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Original Articles

MT1-MMP silencing by an shRNA-armed glioma-targeted conditionally replicative adenovirus (CRAd) improves its anti-glioma efficacy *in vitro* and *in vivo*

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ABSTRACT

MMP14 (MT1-MMP) is a cell membrane-associated proteinase of the extracellular matrix, whose biological roles vary from angiogenesis to cell proliferation and survival. We recently found a direct correlation between MMP14 expression levels in brain tumors of glioma patients and the disease progression. By using gene silencing as an experimental approach we found that MMP14 knockdown decreases production of pro-angiogenic factors such as VEGF and IL8 and thereby suppresses angiogenesis in glioma tumors. Although the clinical relevance of MMP14 down-regulation and its possible implications for glioma therapy in humans remain unclear, we observed a significant improvement in animal survival upon down-regulation of MMP14 in murine intracranial glioma xenografts infected with MMP14 shRNA-expressing CRAd. We further found that down-regulation of MMP14 in gliomas by combinational treatment with CRAd-S-5/3 and Marimastat, a chemical inhibitor of metalloproteinases, augments suppression of pro-angiogenic factors, caused by the replication-competent adenovirus. We also demonstrated that delivery of MMP14-targeting shRNA by a fiber-modified adenoviral vector to the glioma cells effectively suppresses their proliferation *in vitro* and *in vivo*. Thus our data indicate that inhibition of MMP14 expression in tumors in combination with glioma virotherapy could be effectively utilized to suppress angiogenesis and neovascularization of glioma tumors by decreasing production of pro-angiogenic factors.

Introduction

Each year approximately 250,000 people worldwide are diagnosed with brain cancer. Only ~20% of all brain tumors detected at the time of diagnosis are of primary origin, whereas ~80% of them are metastatic. Glioblastoma multiforme (GBM) represents a major form of the disease [1], being among the most prevalent and aggressive primary brain tumors. Although surgical resection, chemotherapy and radiosurgery can prolong GBM patients' survival, the overall prognosis for this disease remains quite dismal [2]. This warrants development of novel clinically-effective approaches for GBM treatment.

To develop effective anti-glioma treatment strategies it is critical not only to understand the molecular basis for glioma tumorigenesis but also to identify regulatory genes, promoting cancer cell resistance to conventional treatment modalities. Genetic analyses of GBM specimens revealed up-regulation of numerous genes, suggesting their involvement in tumorigenesis [3].

One of those genes encodes metalloproteinase type 14 (MMP14, MT1-MMP), a highly conserved extracellular matrix protein essential for critical physiological functions such as angiogenesis, tissue remodeling and wound healing [4]. Under pathological conditions expression of MMP14 can serve as an indicator of aberrant angiogenesis [5] and cancer progression [6,7]. Since previous studies from our [8] and other laboratories [9,10] suggested involvement of MMP14 in cellular mechanisms regulating cancer cell survival, our goal in this study was to specifically down-regulate MMP14 expression in glioma cells by using gene therapy approaches and assess the effect of MMP14 knockdown on signaling in the cancer cells and their ability to survive chemotherapy and form tumor in the brain.

Preclinical testing indicates that tumor cells can attenuate clinical effects of anticancer therapies through activation of internal

Abbreviations: CRAd, conditionally replicated adenovirus; MMP14, metalloproteinase type 14; GBM, glioblastoma; CD46, cluster of differentiation 46.

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cellular programs such as autophagy or by inducing production of pro-angiogenic factors and chemokines. Anti-angiogenic gene therapy has emerged as a unique tumor targeting approach based on suppression of tumor angiogenesis by therapeutic transgenes, delivered and expressed in the context of recombinant viral vectors. Human adenovirus type 5 (Ad5)-based vectors are top candidates for gene delivery since, on the one hand, they have a natural ability to efficiently infect a broad range of target cells and, on the other, can be easily and efficiently propagated *in vitro* to high titers. Abundance of CD46, DSG2 and various integrins on the surface of glioma tumor cells offers a unique opportunity for efficient and highly selective transduction of those cells by Ad group B-based vectors. Experimental evidence from our [11] and other laboratories [12,13] suggests that retargeting of Ad5 to alternate receptors can be effectively used to improve transduction efficiency of various cancer cells including glioma cells. Previously we utilized the promoter element of the survivin gene to improve glioma targeting specificity of oncolytic Ad vectors on the transcriptional level [14]. We also demonstrated that combination of transductional targeting with survivin promoter-controlled tumor-selective replication in oncolytic Ad5 vectors results in robust anti-glioma effects.

