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Advances in Brief

Elevated Levels of M_r 92,000 Type IV Collagenase in Human Brain Tumors[1]

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Abstract

Local invasive growth is one of the key features of primary malignant brain tumors accompanied by remodeling of the vasculature and destruction of normal brain tissue. Tissue invasiveness is an essential biological function used by a tumor to overcome the various barriers to its progression. The expression of metalloproteases has been shown to play a critical role in the invasive process in a number of tumors; however, their expression in human brain tumors has not been previously reported. In this study we showed metalloprotease activities at M_r 240,000, 123,000, 92,000, 72,000, and 67,000 in brain tumor extracts. These enzyme activities were inhibited by EDTA, an inhibitor of metalloproteases. Significant increases in levels of protease bands at M_r 92,000, 123,000, and 240,000 were observed in glioblastoma and metastatic lung tumors. Enzymatic inhibition and Western blotting with M_r 92,000 type IV collagenase antibody confirmed the presence of M_r 92,000 type IV collagenase in all samples. Quantitative analysis by densitometry showed 8–10-fold and 6–8-fold increases in M_r 92,000 type IV collagenase activity in glioblastoma and metastatic lung carcinoma samples, respectively, when compared with normal brain, meningioma, astrocytoma, metastatic colon, and breast carcinoma samples. These findings provide evidence for elevated levels of metalloproteases in glioblastomas and suggest a therapeutic target for minimizing the invasive propensity of gliomas using protease inhibitors.

Introduction

MMPs[3] are an important family of enzymes that are secreted in zymogen from connective tissue cells, inflammatory phagocytes, and a number of transformed cells (1, 2). Most components of the extracellular matrix can be dissociated and degraded after MMPs are activated. MMPs have been suggested to play an important role in various physiological and pathological conditions such as tissue remodeling, reproduction, morphogenesis, various connective tissue diseases, and cancer cell invasion and metastasis. It has been suggested that the collagenases that hydrolyze type IV collagen are localized in basement membranes and play an important role in tumor invasion and metastasis (3, 4). Once activated, the two MMPs, with molecular weights of 72,000 and 92,000, can degrade type IV collagen. Previous studies have reported that many connective tissue cells synthesize these enzymes (5, 6). The proenzymes and their active forms are known to be inhibited by the presence of $TIMP_2$ (7).

Local invasive growth is one of the key features of primary malignant brain tumors and is accompanied by remodeling of the vasculature and destruction of normal brain tissue (8). A M_r 65,000 metalloprotease was previously described as being secreted by fetal astrocytes and glial cell lines in culture (9). The expression of several forms of metalloproteases and metalloprotease inhibitors by fetal astrocytes and glioma cells lines has been reported (9, 10). Furthermore, a metalloprotease secreted by the rat glioma cell line BT5C in serum-free medium was observed to be capable of degrading fetal rat brain aggregates (8, 11). The present study was designed to examine the expression of metalloproteases or type IV collagenases in human brain tumors *in vivo*. The results from this study indicate a significantly increased level of M_r 92,000 type IV collagenase in human glioblastoma and metastatic lung carcinoma samples, compared to several other brain tumors examined.

Materials and Methods

Tissue Processing. Fresh human brain tumor tissue and brain tissue samples were collected in the operating room from patients undergoing craniotomy. The samples included 8 glioblastomas; 15 metastases from lung, colon, and breast carcinomas (5 each); 5 meningiomas; 5 anaplastic astrocytomas; and 5 normal brain samples. The samples were collected, placed immediately on dry ice, transferred to a freezer, and kept at −80°C until used. For further processing, the tissue was thawed, weighed, and homogenized in Tris buffer (50 mM Tris-HCl, pH 7.5, containing 75 mM NaCl) and centrifuged at 5000 × *g* for 20 min. The pellet was discarded, and the supernatant was aliquoted and stored at −80°C.

