

Tenascin-C Stimulates Glioma Cell Invasion through Matrix Metalloproteinase-12

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Abstract

The capacity of glioma cells to invade extensively within the central nervous system is a major cause of the high morbidity rate of primary malignant brain tumors. Glioma cell invasion involves the attachment of tumor cells to extracellular matrix (ECM), degradation of ECM components, and subsequent penetration into adjacent brain structures. These processes are accomplished in part by matrix metalloproteinases (MMP) within a three-dimensional milieu of the brain parenchyma. As the majority of studies have used a two-dimensional monolayer culture system, we have used a three-dimensional matrix of collagen type I gel to address glioma-secreted proteases, ECM, and invasiveness of glioma cells. We show that in a three-dimensional collagen type I matrix, the presence of tenascin-C, commonly elevated in high-grade gliomas, increased the invasiveness of glioma cells. The tenascin-C-mediated invasiveness was blocked by metalloproteinase inhibitors, but this did not involve the gelatinases (MMP-2 and MMP-9) commonly implicated in two-dimensional glioma growth. A thorough analysis of 21 MMPs and six members of a disintegrin and metalloproteinase domain showed that MMP-12 was increased in gliomas by tenascin-C in three-dimensional matrix. Furthermore, examinations of resected specimens revealed high MMP-12 levels in the high-grade glioblastoma multiforme tumors. Finally, a function-blocking antibody as well as small interfering RNA to MMP-12 attenuated the tenascin-C-stimulated glioma invasion. These results identify a new factor, MMP-12, in regulating glioma invasiveness through interaction with tenascin-C. (Cancer Res 2006; 66(24): 11771-80)

Introduction

Gliomas are the most common primary tumors that arise within the central nervous system in adults. The most malignant form, glioblastoma multiforme, is resistant to current modalities of treatment. The hallmark of gliomas is local invasion of single tumor cells to adjacent and distant brain structures. Such invasiveness is mediated in part by the interaction of glioma cells with the extracellular matrix (ECM), followed by degradation of matrix by tumor cell-derived proteases, particularly the matrix metalloproteinases (MMP; refs. 1–7).

The overexpression of the gelatinase subfamily of MMPs, MMP-2 and MMP-9, is well documented in malignant gliomas (2–4, 6, 7). More recently, the membrane-type MMPs have also been found to

be overrepresented in glioma specimens (4, 8, 9). There are 24 human MMP members, however, and the possible roles of nongelatinase or membrane-type MMPs in glioma biology remain uncertain. Moreover, the specific interaction between glioma cells and particular ECM molecules and how ECM may alter MMP expression are, for the most part, unclear.

The roles played by the ECM in tissue repair and neoplastic transformation are complex. In malignant gliomas, many ECM components are overexpressed both in the tumor stroma and at the advancing edge of the tumor within brain parenchyma. These ECM molecules include vitronectin, collagen I, collagen IV, osteopontin, tenascin-C, secreted protein acidic and rich in cysteine, and brain enriched hyaluronan binding (10–21). Glioma invasion is thought to occur along ECM protein-containing structures, such as along tracts of myelinated fibers (22–24). Besides creating a more permissive substrate for invasiveness, ECM proteins can also affect other tumorigenic properties, such as survival, cell cycle progression, and angiogenesis.

Here, we have evaluated whether particular ECM proteins may modulate the invasiveness of glioma cells and whether this involves specific MMPs. We focused on a three-dimensional model of invasiveness, in which glioma cells are encased within a collagen I gel supplemented with test ECM proteins. Previous studies of glioma invasiveness *in vitro* have largely used a two-dimensional monolayer growth but it has become apparent that a three-dimensional matrix allows a more physiologic representation of tumor behavior (25–27). In addition, rather than focusing on a few MMPs, we have extended our analyses to include all MMP members and also a related family of metalloproteinases, a disintegrin and metalloproteinases (ADAM). Our results show that tenascin-C is a permissive substrate for glioma invasiveness and that it does so by up-regulating MMP-12. These results provide new insights and targets to inhibit glioma invasiveness.

Materials and Methods

Cell culture. U178 and U251 cell lines (28) were maintained in MEM supplemented with 10% fetal bovine serum (FBS). For two-dimensional cell culture, a six-well plate was first coated with polyornithine (10 µg/mL) for 30 minutes at room temperature. Wells were then further coated with specific ECM protein (10 µg/mL) at 37°C for 24 hours. Glioma cells were trypsinized with 0.25% trypsin-EDTA and seeded onto the ECM substrate.

For three-dimensional cell culture, collagen I gel was prepared on ice according to the manufacturer's instruction (Chemicon, Temecula, CA). In brief, for a 500-µL volume, 400 µL of cold collagen I solution were mixed with 100 µL of cold 5× DMEM and the pH was neutralized. One million glioma cells were added to the cold 500-µL collagen I solution, supplemented with or without 5-µL test molecules (e.g., tenascin-C). Following pipetting of mix cells into the collagen I solution, 70 µL (140,000 cells) were distributed onto the center of the top compartment of transwell inserts (see below). The plate was then placed immediately at 37°C for 1 hour to allow collagen I to polymerize, trapping cells with it. The collagen I gel was then covered with culture medium.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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doi:10.1158/0008-5472.CAN-05-0470

Collagen I was chosen as the means to generate a three-dimensional matrix because it readily forms a gel when warmed to 37°C and into which glioma cells could be embedded (29). Importantly, collagen I is an ECM molecule expressed around glioma cells *in situ* (10, 11, 13, 14).

To plate cells for RNA analyses, gelatin zymography, or Western blots, 1 million glioma cells were suspended within 500 µL of collagen I gel supplemented with or without ECM proteins (10 µg/mL). This was then placed onto the center of each well of a six-well culture plate and incubated for 1 hour at 37°C to allow polymerization of collagen I. The collagen I gel was then covered with feeding medium.

Invasion assays. Transwell migration chambers (Costar 3422, polycarbonate membrane, 24-well format, 8-µm pore size, Corning, Inc., Corning, NY) were used (30, 31). Following polymerization of the collagen I droplet in the top compartment, 100 µL of DMEM/F-12 medium with N2 supplement were added to the upper chamber and 1 mL of 10% FBS-containing glioma medium was applied to the lower well. Cells were then allowed to invade out of the three-dimensional collagen I matrix, across the membrane, at 37°C for 24 hours. Noninvasive cells were then removed from the top compartment of the transwell with a cotton swab and the invasive cells present on the underside of the membrane were fixed and stained with hematoxylin (30). The number of invasive cells was counted per field ($\times 40$ microscope objective) from four random fields of each membrane.

In some experiments, metalloproteinase inhibitors [BB94, final concentration of 500 nmol/L (British Biotech, Oxford, United Kingdom), 10 µmol/L GM6001 (Calbiochem, Mississauga, Ontario, Canada), and 1 µg/mL tissue inhibitor of metalloproteinase-1 (TIMP-1)] were added within the gel, in medium in the upper chamber of the transwell insert, as well as in the lower well. After 24 hours, the number of invasive cells was quantified. Similarly, a function-blocking antibody to MMP-12 (rabbit anti-human MMP-12; Chemicon), raised to the NH₂ terminus of MMP-12 but which can detect proform, intermediate, and active forms of MMP-12 in Western blots, was incorporated (10 µg/mL) within three-dimensional collagen I gels supplemented with or without tenascin-C in transwell invasion chamber.

