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Role of plasminogen activator and of 92-KDa type IV collagenase in glioblastoma invasion using an *in vitro* matrigel model

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Summary

The invasive nature of human gliomas represents a major factor in preventing their total resection. The exact nature of the underlying mechanisms of tumor cell invasion are still unclear. In this study, we have quantitatively assayed a glioblastoma cell line for its ability to migrate through a polycarbonate filter coated with matrigel which contains a complex of multiple basement membrane components. At 48 h the glioblastoma cell line (U251) showed a rate of invasiveness of 42% and also dependent on the concentration of matrigel. The U251 cell line produced a urokinase type plasminogen activator and a 92-KDa type IV collagenase. Both enzymes were inhibited by the addition of uPA and 92-KDa type IV collagenase antibodies. Those same antibodies reduced the invasion rate of U251 cells from 42% to 12 and 21%, respectively. Similarly, the addition of ε-aminocaproic acid (a plasmin inhibitor) or tissue inhibitor of metalloprotease ($TIMP_2$, a collagenase inhibitor) reduced the invasiveness of U251 cells from 42% to 14% and 10%, respectively. Additionally, the other two glioblastoma cell lines (LG11, UWR1) and astrocytes showed a rate of invasiveness at 41%, 61% and 12%, respectively. Finally, the addition of hyaluronic acid to the matrigel, a constituent of brain extracellular matrix, enhanced the rate of invasion. These findings provide evidence for the role of serine proteases and metalloproteases in facilitating the invasion of extracellular matrix components by glioblastoma cell line and suggest a therapeutic role for protease inhibitors in attempting to minimize the invasive propensity of gliomas.

Introduction

Tumor invasion is a complex phenomenon involving the disruption of basement membranes and the penetration of cells into normal adjacent tissues, caused by the production of various lytic enzymes. Proteolytic enzymes, including plasminogen activators [1] lysosomal hydrolases [2–4], and collagenases [5–9], are elevated as the invasive and/or metastatic potential increases in many malignant cells. Various test systems have been developed to assess the invasiveness of tumor cells, including chick embryonic skin [10], embryonic chick heart [11], cultures of endothelial cells [12, 13], isolated hepatocytes [14], bone or cartilage [15], rat urinary bladder [16] canine or bovine large vessels [17], chick chorioal-

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lantoic membrane [18, 19], bovine lens capsule [20] and human amnion [21, 22].

Local invasive growth is one of the key features of primary malignant brain tumors and it has long been recognized that they not only invade into the surrounding normal brain tissue but also cause the destruction and replacement of the normal anatomical structures. The underlying molecular mechanisms of brain tumor invasiveness are only now being uncovered. Several reports have indicated an increased production of plasminogen activators in solid brain tumors and in tumor cell lines [23–26]. It has also been reported several forms of metalloproteases produced by fetal astrocytes and glioma cell lines are capable of degrading fetal rat brain aggregates [27–30]. The invasive nature of glioblastomas (Spheroids) *in vitro* have been reported in several studies using normal fetal rat brain cell aggregates as target tissue for invasion [31–34]. Reconstituted basement membranes molecules of matrigel formed into barriers have been used to study the invasiveness of tumor cells *in vitro* [35–38]. We used the matrigel assay in order to understand the role of these enzymes, and the effect of their antibodies and inhibitors in preventing glioblastoma invasion. The results from this *in vitro* study indicate that both serine proteases and metalloproteases are required for glioblastoma invasion.

Material and methods

Materials

Fetal bovine serum, Eagle's basal medium, and antibiotics were from GIBCO (Grand Island, NY). Plasminogen was prepared from fresh human plasma obtained from the M.D. Anderson Blood Center and purified on a lysine-agarose column as described [39]. Tissue culture plates were from Falcon (Oxnard, CA); Triton X-100, ε-aminocaproic acid, the vital stain MTT (3-[4,5-dimethyl thiazole-2yl]-2,5-diphenyl tetrazolium bromide; thiazolyl blue) and coomassie brilliant blue-G were from Sigma Chemical Co. (St. Louis, MO). Transwell polycarbonate filters (8.0 μm pore size) were obtained from Costar Corp. (Cambridge, MA). Matrigel was supplied by Collaborative Research Inc.

