Carcinogenesis vol.26 no.12 pp.2069–2077, 2005
doi:10.1093/carcin/bgi183
Advance Access publication July 20, 2005

The chemokine stromal cell derived factor-1 (CXCL12) promotes glioma invasiveness through MT2-matrix metalloproteinase

Jing Zhang, Susobhan Sarkar and V.Wee Yong*

Department of Oncology and Department of Clinical Neurosciences, Hotchkiss Brain Institute, University of Calgary, Calgary, Alberta, Canada

*To whom correspondence should be addressed at: 3330 Hospital Drive, Calgary, Alberta T2N 4N1, Canada. Tel: +1 403 220 3544; Fax: +1 403 293 8731;
Email: vyong@ucalgary.ca

Chemokines have been found to alter tumor growth and metastasis. We have described previously that a particular chemokine receptor, CXCR4, was predominantly expressed on various glioma cell lines and in resected glioblastoma specimens. Herein, we have tested the ligand of CXCR4, stromal cell derived factor-1α (SDF-1α, CXCL12), on the response of human glioma cells. We found that SDF-1α increased the expression of membrane type-2 matrix metalloproteinase (MT2-MMP), but not the other MT-MMPs, MMP-2 or MMP-9. The SDF-1α enhanced MT2-MMP expression was blocked by a CXCR4 antagonist, AMD3100. Functional invasion assays showed that SDF-1α stimulated glioma cells to invade through matrigel-coated chambers and this effect was inhibited in glioma cells by the stable downregulation of MT2-MMP expression using small interfering RNA (siRNA). *In vivo* and at asymptomatic stages following intracerebral implant of cells, mice harboring MT2-MMP siRNA downregulated clones had smaller and less invasive tumors compared with mice implanted with non-specific siRNA control cells. Analyses at symptomatic stages demonstrate that mice with MT2-MMP siRNA clones survive longer than mice harboring control cells. These results highlight MT2-MMP as an effector of CXCR4 signaling in glioma cells, and they reveal the novel role of MT2-MMP in modulating tumor activity.

Introduction

Malignant gliomas are the most common primary brain tumors in humans, accounting for >50% of neoplasms that arise within the central nervous system (CNS). A major pathophysiological feature of malignant gliomas is their ability to diffusely invade into surrounding brain tissues. This dissemination of tumor cells throughout the CNS, leading to new growth foci, confers these tumors incurable by surgical removal even when combined with radiation and chemotherapies. The invasive process of glioma cells involves the attachment of tumor cells to extracellular matrix (ECM) components, degradation of the ECM, and subsequent cell penetration into adjacent brain structures. This process is accomplished in part by tumor-secreted matrix metalloproteinases (MMPs) that degrade the ECM at tumor fronts to remove barriers (1–3).

Abbreviations: MT-MMP, membrane type matrix metalloproteinase; SDF-1α, stromal cell derived factor-1α; siRNA, small interfering RNA.

The MMPs represent a large family of proteolytic enzymes that play key roles in cancer progression, proliferation, angiogenesis and tumor metastasis. Gelatinase A and B (MMP-2 and MMP-9, respectively) have been well studied in glioma malignancy, particularly because these enzymes are easy to measure by gelatin zymography. In this regard, a large body of literature reports that MMP-2 and -9 are upregulated in glioma specimens *in vitro* and *in situ* (3–7).

The membrane-type MMPs (MT-MMPs) constitute six members of the MMP family that are anchored to cell surfaces either through a transmembrane domain or a glycosylphosphatidylinositol linkage. MT-MMPs are upregulated in many tumor types including gliomas (8–13). Functionally, MT1-MMP (MMP-14) has been noted to promote cell invasion in tumor types such as melanoma (14,15), breast cancers (16) and gastric carcinoma (17,18).

In recent investigations, we determined that although many tumor types contain high levels of MT-MMPs, these were further elevated in glioma cell lines compared with carcinomas; indeed, there were positive correlations between glioma grade and the mRNA levels of MT1-MMP, type-2 matrix metalloproteinase MT2-MMP (MMP-15) and MT6-MMP (MMP-25) (13). Nonetheless, how these MT-MMPs are regulated in glioma cells, or their functional roles, remain unclear.

Chemokines are a large family of small, secreted chemotactic cytokines which play critical roles in many normal and pathophysiological processes, such as infections and autoimmune diseases. Chemokines are also implicated in the pathogenesis of a variety of tumors (19–21). Several chemokines have been described on glioma cells, including macrophage chemoattractant protein-1 (CCL2) (22) and interleukin-8 (CXCL8) (23). To address which chemokine may play particularly important roles in glioma characteristics, we analyzed glioma cell lines for specific receptors that are commonly expressed across a majority of glioma lines. In this regard, we described the predominance of CXCR4 and showed that its ligand, CXCL12 (stromal cell derived factor-1, SDF-1), is a survival and chemotatic factor for glioma cells in culture (24). While the Akt/protein kinase B pathway was activated in glioma cells and may mediate survival of cells consequent to SDF-1 stimulation, the mechanisms of SDF-1–CXCR4 interaction in glioma that lead to the altered motility of cells remain unclear.

In this study, we have investigated the mechanisms consequent to SDF-1–CXCR4 interactions that mediate glioma invasiveness. We report that SDF-1α stimulated the invasiveness of CXCR4-bearing glioma cells, and also increased MT2-MMP expression at both mRNA and protein levels of glioma cells; other MT-MMPs analyzed were unaffected. By specifically downregulating MT2-MMP expression using small interfering RNA (siRNA), the basal or SDF-1α-enhanced invading capacity of glioma cells was inhibited *in vitro*. Furthermore, clones underexpressing MT2-MMP formed smaller sized and less invasive tumors in nude mice. These

Downloaded from https://academic.oup.com/carcin/article/26/12/2069/2390749 by guest on 20 August 2022

 © Oxford University Press 2005; all rights reserved.

results demonstrate that MT2-MMP lies downstream of SDF-1α in glioma biology, and they reveal a previously unknown role of MT2-MMP in glioma activity.

Materials and methods

Glioma cell culture and resected glioma specimens

LN827 and U373 are established human glioma cell lines that were used in most experiments in this study; in some cases, LN215, LN308 and LN992 were also utilized. These cell lines were chosen based on their expression of CXCR4 transcripts as determined previously (24). All lines were maintained in medium containing 10% fetal calf serum (FCS) (24), which was also the medium used during *in vitro* experiments. Six resected glioma specimens were from patients with glioblastoma multiforme, and histological confirmation of the grade of malignancy was obtained by sampling sections from these tumors (24). We were not informed of the treatment history or clinical presentation (e.g. recurrent tumor) of these patients, in order to respect privacy laws. Non-transformed brain tissues were from patients with epilepsy and these were normal appearing tissues that were resected in order to access and remove epileptogenic foci.

Real time quantitative PCR

For RNA isolation, cells at 80% confluency in 6-well plates, treated with recombinant SDF-1α (R&D system) or saline vehicle, were lysed in 1 ml TRIzol (Invitrogen, Burlington, Ontario). RNA was isolated following the manufacturer's instruction and was resuspended in 30 μl RNaseOUT (Invitrogen) water. Trace DNA was removed by DNase digestion at 37°C for 45 min. Two microgram total RNA samples were reverse-transcribed with random primers (Roche, Indianapolis, IN), SuperScript II (Invitrogen) and reverse transcription (RT) buffer at 37°C for 2 h. Five microlitres of RT product were used for 25 μl PCR using *Taq* Polymerase (Invitrogen) and SYBRgreen detect system (BioRad). The primers are as follows: MT1-MMP; forward, 5′-TGC AGC AGT ATG GCT ACC TG-3′ and reverse, 5′-TAG CGC TTC CTT CGA ACA TT-3′; MT2-MMP: forward, 5′-ATG CGT TCC GCC CAG AT-3′ and reverse, 5′-GCC GCT TCA TCC ACT CCT T-3′; MT3-MMP: forward, 5′-ATG ATT TAC AGG GCA TCC AGA AA-3′ and reverse, 5′-TGG AGG CCG AGG AGG TTT-3′; MT5-MMP: forward, 5′-CCA GTA CAT GGA GAC GCA CAA-3′ and reverse, TGC GGA CGG GGA GTG T-3′; vascular endothelial cell growth factor (VEGF): forward, 5′-AAG GAG GAG GGC AGA ATC AT-3′ and reverse, 5′-ATC TGC ATG GTG ATG TTG GA-3′; and hepatocyte growth factor (HGF): forward, 5′-CTT AAA GCC TTG CCA ACA GC-3′ and reverse, 5′-GCC TAG CCA TGC TCT TTC AC-3′.

