Expression and Tissue Localization of Membrane-Type 1, 2, and 3 Matrix Metalloproteinases in Human Astrocytic Tumors

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Three different membrane-type matrix metalloproteinases (MT1-, MT2-, and MT3-MMPs) are known to activate *in vitro* the zymogen of MMP-2 (pro-MMP-2, progelatinase A), which is one of the key MMPs in invasion and metastasis of various cancers. In the present study, we have examined production and activation of pro-MMP-2, expression of MT1-, MT2-, and MT3-MMPs and their correlation with pro-MMP-2 activation, and localization of MMP-2, MT1-MMP, and MT2-MMP in human astrocytic tumors. The sandwich enzyme immunoassay demonstrates that the production levels of pro-MMP-2 in the anaplastic astrocytomas and glioblastomas are significantly higher than that in the low-grade astrocytomas ($P < 0.05$ and $P < 0.01$, respectively), metastatic brain tumors ($P < 0.05$), or normal brains ($P < 0.01$). Gelatin zymography indicates that the pro-MMP-2 activation ratio is significantly higher in the glioblastomas than in other astrocytic tumors ($P < 0.01$), metastatic brain tumors ($P < 0.01$), and normal brains ($P < 0.01$). The quantitative reverse transcription polymerase chain reaction analyses demonstrate that MT1-MMP and MT2-MMP are expressed predominantly in glioblastoma tissues (17/17 and 12/17 cases, respectively), and their expression levels increase significantly as tumor grade increases. MT3-MMP is detectable in both astrocytic tumor and normal brain tissues, but the mean expression level is approximately 50-fold lower compared with that of MT1-MMP and MT2-MMP in the glioblastomas. The activation ratio of pro-MMP-2 correlates directly with the expression levels of MT1-MMP and MT2-MMP but not MT3-MMP. *In situ* hybridization indicates that neoplastic astrocytes express

MT1-MMP and MT2-MMP in the glioblastoma tissues (5/5 cases and 5/5 cases, respectively). Immunohistochemically, MT1-MMP and MT2-MMP are localized to the neoplastic astrocytes in glioblastoma samples (17/17 cases and 12/17 cases, respectively), which are also positive for MMP-2. *In situ* zymography shows gelatinolytic activity in the glioblastoma tissues but not in the normal brain tissues. These results suggest that both MT1-MMP and MT2-MMP play a key role in the activation of pro-MMP-2 in the human malignant astrocytic tumors and that the gelatinolytic activity is involved in the astrocytic tumor invasion. (Am J Pathol 1999, 154:417–428)

Proteolytic extracellular matrix (ECM) degradation is a key step in tumor invasion and metastasis. Although various proteinases are involved in the process, matrix metalloproteinases (MMPs), a gene family of metalloproteinases that can degrade ECM components, are believed to play a major role in the invasion and metastasis. Among the MMPs, MMP-2 (gelatinase A) is considered to be especially important in the malignant behavior of the tumor cells.^{1,2} However, overexpression is not enough for the *in vivo* action of MMP-2 as most MMPs are secreted in inactive zymogens (pro-MMPs). Thus, activation is a prerequisite to its functioning *in vivo*.^{3,4} Pro-MMPs can be activated by various factors such as organomercurials, serine proteinases, hypochlorous acid, and acid exposure.^{3,4} Serine proteinases such as plasmin, plasma kallikrein, and neutrophil elastase may be generally important as *in vivo* activators for pro-MMPs.³ However, pro-MMP-2 is unique in that it is not activated by serine proteinases,⁵ but activated by membrane-type MMP (MT-MMP).⁶ Four different members of MT-MMPs (MT1-, MT2-, MT3-, and MT4-MMPs) have been cloned,^{6–9} but MT4-MMP differs from the other three in that it is only 30% homologous to MT1-MMP⁹ and has no activator function (M. Seiki et al, unpublished data). Correlations between

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the MT1-MMP expression and pro-MMP-2 activation have been reported in various human cancer tissues,^{2,10-16} suggesting the involvement of MT1-MMP in pro-MMP-2 activation in such tumors. However, information about MT2-MMP and MT3-MMP expression and their roles in human tumors is limited.

A characteristic of malignant astrocytic tumors is their ability to infiltrate and invade the surrounding normal brain tissue. Glioma invasion involves cell adhesion and proteolytic degradation of the ECM,¹⁷ and MMP-2 has been shown to correlate with the invasive activity of the human glioma cell lines.^{18,19} Active MMP-2 species are also detected in human malignant astrocytic tumor tissues,^{12,20} and MT1-MMP expression is known in the tumors.¹² However, these studies determined neither the relationship between the expression and pro-MMP-2 activation nor the expression of MT2-MMP and MT3-MMP in the tumors. Thus, the question of which MT-MMP is responsible for pro-MMP-2 activation in astrocytic tumors remains unanswered. In addition, tissue localization of the activity has not been studied in the astrocytic tumor tissues.