TaqMan real-time PCR analysis. To determine the expression profile of MMP and ADAM members, TaqMan real-time PCR analysis was done (9). All MMP members were assessed. Not all ADAMs have the consensus HEXXHXXGXXH motif to bind zinc for catalytic activity, so only a majority of those with this motif were examined (ADAM-10, ADAM-12, ADAM-15, ADAM-17, ADAM-19, and ADAM-28).

Human glioma specimens. Human glioma samples were obtained from the University of Calgary and the Canadian brain tumor bank in London, Ontario. All patients gave signed, informed consent for their tissues to be used. The following grades of tissues were studied: nontransformed brain tissues (apparently normal tissues resected during the course of removal of epileptogenic foci; ref. 28), low-grade gliomas, mid-grade gliomas (also called anaplastic gliomas), and high-grade glioblastoma multiformes. Tumors were classified and graded by neuropathologists at the two institutions supplying tissues. Although the exact location of the tumor in the brain blocks that we analyzed for MMPs (PCR and Western blots) was not described by the donating institutions, samples were considered to be largely from within the tumor rather than infiltrating margins, as the former is more commonly stored and supplied for research. Although these are astrocytic tumors, we are uncertain about whether there is oligodendroglioma presentation. Thus, future studies with better documented tumors would need to resolve whether astrocytic gliomas and oligodendrogliomas have differing MMP expression. Samples were homogenized in RNazol and total RNA was frozen at -20°C until further analyses.

For brain samples used for Western blot analyses, each specimen was placed in chilled mortar and ground using a chilled pestle; liquid nitrogen was added from time to time to keep the samples frozen. Each ground sample in liquid nitrogen was then poured into a 1.5-mL Eppendorf tube on ice, and time was allowed for the liquid nitrogen to evaporate. Lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 1 mmol/L EDTA] containing protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 5 µg/mL aprotinin, and 5 µg/mL leupeptin) was then added, and a rubber pestle was used to further homogenize samples for 10 seconds. The homogenate was kept on ice for

30 minutes and microfuged at 13,000 rpm for 15 minutes at 4°C. The supernatant was mixed with 4× SDS loading buffer for Western blot analysis with rabbit anti-human MMP-12 antibody described above (Chemicon).

Western blot analyses of cell-conditioned medium. Equal number of glioma cells was grown for 24 hours in 1 mL of DMEM/F-12 serum-free medium either in two-dimensional monolayer or three-dimensional collagen I matrix supplemented with or without tenascin-C. Because MMP-12 levels secreted into the culture medium were low, this necessitated each sample to be pooled from four wells of a six-well plate. The medium was then concentrated in excess of 100-fold using centrifugal filter devices (Centricon, ultracel YM-10, Millipore Corporation, Bedford, MA) and then further enriched for MMP-12 using a MMP-12 antibody bound to microbeads (R&D Systems, Minneapolis, MN). Purification was done according to the manufacturer's instruction (MMP-12 purification kit, R&D Systems). Following measurement of total protein content, equal amounts of samples were electrophoresed on 12% SDS-PAGE under reducing conditions and transferred to a polyvinylidene difluoride membrane (Millipore). The latter was then blocked overnight with 10% milk in saline and was then probed for 1 hour with either mouse anti-human MMP-12 antibody (recognizing only the proform of MMP-12; 1:1,000; R&D Systems) or rabbit anti-human MMP-12 antibody described earlier. A secondary antibody (antimouse or anti-rabbit horseradish peroxidase; 1:10,000) was added for 1 hour, and blots were detected by enhanced chemiluminescence detection kit (Amersham Bioscience, Piscataway, NJ).

Gelatin zymography. Whereas the above cell-conditioned media were assayed for total protein content and then loaded onto gels based on equal protein, it would be ideal to have an internal control in the cell-conditioned medium that is not altered by two-dimensional or three-dimensional growth, with or without tenascin-C. Preliminary experiments indicated that pro-MMP-2 and active MMP-2 did not change in these conditions and we thus used gelatin zymography (30, 31) to determine the amount of MMP-2 in conditioned medium of cells to confirm equal loading of samples.

Small interfering RNA to MMP-12. A predesigned small interfering RNA (siRNA; 21 oligonucleotides in length; Ambion, Austin, TX), designated siRNA1, was used to target human MMP-12. The sequence was 5'-GCUUUUUUAACCCACGUUtt-3', targeting exon 8. A second siRNA, designated siRNA2 and targeting exon 3, was used to confirm the siRNA1 results. The sequence of siRNA2 was 5'-CCGUGAGGAUGUUGACUActt-3'. A matrix-assisted laser desorption/ionization-time of flight mass spectrometer was used to identify the correct mass of the single-stranded RNA oligonucleotides. The annealed siRNAs were analyzed by nondenaturing PAGE. A negative control siRNA, composed of a 19-bp scrambled sequence with 3' deoxythymidine overhangs, was used. The sequences have no significant homology to any known gene sequences from mouse, rat, or human.

For transfection with siRNA, glioma cells (U178 and U251) were plated in 12-well plates and were incubated with 50 nmol/L siRNA and LipofectAMINE (Invitrogen, Burlington, Ontario, Canada). After 24 hours, cells were harvested for invasion assays as described above (except that 33,000 cells per migration chamber were used, for economy reasons, rather than 140,000 as described earlier) or for MMP-12 Western blots of cell lysates, or RNA was extracted for PCR of MMP-12. Primer sequences were as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-AGCCTTCTCCATGGTGGTGAAGAC-3' (forward) and 5'-CGGAGTCAACGGATTGGTTCG-3' (reverse); human MMP-12, 5'-ACACCTGACATGAACCGTGA-3' (forward) and 5'-CAATGCCAGATCCAGGTCC-3' (reverse). The annealing temperature used for both *GAPDH* and *MMP-12* genes was 55°C. Semiquantitative analysis was done by monitoring in real time the increase of fluorescence of SYBR green dye on Bio-Rad i-Cycler. Real-time fluorescence measurements were done and a threshold cycle value for *GAPDH* or *MMP-12* gene was calculated. All MMP-12 data were normalized against the GAPDH mRNA levels and expressed relative to the average of four mock-transfected samples from the same experiment.

In another series of experiments (Supplementary Fig. S2) that evaluated the specificity of the MMP-12 siRNA, 10⁵ cells plated for 24 hours in 12-well plates in 10% FBS-containing glioma medium were exposed to 50 nmol/L siRNA and LipofectAMINE. After 24 hours, RNA was pooled from four wells

to obtain a single sample, and this was repeated for four samples per group. TaqMan real-time PCR, as described above, was then used to evaluate levels of MMP transcripts.

Statistics. When two groups were compared, the unpaired Student's *t* test was applied. When multiple groups were evaluated, the one-way ANOVA test with *post hoc* Tukey-Kramer multiple comparisons test was used.

Results

Tenascin-C increases glioma invasiveness in three-dimensional collagen I matrix. The highly invasive nature of malignant gliomas prompted us to investigate ECM proteins permissive for this function. We focused on three-dimensional growth because invasiveness *in situ* would be through a three-dimensional matrix rather than a two-dimensional monolayer system. Glioma cells (U251 and U178) were suspended into collagen I gels supplemented with ECM proteins described to be elevated in glioma specimens, including collagen IV, vitronectin, and tenascin-C, above transwell membranes. Compared with three-dimensional collagen I controls, the supplementation of tenascin-C was found to increase the invasiveness of U251 and U178 glioma cells (Fig. 1A). Collagen IV did not alter glioma invasiveness, whereas vitronectin decreased this

capacity (Fig. 1B). Figure 1C depicts the increased number of glioma cells that invaded across transwell membranes coated with tenascin-C encased in collagen I, compared with collagen I matrix alone. These results show that tenascin-C is a permissive substrate for glioma invasiveness across a three-dimensional matrix.

