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Urokinase directly activates matrix metalloproteinases-9: A potential role in glioblastoma invasion

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ABSTRACT

Previous reports showed that urokinase plasminogen activator (uPA) converts plasminogen to plasmin which then activates matrix metalloproteinases (MMPs). Here, we report that uPA directly cleaved pro-MMP-9 in a time-dependent manner at both C- and N-terminus and generated two gelatinolytic bands. uPA-activated-MMP-9 efficiently degraded fibronectin and blocked by uPA inhibitor B428 and recombinant tissue inhibitor of metalloproteinase-1 (TIMP-1). B428 inhibited basal and PMA-induced active MMP-9 in glioblastomas (GBM) U1242 cell media as well as cell invasion *in vitro*. A combination of MMP-9 and uPA antibodies more significantly inhibited U1242 cell invasion than uPA or MMP-9 antibody alone. Both uPA and MMP-9 were highly expressed in U1242 cell and GBM patient specimens. Furthermore, two active MMP-9 fragments with identical molecular weights to the uPA-activated MMP-9 products were detected in GBM patient specimens. These results suggest that uPA-mediated direct activation of MMP-9 may promote GBM cell invasion.

Extracellular proteolysis is critical for tumor invasion, metastasis and angiogenesis. The two best-characterized groups of extracellular proteolytic enzymes are urokinase plasminogen activator (uPA) and matrix metalloproteinases (MMPs) [1]. MMPs, a family of zinc-dependent enzymes that proteolytically degrade various components of the ECM, play a critical role in a variety of malignant tumor invasive processes [2]. The expression and activation of gelatinase B (MMP-9) are involved in tumor progression [2]. Similarly, increased expression of uPA and MMP-9 has been found in human malignant brain tumors *in vivo* [3,4] and these proteases play an important role in human glioblastomas (GBM) invasion and tumorigenesis [5,6]. MMP-9 is secreted as an inactive precursor and requires activation by other proteases or autocatalysis [7] which is critical for its activity and biological function.

Previous study reported that uPA interacts with MMP-9 indirectly through uPA/plasminogen/plasmin system, in which uPA activates plasminogen to plasmin. The latter subsequently acts as a potential activator of pro-MMP-9 in various human cancer cell lines [6,8–10]. Other reports demonstrated that plasmin is not a direct [10] or an efficient [11] activator of pro-MMP-9. These findings led us to investigate whether uPA can directly activate MMP-9.

In this study, we provide evidence that uPA directly activates pro-MMP-9 at both N-terminus and C-terminus *in vitro*. Neutralization of uPA and MMP-9 activities with specific antibodies attenuated GBM U1242 cell invasion. Our results suggest that uPA-evoked MMP-9 activation and increased invasion may be partly mediated through the plasminogen/plasmin-independent pathway.

Materials and methods

Biochemical cleavage assay of MMP-9 by uPA. Purified recombinant uPA (0.02 μM, a gift from Drs. Jack Henkin and Andrew Mazar of Abbott Laboratories, Abbott Park, IL) was incubated with purified pro-MMP-9 (pMMP-9, 0.2 μM), purified MMP-9/TIMP-1 (0.2 μM), and purified MMP-9–lipocalin complex (0.2 μM), MMP-9 monomer (mMMP-9, 0.2 μM) in the presence or absence of purified recombinant N-terminal domain of TIMP-1 (N-TIMP-1, 0.2 μM) [12] in glycine buffer (0.1 M glycine, pH 8.0) at 37 °C for 24 h. To compare this novel activation with other known MMP-9 activators mediated activation, recombinant MMP-3 catalytic domain (0.02 μM), and MMP-26 (0.02 μM), and uPA-activated plasmin (0.02 μM) were incubated with latent MMP-9 (0.2 μM) in the incubation buffer [15] at 37 °C for 24 h. The purified MMPs (except MMP-26 [13]) were purchased from Calbiochem, San Diego, CA. The selective uPA inhibitor 4-iodobenzo[*b*]thiophene-2-carboxamidine (B428, 7.5 μM) was added to inhibit the cleavage. To verify whether latent forms of MMP-9, MMP-9/TIMP-1 complex and MMP-9/NGAL complex can be activated, 4-aminophenylmercuric acetate (APMA, 1 mM), a well-known pro-MMP activator *in vitro*, was added and incubated for 4 h. For the time-dependent assay of MMP-9 cleavage by uPA, MMP-9 (0.2 μM) was incubated with uPA (0.02 μM) for the indicated time periods.