Flow cytometry

Cells at 80% confluence in 100 mm dishes were detached with 2 ml of Versene (Invitrogen) for 10 min and were washed with cold Hanks' balanced salt solution twice. Cells were then incubated with a monoclonal anti-human MT2-MMP antibody directly conjugated to phycoerythrin (R&D systems) for 45 min on ice in the dark. The cells were washed, fixed and analyzed by flow cytometry.

Western blot

To determine the total cellular content of MT2-MMP, glioma cells were treated with SDF-1α (100 ng/ml) for 24 h. Cell lysates were then generated in lysis buffer (24), collected, freeze-thawed twice, and spun at 13 000 r.p.m. at

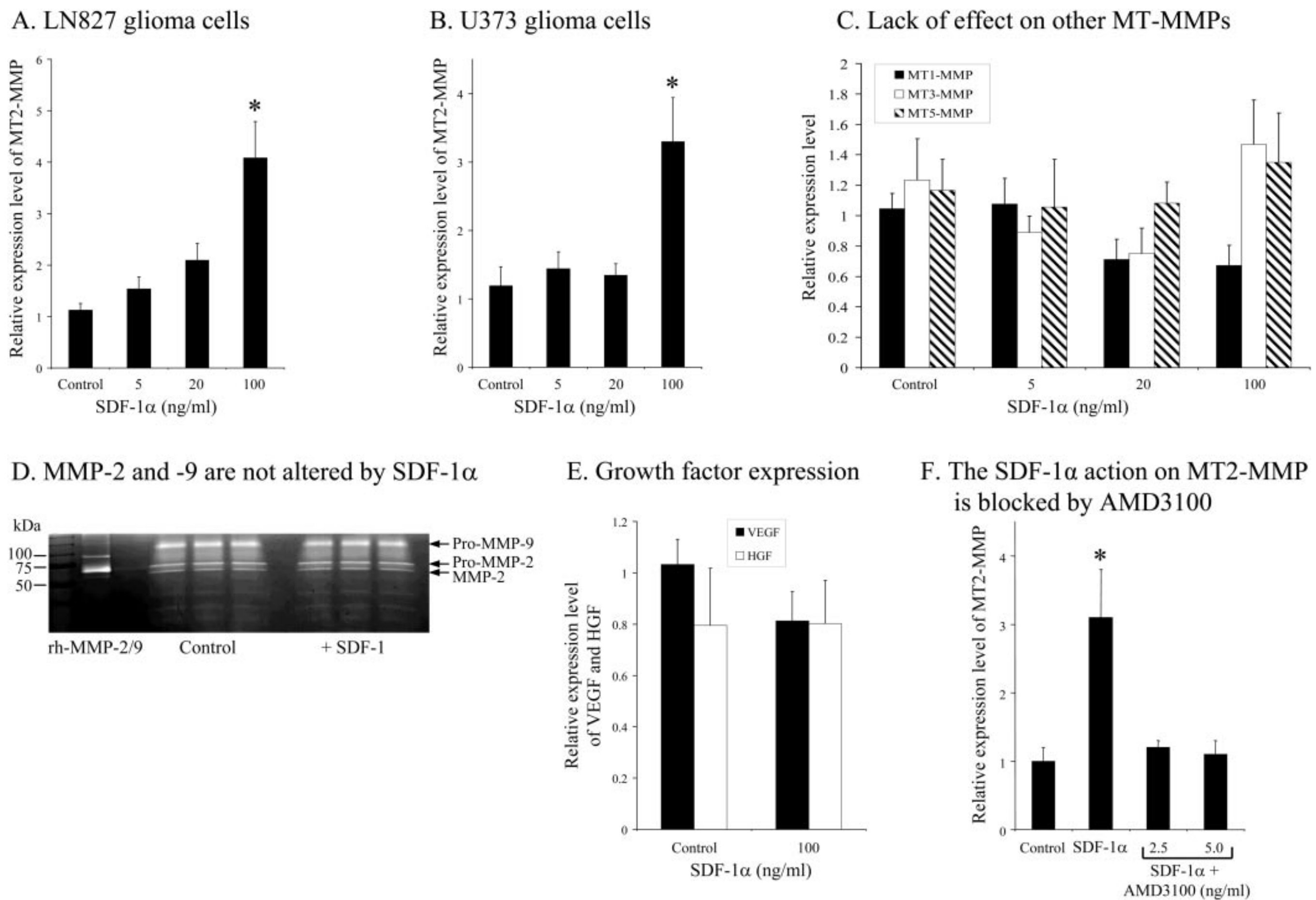

Fig. 1. The treatment of glioma cells with SDF-1α selectively increased MT2-MMP transcripts. LN827 (**A**) and U373 (**B**) human glioma lines increased their expression of MT2-MMP when treated with 100 ng/ml SDF-1α for 24 h. In contrast, transcripts for MT1-, MT3- and MT5-MMP (**C**), or protein levels for MMP-2 and -9 (**D**), did not vary following SDF-1α treatment; the data shown are for LN827 cells but similar data were also found for the U373 line. Similarly, VEGF and HGF transcripts did not alter in response to SDF-1α in the LN827 line (**E**). The SDF-1α upregulation of MT2-MMP in LN827 cells was blocked by a CXCR4 receptor antagonist, AMD3100 (**F**); $P < 0.05$ comparing between the two concentrations of AMD3100 group versus SDF-1α only. Values are mean ± SEM of 8–24 samples (A–C, E), or six samples (F). The zymography for MMP-2 and -9 (D) was performed on triplicate samples of the LN827 cell line, with or without 100 ng/ml SDF-1α; a recombinant human (rh) MMP-2 and -9 mixture was used as a standard. $^{*}P < 0.01$ compared with controls (1 way ANOVA with Tukey's *post hoc* comparisons).

Downloaded from https://academic.oup.com/carcin/article/26/12/2069/2390749 by guest on 20 August 2022

4°C for 10 min (Beckmann JA21). The total protein concentration was determined using the Bio-Rad protein assay. Fifty micrograms of total protein were loaded into each lane of 10% SDS–PAGE gels. Proteins were transferred onto polyvinylidene difluoride membranes (Immobilon, Millipore) for 2 h at 250 mA at 4°C. Membranes were then blocked in phosphate-buffered saline (PBS) containing 0.1% of Tween-20 and 10% skim milk for 30 min. A monoclonal mouse anti-human MT2-MMP antibody (R&D systems) (1:1000) was applied overnight at 4°C followed by goat anti-mouse IgG for 1 h at room temperature. All antibodies were diluted in PBS containing 0.1% Tween-20 and 3% milk. ECL (Amersham Biosciences) was used for immunodetection.

Gelatin zymography

To detect MMP-2 and -9, gelatin zymography of the conditioned medium of cells was performed as described previously (24).

RNA interference

Plasmid psiRNA-hH1neo (InvitroGen, San Diego, CA) was used to express small hairpin RNA (shRNA) targeting human MT2-MMP. Two 21 oligonucleotides from different coding areas of full length human MT2-MMP cDNA were chosen to design inverted insert separated by a short spacer region of 7 nt (TCAAGAG). The sequences are as follows: Oligo A (262–274): 5′-AAG GAG GCC GAC ATC ATG GTA-3′; Oligo B (1250–1271): 5′-ACG CAG CCT ACA CCT ACT TCT-3′; negative control: 5′-AAG ATC GAC TTA CGA CGT TAG-3′. The insert was cloned into a BbsI site, which is downstream of the H1 promoter, a RNA polymerase III promoter. All the constructs were confirmed by DNA sequencing before transfection. As non-specific siRNA control for the MT2-MMP siRNA sequence, a pair of siRNA that did not conform to any sequences in GeneBank was also utilized.

