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Matrix Metalloproteinases in Human Gliomas
: Activation of Matrix Metalloproteinase-2 (MMP-2) May be Correlated with Membrane-Type-1 Matrix Metalloproteinase (MT1-MMP) Expression

To evaluate possible roles of matrix metalloproteinase (MMP)-1, -2, tissue inhibitor of metalloproteinase (TIMP)-1, -2 and membrane-type-1 matrix metalloproteinase (MT1-MMP) in invasion of human gliomas, expressions of these proteins were investigated in ten cases of human glioma and two meningioma tissues and eight human glioma cell lines. In gelatin zymography, MMP-2 activities of glioblastomas were higher than astrocytomas. The activated form of MMP-2 was seen in five of six cases of glioblastomas, but not in astrocytomas. MMP-9 activity was detected in all cases of malignant astrocytomas but the reactivity of MMP-9 was weaker than that of MMP-2. MT1-MMP mRNA expression in glioblastomas was higher than that in astrocytomas. Five cases of glioblastomas with activated form of MMP-2 had MT1-MMP expressions. In vitro, human glioma cell lines with high expression of MT1-MMP also showed high MMP-2 activity. TIMP-1 transcripts were constitutively present in almost all glioma tissues and cell lines, whereas TIMP-2 mRNA were weak especially in malignant gliomas. Imbalance of TIMP-2/MMP-2 was observed using immunoprecipitation analysis in a glioma cell line. High expression of MMP-2 and MT1-MMP is possibly involved in invasiveness of malignant glioma.

Key Words: *Glioma; Matrix Metalloproteinases (MMPs); Tissue Inhibitors of Metalloproteinases (TIMPs); Membrane-Type-1 Matrix Metalloproteinase (MT1-MMP); Neoplasm Invasiveness*

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INTRODUCTION

Local invasiveness is one of the key features of primary malignant brain tumors. It accounts for the high incidence of recurrence even following drastic surgical resection. The underlying molecular mechanisms of invasiveness of the brain tumor are complex, involving a series of sequential steps. Proteolytic degradation of the extracellular matrix (ECM) is the most critical step in local invasion. Several proteases are thought to be involved in the degradation of ECM components, among which matrix metalloproteinases (MMPs) have been implicated as key rate-limiting enzymes in ECM degradation and are believed to play a major role in tumor invasion (2).

Matrix metalloproteinase (MMP)-2 and MMP-9 are specific for the degradation of type IV collagen (1), and among all the MMPs, they are perhaps the most widely distributed and have been identified in a number of cell types in normal and malignant cells (2, 3). Increasing evidence shows an association between deregulated production, activation of MMP (in particular MMP-2 and MMP-9), and aggressive behavior in a variety of human cancers such as breast, lung, bladder and malignant gliomas (4-7). These enzymes are produced as latent proenzymes followed by the removal of the proenzyme domain via proteolysis (8). Once secreted and activated, they are inhibited by a family of endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs) (9, 10). Therefore, the balance between the levels of activated MMPs and their inhibitors determines overall MMP activity (11).

Sato et al. have recently discovered a novel membrane-type MMP (MT-MMP) that is a specific activator of the latent form of MMP-2 (12). MT-MMP has a unique structure of transmembrane domain at the C-terminus (13). Recent studies demonstrated that MT1-MMP func-

tions not only as an activator of MMP-2 but also as a receptor for MMP-2/TIMP-2 (14) complex, regulating proteolytic activation. Overexpression of MT1-MMP has been demonstrated in a variety of adult malignant tumors, including lung, breast, gastric and cervical carcinomas. MT1-MMP overexpression correlates with the activation rate of MMP-2 and tumor invasion of these tissues (15-18).

In the present study, we investigated the expression patterns of MMPs (MMP-2, -9, and MT1-MMP) and TIMPs (TIMP-1 and -2) in brain tumor tissues and cell lines.

