Tissue Inhibitor of Metalloproteinase-3 Expression from an Oncolytic Adenovirus Inhibits Matrix Metalloproteinase Activity In vivo without Affecting Antitumor Efficacy in Malignant Glioma

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Abstract

Oncolytic adenoviruses exhibiting tumor-selective replication are promising anticancer agents. Insertion and expression of a transgene encoding tissue inhibitor of metalloproteinase-3 (TIMP-3), which has been reported to inhibit angiogenesis and tumor cell infiltration and induce apoptosis, may improve the antitumor activity of these agents. To assess the effects of TIMP-3 gene transfer to glioma cells, a replication-defective adenovirus encoding TIMP-3 (Ad.TIMP-3) was employed. Ad.TIMP-3 infection of a panel of glioma cell cultures decreased the proliferative capacity of these cells and induced morphologic changes characteristic for apoptosis. Next, a conditionally replicating adenovirus encoding TIMP-3 was constructed by inserting the TIMP-3 expression cassette into the E3 region of the adenoviral backbone containing a 24-bp deletion in E1A. This novel oncolytic adenovirus, AdΔ24TIMP-3, showed enhanced oncolytic activity on a panel of primary cell cultures and two glioma cell lines compared with the control oncolytic virus AdΔ24Luc. In vivo inhibition of matrix metalloproteinase (MMP) activity by AdΔ24TIMP-3 was shown in s.c. glioma xenografts. The functional activity of TIMP-3 was imaged noninvasively using a near-IR fluorescent MMP-2–activated probe. Tumoral MMP-2 activity was significantly reduced by 58% in the AdΔ24TIMP-3–treated tumors 24 hours after infection. A study into the therapeutic effects of combined oncolytic and antiproteolytic therapy was done in both a s.c. and an intracranial model for malignant glioma. Treatment of s.c. (U-87MG) or intracranial (U-87ΔEGFR) tumors with AdΔ24TIMP-3 and AdΔ24Luc both significantly inhibited tumor growth and prolonged survival compared with PBS-treated controls. However, expression of TIMP-3 in the context of AdΔ24 did not significantly affect the antitumor efficacy of this oncolytic agent. (Cancer Res 2005; 65(20): 9398-405)

Introduction

Oncolytic viruses are receiving widespread attention as novel therapeutic agents for the treatment of various malignancies (1, 2). Currently, further development of these agents includes combining the oncolytic effects of the virus with the added antitumor activity of a therapeutic transgene. Such “armed” therapeutic viruses carrying a variety of transgenes encoding prodrug-converting enzymes, immune stimulatory molecules, or apoptosis-enhancing proteins have already been shown to exhibit increased antitumor efficacy (3–12). However, contradictory evidence exists regarding the value of additional prodrug-converting enzymes or cytotoxic genes in the context of an oncolytic virus, as they may induce premature killing of the infected tumor cells, thereby limiting the production of progeny virus and counteracting virus-induced oncolysis (13–15). An alternative approach is to insert transgenes that exert their inhibitory effects on the tumor indirectly. This will ensure that sufficient viral replication and enzyme production is maintained.

Matrix metalloproteinases (MMP) offer an interesting target for such an approach. MMPs belong to a family of structurally related proteolytic enzymes that mediate degradation of components of the extracellular matrix and basement membrane. Moreover, they mediate the release of sequestered latent growth and angiogenic factors and the activation of latent growth factors (16). Up-regulation of MMP activity has been linked to tumor growth and invasion and tumor-associated angiogenesis.

Malignant gliomas constitute a relevant target for anti-MMP treatment. Their highly invasive and vascularized nature contributes to the poor prognosis of glioma patients. Immunohistochemical localization studies in surgical specimens have confirmed that MMPs are overexpressed in these tumors (17–19) and correlated to malignant progression (20, 21). Interference with these proteases might therefore inhibit local tumor dissemination and neovascularization.

Tissue inhibitors of metalloproteinases (TIMP) are a family of natural inhibitors that control the activity of MMPs and are often concomitantly expressed. Four members of this family have been characterized thus far (i.e., TIMP-1, TIMP-2, TIMP-3, and TIMP-4), which are expressed by a variety of cell types and can be found in most tissues throughout the body (22). In human gliomas, TIMP expression is reported to be downregulated with increasing malignancy, resulting in an imbalance between the production of MMPs and TIMPs and a shift toward a proproteolytic state with invasive phenotype (21, 23, 24). TIMP-3 is unique within the TIMP
family as it remains closely associated to the extracellular matrix after being secreted by the cell.