For transfection, glioma cells seeded in 6-well plates (2×10^5 cells/well) and were transfected by Lipofectamine (Invitrogen). G418 (500 μg/ml, Calbiochem) was added to the cells 48 h after transfection to select for positive clones.

Cell invasion assay

Human glioma cell invasion was evaluated using matrigel coated boyden chamber with 8 μm pore size (BD Biosciences, Two Oak Park, Bedford, MA). Matrigel was diluted with cold DMEM (1:3) and then gelled in 24-well insert by incubating at 37°C for at least 1 h. A 0.5 ml aliquot of glioma cell suspension at 5×10^4 cells/ml in 2.5% FCS-containing glioma medium was seeded on the upper chamber of each well, while 1 ml of 10% FCS-containing glioma medium was added to the lower chamber; the higher serum content in the lower chamber acted as a chemotactic gradient. Cells were left to invade for 20 h. Non-invading cells that remained on the upper surface of the filter were removed by a cotton swab, and cells that remained adherent to the underside of membrane were fixed in 95% ethanol/5% acetic acid for 15 min followed by hematoxylin staining for 10 min. The membrane was then cut off, mounted and the number of migrated cells was counted using a microscope. Six contiguous fields through the equatorial axis viewed using a 40× objective were examined to obtain a representative number of cells in each set.

Cell growth curve

Ten thousand glioma cells were seeded into each well of 24-well plates. At 1, 2, 3 and 4 days, adherent cells were detached by 0.25% trypsin, collected and total cell numbers were counted using a Z2 Coulter Counter. Cell numbers were plotted against the time points, giving rise to the growth curve of cells.

Implantation of tumor cells: assessment of survival of recipients and tumor size

Two MT2-MMP downregulated LN827 cell clones (clone 1 and 2), wild-type (wt) LN827 cells, and non-specific siRNA control cells were trypsinized, washed in PBS once and resuspended in PBS at a concentration of 2.5×10^7/ml. Four microlitres (1×10^5 cells) of each preparation were injected stereotaxically into the right striatum of each anesthetized nude mouse (*n* of six per group per experiment). Animals were then sutured and returned to their cages and allowed free access to food and water. Animals were weighed every other day and any symptoms of neurological deficit were recorded. The occurrence of death was recorded so as to obtain data for the Kaplan–Meier survival curve. In accordance with ethical practice, mice that were moribund (negligible limb movement and loss of body wt >20%) were also considered dead and were killed. Two survival curves, representing two separate experiments conducted at different times, were tabulated (Figure 6E and F).

In another set of experiments, six mice each of the non-specific siRNA control group or implanted with MT2-MMP downregulated clones were killed 21 days after implantation to address tumor growth in the brain prior to the appearance of symptoms.

To assess tumor growth in the brain, the whole brain was removed from mice at sacrifice, cut into defined blocks, fixed in formalin and embedded in paraffin. Six micrometer sections were taken every 200 μm apart through the entire brain. The sections were then stained with hematoxylin and eosin, dehydrated through graded series of alcohol and xylene, and mounted with Acrotol. The presence of tumor was determined by the hyper-cellularity of tissue, and by the darker blue staining of tumor cells in hematoxylin and eosin-treated slides (Figure 6). To measure tumor size, at least 16 stained sections from a single animal were evaluated qualitatively and the section containing the largest tumor was then subjected to ImagePro analysis; here, the entire boundary of the tumor in that section was traced out and the area thus computed. It is not possible to determine the volume of the tumor across multiple sections per animal since the shape of the tumor was not uniform across these sections.

Statistics

The one-way ANOVA test was used to compare multiple group data while the unpaired Student's *t*-test was used to compare two groups of data. $P < 0.05$ was considered as statistically significant.

Results

Selective increase of MT2-MMP following SDF-1α simulation of glioma cells

We tested the responsiveness of the LN827 and U373 glioma cell lines to SDF-1α. Figure 1 demonstrates that SDF-1α

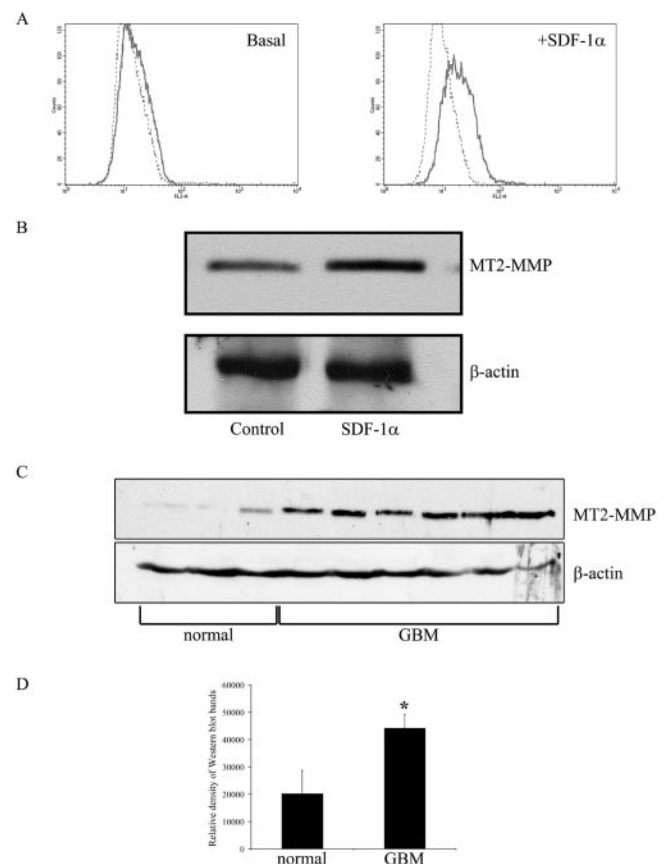

Fig. 2. The protein expression of MT2-MMP is elevated through SDF-1α treatment *in vitro* and in resected gliobastoma specimens. (**A**) The flow cytometry profile of LN827 cells under basal condition or when stimulated with 100 ng/ml SDF-1α is displayed. The dotted line in each flow panel represents staining using an isotype control while the fully stained cells in each case are represented by solid lines. (**B**) Total cellular content using western blot analyses show that MT2-MMP (72 kDa) is elevated in LN827 glioma line in response to 100 ng/ml SDF-1α compared with control; β-actin was used to normalize between samples. (**C**) The increased expression of MT2-MMP is also evident in resected glioma specimens as western blot analyses show that MT2-MMP expression was detected in three non-transformed specimens and this was elevated in six gliobastoma multiforme (GBM) specimens. Normalizing for actin, the group means confirmed the quantitative increase of MT2-MMP in glioblastoma compared with controls (**D**). $^*P < 0.05$. See online Supplementary material for a colour version of this figure.

Downloaded from https://academic.oup.com/carcin/article/26/12/2069/2390749 by guest on 20 August 2022

increased the expression of transcripts encoding MT2-MMP in both lines. This occurred at 100 ng/ml but not at lower concentrations, in agreement with our previous observations that high concentrations of this chemokine are required to produce responses in glioma lines (24).

In contrast to MT2-MMP, other MT-MMP transcripts measured were not altered by SDF-1α. This was the case for MT1, MT3 and MT5-MMP (Figure 1C); we could not reliably assess MT4 and MT6-MMP in these cells due to technical difficulties.

Further selectivity of SDF-1α for MT2-MMP was demonstrated by the inability of this chemokine to alter the protein levels of MMP-2 and -9 (Figure 1D), which are commonly studied in glioma biology as noted earlier (3–7), and of two growth factors that are of significance to glioma cells: VEGF and HGF (Figure 1E).

Thus, of various transcripts investigated, SDF-1α selectively increased MT2-MMP in glioma cells. The presence of the CXCR4 antagonist, AMD3100 (AnorMed, British Columbia, Canada), added 1 h before SDF-1α, abolished this effect, suggesting that the SDF-1α enhanced MT2-MMP transcript is through the CXCR4 receptor (Figure 1F).