Gelatin Zymography. To identify the various types of collagenolytic enzymes in the brain tumor samples gelatin zymograms were perfomed, as described previously (12). Briefly, 50 μg of tissue extracts were electrophoresed on an 8% SDS-PAGE containing gelatin (2 mg/ml) from swine skin (Sigma Chemical, St. Louis, MO). After electrophoresis, the gels were rinsed twice in 2.5% Triton X-100 and incubated at 37°C for 20 h in 0.15 M NaCl, 10 mM $CaCl_2$, and 50 mM Tris-HCl buffer (pH 7.5) containing 0.05% NaN_3. The gels were stained with 0.05% Coomassie blue and destained in 10% isopropanol and 10% acetic acid in H_2O. Gelatinolytic enzymes were detected as transparent bands on the blue background of the Coomassie blue-stained slab gel. To further characterize these collagenolytic bands, the gels were incubated in Tris buffer containing 10 mM EDTA. To further confirm the specifity of the collagenolytic bands, the samples were incubated with antibodies against the M_r 72,000 and M_r 92,000 type IV collagenases and with $TIMP_2$ antibodies before electrophoresis for 45 min at room temperature.

Western Blotting. Normal brain and brain tumor extracts (50 μg) were electrophoresed in an 8% (SDS-PAGE) gel, followed by transfer of the proteins onto nitrocellulose paper according to the method of Towbin *et al.* (13). The nitrocellulose paper was then incubated in blocking buffer for 2 h at room temperature (1.5% bovine serum albumin, 0.15 M NaCl, 0.1 mM phenylmethylsulfonyl fluoride, 20 mM Tris-HCl, pH 7.6) and washed with antibody buffer (0.3% bovine serum albumin, 0.15 M NaCl, 20 mM Tris-HCl, pH 7.6) three times at 10-min intervals. Strips were then incubated with anti-M_r 92,000 collagenase for 2 h at room temperature, washed as above, incubated with a second antibody (peroxidase conjugated) for 2 h at room temperature, and finally washed with Tris-HCl buffers as described above, incubated with the substrate 2,4-chloronaphthol, and kept in the dark for 15–30 min to develop color.

Quantitative Analysis. We measured quantitatively the M_r 92,000 collagenolytic activity by densitometric analysis. After electrophoresis, the samples from different patients grouped according to histology were washed with 2.5% Triton X-100 and incubated at 37°C at shorter time intervals than those shown in Fig. 1, to ensure that the intensity of the band fell within the linear

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[3] The abbreviations used are: MMP, matrix metalloprotease; $TIMP_2$, tissue inhibitors of metalloproteases; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ECM, extracellular matrix.

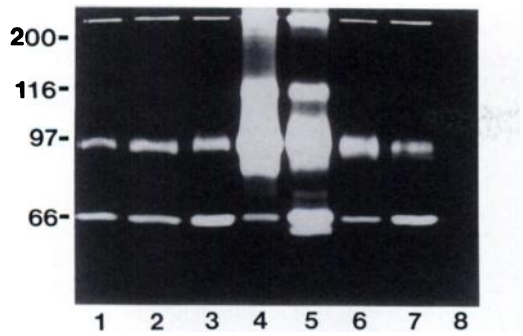

Fig. 1. Gelatin zymography of various brain tumors. Fifty μg of protein from normal brain and brain tumor extracts (meningioma, anaplastic astrocytoma, glioblastoma, metastatic, lung, colon, and breast) were run on 8% SDS-PAGE containing gelatin (2 mg/ml). *Lane 1*, normal brain; *Lane 2*, meningioma; *Lane 3*, anaplastic astrocytoma; *Lane 4*, glioblastoma; *Lane 5*, metastatic lung; *Lane 6*, metastatic colon; *Lane 7*, metastatic breast; *Lane 8*, urokinase.