Overexpression of TIMP-3 has been shown to restrict invasiveness of various tumor cell types in vitro (25, 26). In addition, TIMP-3 overexpression showed inhibitory effects on tumor growth and angiogenesis in neuroblastoma, melanoma, and hepatocellular carcinoma tumor models (27–30). Moreover, evidence is accumulating for a role of TIMP-3 in inducing apoptosis in various cell types by stabilization of death receptors such as Fas on the cell surface (31–33). Taken together, these characteristics make TIMP-3 an interesting candidate as therapeutic transgene in the context of replication-competent, tumor-selective adenoviruses for the treatment of malignant glioma.

In this report, the effects of TIMP-3 overexpression on proliferation of low-passage primary and established glioma cells was studied. In addition, the in vitro and in vivo antitumor effects of concomitant adenoviral replication and TIMP-3 expression were studied using the novel tumor-selective adenovirus encoding TIMP-3, AdΔ24TIMP-3. We show for the first time that the effects of TIMP-3 expressed from an oncolytic virus can be directly imaged using an optical noninvasive in vivo imaging technique for MPP-2 activity.

Materials and Methods

Cell culture. The Ad5 E1-transformed human embryonal kidney cell line 293, the human lung carcinoma cell line A549, and the glioma cell lines U-87MG and U-251MG were purchased from the American Type Culture Collection (Manassas, VA). U-87MGEGFR was a generous gift of Dr. H.J. Su Huang (University of California at San Diego) and colo205 colon carcinoma cells were a kind gift of Dr. E. Boven (VU University Medical Center, Amsterdam, the Netherlands). This cell line was established by retroviral transfer of mutant epidermal growth factor receptor (EGFR) into the parental glioma cell line, enhancing its tumorigenic capacity (34). Primary glioma cell cultures were derived by mechanical dissociation from fresh tumor material collected during brain tumor surgery at the Department of Neurosurgery of the VU University Medical Center and the Academic Medical Center according to the method of Darling (35). Primary cell cultures are designated as VU or AMC and used before passage 8. All tumor samples included in this study were classified as astrocytoma grade 3 or glioblastoma multiforme. All cells were cultured in DMEM supplemented with 10% FCS and antibiotics (Life Technologies, Paisley, United Kingdom).

Recombinant adenoviruses. A recombinant E1-deleted, replication-deficient adenovirus expressing cytomegalovirus (CMV) immediate-early promoter–driven luciferase, AdLuc, was provided by Dr. R.D. Gerard (University of Texas Southwestern Medical Center, Dallas, TX). The replication-deficient adenoviral vector that expresses CMV promoter–driven enhanced green fluorescent protein (AdGFP) has been described previously (36). The replication-deficient adenoviral vector that expresses CMV promoter–driven TIMP-3 (AdTIMP-3) has been characterized previously (37) and the conditionally replicative adenovirus with an expression cassette for luciferase in the E3 region, AdΔ24Luc, has been described elsewhere (38).

To construct the conditionally replicative adenovirus with an expression cassette for TIMP-3 in the E3 region, the CMV-TIMP-3 expression cassette was released from an adenoviral shuttle vector encoding TIMP-3 under the CMV promoter pCMV-TIMP-3 by digestion with AvrII and BgiII. The 1.75-kb fragment was inserted into BamHII/XbaI-digested pAB8.4 (Microbis Biosystems, Toronto, Canada). The resulting construct, pABs-I-TIMP-3, was digested with PvuI and the 3.1-kb fragment carrying the CMV-TIMP-3 cassette and kanamycin resistance gene was inserted into PvuI-digested pHG11 (Microbis Biosystems). A clone with the CMV-TIMP-3 insert was isolated and the kanamycin resistance gene was removed by digestion with SvaI followed by self-ligation, yielding pHG11-TIMP-3. The conditionally replicative adenovirus was made by homologous recombination in 293 cells between the pXC1 (Microbis Biosystems) derivative pXC1-Δ24, which carries a 24-bp deletion corresponding to amino acids 122 to 129 in the CR2 domain of E1A necessary for binding to the Rb protein (ref. 39; a kind gift of Dr. R. Alemany, Gene Therapy Center, UAB, Birmingham, AL), with pHG11-TIMP-3, resulting in the conditionally replicative adenovirus AdΔ24TIMP-3 with the E1A CR2 mutation and the TIMP-3 expression cassette in E3.