To determine whether the effect on MT2-MMP transcripts was also reflected in alteration of protein content, and because MT2-MMP would act extracellularly, we analyzed the cell surface expression of MT2-MMP on glioma cells using flow cytometry. Figure 2A reveals that MT2-MMP on the surface of glioma cells was increased following SDF-1α application. Total cellular content of MT2-MMP was also upregulated by SDF-1α as assessed using western blot analysis (Figure 2B).

In correspondence with cell lines in culture, resected glioma specimens also demonstrated significant elevations of MT2-MMP when compared with non-transformed brain samples (Figure 2C and D).

SDF-1α stimulates invasiveness of glioma cells in vitro through MT2-MMP

In matrigel invasion assays, SDF-1α increased the capacity of glioma cells to invade and this was the case for both lines tested, LN827 and U373 (Figure 3).

To evaluate whether SDF-1 and MT2-MMP were related functionally for invasive capacity, stable clones with downregulated expression of MT2-MMP at the level of transcripts (Figure 4A) and protein (Figure 4B) were generated for the LN827 line using RNA interference. The siRNA to MT2-MMP did not modulate the expression of other MT-MMPs evaluated (Figure 4C), or of the gelatinases, MMP-2 and -9 (Figure 4D). The MT2-MMP siRNA clones grew normally and growth curves over 4 days *in vitro* matched those of control cells with non-specific siRNA (Figure 4E).

The capacity of MT2-MMP downregulated clones to invade across matrigel was investigated. Compared with non-specific siRNA control cells, MT2-MMP siRNA clones 1 and 2 both had decreased capacity to invade under basal conditions (Figure 5A). Furthermore, when stimulated with SDF-1α, whereby control cells increased their invasiveness, MT2-MMP downregulated clones 1 and 2 did not (Figure 5B).

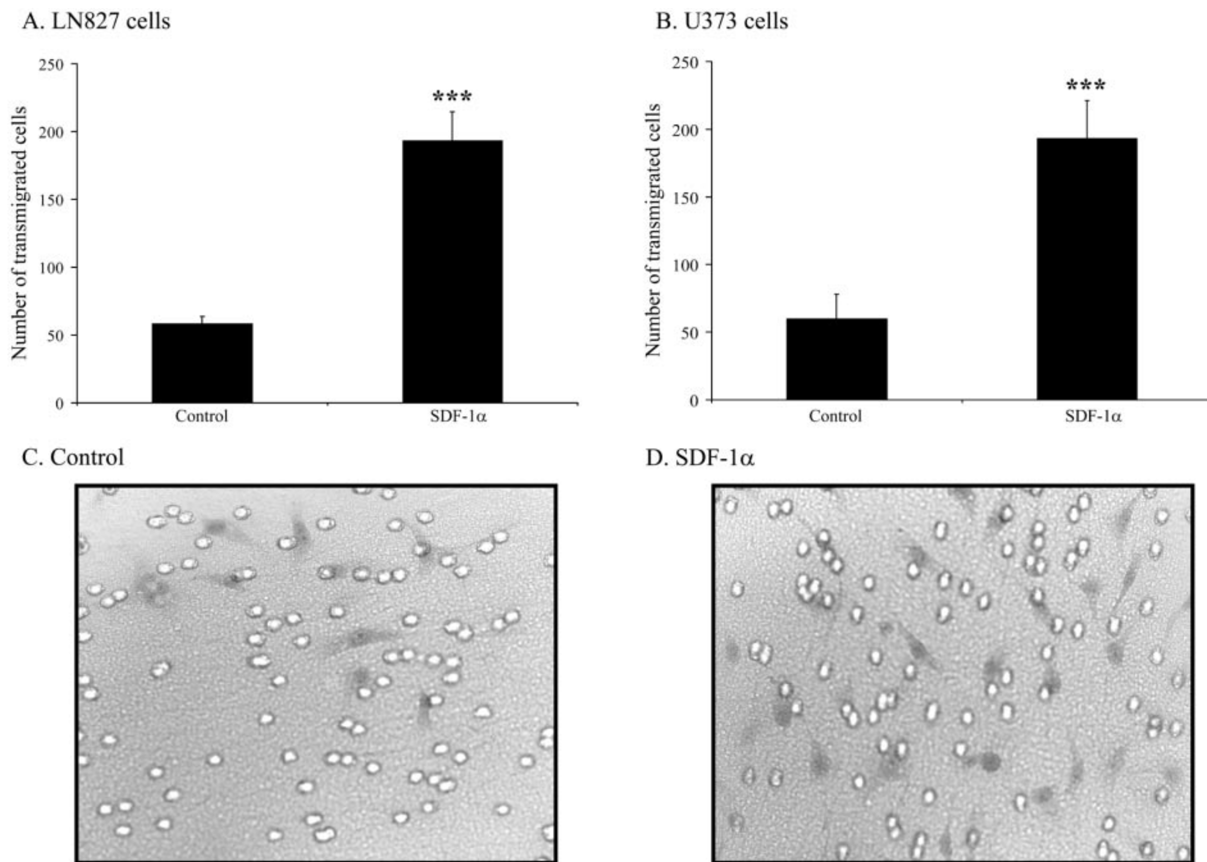

Fig. 3. The invasiveness of glioma cells *in vitro* is increased by SDF-1α. (**A** and **B**) represent the increased invasive capacity of the LN827 and U373 lines, respectively, when treated with 100 ng/ml SDF-1α for 24 h. The pictorial representation of LN827 glioma cells that have invaded across matrigel to the other side of the filter is depicted in **C** (control) and **D** (+ SDF-1α). $^{***}P < 0.001$ compared with controls.

Downloaded from https://academic.oup.com/carcin/article/26/12/2069/2390749 by guest on 20 August 2022