Cell culture

The human glioblastoma cell line (U251) was grown in Eagle's basal medium (MEM) supplemented with 1.0% glutamine, 1.0% vitamins, 1.0% nonessential amino acids, 80.5 μg/ml streptomycin, 80.5 units/ml penicillin and 10% fetal or newborn calf serum at pH 7.2–7.4. Stock cultures are maintained by removing cells from a flask with 0.25% trypsin in Mg^{2+} and Ca^{2+} free Hank's basal salt solution (HBSS) containing 20.0 mg/ml sterile Na_2 EDTA. The trypsin activity was inactivated by addition of complete MEM and the cells were counted and reseeded into either 75 cm^2 flasks ($1–2 \times 10^6$ cells) or 25 cm^2 flasks ($1–2 \times 10^5$ cells). When the cells reached confluence, they were rinsed 3 times with serum-free medium and then incubated for 24 hours in the same medium. The medium was collected and centrifuged at 5,000 × g to remove cellular debris. The cell layer was extracted in Tris buffer, and the medium and cell layers stored at – 80° C until further use.

Invasion assay

Studies of glioblastoma invasion was carried out using a method described previously [40] but with modifications. Soluble extracellular matrix (matrigel), which contains laminin, type IV collagen, heparin sulfate, and entactin diluted in ice-cold serum free medium to achieve a final protein concentration of 1.0 mg/ml (unless otherwise stated) was allowed to gel on a polycarbonate filter of 'Transwell' (Costar Cambridge, MA). The soluble matrigel was mixed with exogenous plasminogen to achieve a final level of 20 μg/ml as described previously [41]. The plasminogen (20 μg/ml) supplemented Matrigel, and 100 μl (1 mg/ml) was applied to Transwell plastes as described [42]. Gelation occurred after a 30 min incubation at 37° C. The glioblastoma cell line was harvested with trypsin and washed (3 ×) with serum-free medium, 2.5×10^5 cells were sus-

pended in 1.0 ml of medium. The cells were added to the Transwell, which was subsequently inserted into an outer well containing culture medium. The cells were incubated at 37° C for 2 days. Then both upper and lower compartments were replenished with fresh serum-free medium containing 0.2 mg/ml of the vital stain MTT and incubated at 37° C for 3 h. The amount of vital stain converted to water-insoluble crystals was considered representative of the number of cells [43]. Crystals on the top aspect of the polycarbonate filter, generated by noninvasive cells, were removed by cotton swabs and dissolved in dimethyl sulfoxide. The filters were subsequently incubated with dimethyl sulfoxide to solubilize the crystals generated by the invasive cells. The color intensity of the solubilized crystals was determined at 570 nm using a multiplate reader. The absorbance reading corresponding to the solubilized crystals on the lower aspect of the polycarbonate filter was divided by the sum of this value and the activity in the upper compartment. Cell invasion (%) was calculated by multiplying this value by 100.

Inhibition of U251 cell invasion by various concentrations of matrigel, inhibitors and antibodies to serine proteases and metalloproteases

The U251 cells were incubated various concentrations of matrigel coated on polycarbonate filters and in the presence of ε-aminocaproic acid (inhibitor of plasmin), TIMP (tissue inhibitor of metalloproteases), urokinase (uPA) antibody, tPA antibody, and 92-KDa type IV collagenase antibody, and the invasion assay was performed as described above. uPA and tPA antibodies were purchased from American Diagnostica. TIMP and type IV collagenase antibodies were characterized by Western blotting [44, 45].

Fibrin zymography

The enzymatic activity and molecular weight of electrophoretically separated forms of plasminogen activators (PAs) in U251 serum-free medium and cell extracts were determined by our modification [25] of the sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) procedure of Laemmli as described in detail previously [46]. Briefly, the SDS gel contained acrylamide to which purified plasminogen and fibrinogen were added as substrates prior to polymerization. After polymerization, the samples were electrophoresed, and the various types of PAs separated based on differences in molecular weight. The gel was then washed with 2.5% Triton X-100 twice for 30 min each and incubated at 37° C overnight with glycine buffer (pH 7.5). Upon staining with coomassie blue and destaining, the final gel had a uniform blue background except in those regions to which PA had migrated and activated the plasminogen to plasmin. To confirm the specificity of PA enzyme activity, the samples were incubated with their specific antibodies (uPA and tPA) at room temperature for 30 min and before electrophoresis on 10% SDS-PAGE. The sample buffer was added without reducing agent and loaded (without boiling).