MATERIALS AND METHODS

Surgical specimens

Fresh human brain tumor tissues and normal baboon brain tissue samples were obtained in the operation room from patients who underwent therapeutic removal of brain tumors. The samples were frozen in liquid nitrogen immediately after surgical removal and stored at -70℃. The samples include six cases of glioblastomas, two cases of anaplastic astrocytomas, two cases of astrocytomas and two cases of meningiomas.

Human glioma cell lines

Eight human glioma cell lines used in this study (HS683MG, T98G, A172, U251MG, U87MG, U373MG, D54MG, and LG-11 derived from human malignant glioma) were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotic agents (penicillin, 10 U/mL and streptomycin 100 μg/mL) in 5% CO_2 at 37℃.

RNA extraction and northern blot analysis

Total RNA (15 μg each) prepared according to the manufacturers was fractionated in a 1.0% denaturing agarose gel containing 6.3% formaldehyde, transferred to nylon membrane (Schleicher & Schuell, Dassel, Germany) and UV cross-linked using a UV cross linker (Stratagen, La Jolla, CA, U.S.A.). The membranes were hybridized to 0.6 kb TIMP-1 and 0.9 kb TIMP-2 cDNA overnight at 42℃, and 1.2 kb MT1-MMP cDNA was labeled with (α-^{32}P)dCTP using random priming kit (Amersham Pharmacia, Uppsala, Sweden). The Northern blots were washed successively for 30 min at room temperature in 2×SSC plus 0.1% SDS and for 5-10 min at 65℃ in 0.1 ×SSC plus 0.1% SDS. Finally, membrane was autoradiographed by exposure to hyperfilm at -70℃.

Western blotting for MT1-MMP protein in human glioma cell lines

Cell extracts were prepared from 100 mm tissues culture plate of confluent cell lines. Cell pellets were lysed in cell lysis buffer and then sonicated for 10 sec. The supernatants were prepared by microcentrifugation at 12,000 *g* at 4℃ for 10 min, and aliquots of the supernatants were used to measure protein concentrations. The supernatants were mixed 1:1 with 2× sample buffer [0.188 mM Tris-HCl (pH 6.8), 3% SDS (w/v), 0.0075% bromophenol blue, 30% glycerol and 3% β-mercaptoethanol], and then equal amounts of protein (30 μg) were loaded onto 10% polyacrylamide gel. The SDS-PAGE separated proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany), and the membrane was blocked with 5% skim milk in TBST for 1 hr at room temperature. Immunoblots were visualized by enhanced chemiluminescence (Amersham Pharmacia), according to protocol. Hyperfilm with cassette closure times of 1-3 min proved in adequate exposure to visualize the bands.

Gelatin zymography

MMP-2 and MMP-9 activities were examined by gelatin zymogram analysis. The supernatants of cell lines were collected after 24 hr incubation in serum free medium. Cell lines and brain tumor tissues were treated with lysis buffer to obtain the lysates, sonicated for 10 sec in Tris buffer (50 mM Tris-HCl, 75 mM NaCl, pH 8.0) and centrifuged at 8,000 *g* for 10 min. The supernatant was aliquoted, and the protein content was determined using BCA protein assay method. Enzymatic activity was identified as a clear band on the gel. Briefly, 20 μL conditioned medium from each cell line and tissue extracts were electrophoresed on 10% SDS-PAGE, containing 0.1% gelatin. After electrophoresis, the gels were rinsed with renaturing buffer (2.5% Triton X-100) at 30 min and incubated at 37℃ for 15 hr in developing buffer [50 mM Tris-HCl (pH 7.4), 20 mM NaCl, 10 mM $CaCl_2$, 0.1% NaN_3] and were stained with 0.5% Coommassie blue in 10% isopropanol and 10% acetic acid and destained in 10% isopropanol and 10% acetic acid in H_2O. Gelatinolytic activities were detected as clear bands on the blue background of the gel.