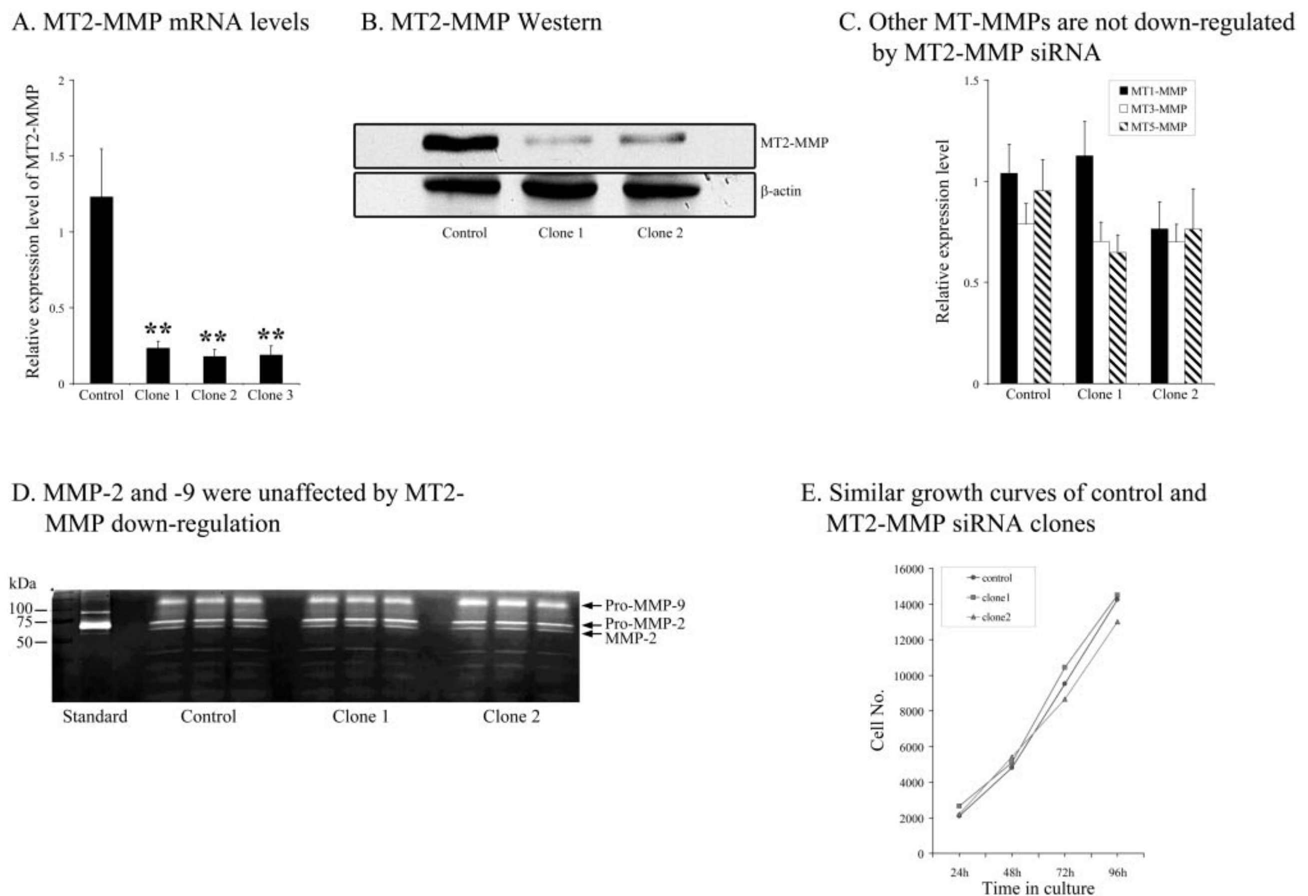

Fig. 4. siRNA to MT2-MMP decreased MT2-MMP expression but not that of other MT-MMPs. The downregulation of MT2-MMP is evident in LN827 clones at the level of transcript (**A**) and protein (**B**). $^{**}P < 0.01$. These clones did not show any reduction in transcripts for MT1-, MT3- and MT5-MMPs (**C**, *n* of six each, ANOVA, $P > 0.05$ comparing between control, clone 1 and 2 for each MT-MMP) in PCR analyses, or of MMP-2 and -9 protein (**D**). In D, the conditioned medium of triplicate cultures of LN827 control or clones was analyzed by gelatin zymography. MT2-MMP downregulation also did not alter growth characteristics as the number of cells collected at 24–96 h after plating did not differ between control and siRNA clones (**E**). In all cases, controls refer to non-specific siRNA controls. See online Supplementary material for a colour version of this figure.

Collectively, these results suggest that the effect of SDF-1α in promoting glioma invasiveness was through an MT2-MMP mediated mechanism. Furthermore, under basal conditions, the invasiveness of glioma cells was also decreased in cells that had low expression of MT2-MMP.

MT2-MMP siRNA clones have reduced tumorigenicity in vivo

We implanted the LN827 glioma cells into the striatum of nude mice. One batch of mice was killed 21 days after implantation, before animals were symptomatic and before any weight loss was evident. Using hematoxylin and eosin stain, tumors were detected in all animals examined (*n* of six non-specific siRNA controls, and *n* of six mice given MT2-MMP siRNA clone 2). The nature of the tumors growing intracerebrally was qualitatively different between control non-specific siRNA cells and clone 2 cells. While the control glioma cells had invasive fingers which arborized from the main tumor mass in several directions (Figure 6A and C), the MT2-MMP siRNA cells grew as a discrete mass with well-delineated borders (Figure 6B and D).

In the mice killed at 21 days after implantation, tumor size was generally smaller in the brain of animals implanted with the MT2-MMP siRNA clones. Measurement of maximal tumor area in sections from six brains implanted with non-specific siRNA control cells revealed a size of 0.50 ± 0.09 mm^2 (mean ± SEM); in contrast, this was 0.31 ± 0.08 in clone 2 implanted brains (*n* of six mice), representing a decrease in tumor size of 40%.

Mice were also monitored for survival in response to the implanted cells. Figure 6E shows that while non-specific siRNA control cells produced death in mice (6/6) within 40 days of implantation, animals implanted with MT2-MMP siRNA clones 1 and 2 (*n* of six in each group) had a longer survival period.

In a second survival experiment (Figure 6F), when all mice in the non-specific siRNA control group were dead at 45 days of implantation, six of six mice given MT2-MMP siRNA clone 2 were still alive at day 58, when the experiment was terminated.

In both survival experiments, we did not determine tumor size at the end-points of death from tumor since the brains generally had large tumor mass in all groups that accounted for their death.

In summary, siRNA downregulation of MT2-MMP in the LN827 line rendered the glioma cells with less evidence of invasive capacity *in vivo*, and survival of mice was prolonged.

Discussion

There is substantial evidence that the family of MMPs is important for various aspects of glioma activity. In particular, the gelatinase MMP members (MMP-2 and -9) have received

Downloaded from https://academic.oup.com/carcin/article/26/12/2069/2390749 by guest on 20 August 2022

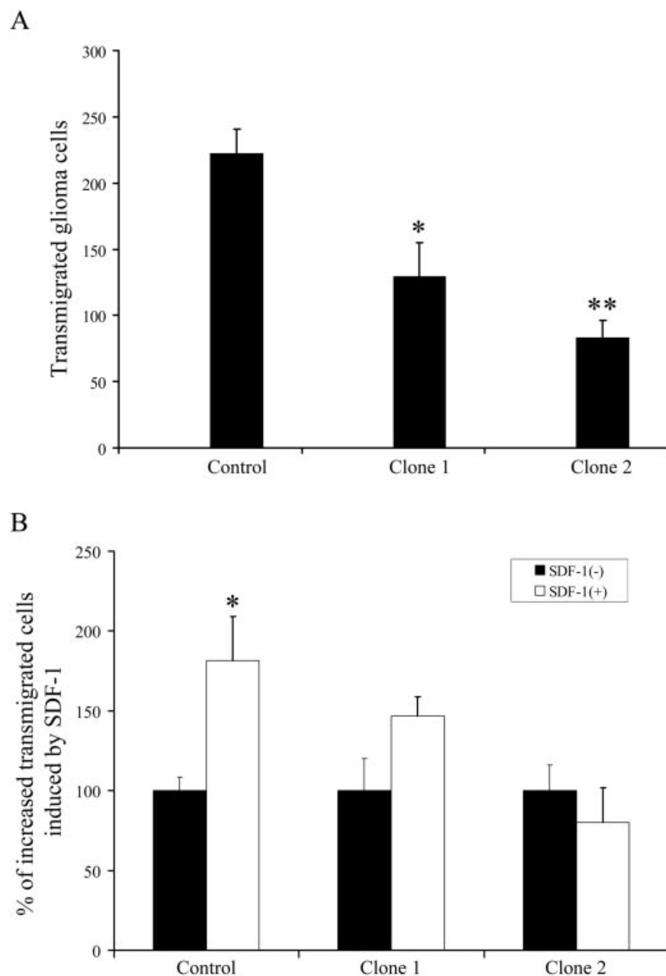

Fig. 5. MT2-MMP siRNA clones have decreased invasive capacity in basal and SDF-1-stimulated conditions. (**A**) Invasive capacity under basal conditions. (**B**) Invasion across matrigel when LN827 clones are activated by SDF-1α. While non-specific siRNA transfected control cells enhanced their invasiveness in response to SDF-1α, this did not occur in MT2-MMP downregulated cells. $^{*}P < 0.05$; $^{**}P < 0.01$ compared with control cells.

attention and their levels are upregulated in glioma tissues compared with normal samples; glioma cell lines express high amounts of MMP-2 and -9 that correlate with several properties of their increased tumorigenicity (3,7,25,26). Functionally, inhibitors of metalloproteinase activity or the downregulation of MMP-2 and -9 by transfections (27) reduce the invasiveness of glioma cell lines *in vitro* (4,27–29) and they attenuate the growth of glioma cells implanted into the flank (30) or brain of mice (29). Impressively, parenterally administered inhibitors of metalloproteinase activity reduce the spread and size of intracranial gliomas in rodents (31–33). Tumor cells are less likely to grow in the brain of MMP-2 deficient mice (34).