Gelatin zymography

To determine whether the glioblastoma cells secreted various types of collagenolytic enzymes, zymography was performed based on methods previously described [47, 48]. Serum-free medium and cell extracts were electrophoresed in 8% polyacrylamide gel 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 coomassie blue-stained slab gel. To confirm the specificity of the 92-KDa type IV collagenase, the samples were incubated with 92-KDa type IV collagenase antibody for 30 min at room temperature as described before electrophoresis [45].

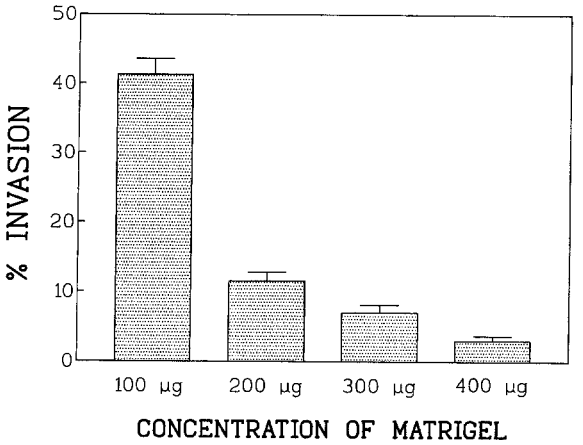

Fig. 1. Inhibition of cell invasion with various concentrations of matrigel. U251 cells (2.5×10^5) were plated on polycarbonate filters coated with various concentrations (1 mg/ml, 2 mg/ml, 3 mg/ml and 4 mg/ml) of matrigel containing 20 µg/ml of plasminogen and analyzed for cell invasion after 48 h. The data are represented as mean values ± SD of four different experiments.

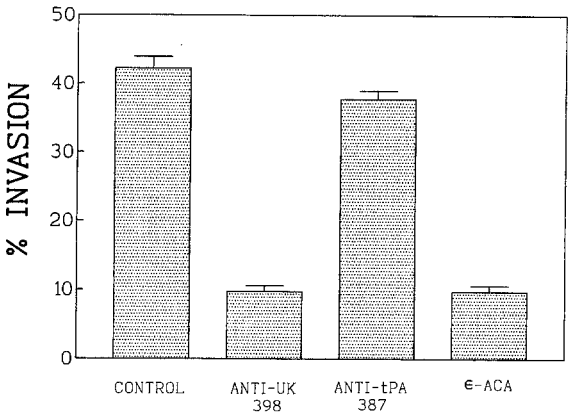

Fig. 2. Effect of antibodies to uPA and tPA and ε-aminocaproic acid on invasion of matrigel by U251 cells. U251 cells were plated along with 25 µg/ml of antibodies against uPA and 50 µg/ml against tPA and 5 mM ε-aminocaproic acid on matrigel coated polycarbonate filters and analyzed for invasion after 48 h. The data are shown as mean values ± SD of four different experiments.

Effect of hyaluronic acid on the invasiveness of U251 cells

The effect of hyaluronic acid on the invasiveness of U251 cells was analyzed by the impregnation of matrigel with various concentrations of 100, 200, 500, 750, and 1000 µg/ml hyaluronic acid and the invasion assay was performed as described above.

Results

U251 cell invasion is dependent on the matrigel concentration

Our recent results have been reported [41] that U251 cell invasion was dependent on the concentration of plasminogen added to the matrigel. We used the same assay to assess the invasive potential of the U251 cells through different concentrations of matrigel. We found that the penetration of U251 cells through the matrigel was dependent on the concentration of matrigel (Fig. 1). It was clearly demonstrated that 200 µg (2 mg/ml), 300 µg (3 mg/ml) and 400 µg (4 mg/ml) concentrations of matrigel reduced the invasiveness of U251 cells from 42% to 13% (~ 3.2 fold), from 42% to 7% (~ 6 fold), and from 42% to 3% (~ 12 fold) respectively when compared to the 100 µg (1 mg/ml) concentration of matrigel.