Glioma invasiveness stimulated by tenascin-C is metalloproteinase dependent. To determine whether the tenascin-C-stimulated glioma invasiveness is metalloproteinase dependent, metalloproteinase inhibitors (BB94, GM6001, and TIMP-1) were used. Addition of BB94 caused a significant decrease of invasiveness of U178 and U251 glioma cells in collagen I gels as well as in collagen I gels supplemented with tenascin-C (Fig. 2). Similarly, the addition of GM6001 or TIMP-1 reduced tenascin-C-stimulated glioma invasiveness in three-dimensional matrix (Fig. 2). These results show that the tenascin-C-stimulated glioma invasiveness in a three-dimensional matrix was dependent on metalloproteinases.

Tenascin-C does not alter MMP-2 or MMP-9 expression by glioma cells. From the observation that the tenascin-C-stimulated glioma invasiveness was metalloproteinase dependent, we sought to elucidate which metalloproteinase member was critical to this process. We first investigated whether MMP-2 or MMP-9 was

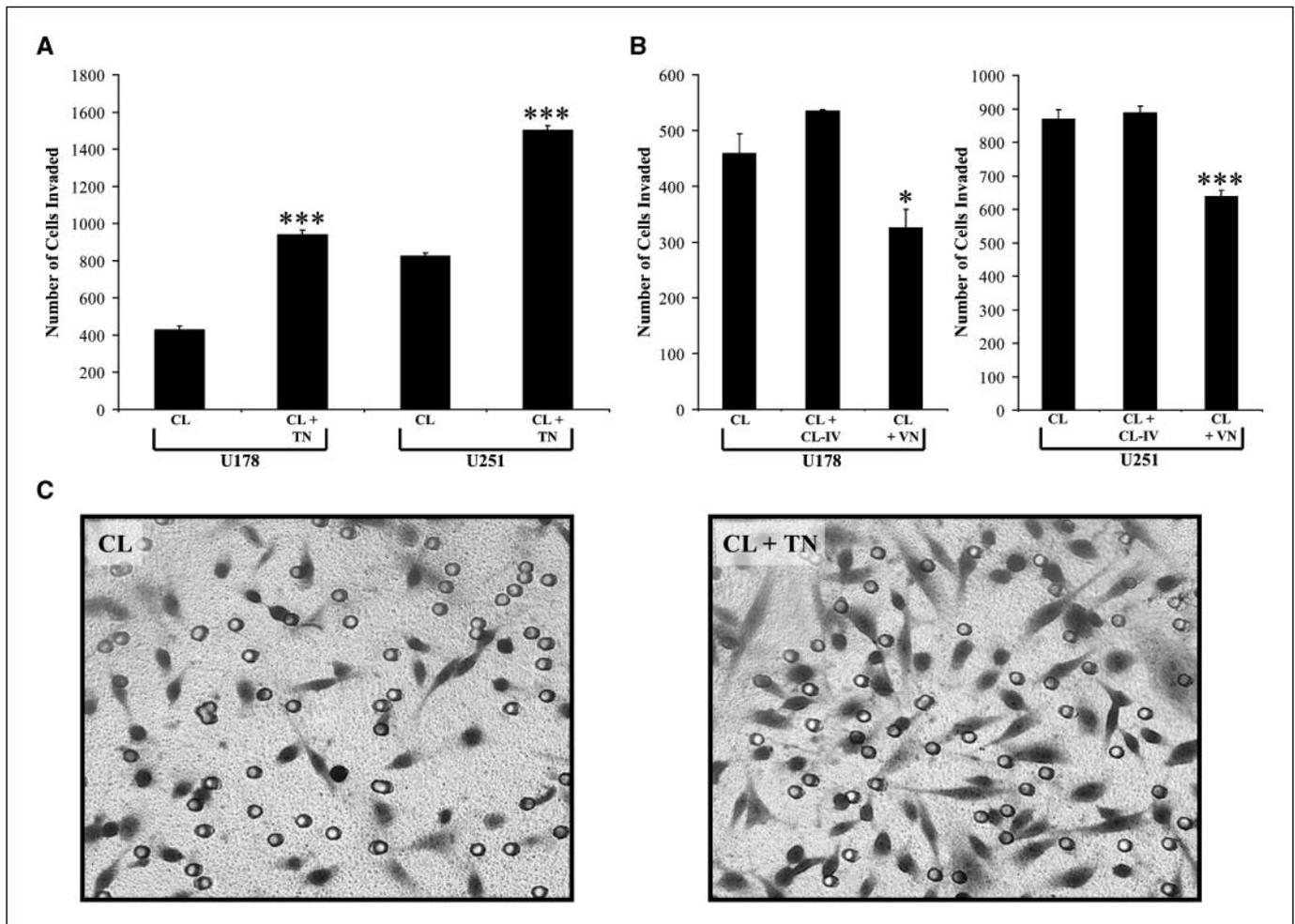


Figure 1. Tenascin-C increases glioma invasiveness in three-dimensional collagen I matrix. A, tenascin-C (TN) embedded within the collagen I (CL) gel increases the invasiveness of both U178 and U251 glioma cell lines, and this is pictorially depicted in (C) for the U178 cell line. In contrast, collagen IV (CL-IV) did not affect invasion, whereas vitronectin (VN) caused a reduction (B). Columns, mean of four wells (the result was reproduced across at least four different sets of experiments); bars, SE. *, $P < 0.05$; ***, $P < 0.001$, compared with collagen I alone.