Viruses were plaque purified, propagated on 293 cells for replication-deficient vectors or on A549 cells for replicative viruses, and purified by CsCl gradient according to standard techniques. The E1Δ24 mutation and CMV–TIMP-3 insertion were confirmed by PCR on the final products. Particle titers of all adenoviruses were determined by absorbance measurements at 260 nm, and functional titers (PFU) were determined by end point dilution titration on 293 cells according to standard techniques. Particle/PFU ratios were 4 for AdGFP, 12 for AdLuc, 30 for AdTIMP-3, 11 for AdΔ24Luc, and 15 for AdΔ24TIMP-3. In all experiments, infections were normalized based on PFU titers.

Proliferation assays. Primary and established glioma cells were plated subconfluently at 5 × 10⁴ cells per well in 96-well plates. Infection was done in triplicate in a multiplicity of infection (MOI) range of AdTIMP-3 or as control AdGFP to allow visualization of infection efficiency. Proliferation was quantified using the tetrazolium salt WST-1 (Roche Diagnostics, Mannheim, Germany) as described by the manufacturer. WST-1 assays were done on day 10 after infection on slow-growing primary cells and on day 6 after infection on the cell lines. Proliferation is expressed as a percentage of AdGFP-infected controls.

Hoehst staining. Hoehst staining was done on UV-135 primary glioma cells. Cells were cultured on glass slides and infected at MOI 300 with AdLuc or AdTIMP-3. On day 3 after infection, cells were fixed with 1% formaldehyde in PBS. Apoptotic cells were visualized by staining with 10 µg/mL Hoehst-33342 (Sigma-Aldrich, Zwijndrecht, the Netherlands) and fluorescent microscopy.

Cytotoxicity assays. Glioma cells were plated confluenty at 10⁴ cells per well in 96-well plates and infected in triplicate with a dose range of AdΔ24TIMP-3 or AdΔ24Luc. Viability was assessed by WST-1 assay at day 8 after infection for the cell lines and at day 12 after infection for the primary cell cultures. For coinfection experiments, U-87MGEGFR cells were plated at 10⁴ cells per well and infected with combinations of AdLuc with AdΔ24Luc or AdTIMP-3 with AdΔ24Luc. Viability was assessed daily by WST-1 assay for five consecutive days. Results are presented as percentage of uninfected controls.

Immunohistochemistry. Immunohistochemical staining for TIMP-3 protein was done on formaldehyde-fixed U-251MG cells. Furthermore, staining for TIMP-3, adenovirus, and vasculature was done on acetone-fixed cryosections obtained from U-87MG s.c. tumors and brains of U-87MGEGFR tumor-bearing mice. Briefly, immunohistochemical staining for TIMP-3 was done using the rabbit-anti-human TIMP-3 antibody AR802 (Chiron International, Temecula, CA); staining for vasculature was done using the rabbit anti-von Willebrand Factor antibody (DAKO, Glostrup, Denmark); and staining for adenovirus was done using the goat anti-adenoviral hexon protein antibody 1056 (Chemicon International). Bound primary antibodies were detected using horseradish peroxidase–conjugated goat anti-rabbit or rabbit-anti-goat antibodies and exposure to the chromogens 3-amino-9-ethylcarbazole or diaminobenzidine followed by counterstaining with Mayer’s hematoxylin. Negative controls were assessed by omitting the primary antibody. In addition, uninfected tumors were used as negative controls for hexon staining and colo205 colon carcinoma cells were used as a negative control for anti-TIMP-3 staining.

In vivo near-IR fluorescence imaging of matrix metalloproteinase-2 activity. For imaging of inhibitory activity of AdΔ24TIMP-3 on MMP-2 activity in vivo, 1 × 10⁶ U-87MGEGFR cells were injected s.c. into both mammary pads of adult female athymic mice (CD1 nude, Charles River, Wilmington, MA). Tumors were allowed to grow to 4- to 6-mm diameter upon which the right tumor of each of six animals was intratumorally injected with 3 × 10⁴ plaque-forming unit (pfu) AdΔ24TIMP-3 and the contralateral tumors received PBS injections. As an additional control, three animals were treated with the same dose of control virus, AdΔ24Luc, and PBS in contralateral tumors. After 24 hours, the imaging probe (2 nmol per animal) containing a preferential MMP-2 peptide substrate that was synthesized and characterized as described previously (40) was injected i.v. MMP-2
enzymatic activity results in cleavage of the substrate and conversion of the probe into a fluorescent product and strongly increases in near-IR fluorescence signal. The near-IR fluorescence signal was imaged 15 hours after i.v. administration. Near-IR fluorescence reflectance acquisition was obtained with 615- to 645-nm excitation and 680- to 720-nm emission using a previously described imaging system (41). After data acquisition, quantification and visualization of fluorescent signals were done with a home-written program with image display and analysis suite developed in IDL (Research Systems, Boulder, CO). Regions of interest were defined and the sum and SD of the white light surface images were fused with the corresponding white light surface images as a transparent pseudocolor overlay. Tumors were removed and frozen directly after imaging for histologic analysis.