In the present study, we examined the expression of MT1-, MT2-, and MT3-MMPs, correlation between their expression and pro-MMP-2 activation, and tissue localization of these MMPs and gelatinolytic activity in the human astrocytic tumor tissues. The results suggest that overexpression of pro-MMP-2 and its activation mediated by MT1-MMP and MT2-MMP are important in the invasive behavior of the malignant astrocytic tumors.

Materials and Methods

Clinical Samples and Histology

Fresh human brain tumor tissues were obtained from 35 patients with astrocytic tumor and 4 patients with metastatic brain tumor (metastatic lung adenocarcinoma) who underwent therapeutic removal of brain tumors. Normal brain tissues were obtained from 5 patients undergoing temporal lobectomy for the epilepsy. The samples were snap-frozen in liquid nitrogen immediately after surgical removal and stored at -80°C to obtain total mRNA and proteins. They were also fixed with periodate-lysine-paraformaldehyde or 4% paraformaldehyde fixative for 18 to 24 hours at 4°C for immunohistochemical study. Histological diagnosis was made by standard light-microscopic evaluation of the sections stained with hematoxylin and eosin (H&E). The classification of human brain tumors used in this study is based on the revised World Health Organization criteria for tumors of the central nervous system.²¹ A total of 35 astrocytic tumors consisted of 9 low-grade astrocytomas, 9 anaplastic astrocytomas, and 17 glioblastomas. All of the tumor tissues were obtained at primary resection, and none of the patients had been subjected to chemotherapy or radiation therapy before resection.

Tissue Homogenates and Sandwich Enzyme Immunoassay for MMP-2

Tissue samples of the brain tumors (35 astrocytic tumor and 4 metastatic brain tumor cases) and control normal brain (5 cases) stored at -80°C were homogenized in 50 mmol/L Tris/HCl buffer, pH 7.5, containing 0.15 mol/L NaCl, 10 mmol/L CaCl_2 , and 0.05% Brij35 on ice. The homogenates were then centrifuged at 4°C for 20 minutes at $10,000 \times g$, and protein concentrations in the supernatants were determined by the dye-binding method according to the manufacturer's instructions (Bio-Rad, Hercules, CA). Concentrations of MMP-2 in the tissue homogenates were measured in the corresponding sandwich enzyme immunoassay (EIA) system for MMP-2 as described previously.²² The EIA system measures pro-MMP-2 and its complex form with tissue inhibitor of metalloproteinases-2 (TIMP-2) but not active MMP-2 species. The values, nmol/g of protein, were determined by using the molecular weight of pro-MMP-2, 70,930, which was calculated by amino acid sequence.

Gelatin Zymography

Gelatinolytic activity in the above-mentioned tissue homogenates was examined by gelatin zymography. The supernatants (50 μg of protein/lane) were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 0.2% gelatin-containing gels as described previously.²³ The supernatants were incubated at 37°C for 30 minutes in SDS sample buffer without reducing agent and then electrophoresed on 9% polyacrylamide gels at 4°C . After electrophoresis, gels were washed in 2.5% Triton X-100 to remove SDS, incubated for 36 hours at 37°C in 50 mmol/L Tris/HCl, pH 7.5, containing 0.15 mol/L NaCl, 10 mmol/L CaCl_2 , and 0.02% NaN_3 , and then stained with 0.1% Coomassie brilliant blue R250. Ratios of pro-MMP-2 activation were estimated by computer-assisted densitometric scanning of M_r 62,000 and M_r 68,000 proteolytic bands, which correspond to active and latent species of MMP-2, respectively.²³

RNA Extraction and Quantitative Reverse Transcription Polymerase Chain Reaction

Total RNA was isolated from surgical specimens by ISO-GEN (Nippon Gene, Toyama, Japan). RNA extracted was treated with RNase-free DNase (Boehringer Mannheim, Mannheim, Germany) to eliminate DNA contamination in the samples and converted to a single-stranded cDNA using a random hexamer of oligonucleotide (Takara, Otsu, Japan). Randomly primed cDNAs were prepared from 5 μg of total RNA by M-MLV reverse transcriptase (Gibco BRL, Gaithersburg, MD) followed by PCR amplification. Nonradioisotopic quantitative RT-PCR was done as previously described.²⁴ cDNAs obtained from surgical specimens were amplified using specific primers of human MT1-MMP (forward primer 5'-TCGGCCCCAAG-CAGCAGCTTC-3', reverse primer 5'-CTTCATGGTGTCT-GCATCAGC-3'), MT2-MMP (forward primer 5'-CAG-