Immunoprecipitation

For MMPs/TIMPs imbalance study, confluent cultures of D54MG cells were incubated with serum-free medium, containing 250 μCi/mL [^{35}S]methionine for 18 hr. For immunoprecipitation (IP) analysis, equal volume of

culture supernatants were incubated with TIMP-1 and TIMP-2 monoclonal antibodies (Calbiochem, San Diego, CA, U.S.A.) for 2 hr at 4℃. After incubation, antibodies were precipitated with protein G-Sepharose 4B (Sigma Chemical Co., St Louis, MO, U.S.A.). The immunoprecipitants bound to protein G beads were washed 3 times with IP buffer (150 mM NaCl, 50 mM Tris-HCl, 0.2% SDS and 0.5% sodium deoxycholate), once in high salt buffer [10 mM Tris-HCl (pH 7.4), 0.5 M NaCl] and once in law salt buffer [10 mM Tris-HCl (pH 7.4)]. Then samples of immunoprecipitants and supernatants after IP were analyzed by gelatin zymography.

RESULTS

MMP-2 and MMP-9 activities in human gliomas and glioma cell lines

MMP-2 activity was present in all brain tumor tissues, but it was expressed highly in glioblastomas. MMP-2 activity was lower in astrocytomas and meningioma than in malignant brain tumors. In contrast, MMP-9 activity was detected only in malignant gliomas and not in low-grade astrocytomas and meningioma. Human brain tumor tissues revealed that almost all samples examined contained a latent form of MMP-2, whereas the activated form of MMP-2 was only seen in two cases of glioblastomas. Glioma cell lines showed high MMP-2 activity and relatively low MMP-9 activity (Fig. 1).

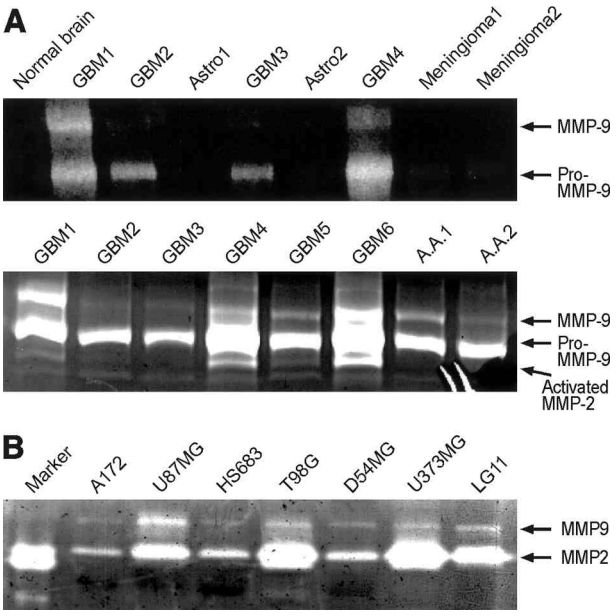

Fig. 1. Gelatin zymographic analysis of gelatinase A (MMP-2) and gelatinase B (MMP-9) activities in human brain tumors and normal baboon brain tissue (A) and brain tumor cell lines (B). Major gelatinolytic activities of 92 kDa, 72 kDa and 63 kDa, which correspond to pro-MMP-9, pro-MMP-2 and active MMP-2, respectively, are indicated. Detailed experimental procedures are described under Materials and Methods (A: GBM, glioblastoma; Astro, astrocytoma; A.A., anaplastic astrocytoma; B: Marker, MMP-2 standard marker).