In vivo adenovirus anti-glioma efficacy studies. For assessing the therapeutic effects of AdΔ24TIMP-3, studies were done in both s.c. and intracranial models for glioma. S.c. tumors were established on the flanks of adult female athymic nu/nu mice (Harlan, Horst, the Netherlands) by s.c. injection of 5 × 10⁶ U-87MG cells. When tumors reached an average of 150 to 175 mm³, mice were randomly divided over three groups of mice and received intratumoral injections on days 0, 2, and 4 of 3 × 10⁶ pfu AdΔ24Luc or AdΔ24TIMP-3 in 50 µL PBS. The third group received equal volumes of PBS injections. Tumors were measured thrice weekly with a digital caliper and tumor volumes were calculated from the average of tumor width and length according to the formula 4/3πr³. Mice were euthanized when the tumor reached a volume of 2,000 mm³. Tumor specimens were removed and frozen for histologic analysis. Intracranial glioma xenografts were established in adult female athymic mice (CD1 nude, Charles River) by stereotactic injection of 9 × 10⁴ U87EGFR cells in 3 µL Hanks buffer into the right frontal lobe, 2.5 mm lateral to the bregma at a depth of 3 mm. Injections were done under anesthesia by i.p. injection of 2 mg ketamine and 0.4 mg of xylazine in 0.9% saline. After 4 days, 2 × 10⁶ pfu AdΔ24TIMP-3 (n = 10) or AdΔ24Luc (n = 9) in 3 µL PBS was inoculated stereotactically into the same coordinates. Control animals (n = 9) received stereotactic inoculation of 3 µL PBS containing 3% glycerol that corresponds to the glycerol concentration in the virus dilutions. Animals were monitored daily and sacrificed upon appearance of symptoms evident for moribund decline due to intracerebral tumor growth, such as paralysis and lethargy. Brains were removed and frozen for histologic analysis.

Statistical analysis. Data from in vitro experiments are presented as means ± SD. Data from MMP-2 imaging study are shown in a box plot indicating 25th and 75th percentiles with lines indicating median and mean. Statistical analysis between groups was conducted with the two-tailed Student’s t test.

Tumor growth rates of s.c. tumors were determined from time of individual tumors to reach thrice initial tumor volume. Statistical significance between treatment groups and controls in their tumor growth rates was estimated by the two-tailed nonparametric Mann-Whitney test. For mice with intracerebral tumors, statistical significance of differences in survival between treatment groups and controls was assessed by the Wilcoxon test.

Results

Effects of tissue inhibitor of metalloproteinase-3 on proliferation of glioma cells. TIMP-3 has been reported to inhibit proliferation and induce apoptosis in various tumor cells (31, 32). To study these effects of TIMP-3 in malignant glioma cells, a panel of low-passage primary glioma cell cultures obtained from patient tumor material and three established glioma cell lines (U-251MG, U-87MG, and U-87EGFR) were infected with a concentration range of Ad.TIMP-3 or Ad.GFP. Proliferation, as measured by WST-1 assay, was assessed on day 10 after infection of slow-growing primary cells and day 6 after infection of cell lines. In Fig. 1A the effects of TIMP-3 overexpression on glioma cell proliferation are shown at the MOI that gave 80% to 100% infection. Ad.TIMP-3 infection significantly inhibited the proliferation of four of five primary glioma cell cultures and of all three cell lines compared with Ad.GFP-infected controls, with the inhibitory effect ranging from 19% to 71%. Hoechst staining in parallel cultures of U-135 showed the significantly increased presence of condensed nuclei, suggestive for apoptosis, in Ad.TIMP-3-infected cells compared with Ad.Luc controls (P = 0.013; Fig. 1B).