CCCAGCCGCCATATGTC-3', reverse primer 5'-CTT-TCACTCGTACCCCGAAC-3'), MT3-MMP (forward primer 5'-ACAGTCTGCGGAACGGAGCAG-3', reverse primer 5'-GTCAATTGTGTTTCTGTCCAC-3'), and GAPDH (forward primer 5'-CCACCCATGGCAAATTCATGGCA-3', reverse primer 5'-TCTAGACGGCAGGTCAGGTCCACC-3'). PCR conditions for MT-MMP amplifications were 20 to 36 cycles at 2-cycle intervals, at 94°C for 1 minute, 50°C for 1 minute, and 72°C for 1 minute, followed by incubation at 72°C for 3 minutes. The conditions for GAPDH amplifications were the same as those for MT-MMPs except for 1 minute of annealing at 60°C. The products were electrophoresed on 3% agarose gels, including 0.1 µg/ml ethidium bromide. The intensity of ethidium bromide fluorescence was measured using a charge-coupled device imaging system (FAS II, TOYOBO, Tokyo, Japan) and Digital Image File Fujix DF-20 (Fuji Photo Film, Tokyo, Japan). The reaction cycle/PCR product of each reaction mixture was plotted on semilogarithmic graphs for each sample. To obtain the control curves, serial dilutions of MT-MMP and GAPDH plasmid cDNAs⁶⁻⁸ were prepared, and PCR was performed in the same way as surgical specimens, as we have previously described.¹⁶ The sample concentration/PCR products of each reaction mixture were plotted on semilogarithmic graphs. To standardize the condition of gel staining, a constant amount of control DNA marker (Promega, Madison, WI) was electrophoresed every time. The PCR procedure was performed at least three times for each sample.

Northern Blot Analysis

To confirm the quantification of RT-PCR, six specimens of glioblastoma and three specimens of normal brain were used for Northern blotting. The RNA samples (30 µg/lane) were electrophoresed on 1% agarose gels containing 2.2 mol/L formaldehyde and transferred onto Hybond N⁺ membranes (Amersham International, Tokyo, Japan). The membranes were hybridized with ³²P-labeled probes for MT1-MMP (a 1.2-kb cDNA fragment corresponding to nucleotides 1647 to 2889),⁶ MT2-MMP (a 1.2-kb fragment to nucleotides 273-1526),⁷ MT3-MMP (a 2.1-kb fragment to nucleotides 1-2107)⁸ and GAPDH as previously described.^{6,8} As a control, total RNA was extracted from OSC-19 cells (a highly metastatic oral squamous cell carcinoma cell line), which are known to express MT-MMPs,^{8,15} and the samples were processed in a similar way. The blotted membranes were scanned by Bioimage analyzer BAS 1000 (Fuji Photo Film).

In Situ Hybridization

To verify the origin of cells expressing MT1-MMP and MT2-MMP mRNA, the glioblastoma samples (five cases) that showed MT1-MMP and MT2-MMP expression by Northern blotting were used for *in situ* hybridization by modification of the methods previously described.²⁵ Briefly, the cDNA fragments encoding MT1-MMP nucleotides 2483 to 2884 (401 bp) and MT2-MMP nucleotides 1249 to 1716 (467 bp) were subcloned into Bluescript KS

Figure 1. Amounts of pro-MMP-2 in the tissue homogenates of astrocytic tumors, metastatic brain tumors, and normal brains. The supernatants from normal brain (NB), low-grade astrocytoma (LGA), anaplastic astrocytoma (AA), glioblastoma (GB), and metastatic brain tumor (Meta) tissues were prepared, and pro-MMP-2 was measured by EIA. Values (nmol/g of protein) were calculated as described in Materials and Methods. Bars indicate the mean value. **P* < 0.05; ***P* < 0.01.

(Stratagene, La Jolla, CA), and sense and antisense digoxigenin-labeled RNA probes were prepared with T3 or T7 RNA polymerase using DIG RNA labeling kit (Boehringer Mannheim). Paraffin sections of the tissues, which were treated with 10 µg/ml proteinase K (Promega Biotech, Oakland, CA) and 0.0025% acetic anhydride (Eastman Kodak, Rochester, NY) in 0.1 mol/L triethanolamine, pH 8.0 (Eastman Kodak), were hybridized with antisense RNA or sense RNA for ~12 hours at 50°C. The slides were then treated with 20 µg/ml RNase A and washed under stringent conditions (2X SSC, 0.5X SSC, and 0.1X SSC, twice for 30 minutes each at 50°C). They were incubated with alkaline-phosphatase-conjugated Fab fragments from sheep anti-digoxigenin antibody (1:1000 dilution; Boehringer Mannheim) at room temperature for 1 hour. Color was developed with nitro blue tetrazolium chloride (Boehringer Mannheim) and 5-bromo-4-chloro-3-indolyl phosphate (DIG nucleic acid detection kit, Boehringer Mannheim). Counterstaining was performed with safranin O. The regions of sequence used to produce riboprobes were selected carefully to avoid stretches of sequence that might cross-hybridize. A computer scan for regions of homology to the MT1-MMP and MT2-MMP probes to the published cDNA sequences for other MMPs, including MT-MMPs, showed that the MT1-MMP and MT2-MMP probes had the highest homology to MT2-MMP (45%) and MT1-MMP (36%), respectively. However, no cross-hybridization to these MT-MMPs was observed using the hybridization protocol described in the present study.