ε-aminocaproic acid and uPA antibodies blocked matrigel invasion by U251 cells

The requirement of exogenous plasminogen for optimal U251 cell invasion suggested that plasminogen activators are involved in the production of the serine protease plasmin, which degrades the matrigel and enhances the invasion of cells. Figure 2 shows that an inhibitor of plasmin, ε-aminocaproic acid (5 mM) significantly inhibits the invasion of U251 cells through the matrigel decreasing it from 42% to 14% (~ 3 fold) in a dose-dependent manner (data not shown). Furthermore, antibodies to uPA (Fig. 2) inhibited the invasion of U251 cells through matrigel, reducing from 42% to 12% (~ 3.2 fold), but anti tPA did not inhibit the invasion of U251 cells (Fig. 2).

Secretion of PA by U251 cells

Serum free medium and cell extracts were assayed for PA activity by fibrin zymography and inhibition of enzyme activity using specific uPA and tPA anti-

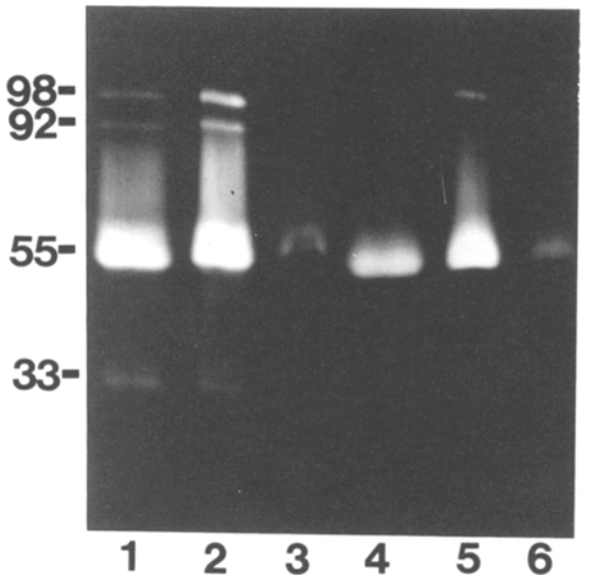

Fig. 3. Fibrin zymography of U251 serum free medium (CM) and cell extracts and inhibition of enzyme activity by antibodies to uPA and tPA. U251 CM, and cell extracts were incubated with anti-uPA (50 μg/ml) and anti-tPA (50 μg/ml) at room temperature for 30 min before electrophoresis. The gels were analyzed as described in Methods. Lanes 1) U251 CM; 2) U251 CM + anti tPA; 3) U251 CM + anti uPA; 4) U251 cell extracts; 5) U251 cell extracts + anti-tPA; and 6) U251 cell extracts + anti-uPA.

bodies. Figure 3 shows the presence of Mr 55,000 and 33,000 uPA and complex bands of uPA with serpins (PNI and PAI-1) in U251 serum free media and cell extracts. Anti-uPA (50 μg/ml) inhibited more than 90% of enzyme activity and its complex bands both in media and cell extracts (lanes 3 and 6) and no inhibition with tPA antibody (lanes 2 and 5) which is consistent with the fact that this cell line does not produce tPA. We have verified that the electrophoretic process did not disassociate the antigen-antibody complex.