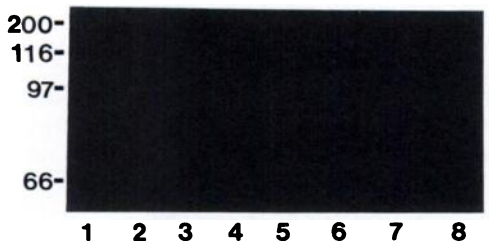

Fig. 2. Inhibition of metalloproteases on gelatin zymography of various brain tumor extracts with 10 mM EDTA. *Lane 1*, normal brain; *Lane 2*, meningioma; *Lane 3*, anaplastic astrocytoma; *Lane 4*, glioblastoma; *Lane 5*, metastatic lung; *Lane 6*, metastatic colon; *Lane 7*, metastatic breast; *Lane 8*, urokinase.

range, yielding a better quantitative estimation. The intensity of the band was quantitated by densitometry.

Other Assays. Protein content was determined by using Coomassie brilliant blue-G as the color indicator (14).

Results

Gelatin Zymography. Metalloprotease activity was detected in various brain tumor extracts with SDS-PAGE gels containing gelatin (Fig. 1). Normal brain and brain tumor extracts (meningioma; anaplastic astrocytoma; glioblastoma; and metastatic lung, colon, and breast carcinomas) had nearly the same protease pattern. All brain tumor extracts contained protease bands at M_r 240,000, 123,000, 92,000, 67,000, and 65,000. But M_r 77,000 and 72,000 protease bands were present in only a few cases. Significant increases of M_r 92,000, 123,000, and 240,000 protease bands were observed in glioblastoma and metastatic lung tumor. Standard urokinase was not detected on gelatin zymography due to the lack of its specific substrate in the gel (Fig. 1, *Lane 8*).

Inhibition of Metalloproteases by EDTA. To confirm that the tumor extracts contained metalloprotease activity, after electrophoresis the gels were washed with 2.5% Triton X-100 and incubated with 10 mM EDTA. Fig. 2 shows that the proteolytic bands on the gelatin zymogram were completely inhibited by EDTA, which is an inhibitor of metalloproteases.

Inhibition of Metalloproteases by Their Antibodies. To further characterize the prominent M_r 92,000 enzymatic band, the glioblastoma samples were incubated with 50 μg/ml of M_r 92,000 type IV anti-collagenase, M_r 72,000 type IV anti-collagenase antibodies, or anti-$TIMP_2$ antibodies. Fig. 3 shows that the M_r 92,000 type IV collagenase and its complex with $TIMP_2$ M_r 123,000 band is completely inhibited by the M_r 92,000 type IV anti-collagenase (Fig. 3, *Lane 2*). The M_r 72,000 type IV anti-collagenase and anti-$TIMP_2$ had no effect on their corresponding lytic bands (Fig. 3, *Lanes 3* and *4*). A M_r 72,000 collagenase antibody raised from the amino-terminal fragment of type IV collagenase did not inhibit collagenase activity. In *Lane 4* the metalloprotease activities were increased due to the presence of anti-$TIMP_2$, which blocks the $TIMP_2$ present in the samples.

Quantitative Analysis of the M_r 92,000 Type IV Collagenase from Brain Tumor Extracts. Levels of M_r 92,000 type IV collagenase were assayed in the brain tumor extracts, obtained from different patients with brain tumors, and quantitated by densitometric analysis. Fig. 4 shows an 8–10-fold increase in type IV M_r 92,000 collagenase in glioblastomas and a 6–8-fold increase in metastatic lung tumors as compared to normal brain, meningiomas, and anaplastic astrocytoma samples. There were no significant increases in the levels of M_r 92,000 type IV collagenase in meningioma, anaplastic astrocytoma, and metastatic colon and breast carcinoma samples as compared to normal brain.

Western Blot Analysis. The M_r 92,000 type IV collagenase in tumor extracts was analyzed by Western blotting using M_r 92,000 type IV collagenase antibody. Fig. 5 shows that a specific but faint M_r 92,000 band was present in all cases. A more prominent band was present in the glioblastoma and metastatic lung carcinoma samples. The faintness of the band in other cases may have been due to a lower concentration of M_r 92,000 type IV collagenase present in these samples. Fig. 5 also shows a strong band of the low-molecular-weight form of M_r 92,000 type IV collagenase in the glioblastoma sample and the same form to a lesser extent in the lung metastasis sample. This band was absent, however, from the other samples. This indicates that the M_r 92,000 type IV collagenase was present as an active enzyme but with a reduced molecular weight of 85,000–82,000 (Fig. 5).