In clinical trials in humans, inhibitors of metalloproteinase activity may have an impact, albeit small, on the response of patients (35,36). Nonetheless, in a systematic review of clinical trials of various small molecule inhibitors to oncogenic and survival pathways in gliomas, it was noted that the combination of temozolomide with marimastat, an MMP inhibitor, provided the best outcome in Phase II trials (37). This is encouraging and it is possible that the efficacy of anti-metalloproteinase inhibition will improve in glioma patients if one could identify all the MMP members that mediate the invasiveness of glioma cells, the major cause of recurrrence and morbidity of gliomas. In previous work, we and others have noted that gliomas express very high levels of several MT-MMPs (8–13). Functional information, however, has largely been presented only for MT1-MMP in gliomas. In this regard, glioma cells secrete MT1-MMP that helps them migrate on myelin (38), and the overexpression of MT1-MMP in glioma cells increased motility through a mechanism that involved cell surface tissue transglutaminase (39). MT1-MMP may also mediate the angiogenesis that is facilitated in glioma–endothelial cell co-culture (40). Furthermore, a mechanism for epidermal growth factor receptor-mediated invasiveness of glioma cells appears to be through MT1-MMP induction (41).

In the current examination, we have uncovered a hitherto unknown role for MT2-MMP in glioma activity. We found that MT2-MMP downregulation decreases the basal rate of invasiveness of glioma cells across matrigel, and that clones stably underexpressing MT2-MMP grew less well in the brains of nude mice and did not confer death to animals to the same extent as control cells (Figure 6). This was evident in two experiments (Figure 6E and F) and, although the lack of pathogenicity of clone 2 MT2-MMP siRNA cells was more pronounced in the second set (F) than in an earlier series (E), the trend was similar towards a longer survival time in MT2-MMP siRNA clones compared with non-specific siRNA controls. It would be of interest to evaluate whether CXCR4 or SDF-1α expression is altered *in vivo* in the brains of these transfectants. Overall, the specific inhibition of MT2-MMP may improve the targeting of glioma cells in future trials.

Significantly, we found that the regulation of MT2-MMP in responsive lines was through another system important in glioma biology, the chemokines. While originally described in the context of directing leukocyte trafficking, chemokines are now known to have important growth and tumor promoting activity (19–21). A pivotal observation has been the finding that the CXCR4 receptor and its ligand, SDF-1α, play a major role in guiding metastatic breast cancer cells to the lung (20). The interaction of CXCR4–SDF-1α is now documented to be important in at least 23 tumor types (21). Attention in glioma cells has also focused on SDF-1α since the CXCR4 receptor is commonly expressed in glioma specimens (24), where its levels may be regulated by cytokines (42). Indeed, SDF-1 and CXCR4 have been found co-localized in human glioblastoma tissues, in regions of angiogenesis, and a role for this system in angiogenesis has been proposed (43,44). *In vitro*, SDF-1α induces proliferation in a dose–dependent manner in glioma cell lines (45). Thus, the current results that SDF-1α regulates MT2-MMP expression, but which is blocked by AMD3100, to mediate glioma invasiveness emphasize the importance of inhibiting the CXCR4 receptor that we have documented to be commonly expressed in glioma lines (24). Along this line, the systemic administration of the AMD3100, inhibits the growth of U87 glioblastoma xenografts implanted intracranially in mice (46).

We note that the SDF-1α enhanced expression of MT2-MMP was not a universal phenomenon of all glioma lines tested. While this occurred in the LN827 and U373 lines (Figure 1), and also in the LN215 line, no upregulation was seen in the LN308 or LN992 lines (data not shown). All these lines have CXCR4 transcripts (24) but it is possible that the CXCR4 signaling cascade is not coupled to the receptor in non-responsive lines.

Downloaded from https://academic.oup.com/carcin/article/26/12/2069/2390749 by guest on 20 August 2022

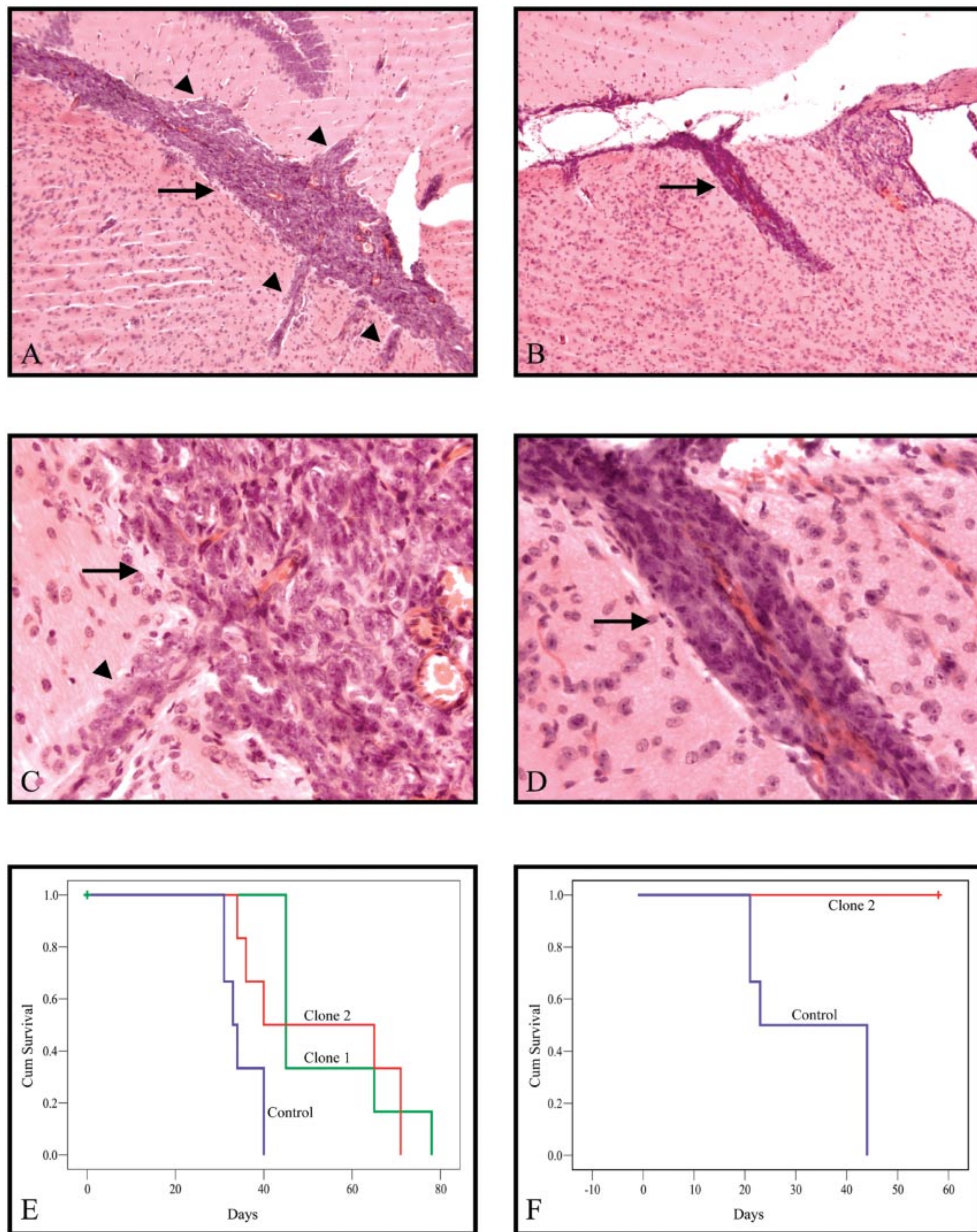

Fig. 6. Reduced tumorigenicity of MT2-MMP siRNA LN827 clones *in vivo*. In mice killed at 21 days, before manifestation of symptoms, the brain of non-specific RNA control cells reveals tumors (arrow) that were large and with several invasive fingers (arrowheads) (**A**, 100× original magnification; **C**, 400× original magnification). In contrast, MT2-MMP siRNA tumors were smaller and had well-delineated uniform borders indicative of less invasive capacity (**B**, 100× original magnification; **D**, 400× original magnification). Survival curves indicate a longer survival period in mice implanted with MT2-MMP siRNA clones 1 and 2 compared with non-specific siRNA controls (*n* of six each) (**E**); the non-specific siRNA cells did not differ from wild-type LN827 cells in causing demise to mice (data not shown). Finally, in a second set of experiments where all six mice implanted with non-specific siRNA control cells were dead at 45 days, six of six mice given MT2-MMP siRNA clone 2 were still alive at termination of experiment (day 58) (**F**).