Characterization of tissue inhibitor of metalloproteinase-3–encoding oncolytic adenovirus. To study the combined effects of adenoviral oncolysis and TIMP-3 overexpression, a conditionally replicating adenovirus was constructed encoding TIMP-3 (AdΔ24TIMP-3). The adenoviral backbone AdΔ24 carries a mutation encoding a deletion of eight amino acids in the pHb-binding CR2 domain of E1A, which induces the tumor selectivity of the virus (39) and the TIMP-3 expression cassette was inserted into the E3 region. To visualize the expression of recombinant TIMP-3 expression in vitro, U-251MG cells were infected with Ad.TIMP-3 or AdΔ24TIMP-3 at MOI 50 and immunohistochemical staining for TIMP-3 was done 24 hours later. As depicted in Fig. 2A, infection with Ad.TIMP-3 led to detectable levels of TIMP-3 production in ~10% of cells. Infection with AdΔ24TIMP-3 led to higher levels of TIMP-3 expression in infected cells and rounding of the infected cells, a characteristic early cytopathic effect of the oncolytic virus.

Next, the oncolytic activity of AdΔ24TIMP-3 was compared with the control virus encoding luciferase, AdΔ24Luc, on a panel of...
glioma cells. Confluent cultures of three primary low-passage glioma cell cultures were infected with a concentration range of Ad\textsubscript{D\textsubscript{24}Luc} or Ad\textsubscript{D\textsubscript{24}TIMP-3} and viability was determined by WST-1 assay 12 days after infection. As shown in Fig. 2B, a modest increase in oncolytic activity was seen in Ad\textsubscript{D\textsubscript{24}TIMP-3}-infected cells compared with the control virus Ad\textsubscript{D\textsubscript{24}Luc}-infected cells in all three cell cultures tested. In addition, the efficacy of these oncolytic viruses was tested in two glioma cell lines, U-87MG and U-87\textsubscript{yEGFR}, which are frequently used for in vivo tumor growth (Fig. 2C). Similar to the results found in primary cell cultures, Ad\textsubscript{D\textsubscript{24}TIMP-3} was more potent than the control virus Ad\textsubscript{D\textsubscript{24}Luc} in both of these cell lines. In parallel cultures of Ad\textsubscript{D\textsubscript{24}Luc}- and Ad\textsubscript{D\textsubscript{24}TIMP-3}-infected U-87\textsubscript{yEGFR} cells, total virus production was determined by end point titration and showed that viral replication speed was not hampered by the concomitant TIMP-3 production during the lytic cycle (data not shown).

To verify that the superior oncolytic efficacy of Ad\textsubscript{D\textsubscript{24}TIMP-3} compared with Ad\textsubscript{D\textsubscript{24}Luc} resulted from TIMP-3 overexpression, a mix experiment was done using the replication-defective Ad\textsubscript{TIMP-3} in combination with replication-competent Ad\textsubscript{D\textsubscript{24}Luc}. U-87\textsubscript{yEGFR} cells were coinfected with Ad\textsubscript{TIMP-3} or the control vector Ad\textsubscript{Luc} at MOI 200, in combination with Ad\textsubscript{D\textsubscript{24}Luc} at MOI 2. Viability was determined by WST-1 assay on days 1, 2, 3, 4, and 5 after infection.

As an additional control, the individual effects of Ad\textsubscript{TIMP-3} and Ad\textsubscript{Luc} were also assessed. As shown in Fig. 2D, infection with Ad\textsubscript{TIMP-3} alone decreased viability up to 53% on day 5 after infection, whereas Ad\textsubscript{Luc} had no significant effect. Coinfection of Ad\textsubscript{TIMP-3} with Ad\textsubscript{D\textsubscript{24}Luc} led to significantly more cell kill than Ad\textsubscript{D\textsubscript{24}Luc} in combination with Ad\textsubscript{Luc} (76.7 ± 3.5% versus 24.3 ± 6.9% on day 5).