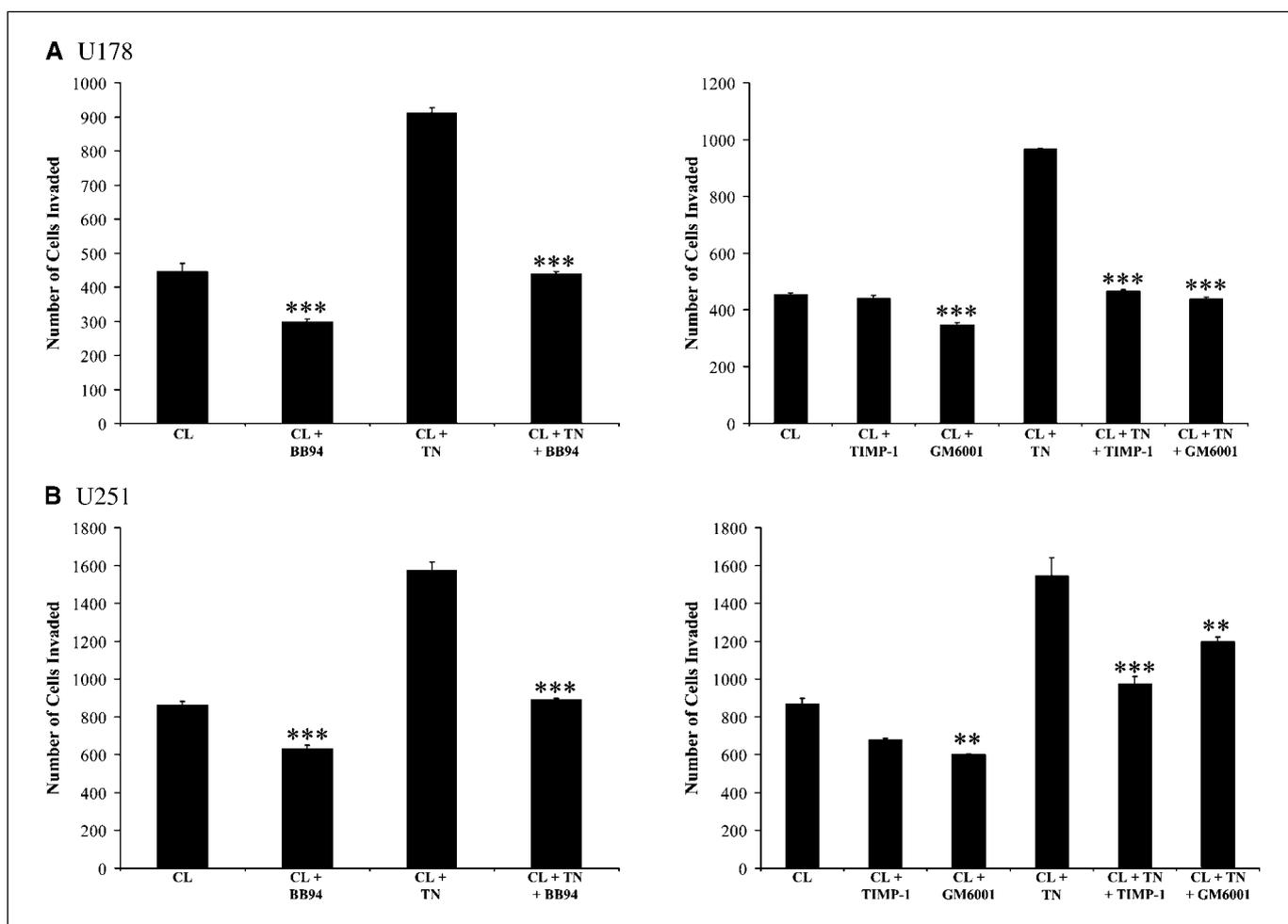


Figure 2. The tenascin-C-mediated invasiveness of glioma cells is metalloproteinase dependent. The addition of BB94 (500 nmol/L), GM6001 (10 μ mol/L), or TIMP-1 (1 μ g/mL) caused a significant decrease of invasion by U178 (A) or U251 (B) glioma cells in three-dimensional collagen I supplemented with tenascin-C. Similarly, these inhibitors also attenuated the invasiveness of cells in basal collagen I matrix. **, $P < 0.01$; ***, $P < 0.001$, compared with the respective collagen I or collagen I + tenascin-C controls. Columns, mean of four wells (the results were reproduced across at least four different sets of experiments); bars, SE. In the absence of inhibitors, invasiveness in three-dimensional collagen I + tenascin-C was significantly different from three-dimensional collagen I alone. Note that (A) and (B) are displayed separately as the data were accumulated from different experiments.

involved because a large literature has implicated these two MMPs in glioma invasiveness *in vitro*, at least across a two-dimensional barrier (2, 3, 5, 6). Analyses of conditioned medium show that whereas the three-dimensional collagen I matrix increased MMP-9 levels in U251 cells (but not in U178 cells) compared with growth in two-dimensional monolayer, tenascin-C in three-dimensional collagen I matrix did not further alter MMP-2 or MMP-9 relative to three-dimensional collagen I alone (Supplementary Fig. S1).

As previous studies implicating MMP-2 and MMP-9 in glioma invasiveness have largely used a two-dimensional culture system, we grew cells in two-dimensional monolayer on polyornithine coated with or without tenascin-C; conditioned medium, after 24 hours, was subjected to gelatin zymography. Similar to results in three-dimensional culture, tenascin-C did not alter MMP-2 or MMP-9 expression in two-dimensional monolayer compared with the polyornithine coating control (Supplementary Fig. S1). Analyses of RNA transcripts by TaqMan PCR confirmed the zymography data, in that MMP-2 and MMP-9 did not vary in response to tenascin-C in either two-dimensional or three-dimensional growth (Fig. 3); whereas there was a trend toward a decrease of MMP-9 transcripts in three-dimensional collagen I + tenascin-C versus

three-dimensional collagen I alone, this did not translate to a change in protein expression (Supplementary Fig. S1).

From these observations, we concluded that whereas glioma cells expressed MMP-2 or MMP-9 in either two-dimensional monolayer or three-dimensional matrix, tenascin-C did not further alter MMP-2 or MMP-9 expression compared with non-tenascin-C controls. The increased invasiveness of glioma cells promoted by tenascin-C in a three-dimensional matrix was thus unlikely to be due to MMP-2 and MMP-9.

Tenascin-C increases MMP-12 expression by glioma cells in three-dimensional collagen I matrix. To identify which MMP or ADAM family members were involved in the process of tenascin-C-stimulated glioma cell invasion, glioma cells were grown for 24 hours in either two-dimensional monolayer or three-dimensional collagen I gels, with or without tenascin-C, and RNA extracts were then subjected to TaqMan real-time PCR analysis. Twenty-one MMP genes (Fig. 3) and six ADAM members with catalytic activity (ADAM-10, ADAM-12, ADAM-15, ADAM-17, ADAM-19, and ADAM-28) were evaluated. Specifically, we were interested in metalloproteinase members that were up-regulated when cultures were switched from a two-dimensional to a three-dimensional

format, and where tenascin-C would further cause increased expression in three-dimensional compared with collagen I three-dimensional matrix. This criterion is based on the assumption that a three-dimensional matrix would impose more restriction on glioma invasiveness than a two-dimensional monolayer, thus requiring more relevant proteases to be expressed. Figure 3 shows that, in U178 glioma cell line, among the MMPs profiled, only MMP-12, MMP-21, and MMP-25 fulfilled these criteria. MMP-8 and MMP-13 were elevated by tenascin-C in three-dimensional compared with collagen I matrix alone, but these levels were not increased beyond those of two-dimensional growth. RNA levels of MMP-1, MMP-7,

MMP-10, MMP-11, MMP-14, MMP-15, and MMP-17 were increased in three-dimensional collagen I control when compared with two-dimensional growth; however, they were not increased further by tenascin-C in three-dimensional matrix.

Of the ADAM members profiled, RNA levels remained unaltered by growth in two-dimensional or three-dimensional format and tenascin-C did not further change their expression levels (data not shown). Because of the extensive analyses needed to sample multiple MMPs and ADAMs, we used only one time point here. Other time points could be instructive as well, and this will be addressed in future experiments.