Some observations suggest that activation of angiogenesis could be an integral part of the adaptive stress response of cancer cells to viral infection and replication [15,16]. Several anti-angiogenic molecules have been utilized to augment the therapeutic effect of oncolytic vectors by suppressing tumor angiogenesis [17].

In this study we investigated the effect of MMP14 knockdown in combination with glioma virotherapy by using a double-modified cancer-targeted oncolytic agent CRAd-S-5/3. Our results suggest that treatment of glioma tumors with MMP14-silencing shRNA (small hairpin RNA)-encoding construct, delivered to the tumor cells by the oncolytic CRAd agent as an expression vector, improves the CRAd-mediated cytotoxicity both *in vitro* and *in vivo* and offers a new potential treatment option for GBM.

Materials and methods

Reagents

Marimastat, obtained from Sigma-Aldrich (St. Louis, MO), was dissolved in DMSO to a stock concentration of 10 mM and stored at –20 °C. The anti-MMP14 antibodies were obtained from Abcam (ab38971), CD11b antibodies (#NB110-89474AF488) were obtained from Novus Bio, OLIG2 (#GTX62440) from GeneTex; CD45 (clone 2B11 + PD7/26) from Dako Cytomation and GAPDH-specific primary antibodies (sc25778) from Santa Cruz Biotechnology.

Cell lines

The U251 and U87 human glioma cell lines were obtained from ATCC (Manassas, VA). Each cell line was grown in DMEM (Life Technologies, Inc., Rockville, MD) supplemented with 10% FBS and maintained at 37 °C in 5% CO_2 atmosphere. The primary human glioma cells obtained from patients were established at the Swedish Neuroscience Institute. Primary cells were maintained in stem cell conditioned media (NSA) that contain B27 and N2 supplements as well as 1% penicillin–streptomycin mixture. These cells were initiated from primary GBM tissue and used for experiments at passages 3–5.

Primary GBM specimens

Primary paraffin embedded specimens, diagnosed as grade IV were obtained from RONC and US Biomax Inc (Rockville, MD, USA). Pathological information and molecular subtyping are described in Table 1.

Table 1
Clinical information for primary GBM samples used in the paper.

1	Age of diagnosis	Gender	Overall survival	Molecular subtype
GBM0	66	M	220	Proneural
GBM1	53	M	317	Classical
GBM2	63	M	310	Classic–mesenchymal
GBM3	61	F	415	Mesenchymal

Knockdown of MMP14 using shRNAs

U87 and U251 cells stably-transfected with MMP14shRNA construct were described previously [8]. The pGFP-V-RS plasmids encoding non-effective "scrambled" shRNA (N13, catalog #TR30013), MMP14 shRNA (clone 73, CUACCACAAGGA CUUUGCCUCUGAAGGCC), MMP14 shRNA5 (clone76, ACUCUGCCGAGCCUUGG ACUGUCAGGAAU), and MMP14 shRNA4 (clone 74, CAGUUCGCCGACUAAGCAG AAGAAAGAUC) were obtained from (Origene, MD). Target cells were transfected according to the protocol provided by the vendor. U87 and U251 cells stably-expressing MMP14-targeting (sh4 or sh5) or non-effective control (Scrambled) shRNAs were seeded at 50% confluence, incubated overnight, and total RNA or proteins were extracted for real time PCR or western blotting.

Recombinant adenoviral vector construction

The following replication-competent adenoviral vectors were used in the current study: CRAd-S-5/3, CRAd-S-5/3shMMP14 and CRAd-S-5/3shScrambled. The CRAd-S-5/3 vector was described before [11]. The genome of the conditionally replicative adenoviral vector CRAd-S-5/3shMMP14, encoding U6-promoter driven shRNA against human MMP14, was constructed by a two-step homologous recombination (HR) in *Escherichia coli* (BJ5183 strain) [18,19], using a previously described Ad5 backbone vector Ad5Δ*E1*/Δ*E3*/ΔF [20] and the *E1* shuttle vector pScs/PA/S with the wild type *E1* genes placed under control of the Survivin gene promoter to render Ad5 replication selective for cancer cells including glioma [21,22].