**In vivo imaging of tissue inhibitor of metalloproteinase-3 activity.** Using an optical noninvasive imaging technique for recording in vivo MMP-2 activity (40), we determined whether the exogenous TIMP-3 expression from Ad\textsubscript{D\textsubscript{24}TIMP-3} could reach sufficiently high production levels to inhibit endogenous MMP activity in tumors. Nude mice bearing bilateral s.c. U87\textsubscript{yEGFR} tumors between 4 and 6 mm in diameter received three injections of PBS into their left tumor and three injections of 10\textsuperscript{8} pfu Ad\textsubscript{D\textsubscript{24}TIMP-3} or 10\textsuperscript{8} pfu Ad\textsubscript{D\textsubscript{24}Luc} into their right tumor. After 24 hours, the MMP-2-sensitive near IR fluorescence probe (2 nmol/animal) was systemically injected. Early time point was chosen to exclude differences in tumor size resulting from viral oncolysis and because TIMP-3 is abundantly produced by the infected cells at this time point, as shown in vitro (Fig. 2A). The mice were imaged 15 hours after probe administration. Figure 3A shows the white light, near-IR fluorescence, and color-encoded images of...
representative examples of AdΔ24Luc- and AdΔ24TIMP-3-treated animals. Quantitative analysis was done by determining total fluorescence within regions of interest in all treated animals and results are summarized in Fig. 3B. As readily visible from the near-IR fluorescence images, there was significantly less MMP-2 near-IR fluorescence signal in AdΔ24TIMP-3-treated tumors compared with PBS-treated contralateral controls. The difference in MMP-2 near-IR fluorescence signal between these two groups was statistically significant and resulted in an average inhibition by AdΔ24TIMP-3 of 58.3 ± 13.5% (P = 0.007). No significant inhibition was seen in the AdΔ24LUC-treated tumors compared with PBS-treated contralateral controls (P = 0.34). Tumors were removed after imaging and tumor cryosections were stained for hexon and TIMP-3 (Fig. 3C). Widespread staining for hexon proteins was found in both AdΔ24LUC- and AdΔ24TIMP-3-infected tumors (top). Although endogenous TIMP-3 expression was detected in U-87MG tumors, a strong increase in TIMP-3 staining can be observed in the AdΔ24TIMP-3-infected tumors (bottom).

Therapeutic evaluation of AdΔ24TIMP-3. The therapeutic effects of AdΔ24TIMP-3 in vivo were assessed in both a s.c. and an intracranial xenograft model for malignant glioma. In the first experiment, established U-87MG s.c. xenografts were treated with a total dose of 9 x 10⁸ pfu AdΔ24LUC or AdΔ24TIMP-3 or with the same volume of PBS. Treatment with intratumoral injections of AdΔ24LUC or AdΔ24TIMP-3 both significantly suppressed tumor growth compared with PBS controls with a tumor growth delay of 7.1 days (P = 0.007) and 10.1 days (P = 0.001), respectively (Fig. 4A). Interestingly, in two of nine AdΔ24TIMP-3-treated tumors, complete regression and long-term survival was observed, whereas none of the AdΔ24LUC- or PBS-treated tumors regressed. However, mean tumor growth rate of AdΔ24TIMP-3-treated mice was not significantly reduced compared with AdΔ24LUC-treated mice (P = 0.31). Tumors removed on day 10 after infection for early time point analysis showed widespread areas of adenoviral replication as detected by anti-adenovirus hexon staining. Parallel sections stained for TIMP-3 showed high level TIMP-3 expression in infected cells (Fig. 4B). Staining of cryosections of AdΔ24LUC- and AdΔ24TIMP-3-infected U-87MG tumors removed at time of sacrifice for adenoviral hexon proteins and TIMP-3 displayed only very few hexon-positive cells per section and high levels of endogenous TIMP-3 expression (data not shown). Moreover, vWF staining for endothelial cells and quantification of vascular density showed no significant differences in vascularization among the three groups (data not shown). A second in vivo experiment was done in an intracranial brain tumor model in nude mice using U-87MGEGFR cells. These tumors overexpress mutant EGFR, which has been shown to result in a highly vascularized and more aggressive phenotype compared with U-87MG tumors. In addition, these tumors have been shown sensitive to antiangiogenic treatment (42, 43). Mice were treated with intratumoral injection of PBS or 2 x 10⁹ pfu AdΔ24LUC or AdΔ24TIMP-3. As shown in Fig. 4C, treatment with AdΔ24LUC or AdΔ24TIMP-3 both significantly

Figure 3. A, in vivo near-IR fluorescence imaging of MMP-2 activity in U-87MGEGFR tumor-bearing mice treated with AdΔ24LUC (top row) or AdΔ24TIMP-3 (bottom row). Tumors (encircled with dotted lines) were injected with PBS or virus (arrows) 39 hours before imaging. White-light images (left), raw image acquisition of near-IR fluorescence signal obtained at 700-nm emission (middle), and the color-coded near-IR fluorescence signal superimposed onto white-light images (right).