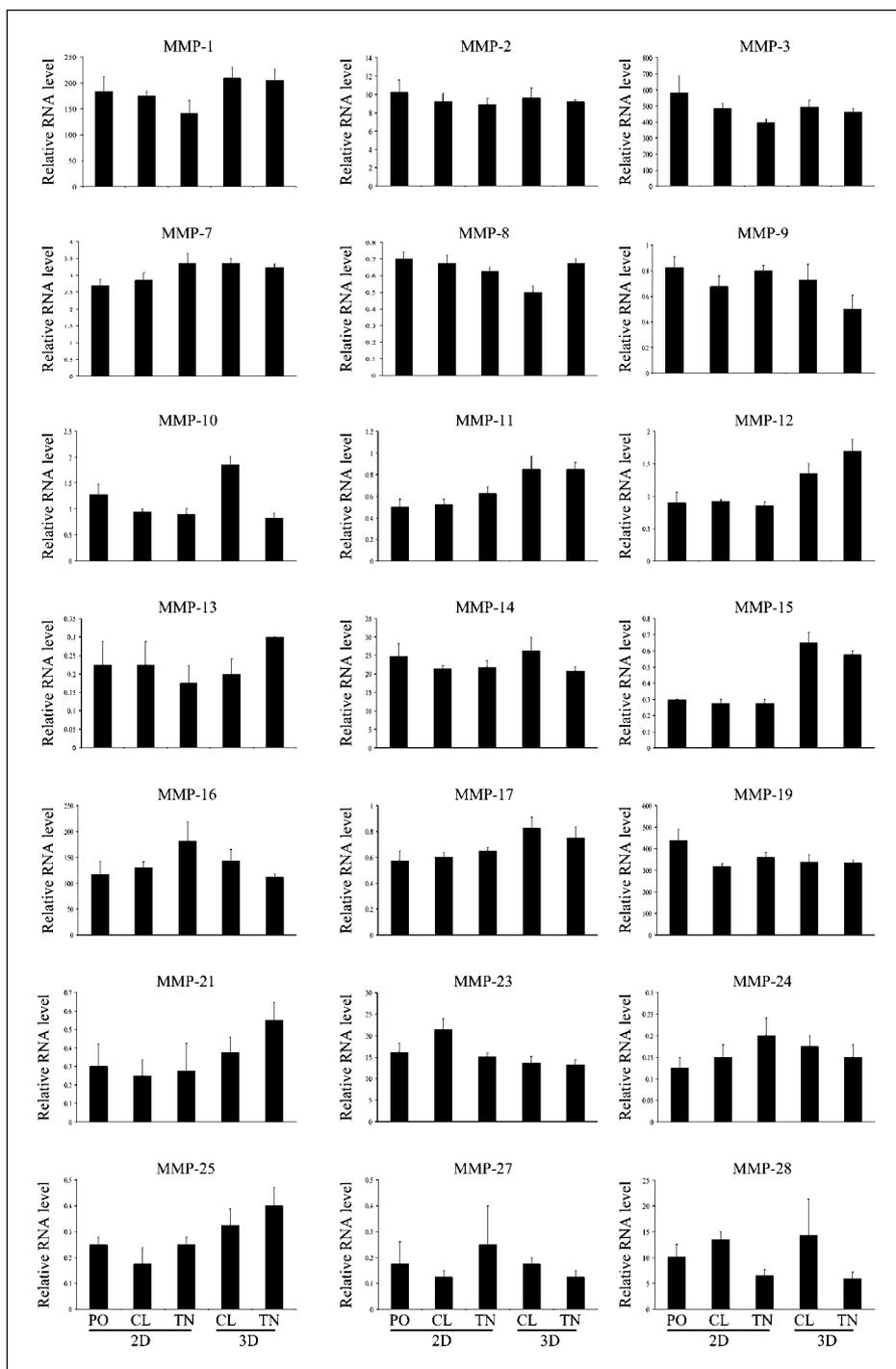


Figure 3. Tenascin-C increases a limited number of MMPs in glioma cells in three-dimensional collagen I matrix. TaqMan real-time PCR analyses showed that among various MMPs analyzed, only MMP-12, MMP-21, and MMP-25 were elevated in three-dimensional (3D) versus two-dimensional (2D) basal growth, and the level of MMP was further enhanced in three-dimensional growth by tenascin-C. Columns, mean of four cultures; bars, SE.

Overall, these results indicate that MMP-12, MMP-21, and MMP-25 were of further interest as proteases that may facilitate tenascin-C-mediated invasiveness of glioma cells.

Human resected glioma specimens have high levels of MMP-12. We further determined the relevance of MMP-12, MMP-21, and MMP-25 to glioma invasiveness by addressing whether these were present in resected glioma specimens and whether there would be a concordant increase in their expression with increasing grade of malignancy. We analyzed a total of 16 resected glioma specimens and compared these to 7 nontransformed human brain tissues. For MMP-12, elevation was observed compared with controls, and this was most marked in the glioblastoma multiforme samples (Fig. 4A). For MMP-21, Fig. 4B shows that this MMP was increased in mid-grade glioma specimens but then declined in glioblastoma multiformes. Finally, we have previously reported that MMP-25 (membrane-type 6 MMP) was elevated in both low-grade and glioblastoma multiforme specimens compared with controls (9).

To address whether the elevation of MMP-12 transcripts (Fig. 4A) was also reflected in an increase in protein, we subjected four

glioblastoma multiforme samples and four nontransformed control specimens to Western blot analyses. Figure 4C shows that whereas the proform of MMP-12 (55 kDa) was not detected in all samples, the intermediate and active forms of MMP-12 (~45 and 22 kDa) were apparent in glioblastoma multiforme but not in the majority of control cases.

The cellular source of MMP-12 in glioblastoma multiforme remains undefined as antibodies to human MMP-12 did not stain convincingly in tissue sections. Whereas glioma cells are a possible source, the microglia/macrophage known to be elevated in glioma specimens (32) represents another possibility as these leukocytes are rich sources of MMP-12 (33).

Tenascin-C stimulates glioma cell invasion through MMP-12.

The results above suggest that MMP-12 is involved in the tenascin-C-stimulated glioma invasiveness across a three-dimensional matrix. To confirm this, we first determined whether MMP-12 protein could be detected in the conditioned medium of cells in culture, and whether this was altered by tenascin-C exposure. Figure 5A, focusing on pro-MMP-12, shows that a faint pro-MMP-12 band was present in the medium from two-dimensional monolayer