MT1-MMP expression in human gliomas and glioma cell lines

In various brain tumor tissues, the levels of MT1-MMP transcripts were compared by Northern blot. As shown in Fig. 2, various amounts of a single transcript of 4.5 kb band was detected. MT1-MMP mRNA expression was significantly higher in malignant brain tumors than in low-grade gliomas and meningioms. One case of recurrent glioblastoma (GBM 1) after surgery and radia-

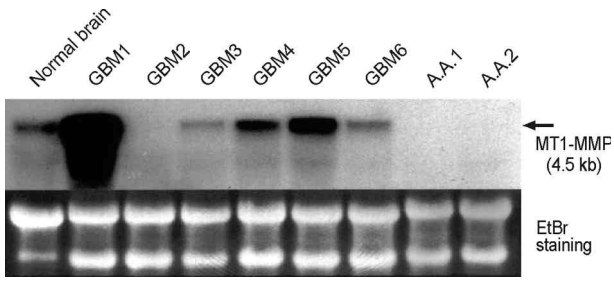

Fig. 2. Northern blot analysis of MT1-MMP RNAs in human brain tumors and normal baboon brain. Arrow indicates the molecular size of the transcripts for MT1-MMP (4.5 kb). Detailed experimental procedures are described under Materials and Methods (A: GBM, glioblastoma; A.A., anaplastic astrocytoma; ErBr, ethidium bromide).

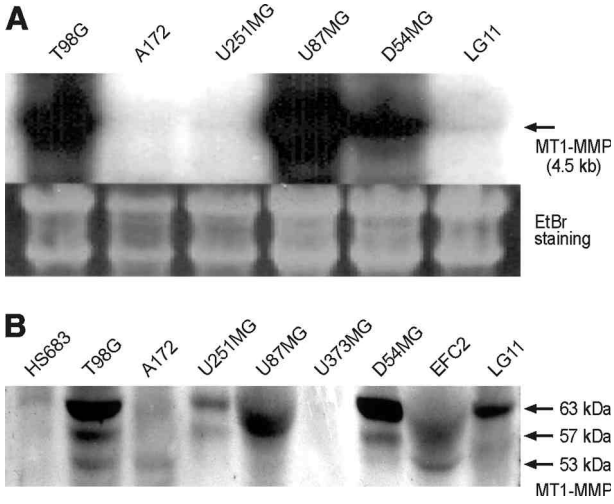

Fig. 3. Northern (A) and Western blot analysis (B) of MT1-MMP on various human glioblastoma cell lines. Arrows indicate the molecular size of the transcripts (4.5 kDa) (A) and proteins (B). Major band of MT1-MMP of protein is 63 kDa size as indicated, and 57 kDa and 53 kDa size bands may be splicing forms of major 63 kDa band.

tion therapy showed a significantly high level of MT1-MMP expression with MMP-2 and MMP-9 activity. Interestingly, MT1-MMP expression was higher in 2 cases of glioblastomas (GBM 1, 2), which showed activated MMP-2 expression. The expression of MT1-MMP in gliobalstoma cell lines U87MG, T98G and D54MG with high MT1-MMP expression also showed high MMP-2 activity (Fig. 3).

Northern blot analysis of TIMP-1 and TIMP-2 mRNA in human gliomas and glioma cell lines

The transcripts were found to be 0.9 kb for TIMP-1, and two specific transcripts of TIMP-2 could be distinguished (3.5 kb and 1.0 kb) by Northern blot. The TIMP-1 transcripts of brain tumor tissues were constitutively present in all glioma tissues except one case of GBM (Fig. 4A). However, the expression of TIMP-2 was very low or not detected in malignant gliomas. In glioma cell lines, TIMP-1 expression was relatively high, compared to TIMP-2 (Fig. 4B). The overall status of MMPs/TIMPs in glioma tissues and cell lines are summarized in Tables 1 and 2.

Identification of imbalance of TIMP-1/MMP-9 and TIMP-2/MMP-2 in glioma cell line

To investigate the imbalance of MMPs/TIMPs in glioma cell line, we observed the TIMP-2/MMP-2 and TIMP-1/MMP-9 complex in conditioned medium of D54MG cells by immunoprecipitation using TIMP-1 and TIMP-2 antibodies. MMP-2 activity was detected not only in immumoprecipitant samples but also in the supernatants after immunoprecipitation. However, MMP-9 activity was detected only in immunoprecipitants and not in supernatants (Fig. 5).