B, quantitative image analysis of tumoral near-IR fluorescence signals are shown in a box plot indicating 25th and 75th percentiles. Solid line, median; dashed line, mean fluorescence as percentage of PBS controls. *, P < 0.01 compared with AdΔ24LUC-infected controls.

C, immunohistochemical staining for adenoviral hexon proteins and TIMP-3 expression in AdΔ24LUC- and AdΔ24TIMP-3-infected U-87MGEGFR tumors excised after imaging. Original magnification, ×20.
prolonged survival of tumor-bearing mice compared with PBS-treated mice, which all perished by day 18 (P < 0.005). Moreover, treatment with AdΔ24Luc or AdΔ24TIMP-3 resulted in 33% and 20% long-term survivors (up to day 120), respectively. However, no significant difference in survival was found between the two viral treatments (P = 0.78). Staining of cryosections of U87EGFR tumors removed at time of sacrifice for TIMP-3 and vWF showed a high level of endogenous TIMP-3 expression and the highly vascularized nature of these tumors (Fig. 4D). No change in TIMP-3 expression was observed in the AdΔ24Luc- or AdΔ24TIMP-3-treated tumors. Moreover, quantitative analysis of vessel density showed no significant difference in vessel density between AdΔ24Luc- or AdΔ24TIMP-3-treated tumors (data not shown). Analysis of cryosections of brains of long-term survivors showed the presence of a small tumor remnant (~1 × 2 mm) with extensive calcification. Stainings for TIMP-3 and adenovirus hexon were negative (data not shown) and staining for vWF showed the presence of few remaining vessels surrounding the tumor remnant (Fig. 4D).

Discussion

Overexpression of TIMP-3 in tumor cells has been shown to suppress tumor growth in experimental models for melanoma and neuroblastoma in squamous cell, breast, and colon carcinoma (27, 29, 44, 45). The proposed mechanisms by which these effects are achieved include the ability of TIMP-3 to induce apoptosis directly (32), by inhibition of tumor angiogenesis (28), and by its ability to inhibit remodeling of tumor extracellular matrix thereby preventing tumor growth and expansion as well as invasion and metastasis (25, 26). In the present study, we have examined the effect of TIMP-3 overexpression on patient-derived primary glioma samples and three established glioma cell lines. In addition, we studied the effects of TIMP-3 in the context of the tumor selective oncolytic adenovirus AdΔ24, allowing virus-induced oncolysis to be combined with local TIMP-3 treatment in in vitro and in vivo models of malignant glioma.

Our data show that overexpression of TIMP-3 inhibited the proliferative potential of three cell lines (36-71%) and four of five low-passage primary glioma cell cultures (19-54%) and induced morphologic changes suggestive for apoptosis. These results are in accordance with previous studies, which have suggested that TIMP-3 can induce apoptotic cell death and inhibition of monolayer cell growth in various tumor cell lines (25, 26, 45, 46). Based on these results as well as the reported inhibitory effects of TIMP-3 on angiogenesis (27, 28, 44, 47), we proceeded to construct a conditionally replicating adenovirus encoding TIMP-3. With this novel oncolytic adenovirus, AdΔ24TIMP-3, the combined effects of oncolytic viral therapy and local TIMP-3 production could be studied. AdΔ24TIMP-3 showed enhanced oncolytic activity on a panel of primary glioma cell cultures and glioma cell lines when compared with the control virus AdΔ24Luc. In vivo functional activity of TIMP-3 expressed in the context of a replicative virus was shown using a novel optical imaging method using an MMP-2-sensitive probe, which produces a near-IR fluorescent signal (40). Although established U-87EGFR
tumors express endogenous TIMP-3, as noted by immunohistochemical staining for TIMP-3, intratumoral injection of AdΔ24TIMP-3 strongly reduced the MMP-2 activity in these tumors. Herewith, we were able to show that functional transgene expression from a replicative adenovirus can reach sufficiently high levels to achieve a biological effect that can be imaged noninvasively. To assess whether this inhibition would lead to antitumor effects in vivo, AdΔ24TIMP-3 and AdΔ24Luc were tested in both a s.c. and an intracranial mouse xenograft model for malignant glioma. In both models, treatment with these oncolytic viruses significantly inhibited tumor growth and prolonged survival of these animals compared with PBS-treated controls. In the s.c. U-87MG tumors, a trend toward enhanced antitumor efficacy of AdΔ24TIMP-3 compared with AdΔ24Luc was observed; however, the mean tumor growth rates of AdΔ24Luc- and AdΔ24TIMP-3-treated tumors did not differ significantly. Furthermore, no significant difference in the survival rate of mice bearing intracranial U87MG tumors treated with the luciferase- or TIMP-3-encoding viruses was found.