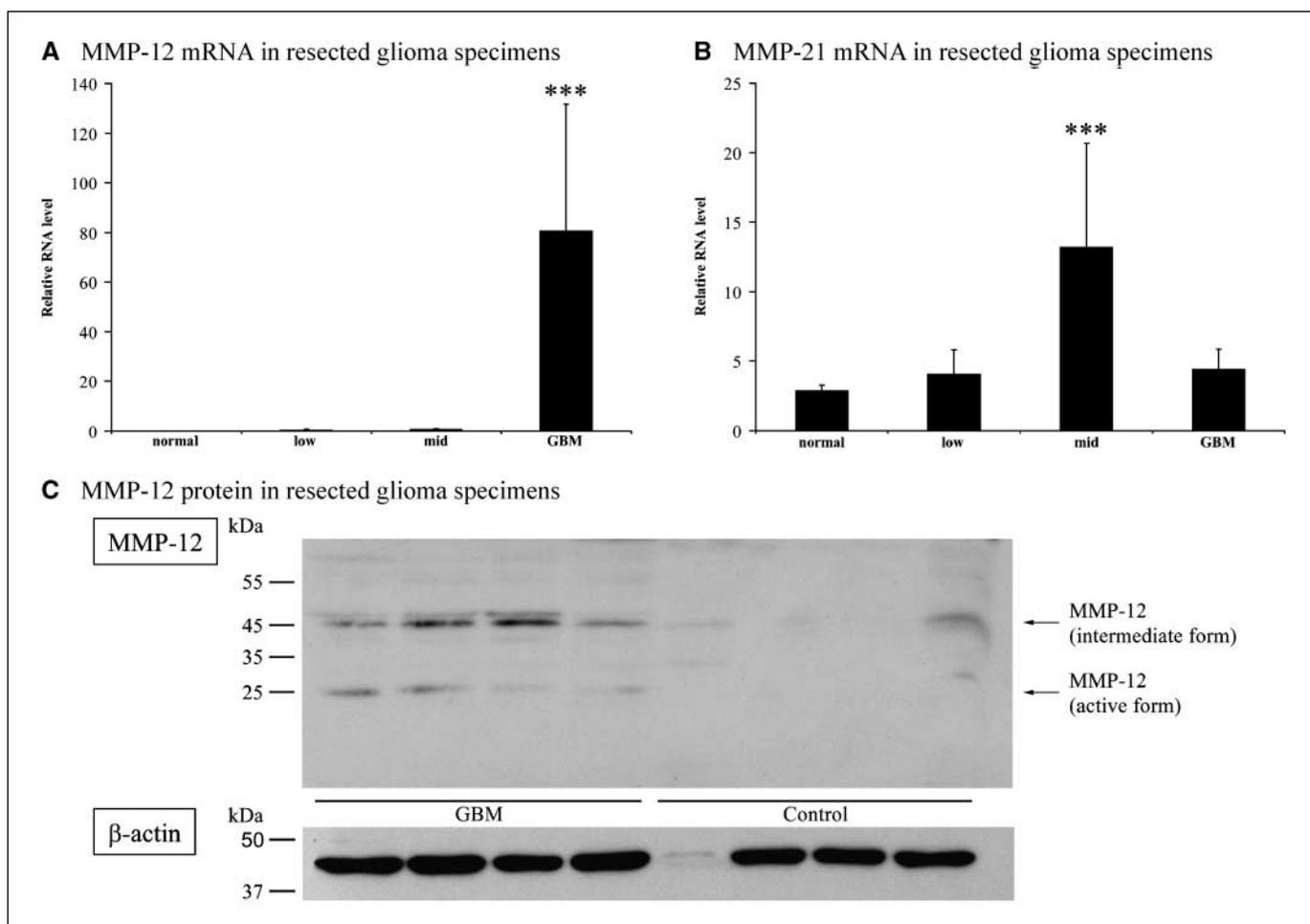


Figure 4. Human resected glioma specimens have high levels of MMP-12. Elevated MMP-12 transcripts were detected only in specimens of high-grade glioblastoma multiforme (GBM) compared with low-grade glioma, mid-grade glioma, or normal brain (A). In contrast, increased level of MMP-21 was observed in mid-grade glioma specimens (B). ***, $P < 0.001$, compared with normal brains. For (A) and (B), seven nontransformed control specimens, three low-grade gliomas, four mid-grade gliomas, and nine glioblastoma multiforme samples were used. C, expression of MMP-12 in resected human glioma specimens at the protein level. Western blot analysis of four glioblastoma multiforme specimens and four nontransformed control specimens showed expression of intermediate and active forms of MMP-12 in glioma specimens, which were not readily evident in nontransformed control specimens. In addition, the proform of MMP-12 (55 kDa) was not detected in all samples. The equal loading of specimens is indicated by the β -actin blot. Note that the first control sample seems to have been degraded as actin expression was not evident.

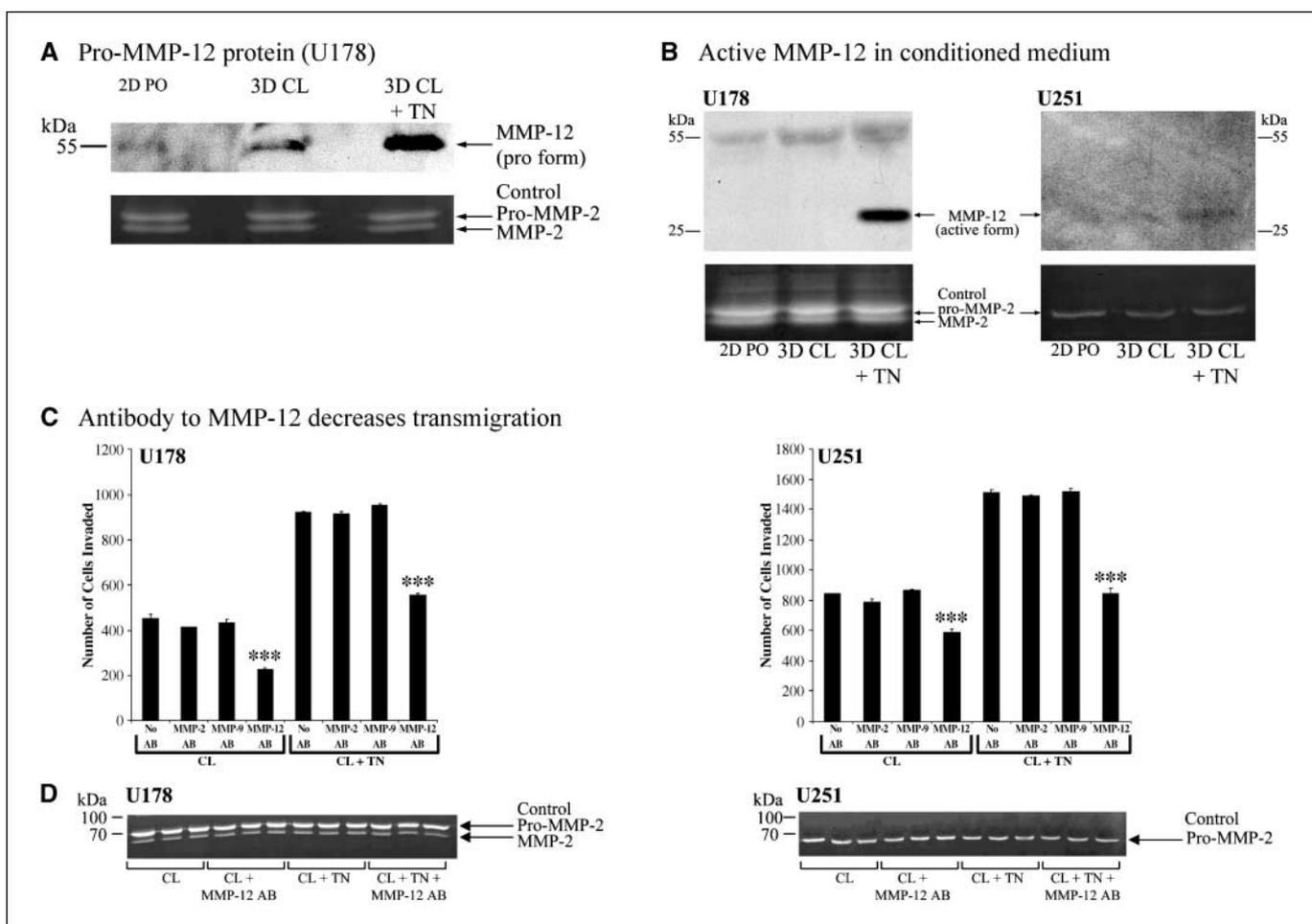


Figure 5. Tenascin-C increases MMP-12 protein expression, which stimulates glioma invasiveness. *A*, Western blot analysis of conditioned medium with the mouse anti-human MMP-12 antibody shows increased expression of pro-MMP-12 (55 kDa) in U178 glioma cell line when grown in three-dimensional collagen I matrix supplemented with tenascin-C, compared with three-dimensional collagen I or two-dimensional polyornithine (PO). The amount of proform or active form of MMP-2 in the conditioned medium denotes the equal loading of the samples because MMP-2 level remained unaltered with tenascin-C or collagen I. *B*, Western blot analysis with the rabbit anti-human MMP-12 antibody raised to the NH₂ terminus reveals elevated level of the active form of MMP-12 in the conditioned medium of U178 or U251 cell lines when cells were in three-dimensional collagen I + tenascin-C, compared with three-dimensional collagen I or two-dimensional polyornithine. U178 showed expression of the proform of MMP-12 as well. MMP-2 expression in the conditioned medium was used as an internal control. Note that whereas U178 contains the proform and active form of MMP-2, U251 shows only the proform of MMP-2 as we have previously described (30). *C*, the tenascin-C-stimulated glioma invasiveness was blocked by a neutralizing antibody to MMP-12. Similarly, the basal invasiveness in a collagen I matrix without tenascin-C was also inhibited by the anti-MMP-12 antibody. In contrast, function-blocking antibodies to MMP-2 or MMP-9 did not affect glioma invasiveness. *Columns*, mean of four wells (the results were reproduced in a second set of experiments); *bars*, SE. ***, $P < 0.001$, compared with the respective no antibody control. *D*, gelatin zymography for MMP-2 in conditioned medium shows that the blocking of MMP-12 did not produce nonspecific effects, in that the MMP-2 internal control of samples was constant across all conditions.