Antibodies, Immunohistochemistry, and Immunoblotting

The monoclonal antibodies to MT1-MMP and MT2-MMP were developed by use of the synthetic peptides,

Figure 2. Gelatin zymography of the tissue homogenates from brain tumors and normal brains and activation ratios of pro-MMP-2. **A:** Gelatin zymography. The supernatants of tissue homogenates from normal brains (lanes 1 and 2, NB), low-grade astrocytomas (lanes 3 and 4, LGA), anaplastic astrocytomas (lanes 5 and 6, AA), glioblastomas (lanes 7 to 11, GB), and metastatic brain tumors (lanes 12 and 13, Meta) were subjected to gelatin zymography as described in Materials and Methods. Thirteen representative samples are shown. Major gelatinolytic activities of M_r 68,000 and M_r 62,000, which correspond to pro-MMP-2 and active MMP-2, respectively, are indicated. **B:** Activation ratios of pro-MMP-2. The activation ratios (percent) were measured by a computer-assisted densitometric analysis of the gels as described in Materials and Methods. Bars indicate mean value. $^{**}P < 0.01$.

REVPYAYIREGHEK (corresponding to the amino acids at positions 160 to 173 in human MT1-MMP) and DTDN-FQLPEDDLRG (corresponding to the amino acids at positions 281 to 294 in mouse MT2-MMP), respectively, and provided by Dr. Kazushi Iwata at Fuji Chemical Industries (Takaoka, Japan). The monospecific reactivity of the antibodies and their applicability to immunohistochemistry were determined previously by us.¹⁵ The paraffin sections were immunostained using the monoclonal antibodies to MT1-MMP (30 μ g/ml; clone 114-6G6) and MT2-MMP (30 μ g/ml; clone 162-22G5) or nonimmune mouse IgG (30 μ g/ml). After reactions with biotinylated horse IgG to mouse IgG (Vector Laboratories, Burlingame, CA) and an avidin-biotin-peroxidase complex (DAKO, Glostrup, Denmark), the color was developed with 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO). Immunostaining for MMP-2 was performed in a similar way using a monoclonal antibody to MMP-2 (2 μ g/ml; clone 75-7F7) that recognizes both zymogen and active forms.²² Counterstaining was performed with hematoxylin.

Supernatants of the homogenates (50 μ g/lane) from six glioblastomas and three normal brains were resolved by SDS-PAGE (10% total acrylamide) under reduction

Figure 3. PCR of plasmid cDNAs for MT-MMPs and GAPDH and correlation between concentrations of plasmid cDNAs and fluorescence intensity. **A:** Serial 1:10 dilutions of the plasmid cDNAs were used for PCR, and PCR products at 28 cycles were electrophoresed as described in Materials and Methods. Lanes 1 to 7 for MT1-MMP (MT1), $10^{-19.7}$ to $10^{-13.7}$ mol; lanes 1 to 7 for MT2-MMP (MT2), $10^{-19.6}$ to $10^{-13.6}$ mol; lanes 1 to 7 for MT3-MMP (MT3), $10^{-19.6}$ to $10^{-13.6}$ mol; lanes 1 to 7 for GAPDH, $10^{-20.1}$ to $10^{-14.1}$ mol. **B:** Correlation between concentrations of plasmid cDNAs and fluorescence intensity. Fluorescence intensity of PCR products was plotted against the concentrations of logarithmically diluted MT-MMP and GAPDH cDNAs. Linear correlations are obtained. $\circ$, MT1-MMP; $\square$, MT2-MMP; $\bullet$, MT3-MMP; $\blacksquare$, GAPDH.

and transferred onto nitrocellulose filters (Amersham International, Little Chalfont, UK). The filters were reacted with 20 μ g/ml monoclonal antibodies to MT1-MMP (clone 114-6G6) and MT2-MMP (clone 162-22G5) or 20 μ g/ml nonimmune mouse IgG. After reactions with biotinylated horse IgG to mouse IgG (Vector Laboratories) and an avidin-biotin-peroxidase complex (DAKO), the color was developed with 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.) as described previously.²

In Situ Zymography

The fresh specimens of the glioblastoma and normal brain tissues (five and two cases, respectively) were embedded without fixation in Tissue-Tek OCT compound (Miles, Elkhart, IN). Serial frozen sections were made by a cryostat (MICROM, Walldorf, Germany) and mounted onto the gelatin films that were coated with 7% gelatin

Figure 5. mRNA expression of MT1-, MT2-, and MT3-MMP in brain tumor and normal brain tissues by RT-PCR. Total RNA was extracted from the normal brains (lanes 1 and 2, NB), low-grade astrocytomas (lanes 3 and 4, LGA), anaplastic astrocytomas (lanes 5 and 6, AA), glioblastomas (lanes 7 to 11, GB), and metastatic brain tumors (lanes 12 and 13, Meta) and reverse-transcribed into cDNA followed by a PCR reaction. PCR amplification was performed by running 28 cycles. Thirteen representative samples, which correspond to those in Figure 2A, are shown. Each amplification of MT1-MMP (MT1), MT2-MMP (MT2), and MT3-MMPs (MT3) and GAPDH was performed at least three times.

solution (Fuji Photo Film) or slide glasses. The films with sections were incubated for 24 hours at 37°C in a moisture chamber and stained with 1.0% Amido Black 10B. The gelatin in contact with the proteolytic areas of the sections was digested, and thus zones of enzymic activity were indicated by negative staining. The digested areas in the sections were compared with the serial sections stained with H&E. As a control, glioblastoma tissues were incubated in Dulbecco's modified Eagle's medium containing 0.2% lactalbumin hydrolysate with or without 50 μ mol/L BB94 (British Biotech Pharmaceuticals, Oxford, UK) for 3 hours at 37°C, and the frozen sections were treated in a similar way as described above.