Our previous work using 20 and 50 ng/ml SDF-1α (24) did not result in SDF-1α increasing the invasiveness of glioma cells across matrigel. In the current experiment, 100 ng/ml of this chemokine had to be used to effect invasion across matrigel (Figure 4), and this is correspondent with the finding that 100 ng/ml is required to increase MT2-MMP expression (Figure 1).

While SDF-1α increased the expression of MT2-MMP, it did not alter the expression of the other MT-MMP members examined. It is important to note that the more commonly studied MT1-MMP was unaffected by SDF-1α treatment. The reason for this is not immediately apparent but can be the subject of future investigations. It is tempting to speculate that dependent on the environment, glioma cells will upregulate

Downloaded from https://academic.oup.com/carcin/article/26/12/2069/2390749 by guest on 20 August 2022

different MMP members to help achieve their growth. We found recently that glioma cells exploit their astrocyte environment for the production and activation of MMP-2, and that the consequence of this glioma–astrocyte interaction is the increased ability of glioma cells to invade (47). In another context, when glioma cells interact with the extracellular matrix protein, tenascin, they upregulate selectively MMP-12 (S. Sarkar and V. W. Yong, unpublished data). In response to an environment of SDF-1α, which is produced by astrocytes, neurons and endothelial cells *in vivo* (44,48,49), the current results suggest that MT2-MMP will be upregulated to facilitate the invasive and growth characteristics of gliomas.

In summary, we have demonstrated that the SDF-1α–CXCR4 interaction leads to the selective increase of MT2-MMP which mediates the invasiveness of glioma cells *in vitro* and glioma growth *in vivo*. The mechanism by which SDF-1α elevates MT2-MMP remains to be addressed. Our results link two major families of molecules implicated in glioma biology, the chemokines and the MMPs, and they demonstrate a novel role for MT2-MMP in facilitating glioma invasiveness and growth. The results also emphasize that the targeting of SDF-1α or its receptor, CXCR4, or the manipulation of MT2-MMP activity, may improve the prognosis of patients with malignant gliomas.

Acknowledgements

This study was supported by an operating grant from the Canadian Institutes of Health Research. J.Z. was supported by a fellowship from the Alberta Cancer Foundation. We thank Tammy Wilson, Yan Fan and Fiona Yong for skilled technical assistance. We gratefully acknowledge the gift of AMD3100 from Dr C.Power, University of Alberta.

Conflict of Interest Statement: None declared.

References

1. Sternlicht,M.D. and Werb,Z. (2001) How matrix metalloproteinases regulate cell behavior. *Annu. Rev. Cell. Dev. Biol.*, **17**, 463–516.
2. VanMeter,T.E., Rooprai,H.K., Kibble,M.M., Fillmore,H.L., Broaddus,W.C. and Pilkington,G.J. (2001) The role of matrix metalloproteinase genes in glioma invasion: co-dependent and interactive proteolysis. *J. Neurooncol.*, **53**, 213–235.
3. Rao,J.S. (2003) Molecular mechanisms of glioma invasiveness: the role of proteases. *Nat. Rev. Cancer*, **3**, 489–501.
4. Uhm,J.H., Dooley,N.P., Villemure,J.G. and Yong,V.W. (1996) Glioma invasion *in vitro*: regulation by matrix metalloprotease-2 and protein kinase C. *Clin. Exp. Metastasis*, **14**, 421–433.
5. Forsyth,P.A., Wong,H., Laing,T.D. *et al.* (1999) Gelatinase-A (MMP-2), gelatinase-B (MMP-9) and membrane type matrix metalloproteinase-1 (MT1-MMP) are involved in different aspects of the pathophysiology of malignant gliomas. *Br. J. Cancer*, **79**, 1828–1835.
6. Pagenstecher,A., Wussler,E.M., Opdenakker,G., Volk,B. and Campbell,I.L. (2001) Distinct expression patterns and levels of enzymatic activity of matrix metalloproteinases and their inhibitors in primary brain tumors. *J. Neuropathol. Exp. Neurol.*, **60**, 598–612.
7. Yong,V.W., Power,C., Forsyth,P. and Edwards,D.R. (2001) Metalloproteinases in biology and pathology of the nervous system. *Nat. Rev. Neurosci.*, **2**, 502–511.
8. Yamamoto,M., Mohanam,S., Sawaya,R., Fuller,G.N., Seiki,M., Sato,H., Gokaslan,Z.L., Liotta,L.A., Nicolson,G.L. and Rao,J.S. (1996) Differential expression of membrane-type matrix metalloproteinase and its correlation with gelatinase A activation in human malignant brain tumors *in vivo* and *in vitro*. *Cancer Res.*, **56**, 384–392.
9. Lampert,K., Machein,U., Machein,M.R., Conca,W., Peter,H.H. and Volk,B. (1998) Expression of matrix metalloproteinases and their tissue inhibitors in human brain tumors. *Am. J. Pathol.*, **153**, 429–437.
10. Nakada,M., Nakamura,H., Ikeda,E., Fujimoto,N., Yamashita,J., Sato,H., Seiki,M. and Okada,Y. (1999) Expression and tissue localization of membrane-type 1, 2, and 3 matrix metalloproteinases in human astrocytic tumors. *Am. J. Pathol.*, **154**, 417–428.
11. Llano,E., Pendas,A.M., Freije,J.P., Nakano,A., Knauper,V., Murphy,G. and Lopez-Otin,C. (1999) Identification and characterization of human MT5-MMP, a new membrane-bound activator of progelatinase a overexpressed in brain tumors. *Cancer Res.*, **59**, 2570–2576.
12. Fillmore,H.L., VanMeter,T.E. and Broaddus,W.C. (2001) Membrane-type matrix metalloproteinases (MT-MMPs): expression and function during glioma invasion. *J. Neurooncol.*, **53**, 187–202.
13. Nuttall,R.K., Pennington,C.J., Taplin,J., Wheal,A., Yong,V.W., Forsyth,P.A. and Edwards,D.R. (2003) Elevated membrane-type matrix metalloproteinases in gliomas revealed by profiling proteases and inhibitors in human cancer cells. *Mol. Cancer Res.*, **1**, 333–345.
14. Iida,J., Wilhelmson,K.L., Price,M.A., Wilson,C.M., Pei,D., Furcht,L.T. and McCarthy,J.B. (2004) Membrane type-1 matrix metalloproteinase promotes human melanoma invasion and growth. *J. Invest Dermatol.*, **122**, 167–176.
15. Bartolome,R.A., Galvez,B.G., Longo,N., Baleux,F., Van Muijen,G.N., Sanchez-Mateos,P., Arroyo,A.G. and Teixido,J. (2004) Stromal cell-derived factor-1alpha promotes melanoma cell invasion across basement membranes involving stimulation of membrane-type 1 matrix metalloproteinase and Rho GTPase activities. *Cancer Res.*, **64**, 2534–2543.
16. Ha,H.Y., Moon,H.B., Nam,M.S. *et al.* (2001) Overexpression of membrane-type matrix metalloproteinase-1 gene induces mammary gland abnormalities and adenocarcinoma in transgenic mice. *Cancer Res.*, **61**, 984–990.
17. Ueda,J., Kajita,M., Suenaga,N., Fujii,K. and Seiki,M. (2003) Sequence-specific silencing of MT1-MMP expression suppresses tumor cell migration and invasion: importance of MT1-MMP as a therapeutic target for invasive tumors. *Oncogene*, **22**, 8716–8722.
18. Seiki,M. (2003) Membrane-type 1 matrix metalloproteinase: a key enzyme for tumor invasion. *Cancer Lett.*, **194**, 1–11.
19. Richmond,A. (2002) Nf-kappa B, chemokine gene transcription and tumour growth. *Nat. Rev. Immunol.*, **2**, 664–674.
20. Muller,A., Homey,B., Soto,H. *et al.* (2001) Involvement of chemokine receptors in breast cancer metastasis. *Nature*, **410**, 50–56.
21. Balkwill,F. (2004) Cancer and the chemokine network. *Nat. Rev. Cancer*, **4**, 540–550.
22. Oh,J.W., Schwiebert,L.M. and Benveniste,E.N. (1999) Cytokine regulation of CC and CXC chemokine expression by human astrocytes. *J. Neurovirol.*, **5**, 82–94.
23. Desbaillets,I., Diserens,A.C., Tribolet,N., Hamou,M.F. and Van Meir,E.G. (1997) Upregulation of interleukin 8 by oxygen-deprived cells in glioblastoma suggests a role in leukocyte activation, chemotaxis, and angiogenesis. *J. Exp. Med.*, **186**, 1201–1212.
24. Zhou,Y., Larsen,P.H., Hao,C. and Yong,V.W. (2002) CXCR4 is a major chemokine receptor on glioma cells and mediates their survival. *J. Biol. Chem.*, **277**, 49481–49487.
25. Wild-Bode,C., Weller,M. and Wick,W. (2001) Molecular determinants of glioma cell migration and invasion. *J. Neurosurg.*, **94**, 978–984.
26. Levicar,N., Nuttall,R.K., Lah,T.T. and Nutall,R.K. (2003) Proteases in brain tumour progression. *Acta Neurochir (Wien)*, **145**, 825–838.
27. Kondraganti,S., Mohanam,S., Chintala,S.K. *et al.* (2000) Selective suppression of matrix metalloproteinase-9 in human glioblastoma cells by antisense gene transfer impairs glioblastoma cell invasion. *Cancer Res.*, **60**, 6851–6855.
28. Lakka,S.S., Gondi,C.S., Yanamandra,N., Olivero,W.C., Dinh,D.H., Gujrati,M. and Rao,J.S. (2004) Inhibition of cathepsin B and MMP-9 gene expression in glioblastoma cell line via RNA interference reduces tumor cell invasion, tumor growth and angiogenesis. *Oncogene*, **23**, 4681–4689.
29. Tonn,J.C. and Goldbrunner,R. (2003) Mechanisms of glioma cell invasion. *Acta Neurochir. Suppl.*, **88**, 163–167.
30. Price,A., Shi,Q., Morris,D. *et al.* (1999) Marked inhibition of tumor growth in a malignant glioma tumor model by a novel synthetic matrix metalloproteinase inhibitor AG3340. *Clin. Cancer Res.*, **5**, 845–854.
31. Yoshida,D., Watanabe,K., Noha,M., Takahashi,H., Teramoto,A. and Sugisaki,Y. (2002) Tracking cell invasion of human glioma cells and suppression by anti-matrix metalloproteinase agent in rodent brain-slice model. *Brain Tumor Pathol.*, **19**, 69–76.
32. Bello,L., Giussani,C., Carrabba,G. *et al.* (2002) Suppression of malignant glioma recurrence in a newly developed animal model by endogenous inhibitors. *Clin. Cancer Res.*, **8**, 3539–3548.
33. Pluderi,M., Lucini,V., Caronzolo,D. *et al.* (2003) Long-term inhibition of glioma growth by systemic administration of human PEX. *J. Neurosurg. Sci.*, **47**, 69–78.