First, an intermediate construct Ad5Δ*E1*/Δ*E3*:U6Pr-shRNA MMP14, F5/3 was generated by HR between the backbone vector Ad5Δ*E1*/Δ*E3*/ΔF and the Pac I-linearized shuttle vector pShuttle-ΔE3-ADP-KanF,U6Pr-MMP14shRNA,F5/3, harboring deletion of the *E3* genes except for part of the 12.5K gene and the entire 11.6K ADP gene of the *E3A* region. This latter shuttle was derived from pShuttle-ΔE3-ADP-Kan, F5/3 by cloning the U6Pr-MMP14 shRNA cassette downstream of ADP in place of deleted *E3* genes following its PCR-amplification from the pGFP-V-RS-U6Pr-MMP14shRNA plasmid as a template. The following forward and reverse PCR primers were used for amplification: 5′GGAGCGTCGACCGCGCGCCGAGCGCGCGCCAAG3′ (forward); 5′ACAGGGTCGACAAGCTTGATCTTTCTTCTGCTTAGT3′ (reverse 1) and 5′GACA CACATTCCACAGGGTCGACAAGCTT3′ (reverse 2). The latter reverse primer was designed to overlap with the reverse 1 primer and introduce Sal I cloning site into the PCR product at its 3′ terminus. The forward PCR primer introduced the other (upstream) Sal I site. After Sal I digestion the PCR product was subcloned into the Sal I site of the pShuttle-ΔE3-ADP-Kan, F5/3 vector downstream of the Swa I site-flanked kanamycin (Kan) gene. The latter vector was a derivative of the previously described vector pShuttle-ΔE3-ADP-KanF Ono et al. [23], where Ad5 fiber knob sequence was replaced with the Ad3 fiber counterpart (Borovjagin, unpublished).

The recombinant clones were screened using two sequential PCR-assays detecting Ad5 hexon gene-specific sequences followed by detection of the 3′ knob sequences of the F5/3 fiber chimera. The presence of the Kan gene in the intermediate backbone plasmid allowed selection of recombinants on two antibiotics: Amp (present in the Ad5Δ*E1*/Δ*E*, F5/3 backbone) and Kan (transferred from the E3 shuttle). Subsequently Kan was removed by Swa I digestion and self-ligation of the vector.

Following HR of the Ad5Δ*E1*/Δ*E3*: U6Pr-MMP14shRNA, F5/3 with Pme I-linearized *E1* shuttle pSlSurvivin*E1*, pIX-mCherry and colony selection on Kan (Amp replaced by Kan as a result of HR) the *E1* recombinants were screened by PCR assays testing for the presence of sequences specific for each of the vectors. The accuracy of HR was verified by restriction digest of the selected recombinant Ad genomes with Hind III and Nhe I restriction enzymes and comparison to the theoretically predicted restriction patterns derived from the virtual recombinant vector maps using the NEBcutter V2.0 software (New England Biolabs, Ipswich, MA). Miniprep plasmid DNA isolated from positive BJ clones was re-transformed in stable strain STBL4 or DH5-alpha, and following colony re-screening and plasmid up-scaling, linearized with *Pac I* and transfected into the HEK-293 helper cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

Generation and molecular validation of CRAds

All viruses were generated and plaque-purified in *E1*-complementing HEK293 cells according to standard procedures. All CRAds were propagated in A549 cells, purified by double cesium chloride (CsCl) ultracentrifugation (AdEASY system; QBiogene) and dialyzed against buffer containing 10 mM Tris–HCl, pH 8.0, 50 mM NaCl, 2 mM $MgCl_2$ and 10% (v/v) glycerol. Viral particle titer for the purified preparations of each virus was determined by absorbance at 260 nm, using a conversion factor: 1 $A_{260} = 1.1 \times 10^{12}$ vp/ml. Infectious titers were determined by using Adeno titer X kit (Clontech). Genomic identity of each virus was verified by conventional PCR assays. The lack of contamination with the wild type *E1* sequence-containing genomes (referred to as RCA) in virus preparations was confirmed by an in-house developed PCR assay (Borovjagin, unpublished). The PCR primer sequences used in those assays are available on request.

Bioinformatic analyses of Rembrandt, TCGA and ncomine databases

The levels of MMP14 expression in normal vs brain tumor cells, NPC vs GM, and the relationship between MMP14 expression and brain tumor patient survival were