Statistics

Statistical analyses were performed using the χ^2 test and the two-tailed Mann-Whitney *U* test. *P* values less than 0.05 were considered significant.

Results

Sandwich Enzyme Immunoassay for Pro-MMP-2

To measure the amounts of pro-MMP-2 produced by the tumor tissues, the EIA system was applied to the supernatants of the tissue homogenates. As shown in Figure 1, the production levels of pro-MMP-2 in the anaplastic astrocytomas (0.021 ± 0.010 nmol/g weight; *n* = 9) and glioblastoma samples (0.038 ± 0.023 ; *n* = 17) were

Figure 4. Correlation between the data by quantitative RT-PCR and Northern blotting. **A:** Northern blotting (upper panel) and RT-PCR (lower panel) for MT1-MMP (MT1), MT2-MMP (MT2), and GAPDH were carried out in six glioblastoma samples as described in Materials and Methods. Molecular sizes of the transcripts for MT1-MMP (4.5 kb), MT2-MMP (3.6 kb), and GAPDH (1.2 kb) are indicated. RNA from OSC-19 cells was used as a positive control. RT-PCR products for MT1-MMP (180 bp), MT2-MMP (169 bp), and GAPDH (598 bp) at 28 cycles are shown. **B** and **C:** Correlations between the ratios of MT1-MMP/GAPDH or MT2-MMP/GAPDH by Northern blotting and quantitative RT-PCR. The ratios of MT1-MMP/GAPDH or MT2-MMP/GAPDH were calculated as described in Materials and Methods. Each level is represented by the highest level of MT-MMP mRNA expression as 1 in either Northern blotting or quantitative RT-PCR. Note a direct correlation in both MT1-MMP/GAPDH (*r* = 0.976, **B**) and MT2-MMP/GAPDH (*r* = 0.995, **C**).

Figure 6. The mRNA expression levels of MT1-, MT2-, and MT3-MMPs in astrocytic tumors, metastatic brain tumors, and normal brains. The relative mRNA expression levels (MT-MMP/GAPDH ratios) of MT1-MMP (A), MT2-MMP (B), and MT3-MMP (C) were analyzed by the quantitative RT-PCR method. Each level is represented by the highest level of MT1-MMP mRNA expression as 1. Bars indicate mean value. * $P < 0.05$; ** $P < 0.01$.

remarkably higher than those in the low-grade astrocytomas (0.012 ± 0.008 ; $n = 9$; $P < 0.05$ and $P < 0.01$, respectively), control normal brain tissues (0.006 ± 0.003 ; $n = 5$; $P < 0.01$), and metastatic brain tumors (0.010 ± 0.004 ; $n = 4$; $P < 0.05$).

Activation of Pro-MMP-2

Pro-MMP-2 activation in the supernatants was analyzed by gelatin zymography. Pro-MMP-2 of M_r 68,000 was detected in all of the samples examined, and the levels in glioblastomas and anaplastic astrocytomas appeared to be higher than those of other samples (Figure 2A), confirming the EIA data described above. On the other hand, the active species of M_r 62,000 was found in all of the glioblastoma and metastatic brain tumor samples (17/17 cases and 4/4 cases, respectively) and in 67% of the anaplastic astrocytomas (6/9 cases), but it was absent in the normal brain and low-grade astrocytoma samples (Figure 2A). Computer-assisted image analyses of the proteolytic bands indicated that the activation ratio of pro-MMP-2 (the ratio of the active form to pro-MMP-2 and active forms) is significantly higher in the glioblastomas ($15 \pm 6\%$; $n = 17$) than in the anaplastic astrocytomas ($5 \pm 4\%$; $n = 9$; $P < 0.01$) or metastatic brain tumors ($6 \pm 3\%$; $n = 4$; $P < 0.01$) (Figure 2B).

mRNA Expression of MT1-, MT2-, and MT3-MMPs

To evaluate the expression levels of each MT-MMP, quantitative analyses of the mRNA expression of MT-MMPs and GAPDH were performed according to the modification of the methods reported by our recent study.¹⁶ When RT-PCR for MT-MMPs and GAPDH was carried out using total RNA from glioblastomas by running for 20 to 36 cycles at an interval of 2 cycles, the products emerged between 22 and 26 cycles, increased exponentially with cycles up to 30 to 34, and then reached a plateau (data not shown). Thus, PCR amplification was set at 28 cycles, and calibration lines for cDNA concentrations of MT-MMPs and GAPDH were obtained using serial dilutions of plasmid cDNAs for MT-MMPs and GAPDH (Figure 3A). Fluorescence intensity of each PCR product was proportional to the amounts of cDNAs used as templates (Figure 3B), and calibration lines obtained for cDNAs of MT-MMPs and GAPDH were used for further calculation of MT-MMP/GAPDH cDNA molar ratios, which represent MT-MMP/GAPDH mRNA ratios in surgical specimens. We analyzed each sample at least three times by this method, and the difference in the obtained values was less than 2%.