Downloaded from https://academic.oup.com/carcin/article/26/12/2069/2390749 by guest on 20 August 2022

34. Takahashi,M., Fukami,S., Iwata,N., Inoue,K., Itohara,S., Itoh,H., Haraoka,J. and Saido,T. (2002) *In vivo* glioma growth requires host-derived matrix metalloproteinase 2 for maintenance of angioarchitecture. *Pharmacol. Res.*, **46**, 155–163.
35. Larson,D.A., Prados,M., Lamborn,K.R. *et al*. (2002) Phase II study of high central dose Gamma Knife radiosurgery and marimastat in patients with recurrent malignant glioma. *Int. J. Radiat. Oncol. Biol. Phys.*, **54**, 1397–1404.
36. Groves,M.D., Puduvalli,V.K., Hess,K.R., Jaeckle,K.A., Peterson,P., Yung,W.K. and Levin,V.A. (2002) Phase II trial of temozolomide plus the matrix metalloproteinase inhibitor, marimastat, in recurrent and progressive glioblastoma multiforme. *J. Clin. Oncol.*, **20**, 1383–1388.
37. Tremont-Lukats,I.W. and Gilbert,M.R. (2003) Advances in molecular therapies in patients with brain tumors. *Cancer Control*, **10**, 125–137.
38. Belien,A.T., Paganetti,P.A. and Schwab,M.E. (1999) Membrane-type 1 matrix metalloprotease (MT1-MMP) enables invasive migration of glioma cells in central nervous system white matter. *J. Cell. Biol.*, **144**, 373–384.
39. Belkin,A.M., Akimov,S.S., Zaritskaya,L.S., Ratnikov,B.I., Deryugina,E.I. and Strongin,A.Y. (2001) Matrix-dependent proteolysis of surface transglutaminase by membrane-type metalloproteinase regulates cancer cell adhesion and locomotion. *J. Biol. Chem.*, **276**, 18415–18422.
40. Lafleur,M.A., Handsley,M.M., Knauper,V., Murphy,G. and Edwards,D.R. (2002) Endothelial tubulogenesis within fibrin gels specifically requires the activity of membrane-type-matrix metalloproteinases (MT-MMPs). *J. Cell Sci.*, **115**, 3427–3438.
41. Van Meter,T.E., Broaddus,W.C., Rooprai,H.K., Pilkington,G.J. and Fillmore,H.L. (2004) Induction of membrane-type-1 matrix metalloproteinase by epidermal growth factor-mediated signaling in gliomas. *Neuro-oncol.*, **6**, 188–199.
42. Oh,J.W., Drabik,K., Kutsch,O., Choi,C., Tousson,A. and Benveniste,E.N. (2001) CXC chemokine receptor 4 expression and function in human astroglioma cells. *J. Immunol.*, **166**, 2695–2704.
43. Rempel,S.A., Dudas,S., Ge,S. and Gutierrez,J.A. (2000) Identification and localization of the cytokine SDF1 and its receptor, CXC chemokine receptor 4, to regions of necrosis and angiogenesis in human glioblastoma. *Clin. Cancer Res.*, **6**, 102–111.
44. Salmaggi,A., Gelati,M., Pollo,B. *et al*. (2004) CXCL12 in malignant glial tumors: a possible role in angiogenesis and cross-talk between endothelial and tumoral cells. *J. Neurooncol.*, **67**, 305–317.
45. Barbero,S., Bonavia,R., Bajetto,A., Porcile,C., Pirani,P., Ravetti,J.L., Zona,G.L., Spaziante,R., Florio,T. and Schettini,G. (2003) Stromal cell-derived factor 1alpha stimulates human glioblastoma cell growth through the activation of both extracellular signal-regulated kinases 1/2 and Akt. *Cancer Res.*, **63**, 1969–1974.
46. Rubin,J.B., Kung,A.L., Klein,R.S., Chan,J.A., Sun,Y., Schmidt,K., Kieran,M.W., Luster,A.D. and Segal,R.A. (2003) A small-molecule antagonist of CXCR4 inhibits intracranial growth of primary brain tumors. *Proc. Natl Acad. Sci. USA*, **100**, 13513–13518.
47. Le,D.M., Besson,A., Fogg,D.K., Choi,K.S., Waisman,D.M., Goodyer,C.G., Rewcastle,B. and Yong,V.W. (2003) Exploitation of astrocytes by glioma cells to facilitate invasiveness: a mechanism involving matrix metalloproteinase-2 and the urokinase-type plasminogen activator-plasmin cascade. *J. Neurosci.*, **23**, 4034–4043.
48. Zheng,J., Thylin,M.R., Ghorpade,A. *et al*. (1999) Intracellular CXCR4 signaling, neuronal apoptosis and neuropathogenic mechanisms of HIV-1-associated dementia. *J. Neuroimmunol.*, **98**, 185–200.
49. Bajetto,A., Bonavia,R., Barbero,S., Piccioli,P., Costa,A., Florio,T. and Schettini,G. (1999) Glial and neuronal cells express functional chemokine receptor CXCR4 and its natural ligand stromal cell-derived factor 1. *J. Neurochem.*, **73**, 2348–2357.

Received April 9, 2005; revised July 4, 2005; accepted July 10, 2005

Downloaded from https://academic.oup.com/carcin/article/26/12/2069/2390749 by guest on 20 August 2022