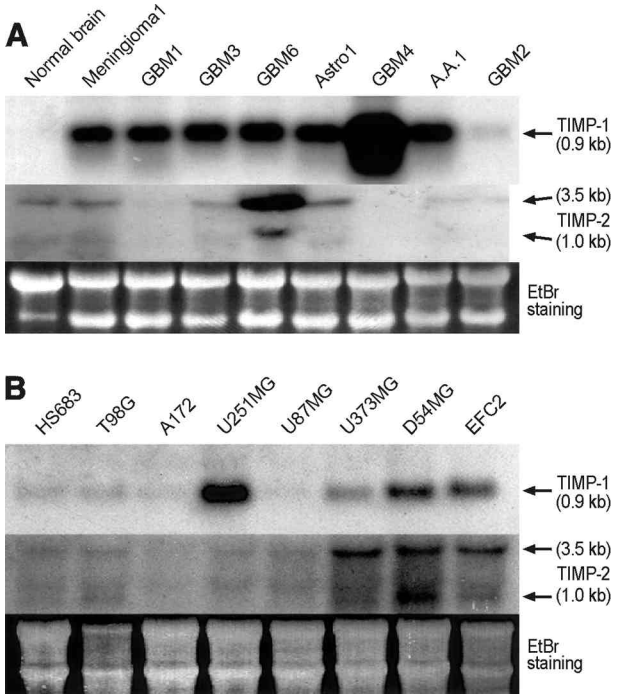

Fig. 4. Northern blot analysis of TIMP-1 and TIMP-2 RNAs in human brain tumors and normal brain **(A)** and human brain tumor cell lines **(B)**. Arrows indicate the molecular size of the transcripts for TIMP-1 (0.9 kb) and TIMP-2 (3.5 kb and 1.0 kb) (A: GBM, glioblastoma; Astro, astrocytoma; A.A., anaplastic astrocytoma; ErBr, ethidium bromide).

Table 1. Summary of the overall status of MMPs/TIMPs in glioma tissues

MMPs or TIMPs / Tumor grade	Pro-MMP-2	Activated MMP-2	MMP-9	MT1-MMP	TIMP-1	TIMP-2
Glioblastoma 1	+++	++	+++	+++	++	+
Glioblastoma 2	++	−	+	−	−	−
Glioblastoma 3	++	+	+	+	++	−
Glioblastoma 4	++	++	++	+	+++	−
Glioblastoma 5	+++	+	+	++	++	−
Glioblastoma 6	++	++	+	+	++	++
Anaplastic astrocytoma 1	++	−	+	−	++	+
Anaplastic astrocytoma 2	++	−	+	−	++	−
Astrocytoma 1	+	−	−	−/+	++	++
Astrocytoma 2	+	−	−	−	++	++
Meningioma 1	+	−	−	−	++	++
Meningioma 2	+	−	−	−	+	+
Normal baboon brain	−	−	−	+	−	+

−: negative, +: weak, ++: moderate, +++: strong degree of expression
MMP-2, 9: matrix metalloproteinase-2, -9; MT1-MMP: membrane-type-1 MMP; TIMP-1, -2: tissue inhibitor of metalloproteinase-1, -2

Table 2. Summary the overall status of MMPs/TIMPs in glioma cell lines

MMPs or TIMPs / Glioma Cell Lines	MMP-2	MMP-9	MT1-MMP	TIMP-1	TIMP-2
A173	++	+	−	+	−/+
D54MG	++	+	++	++	+
HS683	+	+	−	+	+
LG11	+	−/+	+	−	−
U87MG	+++	+	+++	−/+	−/+
U251	+	+	−	+++	−/+
U373MG	++	+	−	+	+
T98G	+++	+	+++	+	−/+

−: negative, +: weak, ++: moderate, +++: strong degree of expression
MMP-2, 9: matrix metalloproteinase-2, -9; MT1-MMP: membrane-type-1 MMP; TIMP1, -2: tissue inhibitor of metalloproteinase-1, -2