Figure 7. Correlation of pro-MMP-2 activation ratio with mRNA expression of MT1-MMP (A), MT2-MMP (B), and MT1-MMP plus MT2-MMP (C) in glioblastomas. The mRNA expression levels (MT-MMP/GAPDH ratios) were calculated as described in Materials and Methods. Direct correlations are observed in A, B, and C with correlation coefficients of $r = 0.893$ ($P < 0.01$), $r = 0.792$ ($P < 0.01$), and $r = 0.953$ ($P < 0.01$), respectively.

Figure 8. *In situ* hybridization for MT1-MMP and MT2-MMP in glioblastoma tissues. *In situ* hybridization was performed as described in Materials and Methods. Note that strong signals for MT1-MMP (A) and MT2-MMP (C) in the neoplastic astrocytes (arrows) with the antisense probes. The sense probes give only a background signal in the glioblastoma tissues (B and D). Safranin O counterstain. E: Glioblastoma tissue stained with H&E. Bar, 50 μ m.

To ascertain the accuracy of the quantitative RT-PCR, the data were compared with those from Northern blotting. According to the methods reported by Yamamoto et al,¹² intensity of the hybridization signals obtained by Northern blotting with the probes for MT-MMPs was normalized to that with the GAPDH-specific probe. A strong MT1-MMP signal was seen in all of the glioblastoma

samples (6/6 cases, Figure 4A), whereas no hybridization was detected in the normal brain samples (data not shown). MT2-MMP mRNA was also found in glioblastomas (5/6 cases, Figure 4A). However, MT3-MMP mRNA was undetectable in all of the specimens examined (0/6 cases) except for the positive control (data not shown). As shown in Figure 4, B and C, direct correlations were

Figure 9. Immunolocalization of MT1-MMP, MT2-MMP, and MMP-2 in glioblastoma tissues. Paraffin sections were immunostained with monoclonal antibodies against MT1-MMP (A), MT2-MMP (B), and MMP-2 (C) or nonimmune mouse IgG (D) as described in Materials and Methods. Note that MT1-MMP (A), MT2-MMP (B), and MMP-2 (C) are immunostained in the glioblastoma cells (arrows), whereas no staining is observed with nonimmune IgG (D). Hematoxylin counterstain; bar, 50 μ m.

obtained between the results from RT-PCR and Northern blot for MT1-MMP/GAPDH and MT2-MMP/GAPDH. Thus, we used the PCR method for the quantification of mRNA expression for MT-MMPs in the tumor and normal brain tissues.

By RT-PCR at 28 cycles, MT1-MMP mRNA was detected in 100% of the glioblastomas (17/17 cases) and metastatic brain tumors (4/4 cases), whereas it was detectable in 22% of the anaplastic astrocytomas (2/9 cases) and undetectable in the low-grade astrocytomas (0/9 cases) and normal brains (0/5 cases) (Figure 5). MT2-MMP was also expressed in 71% of the glioblastomas (12/17 cases) and in 100% of the metastatic brain tumors (4/4 cases), but it was detected in only 22% of the anaplastic astrocytomas (2/9 cases) and 0% of the low-grade astrocytomas (0/9 cases) and normal brains (0/5 cases) (Figure 5). On the other hand, MT3-MMP mRNA was detectable in 71% of the glioblastomas (12/17 cases), 44% of the anaplastic astrocytomas (4/9 cases), 67% of the low-grade astrocytomas (6/9 cases), and 100% of the normal brains (5/5 cases), but not in metastatic brain tumors (0/4 cases) (Figure 5). The quantitative RT-PCR

indicated that the levels (MT-MMP/GAPDH ratios) of both MT1-MMP and MT2-MMP are significantly higher in the glioblastoma samples (0.49 ± 0.27 and 0.26 ± 0.26 , respectively; $n = 17$) than in the anaplastic astrocytomas (0.07 ± 0.14 , $P < 0.01$, and 0.06 ± 0.12 , $P < 0.05$, respectively; $n = 9$), low-grade astrocytomas (0 and 0, respectively; $n = 9$; $P < 0.01$) and normal brain tissues (0, $P < 0.01$, and 0, $P < 0.05$, respectively; $n = 5$) (Figure 6, A and B). However, the mean expression level of MT3-MMP was at least 50-fold lower compared with that of MT1-MMP and MT2-MMP, and there was no correlation between the MT3-MMP expression and histological grades of the astrocytic tumors (Figure 6C).