Inhibition of U251 cell invasion by antibodies to type IV collagenase and an inhibitor of metalloproteases

Considering the significant role of metalloprotease in tumor invasion, we studied whether the invasion of U251 cells through the matrigel is dependent on metalloproteases. U251 cells were incubated in the presence of the inhibitor of metalloproteases

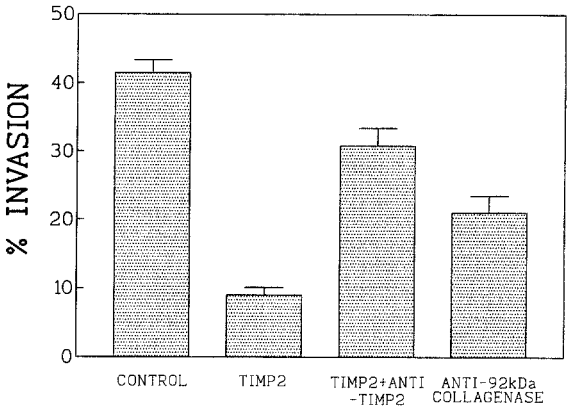

Fig. 4. Effect of $TIMP_2$, anti-$TIMP_2$, and anti type IV collagenase on U251 cell invasion. U251 cells were plated with $TIMP_2$, $TIMP_2$ + anti-$TIMP_2$, and 50 μg/ml Mr 92,000 type IV anticollagenase on matrigel coated polycarbonate filter and analyzed for invasion after 48 h. The data are shown as mean values ± SD of four different experiments.

$TIMP_2$ (10 μg/ml) and anti type IV collagenase 92-KDa (50 μg/ml). Figure 4 shows that $TIMP_2$ inhibited the invasion of U251 cells through the matrigel from 42% to 10% (4.2 fold). It was also confirmed that when $TIMP_2$ and antibodies to $TIMP_2$ were added to U251 cells plated on the polycarbonate filters, the percentage of the cells that invaded the matrigel was increased from 10% to 35% (3.5 fold or almost to normal) compared to $TIMP_2$ alone. Figure 4 also showed that the Mr 92,000 anti-type IV collagenase inhibited the invasion of U251 cells through the matrigel from 42% to 21% (2 fold). Partial inhibition of U251 cell invasion by anti 92-KDa type IV collagenase because these cells secrete another 66-KDa type IV collagenase as shown in Figure 5.

Secretion of metalloproteases by U251 cells

Serum-free medium and cell extracts were assayed for metalloproteases by gelatin zymography and inhibition of enzyme activities by specific antibodies. Figure 5 shows that serum-free medium contained enzymatic bands at Mr 240,000, 123,000, 92,000, 67,000, and 65,000. Cell extracts also contained bands at Mr 123,000, 92,000, 67,000, 65,000, and 52,000 but each band contained less activity than these from the medium. Mr 92,000 anti-type IV col-

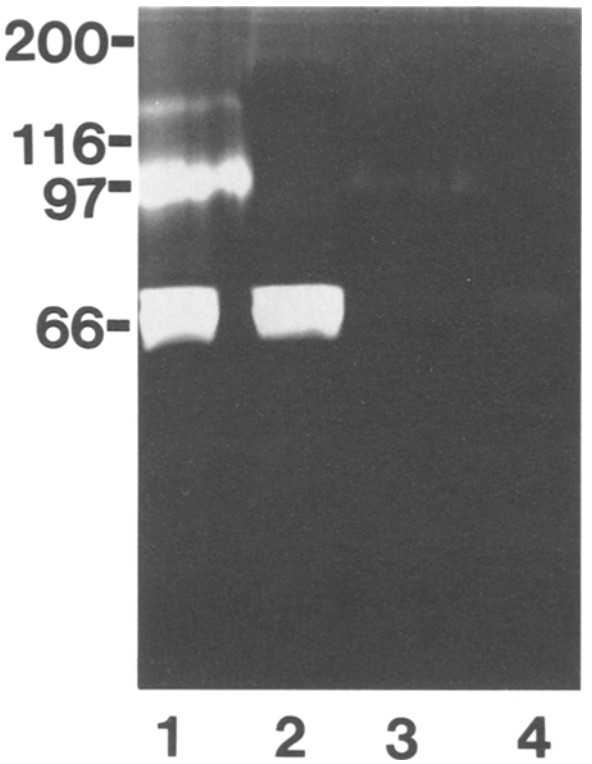

Fig. 5. Gelatin zymography of U251 cell extracts and serum free medium and inhibition by Mr 92,000 anti type IV collagenase. U251 cell extracts and CM were incubated with Mr 92,000 anti type IV collagenase (50 µg/ml) at room temperature for 30 min before electrophoresis. The gels were analyzed as described in Methods. Lanes 1) U251 CM; 2) U251 CM + anti type IV collagenase; 3) U251 cell extracts; 4) U251 cell extracts + anti type IV collagenase.

lagenase antibody inhibited the enzyme activity of Mr 92,000 type IV collagenase and its inhibitor complex of Mr 123,000 in media and cell extracts.