Discussion

Our results showed the presence of several gelatinolytic enzymes in normal brain and in various brain tumor extracts. The elevated levels of M_r 92,000 type IV collagenolytic activity was confirmed by enzymatic inhibition and Western blotting using specific antibodies. Previously, there has been limited information concerning collagenase activity in brain tumors (15). Recent results show, however, that gelatinolytic bands are present in cultured fetal astrocytes and in glioma cell lines (9, 10). The destruction of neural elements by glioma cell lines (8, 11) and biopsy samples (16) has been demonstrated using the spheroid brain aggregate model. This is due at least in part to the effect of metalloproteases, as has been demonstrated with the C6

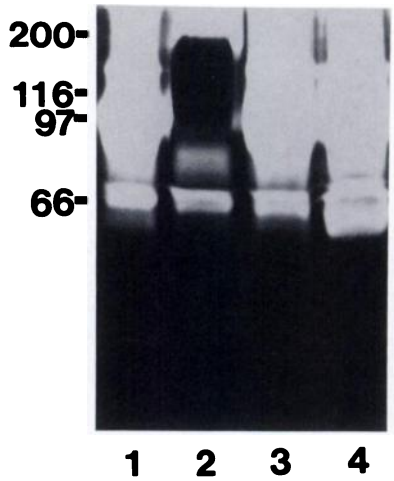

Fig. 3. Inhibition of M_r 92,000 type IV collagenase with anti-type IV collagenase in glioblastoma by gelatin zymography. The samples were incubated with antibodies to M_r 92,000 and 72,000 type IV collagenases and $TIMP_2$ antibodies. *Lane 1*, anaplastic astrocytoma; *Lane 2*, glioblastoma plus M_r 92,000 anti-type IV collagenase; *Lane 3*, glioblastoma plus M_r 72,000 anti-type IV collagenase; *Lane 4*, glioblastoma plus anti-$TIMP_2$.

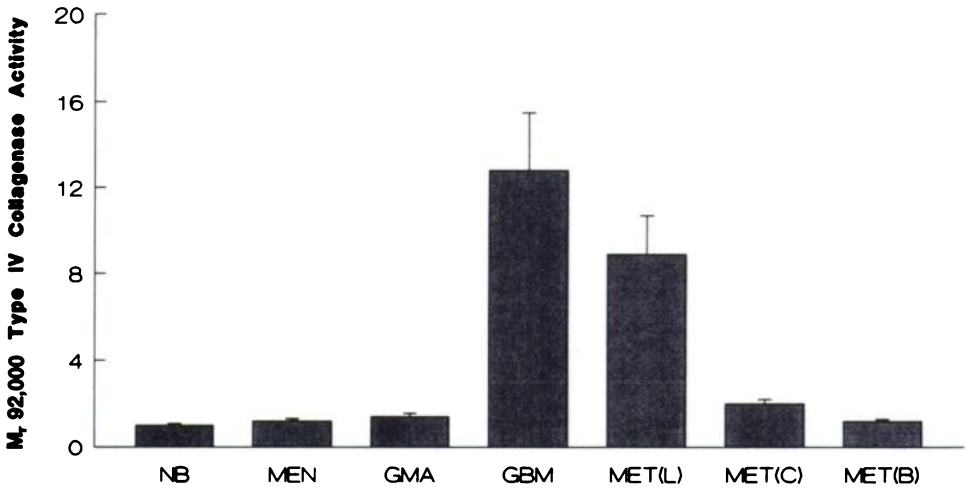

Fig. 4. Quantitative analysis of M_r 92,000 type IV collagenase in brain tumor extracts. In each group the M_r 92,000 band was scanned in three positions by densitometry, and the peak areas were averaged to give the values presented. The data are shown as mean values ± SD of 5 different patient samples from each group. Units of peak area are arbitrary.