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Substrate cleavage assay, TIMP-1 inhibition and silver staining. To further determine the uPA cleavage of MMP-9 is activation, substrate cleavage assays were performed. Fibronectin is one of the major ECM components, which are elevated in the brain of the human glioblastoma patients [14]. For fibronectin cleavage assays, pro-MMP-9 was pre-incubated with uPA, MMP-26, MMP-3, plasmin and APMA and for 24 h to generate active MMP-9 solution. Fibronectin (1 mg/ml, Sigma) was incubated with 2 μl of the active MMP-9 solution in incubation buffer [13] at 37 °C for another 24 h. Fibronectin incubated with uPA (0.02 μM), pro-MMP-9 (0.2 μM), MMP-26 (0.02 μM), or MMP-3 (0.02 μM) alone under the same experimental condition were served as control. To test the effects of TIMP-1 on activity of uPA-activated MMP-9, N-TIMP-1 (0.03 μM) was pre-incubated MMP-9 monomer (0.02 μM) at 37 °C for 6 h, followed by adding uPA (0.002 μM) to the pre-incubated solution and incubated at 37 °C for another 24 h. The silver staining was performed according to our previous report [13].

Edman protein N-terminal sequencing and phenyl isocyanate (PIC) N-terminal labeling and mass spectrum (MS) analysis. Edman N-terminal sequencing was performed as previously reported [13] at the Biomolecular Research Facility, University of Virginia. The reaction solution of uPA and MMP-9, and MMP-9 alone incubated with 2.5 mM PIC (Sigma) in 10 mM Hepes (pH 7.5) for 10 min at room temperature, the reaction was stopped by addition of 1 μl of 100 mM ammonium bicarbonate buffer. Under these conditions PIC has been shown to label only the N-terminal amines [15]. The mixture was loaded onto 9% SDS–PAGE for separation and the protein bands were revealed with silver staining [13]. The interested band was excised from the gel. The detailed method of MS was provided in Supplementary Method 1.

Gelatin and fibrinogen zymography. MMP-9 activity was detected by gelatin zymography as described previously [13]. uPA activity was detected using fibrinogen zymography. Briefly, cell media or protein extracts were resolved under non-reducing conditions on 10% SDS–PAGE gels containing 1 mg/ml fibrinogen and 20 μg/ml plasminogen (Sigma). The gels were rinsed, incubated, stained and destained as described previously [13] except the incubation buffer was 0.1 M glycine (pH 8.0).

Cell invasion assay. The cell invasion assay was performed as previously reported [13]. Briefly, cell suspension (1×10^5 cells) was added to each insert, which were pre-coated with 0.25 mg/ml fibronectin in the presence or absence of specific uPA inhibitor, B428 (7.5 μM), phorbol 12-myristate 13-acetate (PMA, 50 nM), uPA antibody (25 μg/ml, American Diagnostica Inc.) and MMP-9 antibody (25 μg/ml, CalBiochem). DMSO or normal IgG was used as controls. The invaded cells were stained and counted as previous report [13].

Results

uPA directly cleaves latent MMP-9

Our biochemical assays revealed that purified 92 kDa pro-MMP-9 was cleaved directly by uPA, generating two new bands (86 and 80 kDa) showed gelatinolytic activity (Fig. 1). A specific uPA inhibitor B428 (7.5 μM) completely blocked the cleavage of MMP-9 by uPA (Fig. 1). Furthermore, uPA-mediated cleavage of pro-MMP-9 was time-dependent. These results suggest that pro-MMP-9 can be cleaved by uPA directly and generated two new bands with gelatinolytic activity *in vitro*.