Invasion of various glioblastoma cell lines through matrigel

The invasive potential of various glioblastoma cell lines through the matrigel was determined as described above. Figure 6 shows that UWR1 cells invade more when compared to U251 and LG11. The invasion of astrocytes through matrigel was significantly less when compared to glioblastoma cell lines.

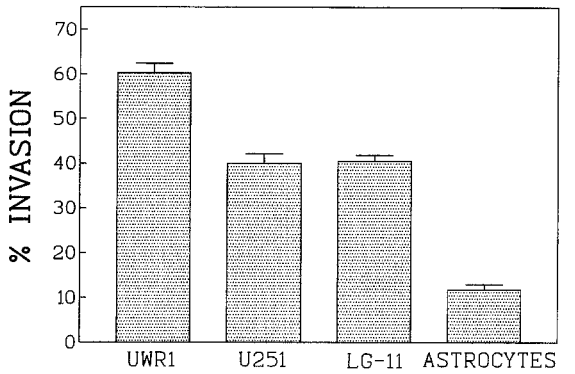

Fig. 6. Invasion of different glioblastoma cell lines through matrigel. UWR1, U251, LG11 and astrocytes were plated on matrigel coated polycarbonate filters and analyzed for invasion after 48 h. The data are shown as mean values ± SD of four different experiments.

Effect of hyaluronic acid on the invasiveness of U251 cells

The effect of hyaluronic acid on the invasive nature of U251 cells was analyzed by the impregnation of matrigel with various concentrations of hyaluronic acid. Enhanced U251 cell penetration (1.7 fold) at 200 µg/ml was found (Fig. 7). Higher concentrations of hyaluronic acid (above 500 µg/ml) inhibited slightly the penetration of U251 cells through matrigel. The enhanced U251 cell penetration through matrigel in the presence of hyaluronic acid, is presumably caused by the increased secretion of uPA

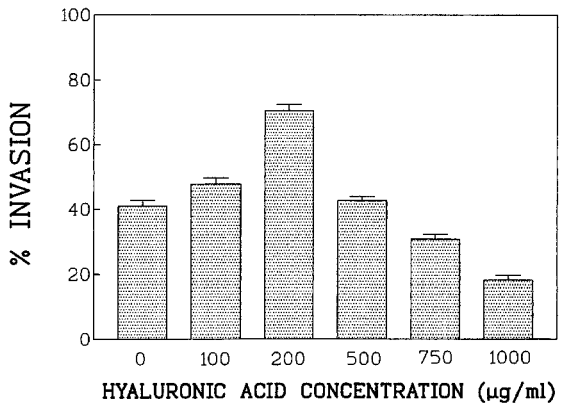

Fig. 7. Effect of different concentrations of hyaluronic acid (HA) on rate of invasion of U251 cells. Different concentrations of HA were added to the matrigel before polymerization. U251 cells were plated on the matrigel contained various concentrations of HA and analyzed for cell invasion at 48 h. The data are presented as mean values ± SD of four different experiments.

and type IV collagenases by the cells as revealed on the fibrin and gelatin zymograms (data not shown).