cells plated onto polyornithine. In three-dimensional collagen I matrix, pro-MMP-12 was readily apparent and its level was further elevated by tenascin-C. As these samples were from the conditioned media, and to ensure that media from equal amounts of cells in all conditions were analyzed, we examined MMP-2 levels in these samples by gelatin zymography; as previously mentioned, MMP-2 levels were not altered in glioma cells in response to tenascin-C. Figure 5A shows that there were equal amounts of MMP-2 in the samples in conditions in which tenascin-C up-regulated pro-MMP-12 in three-dimensional matrix.

We then focused our examinations for the active form of MMP-12 using antibodies directed against the NH₂ terminus. Figure 5B shows that the active form for MMP-12 was not readily detected in two-dimensional or three-dimensional collagen I but was apparent in collagen I + tenascin-C matrix; as before, equal loading was confirmed by a constant MMP-2 band across all samples. Thus,

tenascin-C increases pro- and active MMP-12 protein levels similar to its elevation of MMP-12 transcripts noted earlier.

To ascertain whether MMP-12 is indeed involved in the process of tenascin-C-stimulated glioma invasiveness, we first used a function-blocking antibody to MMP-12. Addition of this antibody (10 μg/mL) within tenascin-C-supplemented collagen I gels caused a significant decrease of invasiveness by U178 or U251 glioma cells compared with controls without any supplemented antibodies (Fig. 5C); no toxicity caused by the MMP-12 antibody was noted and this was confirmed by a consistent MMP-2 level across control and MMP-12 antibody samples (Fig. 5C). In contrast to the MMP-12 antibody, anti-MMP-2 or anti-MMP-9 antibodies did not alter the tenascin-C-stimulated invasiveness (Fig. 5C). These observations show that the elevation of MMP-12 by tenascin-C in three-dimensional cultures was functionally involved in the process of tenascin-C-mediated glioma invasiveness.

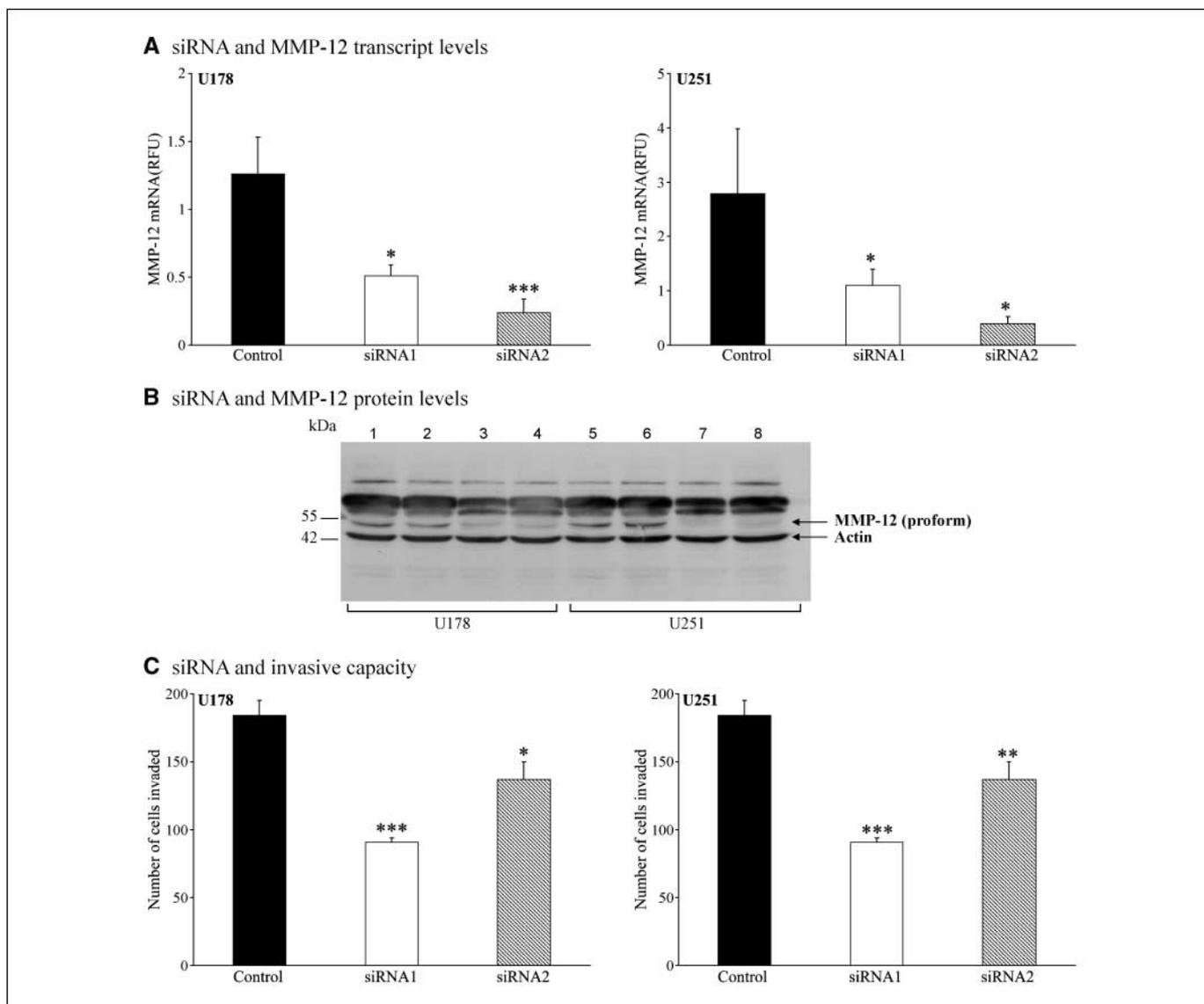


Figure 6. siRNA experiments validate tenascin-C-stimulated glioma cell invasion to be mediated through MMP-12. *A*, both siRNAs targeted toward different regions of MMP-12 reduced the expression of MMP-12 mRNA transcript as detected by SYBR green real-time PCR in U178 or U251 glioma cells compared with control siRNA-treated cells. *B*, protein expression for pro-MMP-12 was also reduced by the siRNA treatment. Whereas the nature of the nonspecific bands above MMP-12 is unclear, they do serve as internal controls alongside actin in denoting equal loading of gels. *Lanes 1 and 5*, wild-type cells; *lanes 2 and 6*, glioma cells treated with scrambled controls; *lanes 3 and 7*, cells treated with siRNA1; *lanes 4 and 8*, siRNA2-exposed cells. The Western blot results were reproduced in three other experiments. *C*, when siRNA-treated cells were allowed to invade through three-dimensional collagen I matrix supplemented with tenascin-C, the invasiveness of glioma cells was attenuated significantly compared with control siRNA-treated cells. In control experiments, the siRNA was not found to affect the viability of cells. *, $P < 0.5$; **, $P < 0.01$; ***, $P < 0.001$, compared with the respective controls.

