist (G. N. F.) revealed that there was no evidence of tumor formation.

Fig. 1. Determination of MMP-9 enzyme activity and mRNA and protein expression in SNB19, vector-, sense MMP-9 construct-, and antisense MMP-9 construct-transfected cells. A, gelatin zymograms of the conditioned medium from parental SNB19 cells and transfected cells. Conditioned medium containing equal amounts of protein (20 μg) was mixed with Laemmli sample buffer and run on 10% SDS-polyacrylamide gels containing 0.1% gelatin. The zymographic results shown here were obtained after the cells were treated with and without PMA. B, Northern blot analysis of MMP-9 expression by SNB19 and transfected cells. Total RNA was isolated, and 10 μg were electrophoresed on 1.2% agarose gels, blotted onto a nylon membrane, and hybridized with a 10^6-labeled MMP-9 cDNA probe. After removal of the radiolabeled probe, the membrane was rehybridized with a GAPDH cDNA probe to check the relative amounts of mRNA that had been loaded onto the gels. C, Western blot analysis of MMP-9 protein expression. SNB19 cells were plated at 1 × 10^5 cells/well in 6-well plates in the presence or absence of PMA. Conditioned medium was collected, and equal amounts of protein (20 μg) from each treatment group were run on 10% SDS-polyacrylamide gels. Separated proteins were transferred onto nitrocellulose membranes and probed with an anti-MMP-9 monoclonal antibody in an enhanced chemiluminescence protocol.

Fig. 2. Invasion of SNB19 and transfected cells through Matrigel. Two hundred μl of a single-cell suspension (1 × 10^6 cells/ml) of cells were placed in the upper wells of individual transwell inserts containing 8-μm pore polycarbonate membranes precoated with Matrigel. Cells were allowed to invade for 48 h at 37°C, and then they were fixed and stained with Hema-3. Cells on the upper surface were removed with a cotton swab, and the cells that migrated to the lower side of the membrane were mounted onto a microscope slide and photographed under a light microscope at ×200 magnification (A). The percentage of invading cells was quantified as described in “Materials and Methods” (B). Data shown are the mean ± SD values from four separate experiments for each group (P < 0.001).
significant difference in tumor size in mice that received an injection of parental, vector-, and sense-transfected clones. However, the tumor size was significantly reduced ($P < 0.001$) in antisense clones compared with parental, vector, and sense clones (Fig. 3 D).

**Discussion**

Invasion by tumor cells involves their attachment to and degradation of the ECM (22). Degradation of the ECM in the vicinity of a tumor by tumor cells is thought to be one of the most important initial steps in the complex process of invasion. In the central nervous system, a well-defined basal lamina exists at the glial membrane limitans externa consisting of ECM macromolecule type IV collagen, laminin, and fibronectin (23). Malignant astrocytomas/glioblastomas are the most invasive of brain tumors and frequently demonstrate extensive infiltration of surrounding tissues. The characteristic local invasiveness of glioblastomas often prevents their total surgical resection and contributes to recurrences at the primary site and on the opposite side of the brain.

Glioblastomas express greater amounts of MMPs than do either low-grade gliomas or normal brain in vivo (13–17). We found that MMP-9 activity in surgical specimens was proportional to the grade of the glioma (16). Others have reported that overexpression of MMP-2 and MMP-9 associated with glioma malignancy was accompanied by overexpression of the TIMP-1 gene (24). In contrast, we found glioma progression to be associated with down-regulation of TIMP-1 and TIMP-2 (25), suggesting that an imbalance between expression and/or activity of MMPs and their specific inhibitors may be involved in tumor development. If the abundant expression of MMP-9 is responsible for the aggressive invasive behavior of glioblastomas, then down-regulation of MMP-9 could reduce the MMP-9-mediated invasiveness in glioblastomas.

Strategies such as expression of antisense RNA enable cell surface

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Fig. 3. **A**, confocal laser scanning images of SNB19 spheroids and rat brain aggregate cocultures scanned at a depth of 100 μm at 24 h. The rat brain aggregates (green fluorescence) and the parental or vector-transfected or antisense-transfected tumor cell spheroids (red fluorescence) are visible. **B**, quantitation of remaining fetal rat brain aggregates by tumor spheroids as described in “Materials and Methods.” Data shown are the mean ± SD values from four separate experiments for each group ($P < 0.001$). **C**, tumor formation by SNB19 or vector-transfected cells but not by antisense-transfected cells in nude mouse brain. Photomicrographs of brain sections stained with H&E show a tumor in the brain tissue of a mouse receiving intracerebral injection of SNB19 glioblastoma cells but show no tumor formation in mice receiving injection of the MMP-9 antisense-transfected cells. **D**, semiquantitation of tumor volume in parental, vector, sense, and antisense clones 4 weeks after intracranial injection of these cells as described in “Materials and Methods.” Data shown are the mean ± SD values from five animals from each group ($P < 0.001$).
events to be bypassed such that the antisense sequence can directly influence the expression of a given gene of interest. Expression of cDNA constructs resulting in antisense RNA provides a direct and unambiguous experimental approach for studying the involvement of MMP-9 in brain tumor progression. In the present study, we stably transfected the glioblastoma cell line SNB19 with an expression vector containing a 528-bp MMP-9 cDNA in the sense and antisense orientations. Our results showed that successful transfection of the SNB19 cells with MMP-9 antisense construct led to decreases in MMP-9 enzymatic activity, protein contents, and mRNA transcripts, suggesting that this antisense construct has profound biological consequences. Parental, vector-, and sense-transfected clones showed no changes in these variables, which indirectly confirms that the anti-sense construct was responsible for the observed decrease in transcripts and protein. The mechanism by which antisense RNA affects the expression of MMP-9 in antisense-transfected cells is unclear, but it may interfere with mRNA transport or hybridization with cytoplasmic MMP-9 mRNA, which presumably would interfere with translation.

Antisense-transfected MMP-9 clones showed reduced invasiveness in Matrigel and spheroid invasion assays, indicating that MMP-9 is required for invasiveness. Antisense-transfected MMP-9 clones were also unable to form tumors in nude mice, unlike parental cells or vector-transfected or sense-transfected clones. In a rat sarcoma model, MMP-9 expression was shown with ribozyme directed against MMP-9 mRNA to be involved in metastasis but not in tumor growth (26). The importance of MMP gene expression is underlined by the studies that demonstrated that synthetic MMP inhibitors limit tumor growth in animal models. Synthetic MMP inhibitors batimastat and marimastat effectively reduced glioma invasion in Matrigel-coated transwell assays and cocultures of tumor spheroids with fetal rat brain aggregates, although higher concentrations were required in coculture systems (27, 28). AG3340, another novel synthetic MMP inhibitor, inhibits the growth of the U87 glioma cell line s.c. implanted in SCID-NOD mice (29).

In summary, our stable transfection of an established human glioblastoma cell line with antisense MMP-9 cDNA produced stable antisense transfecants that were less invasive than their parental cells because of the inhibition of MMP-9 expression. Our results suggest that MMP-9 would be a promising target for gene therapy to reduce the invasiveness of human gliomas.

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References

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