Discussion

Malignant gliomas represent one of the most refractory cancers to therapy and remain incurable. Local invasive growth is one of the key features of primary malignant brain tumors, as is remodeling of the vasculature and the destruction of normal brain tissue. The tumor cells usually show an infiltrative and a degradative pattern of invasion [49, 50]. Tissue invasiveness by a tumor depends in great part on the appropriate proteolytic enzymes necessary to overcome the various barriers to its progression. It has also been reported [28] for example, that astrocytes secrete proteases that may facilitate their migration through the developing central nervous system in a similar manner to the enhanced migration of malignant cells that secrete these proteases [8, 12]. Likewise, several studies have reported that the plasminogen activator-plasmin system plays an important role in tumor invasion/metastasis. For example, invasion can be blocked by preventing plasmin formation through the inhibition of urokinase with a specific antibody or selectively inhibiting plasmin activity with ε-aminocaproic acid, an inhibitor of plasmin. Protease Nexin I (PNI), an inhibitor of plasmin and PAs, inhibits the degradation of smooth muscle cell extracellular matrix by human fibrosarcoma cells [51] and that 9L conditioned medium inhibits the release of labeled extracellular matrix components due to the presence of PNI and plasminogen activator inhibitor (PAI) in the medium [52]. Similarly α2 antiplasmin prevents amnion invasion by B16 cells at low concentration of 140 ng/ml [53]. Of interest is the fact that antibodies to urokinase but not tPA have prevented the amnion invasion by B16 cells [53]. Anti-uPA blocks human HEP-3 cell invasion in the chick chorioallantoic membrane assay [19, 54]. H-ras-transformed 3T3 cells facilitated enhanced lung invasion and experimental metastasis as the result of an enhanced expression of uPA [55]. This is supported by our observation that ε-amino caproic acid and uPA antibodies but not tPA antibodies inhibit invasion by U251 cells through the matrigel (Fig. 2 and 3).

Other experiments have shown a positive correlation exists between type IV collagenase activity and tumor cell invasion [8, 56], as has a metastatic phenotype associated with augmented type IV collagenase activity [57, 58]. Down-regulation of type IV collagenolytic activity by retinoic acid treatment of human melanoma cells has been correlated with a loss of the invasive phenotype [47]. Similarly, TIMPs production by the host or by the tumor cell itself can block the latent or the active metalloproteases, and these inhibitors suppress metastasis by inhibiting tumor cell invasion of the extracellular matrix [59], and the administration of recombinant TIMPs has blocked tumor metastasis in animal models [60, 61]. Our results showed that $TIMP_2$ and type IV collagenase antibodies markedly reduced matrigel invasion by U251 cells (Fig. 4). Gelatin zymography revealed that these cells secrete several types of collagenases and the 92-KDa type IV collagenase antibody inhibited the enzyme activity and its complex with TIMPs in both media and cell extracts (Fig. 5).

The nature of extracellular matrix may play an important role for the secretion of proteases in various glioblastoma cell lines. Indeed, tissue culture flasks coated with fibronectin or laminin had no effect on the growth of U343 MG-A glioma cells, whereas flasks coated with type I and IV collagens 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 [27]. Conversely our results have shown that matrigel invasion by U251 cells was significantly increased in the presence of hyaluronic acid in the matrigel presumably by inducing the cells to secrete higher levels of proteases (Fig. 7).

Several reports have demonstrated an increased production of plasminogen activators in solid brain tumors and in cell lines [1, 23, 25, 26]. Fetal astrocytes and glioma cell lines produced several forms of metalloproteases and metalloprotease inhibitors [28]. A metalloproteinase secreted by the rat glioma cell line BT5C in serum free medium is capable of degrading fetal rat brain aggregates. Several re-

cent reports have identified the components of the extracellular matrix of normal brain and of brain tumors as collagen types I, III, IV, and V, laminin and fibronectin [27, 29, 30, 62–65]. When BT5C rat glioma cells were implanted intracranially into syngeneic rats, they gave rise to tumors with enriched interstitial collagen and fibronectin in the invasion zone of tumor [66]. Serine proteases and metalloproteases are therefore necessary to facilitate the invasiveness of the glioblastoma cells. The two types of proteases have functions that are either independent from each other or closely interconnected. Inhibition of either serine or metalloproteases by their inhibitors of their antibodies causes a reduction in invasiveness ($\sim 70\%$). The interrelation between these two different forms of proteases is apparent from the results of this study and is consistent with data from other studies. Plasminogen activators for instance can indirectly activate collagenases, such as in the case of breast carcinoma through the conversion of plasminogen to plasmin [67]. More recently however, uPA was shown to directly activate latent type IV collagenase in a glioma cell line [68]. uPA is abundantly secreted in our glioblastoma cell line as well as in samples derived from brain tumor patients [25].

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