Activation of Pro-MMP-2 and Its Correlation with Expression of MT1-, MT2-, and MT3-MMPs

When the activation ratio of pro-MMP-2 was plotted against mRNA expression levels of MT1-MMP or MT2-MMP in each case, it showed direct correlations with the

Figure 10. Immunoblotting for MT1-MMP and MT2-MMP. Tissue homogenates from glioblastoma (GB) and control normal brain tissues (NB) resolved by SDS-PAGE were transferred onto nitrocellulose filters, and the filters immunostained with the antibody to MT1-MMP or MT2-MMP. Note that two bands corresponding to latent and active forms of MT1-MMP (M_r 66,000 and M_r 60,000) or MT2-MMP (M_r 68,000 and M_r 62,000) are found in the glioblastoma samples, whereas no such species are recognized in the control normal brain tissues. The molecular weights of the protein standards are phosphorylase b (M_r 94,000), transferrin (M_r 77,000), bovine serum albumin (M_r 68,000), heavy chain of IgG (M_r 55,000), ovalbumin (M_r 43,000), and carbonic anhydrase (M_r 29,000).

expression of MT1-MMP ($r = 0.893$, $P < 0.01$) and MT2-MMP ($r = 0.792$, $P < 0.01$) (Figure 7, A and B). In addition, the correlation was stronger when the activation ratio was compared with the expression level of MT1-MMP plus MT2-MMP in each case ($r = 0.953$, $P < 0.01$) (Figure 7C). However, no correlation was observed between the activation and MT3-MMP expression (data not shown).

In Situ Hybridization

Cells expressing MT1-MMP and MT2-MMP mRNA in the glioblastomas were identified by *in situ* hybridization. The signals for MT1-MMP and MT2-MMP were observed with the antisense RNA probes mainly in the neoplastic astrocytes (5/5 cases and 5/5 cases, respectively) (Figure 8, A and C) and some endothelial cells. The sense probes gave only a background signal in the glioblastoma tissues (Figure 8, B and D).

Immunohistochemistry and Immunoblotting

MT1-MMP was immunolocalized predominantly to the neoplastic astrocytes in all of the glioblastoma cases (17/17 cases; Figure 9A). In anaplastic astrocytomas and metastatic brain tumors, some atypical cells were weakly immunostained, but no staining was seen in the normal brains or low-grade astrocytomas (data not shown). MT2-MMP was also immunostained predominantly in the gli-

oma cells (12/17 cases; Figure 9B). Endothelial cells of blood vessels in the glioblastoma tissues were occasionally immunostained for MT1-MMP and MT2-MMP. MMP-2 was also localized in the neoplastic astrocytes and endothelial cells in all of the glioblastoma samples (Figure 9C). In normal brain tissues, some endothelial cells reacted with the monoclonal antibody against MMP-2 (data not shown). No staining was observed with nonimmune mouse IgG (Figure 9D).

By immunoblotting, both latent and active forms of MT1-MMP (M_r 66,000 and M_r 60,000) and MT2-MMP (M_r 68,000 and M_r 62,000) were identified in the tissue homogenates of the glioblastoma samples (Figure 10). Immunoblotting of the normal brain samples was negative (Figure 10).

Detection of Gelatinolytic Activity by In Situ Zymography

By *in situ* zymography using gelatin films, strong gelatinolytic activity was detected in the glioblastoma tissues (Figure 11, A and B), but no activity was recognized in the normal brain tissues (Figure 11, E and F). The activity was completely blocked in the glioblastoma tissues that had been incubated with BB94 (Figure 11, C and D). Distributions of the activity were consistent with the immunolocalization of MT1-MMP, MT2-MMP, and MMP-2.

Discussion

The present studies have demonstrated that, among the three different MT-MMPs, both MT1-MMP and MT2-MMP are strongly expressed and their expression correlates with the activation of pro-MMP-2 in the human malignant astrocytic tumors. In addition, as the stronger correlation was observed between the activation and the expression level of MT1-MMP plus MT2-MMP, it is suggested that MT1-MMP and MT2-MMP may work synergistically to activate pro-MMP-2 in the tumors. MT1-MMP expression has been reported in various human cancers, including carcinomas of the stomach,² lung,¹⁰ uterine cervix,¹¹ head and neck,¹⁴ and ovary,¹³ and its correlation with pro-MMP-2 activation is known in the lung¹⁰ and stomach carcinomas.² Our previous studies on the expression of the three MT-MMPs in the carcinomas of the breast¹⁵ and thyroid (H. Nakamura et al, manuscript submitted) demonstrated that almost selective expression of MT1-MMP correlates with the pro-MMP-2 activation in the carcinoma tissues. On the other hand, our recent studies have shown high expression of MT1-MMP and MT2-MMP in the human urothelial carcinomas,¹⁶ although the relation of their expression to the pro-MMP-2 activation could not be performed because of the shortage of the samples for the analyses. Thus, the present study is the first to demonstrate that both MT1-MMP and MT2-MMP are involved in the activation of pro-MMP-2 in human malignant tumors.