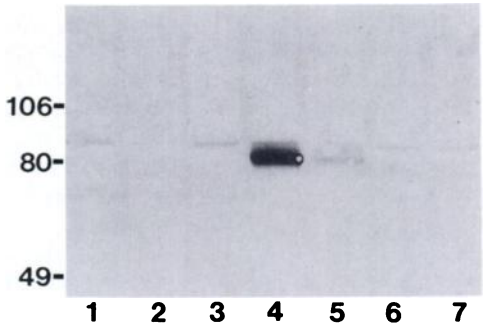

Fig. 5. Western blot analysis of various brain tumors with M_r 92,000 anti-type IV collagenase. *Lane 1*, normal brain; *Lane 2*, meningioma; *Lane 3*, anaplastic astrocytoma; *Lane 4*, glioblastoma; *Lane 5*, metastatic lung; *Lane 6*, metastatic colon; *Lane 7*, metastatic breast.

glioma cells placed on freeze-prepared pieces of rat optic nerve. The C6 cell invasion of the optic nerve was inhibited with 1,10-phenanthroline, an inhibitor of metalloproteases (17).

An accumulating body of evidence supports a positive correlation between type IV collagenase activity and tumor cell invasion (12, 18) and the genetic induction of a metastatic phenotype (19, 20). There are several reports indicating that the biochemical inhibition of type IV collagenase activity or the blockage of collagenase secretion can prevent tumor cell invasion *in vitro* (21, 22). TIMPs suppress the ability of tumors to metastasize by blocking the pro and active forms of the enzyme and thereby the ability of the tumor cells to invade the extracellular matrix (23). Similarly, the administration of recombinant TIMP-1 has blocked metastasis formation in animal models (24). In our own studies glioblastoma cell lines were shown to secrete M_r 72,000 and M_r 92,000 type IV collagenases. The invasiveness of these cell lines through a matrigel model constituted with extracellular matrix proteins was blocked by TIMPs and by antibodies for the type IV collagenase.[4] Moreover, two myelin-derived proteins with molecular weights of 35,000 and 250,000 were found (25) to contain metalloprotease blocking sequence (gly-phe, tyr-tyr). The proteins are thought to be responsible for inhibiting the spread of TB16 melanoma cells and 3T3 fibroblasts on central nervous system structures (17).

Primary malignant brain tumors are particularly invasive, but no detailed characterization of ECM proteins in brain tumors has been published (26). Several recent reports have identified the components of the ECM to include collagen types I, III, IV, and V laminin and fibronectin in normal brain and brain tumors (27–31). Tissue culture flasks coated with fibronectin and laminin had no effect on the growth of U343 MG-A glioma cells, but the flasks coated with types I and IV collagen showed decreased cellular proliferation, formation of stellate cells, and increased production of glial fibrillary acidic protein per cell in comparison with glioma cells growing on plastic (9). It has also been found by gelatin gel analysis that U343 MG-A glioma cells growing on plastic secrete a M_r 65,000 metalloprotease that was not secreted by cells grown on the leptomeningeal ECM. Immunohistolocalization of ECM proteins during brain tumor invasion in BD1X rats showed an overproduction of collagen types I and IV and of fibronectin (32). These studies also indicated that type I collagen and fibronectin were enriched in the zone of invasion (32), where degradation products of collagen were also found, suggesting an enzymatic turnover of matrix components in the invasion zone. Spheroids from biopsy specimens of human glioma contain ECM (33), which in cocultures may be distributed in a similar fashion (34). Several reports have also showed an increased production of protease inhibitors in glioma cell lines and in brain tumors (9, 35, 36). In summary, the increased invasive propensity of glioblastoma compared to normal brain meningioma and anaplastic astrocytoma may be due to (*a*) increased production and secretion of metalloproteases and serine proteases into the extracellular matrix, (*b*) decreased levels of serine protease and metalloprotease inhibitors, (*c*) activation of proenzyme plasminogen to plasmin, and (*d*) activation of metalloproteases by urokinase directly or by a combination of all of these mechanisms.

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