To further test the contention that tenascin-C-mediated glioma invasiveness involves MMP-12, siRNA to MMP-12 was used. Figure 6 reveals that the two siRNAs targeting different regions of MMP-12 reduced MMP-12 transcripts and protein in both U178 and U251 cell lines; concordantly, glioma invasiveness across the collagen I + tenascin-C matrix was also attenuated. Finally, of several MMPs that were measured in RNA samples, the siRNA to MMP-12 reduced only the transcript for MMP-12 (Supplementary Fig. S2).

Discussion

The interaction of tumor cells with their stromal environment is a critical determinant of the growth of that tumor (34, 35). Similarly, glioma invasiveness is modulated by cell-to-ECM interactions and

ECM degradation by proteases, which are the key determinants of the nature of the tumor microenvironment. To address glioma invasiveness in the context of ECM and proteases, a model system is helpful. As tumor cells *in situ* are confined by a three-dimensional environment, monolayer growth assays become only partially useful for studies of glioma invasiveness and three-dimensional restraints become necessary. Indeed, studies of other cell types have emphasized that the use of three-dimensional cultures is more representative of *in vivo* growth when compared with two-dimensional monolayer cultures (25–27). Because the majority of studies of MMPs and ECM relating to glioma invasiveness have used monolayer growth cultures, or the infiltration of glioma cells in a two-dimensional layer across a matrix barrier, we sought to evaluate the interactions of glioma cells with ECM and MMPs when the cells

were encased in a three-dimensional constraint. To this end, we have used a collagen I gel because collagen I is a component of the ECM that glioma cells encounter *in vivo* (10, 11, 13, 14). In addition, glioma cells and additional ECM molecules can be embedded within the collagen I matrix for further studies.

The present study shows that in a three-dimensional collagen I matrix, tenascin-C increases the invasiveness of glioma cells, and that this does not involve the gelatinases (MMP-2 and MMP-9) commonly up-regulated in two-dimensional glioma growth. The tenascin-C-mediated invasiveness was metalloproteinase dependent, as this was blocked by BB-94, GM6001, and TIMP-1. A thorough analysis of 21 MMPs and 6 ADAM members indicated the potential importance of MMP-12, and this relevance is supported by examinations of resected human glioma specimens where high MMP-12 level was detected in the high-grade glioblastoma multiformes. The high variability of MMP-12 in the glioblastoma multiforme cases (Fig. 4A) likely reflects the heterogeneous nature of the clinical material. We are aware of only few studies (36, 37) where elevated MMP-12 expression was detected in glioma specimens. Finally, we show that a function-blocking antibody to MMP-12 and siRNA targeting MMP-12 attenuated the tenascin-C-stimulated glioma invasion. The specificity of the MMP-12 siRNA was suggested by the lack of down-regulation of other MMPs (Supplementary Fig. S2), but we did find MMP-3 and MMP-10 to be elevated; the significance of the latter is unclear but some MMP members do undergo compensatory increases when other are reduced (7). Overall, these results have revealed a new metalloproteinase member, MMP-12, in regulating glioma invasiveness, particularly in the context of tenascin-C within a three-dimensional matrix.

We did not characterize the particular cell types expressing MMP-12 in resected tumor specimens because MMP-12 antibody stainings have tended to be ambiguous. Based on glioma cells expressing MMP-12 in culture (Figs. 3, 5, and 6), it is logical to assume that glioma cells would be producing MMP-12 *in vivo*. An additional source of MMP-12 in glioma specimens *in situ* would be macrophages or microglia, as these are well represented in glioma tissues (32) and are known producers of MMP-12 (33). Regardless of the cell source, the up-regulation of MMP-12 by tenascin-C would conceivably favor the degradation of barriers that retard the movement of glioma cells, thereby favoring invasiveness.

The identification of tenascin-C as a facilitatory factor for glioma invasiveness in a three-dimensional matrix is relevant because it is increased in brain tumors where its expression increases with tumor grade (15, 16, 18, 20, 38). *In vitro* studies have found that tenascin-C increases the migration of glioma cells in monolayer or spheroid cultures (39–41). Our results of tenascin-C inducing the increased invasiveness of glioma cells embedded within a three-dimensional matrix thus extend these findings of an important role of tenascin-C in glioma biology.

This is the first report that reveals a role for MMP-12 in mediating glioma invasiveness. Besides ECM degradation, other functions of MMP-12 could also be important in its role as a tumor promoter. MMP-12 can activate other MMPs, such as MMP-2 and

MMP-3, to exaggerate the cascade of proteolytic processes (42). MMPs can also facilitate signaling, survival, and proliferation (3, 6). Thus, it is possible that the elevation of MMP-12 by tenascin-C *in vivo* could enhance glioma tumorigenicity by degrading ECM barriers for invasiveness, by releasing ECM-bound growth factors following degradation of ECM, and by mediating intracellular signaling relevant to invasiveness, survival, and proliferation.

There is now substantial evidence that the family of MMPs is important for various aspects of glioma activity. Functionally, inhibitors of metalloproteinase activity attenuate the growth of glioma cells implanted into the flank (43) or brain of mice (44). Impressively, parenterally given inhibitors of metalloproteinase activity reduce the spread and size of intracranial gliomas in rodents (45). In clinical trials of glioma patients, inhibitors of metalloproteinase activity seem to have an effect, albeit small, on the response of patients (46, 47). 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, a metalloproteinase inhibitor, provided the best outcome in phase II trials (48). Recently, a randomized, double-blind, placebo-controlled trial found that whereas a MMP inhibitor, marimastat, did not improve survival in patients with glioblastoma following surgery and radiotherapy, there were favorable observations in patients on marimastat in combination with cytotoxic chemotherapy (49). These results are encouraging and it is possible that the efficacy of anti-metalloproteinase inhibition in glioma patients will improve if one could identify all the MMP members that mediate the invasiveness of glioma cells, the major cause of recurrence and morbidity of gliomas.

It is important to point out that glioma cells seem to use a number of MMPs for tumor growth. The microenvironment may dictate which MMP members are used in preference. In previous work, we have noted that when glioma cells interact with astrocytes, the activation of MMP-2 occurs to facilitate glioma invasiveness in two-dimensional cultures (30). Recently, we described that the CXCL12 chemokine, up-regulated in glioma specimens (28), activated its receptor on glioma cells to increase membrane-type 2 MMP expression for increased glioma invasiveness (50). In contact with tenascin-C in a three-dimensional environment, we now describe the predominant expression and usage of MMP-12 for invasiveness.

In summary, we have found that in a three-dimensional growth system, tenascin-C is a favorable substrate for glioma invasiveness and that its effect is mediated through MMP-12. The results have identified MMP-12 and tenascin-C as new therapeutic targets for glioma intervention.

Acknowledgments

Received 2/11/2005; revised 7/12/2006; accepted 10/5/2006.

Grant support: Canadian Institutes of Health Research and the European Union Framework 6 Programme.

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