MT3-MMP mRNA expression was observed by RT-PCR in the human malignant astrocytic tumors, although it was undetectable by Northern blotting. The quantitative

Figure 11. *In situ* zymography of glioblastoma and normal brain tissues. Serial frozen sections were made and stained with H&E (A, C, and E) or subjected to *in situ* zymography (B, D, and F) as described in Materials and Methods. A and B: Glioblastoma tissues; C and D: Glioblastoma tissues treated with BB94 before the reaction on the films; E and F: Normal brain tissues. Note the strong gelatinolytic activity in the glioblastoma tissues (B) and abolishment of the activity in the BB94-treated tumor tissues (D). Bar, 50 μ m.

RT-PCR indicated that the mean expression level in the brain tumors is at least 50-fold lower than that of MT1-MMP and MT2-MMP. Interestingly, however, the expression surely exists in various human brain samples, including normal brains, whereas no expression is present in the metastatic lung adenocarcinomas. This brain-specific expression agrees with the previous finding that the brain is one of the organs where MT3-MMP is selectively expressed.⁸ However, our data demonstrate that the expression level of MT3-MMP is not significantly different between the normal brain and astrocytic tumor tissues without correlations with the pro-MMP-2 activation. Thus, it seems likely that MT3-MMP may have a different role from the activation in the normal brain or human astrocytic tumors. Recent studies on MT3-MMP showed that this MMP has proteolytic activity against ECM components such as type III collagen.²⁶ We have also demonstrated that recombinant MT3-MMP can digest proteoglycan as well as interstitial collagens (Shimada et al, manuscript submitted). It may be possible to speculate that MT3-MMP is involved in the turnover of ECM in the normal brain and astrocytic tumor tissues.

Previous studies reported the mRNA expression and protein production of MMP-2 by human astrocytic tumors in a small number of samples.^{12,20} The present studies

have demonstrated that pro-MMP-2 production is enhanced significantly as tumor grade increases from low-grade astrocytomas to glioblastomas in 35 astrocytic tumor samples. The increase in pro-MMP-2 level in the tumor tissues is ascribed to enhanced production by the astrocytic tumor cells, as MMP-2 was immunolocalized predominantly to the tumor cells. In contrast, the production level of pro-MMP-2 and its activation in the metastatic lung adenocarcinomas were significantly lower than those in the glioblastomas and primary lung carcinomas.^{6,10} The expression level of MT1-MMP also tended to be lower in the metastatic carcinomas than in the glioblastomas¹² and primary lung adenocarcinomas.^{6,10} This may be explained by the lack of stromal cells originated from the lung tissue in the metastatic adenocarcinomas of the brain, as pro-MMP-2 is produced mainly by stromal fibroblasts in the primary lung carcinomas,^{23,27,28} and MT1-MMP is detected in both carcinoma cells and stromal fibroblasts.¹⁰ Another possibility is, however, the presence of the mechanism by which expression of MMP-2 and MT1-MMP is reduced in the metastatic tumors of the brain. Regardless of the mechanisms, suppression of pro-MMP-2 production and activation might be related to a less invasive character of the metastatic

lung adenocarcinomas in the brain²⁹ compared with the highly invasive malignant astrocytic tumors.

In situ hybridization indicated that neoplastic astrocytes and some endothelial cells are responsible for the expression of MT1-MMP and MT2-MMP in the glioblastoma tissues. Immunohistochemically, both MT1-MMP and MT2-MMP were localized to the glioblastoma cells and endothelial cells of blood vessels. In carcinomas of the breast and head and neck, however, the discrepancy in the localization patterns of MT1-MMP by *in situ* hybridization and immunohistochemistry is argued.^{15,28} The former showed predominant distribution of MT1-MMP mRNA in the stromal cells,²⁸ but the latter indicated that MT1-MMP is localized in the carcinoma cells, although some staining is also observed in the stromal cells.¹⁵ However, our data indicate that this is not the case in glioblastomas.

One interesting finding in the present studies is that, with *in situ* zymography, gelatinolytic activity was detected for the first time in the glioblastoma tissue but not in normal brains. This method was originally developed by Galis et al.³⁰ However, because of the simplicity and better resolution, our method using gelatin films is considered to be better than the original, in which frozen sections mounted on the slide glasses were dipped in emulsion containing gelatin.³⁰ As the gelatinolytic activity was abolished by the treatment of the tissues with BB94, a hydroxamate MMP inhibitor, the activity is ascribed to MMP(s). Gelatin is a nonspecific substrate susceptible to many MMPs.³ Among the MMP gene family members, however, MMP-2 and MMP-9 have the highest activity to gelatins,³ and the major gelatinolytic MMPs produced in the glioblastomas are MMP-2 and MMP-9.^{12,17,20} Thus, it is reasonable to speculate that these MMPs are mainly responsible for the activity.

In addition to the activator function for pro-MMP-2, recent studies on the characterization of MT1-MMP have demonstrated that it has ECM-degrading activities, including collagenolytic activity.^{31–33} Like MT1-MMP, MT2-MMP has also been reported to exhibit ECM-degrading activity as well as activation of pro-MMP-2.^{34,35} As MT1-MMP, MT2-MMP, and MMP-2 are co-localized in the glioblastoma cells, the combination of these MMPs may function as a powerful machinery for the pericellular ECM digestion in glioblastomas, which facilitates invasion of the glioma cells in the brain.

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