Various issues can be considered to explain this lack of in vivo efficacy. First, the question arises whether exogenous TIMP-3 production in the context of a replicating adenovirus is maintained sufficiently long enough in vivo to achieve a therapeutic effect. Parallel studies in mice bearing intracranial U-87MG tumors injected with AdΔ24Luc showed strong expression of luciferase at 24 hours and a gradual, up to 30-fold, decrease during the course of 15 days after infection.7 A longer follow-up could not be done due to animals becoming symptomatic. However, these results do suggest that transgene expression from an oncolytic adenovirus is not limited to a single round of replication of the virus. Whether the TIMP-3 production is maintained high enough to significantly affect tumoral and stromal MMP activity during the time span of the in vivo experiments remains unresolved.

Second, reported studies describing the effects of TIMP-3 gene delivery on s.c. tumor growth were done by ex vivo infection of tumor cells before implantation (28, 44, 45) or by injection of TIMP-3 expressing vectors into small established tumors (25-100 mm³); ref. 29). The s.c. tumors in our study were treated at a mean volume of 150 to 175 mm³, at which time the tumors may be less susceptible to TIMP-3 treatment. Indeed, Spurbeck et al. showed that small established tumors (50 mm³) were susceptible to retroviral TIMP-3 treatment, whereas large established tumors (500 mm³) were not (27). It remained unclear, however, whether this was due to lack of efficient tumor cell transduction in the large tumors or the fact that larger, older tumors have already established a mature neovasculature that is not reversed by TIMP-3 treatment. The latter seems more likely as others have reported tumor size dependent effects of (synthetic) MMP inhibitors as well (48).

Third, treatment of small intracranial tumors by intratumoral injection of the oncolytic viruses improved survival but also failed to show additional antitumor activity of TIMP-3. Contrary to s.c. models for malignant glioma, effective treatment of intracranial tumors by MMP inhibitors has only been reported scarcely (49). Moreover, clinical experience with monotherapy of synthetic MMP inhibitors such as Marimastat, Metastat, and Prinomastat in glioma patients has showed little to no tumor response in these studies (50–52). This suggest that although MMPs are up-regulated in malignant gliomas and correlate with their malignant progression (18, 21, 53), the complexity of the MMP system and redundancy in proteolytic enzyme cascades may play a role in circumventing the effects of MMP inhibitors such as TIMP-3. Moreover, in vitro and s.c. tumor models may not accurately predict the outcome of anti-MMP treatment in intracranial tumors due to differences in microenvironment such as type of extracellular matrix and specific characteristics of the endothelial cells in the brain.

Finally, the lack of enhanced in vivo efficacy of TIMP-3 insertion into the AdΔ24 backbone may reside in the fact that the viral oncolysis itself already produces a very potent antitumor effect, which is not significantly affected by additional indirect effects on the tumor. This becomes particularly evident when considering the fact that treatment with an oncolytic adenovirus, with or without TIMP-3, cures up to 33% of mice bearing intracranial U-87MG gliomas, an enhanced tumorigenic and notoriously difficult to treat tumor (34). Among the various treatments studied in this tumor model (42, 43, 54, 55), only one report described the occurrence of cures (up to 20%) after combined gene and radiation therapy (56). Therefore, the impressive antitumor effect achieved after a single injection of our oncolytic adenoviruses in these tumors may be difficult to enhance by combining with the relatively modest antitumor effects that TIMP-3 may confer. Such effects are perhaps more readily detected using tumor models of slow-growing invasive phenotypes.

Overall, we found that TIMP-3 gene transfer was capable of inducing apoptosis in primary glioma cells in vitro. Although expression of TIMP-3 in the context of a conditionally replicating adenovirus also led to enhanced cell killing of primary and clonal human glioma cells in vitro, these properties did not result in significantly greater antitumor activity when combined with adenoviral oncolysis in vivo models for malignant glioma. Nevertheless, our imaging data do show that AdΔ24TIMP-3-induced TIMP-3 expression can reach sufficiently high levels to inhibit in vivo tumoral MMP activity, indicating that AdΔ24TIMP-3 may be of potential interest in more invasive/metastatic tumor models.

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