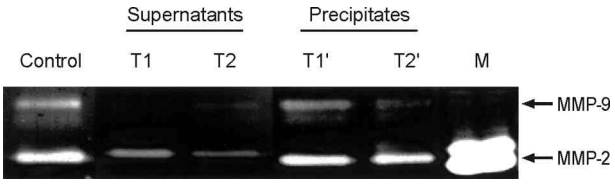

Fig. 5. Gelatin zymographic analysis of supernatants and immunoprecipitants after immunoprecipitation, using anti-TIMP-1 and -2 monoclonal antibodies in D54MG and U87MG glioblastoma cell lines conditioned media. Major gelatinolytic activities of 92 kDa, 72 kDa and 63 kDa, which correspond to pro-MMP-9, pro-MMP-2 and active MMP-2, respectively, are indicated (Control: conditioned medium; T1 and T2: supernatants after immunoprecipitation by TIMP-1 and TIMP-2 monoclonal antibodies, respectively; T1' and T2': immunoprecipitants of TIMP-1 and TIMP-2, respectively).

DISCUSSION

It has been shown that gliomas secrete various MMPs and their endogenous inhibitors, TIMPs (19-22). MMPs have been implicated as an important factor in glioma as they may facilitate invasion into the surrounding brain tissue and also participate in neovascularization. Expressions of MMP-2 and MT1-MMP appear to be related to malignant progression in gliomas (19, 21-22). Altered MT1-MMP expression might partly contribute to MMP-2 activation, which in turn facilitates invasion of these tumors. Since MT1-MMP is a critical molecule in the cellular activation of proMMP-2 (12, 23-24), concomitant up-regulation of MMP-2 and MT1-MMP in glioma cells would result in a considerable accumulation of enzyme activity in glioma tissues. In this study, we found that MT1-MMP protein was expressed at high levels in human malignant glioma tissues and cell lines, and the activated form of MMP-2 is predominantly expressed in glioblastoma tissues, the most aggressive and phenotypically transformed of the gliomas. In particular, MT1-MMP expressions were higher in five cases of glioblastomas which also showed higher activated MMP-2 expression. On the other hand, activated form of MMP-2 was not detectable in anaplastic astrocytomas and astrocytomas. Northern and Western blot analyses revealed that MT1-MMP transcripts and protein expression were high in U87MG, T98G and D54MG, which also showed high MMP-2 activity. Using these data we carefully suggest that MT1-MMP is present in malignant human brain tumors, and MT1-MMP expression correlates with MMP-2 expression and activation during the malignant progression in vivo. Interestingly, one case of recurrent glioblastoma (GBM 1) after surgery and radiation therapy showed very high levels of MT1-MMP expression with MMP-2 and MMP-9 activity.

Several studies have emphasized that the balance between MMPs and their inhibitors are critical for the control of proteolysis of extracellular matrix (25). It has been reported that TIMP-1 and TIMP-2 were expressed in normal brain and in tumor tissues but were significantly lower in highly invasive glioblastomas (11). Low-grade gliomas may produce proportionately more TIMPs than high-grade tumors, thus explaining the different degrees of invasiveness (11). Likewise, our results showed that the TIMP-2 level of malignant gliomas was lower than that of low-grade gliomas, whereas the TIMP-1 level was relatively high in malignant gliomas in agreement with others (19). Accordance with the results of malignant gliomas, the TIMP-1 expression level was higher than TIMP-2 expression in glioblastoma cell lines. We also confirmed the overwhelming effect of MMP-2 in D54MG, a malignant glioma cell line using immunoprecipitation assay. This data suggests that the high MMP-2 activity with low expression of TIMP-2 was an important factor in invasion of malignant gliomas as reported by Mohanam et al. (11).

In conclusion, our results suggest that increased expression of MT1-MMP and activated MMP-2 correlate

with the malignant potential and invasiveness of glioblastomas. The results also demonstrated that high MMP-2 activity with low expression of TIMP-2 was an important factors in the invasion of malignant glioma.

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