To identify the cleavage sites of MMP-9 by uPA, Edman N-terminal sequencing and MS analyses using N-terminal PIC-labeling were performed. N- and C-terminal amino acids of 86 kDa band (band 2B) and 80 kDa (band 2C) were shown in Fig. 1D. MS analyses showed that the N-terminal amino acids of band 2B was very similar to the data generated from Edman N-terminal sequencing, which matched the known sequence of MMP-9. Our results explain why bands 2B and C showed gelatinolytic activity because they still had the zinc-binding motif in the catalytic domain of MMP-9. N-terminal and C-terminal fragments of band 1A indicate that band 1A is a full length MMP-9. These results suggest that latent MMP-9 can be cleaved by uPA at both N-terminus and C-terminus. The N- and C-terminal amino acids of other bands were shown as in Fig. 1D.

Comparison of uPA and other known MMP-9 activators on gelatin and fibronectin cleavage

Since a number of previous studies show that uPA converts inactive plasminogen to active plasmin, the latter further cleaves extracellular matrix components and activates some MMPs [6,8–10], we compared the cleavage of uPA-activated MMP-9 and uPA–plasmin-activated MMP-9. The results revealed that uPA–plasmin-activated MMP-9 fragment was 84 kDa, indicating the cleavage site is different from uPA-activated MMP-9 (Fig. 2). Next, we designed experiment to identify enzymatic activity of uPA-activated MMP-9 against its substrate and compare with MMP-26-, MMP-3- and APMA-activated MMP-9 substrate cleavage. We chose fibronectin as substrate because it is a common extracellular matrix component and elevated in human brain glioblastoma [14]. Biochemical substrate digestion assay showed that uPA-activated MMP-9 cleaved fibronectin and generated at least nine new products compared with control (Fig. 2). MMP-26-activated MMP-9 generated four fragments, MMP-3-activated MMP-9 generated three fragments and APMA-activated-MMP-9 even did not show any cleavage; while uPA, pro-MMP-9, MMP-26 and MMP-3 alone exhibited no catalytic activity to fibronectin (Fig. 2C). The silver staining of uPA-, MMP-26-, MMP-3- and APMA-activated MMP-9 and uPA, pro-MMP-9, MMP-26 and MMP-3 alone exhibited no additional bands under the same experimental condition (molar concentration ratio enzyme:fibronectin = 1:50) (data not shown). These results indicate that uPA-activated MMP-9 cleaved fibronectin more efficiently than MMP-26-, -3- and APMA-activated MMP-9 or uPA, pro-MMP-9, MMP-26, and -3 alone in *in vitro*.

uPA-mediated MMP-9 activation is regulated by TIMP-1 and NGAL

Since MMP-9 preferentially forms complexes with its regulators (TIMP-1 and NGAL), we determined the effects of these regulators on uPA-mediated MMP-9 activation, using purified pro-MMP-9/TIMP-1 and pro-MMP-9/NGAL complexes. Both MMP-9/TIMP-1 and MMP-9/NGAL complexes were not cleaved by uPA compared with control (Fig. 3A), while APMA activated the pro-MMP-9/NGAL complex and reduced the 175 kDa pro-MMP-9/TIMP-1 complex (data not shown). Purified MMP-9 monomer, and the purified recombinant N-TIMP-1 were used to further confirm the regulation of uPA-mediated-pro-MMP-9 cleavage by TIMP-1. The results showed that pre-incubation with both N-TIMP-1 and uPA specific inhibitor B428 for 2 h abolished MMP-9 monomer activation by uPA when compared with control (Fig. 3B). These results suggest that uPA-mediated MMP-9 activation is inhibited by TIMP-1.

uPA-activated MMP-9 promotes U1242 GBM cell invasion

We then explored whether uPA-mediated-MMP-9 activation is involved in GBM cell invasion. First we identified that both uPA and MMP-9 were highly expressed in U1242 GBM cell media (Fig. 4A and B). Treatment of U-1242 GBM cells with uPA specific inhibitor (B428, 7.5 μM) for 24 h inhibited formation of active MMP-9 in U1242 cell media (Fig. 4A). Following treatment of U1242 cells with PMA (50 nM), a stimulator of uPA and MMPs via activation of protein kinase C pathway, secreted uPA, pro-MMP-9 and active MMP-9 were highly increased compared with DMSO (Fig. 4B and C). B428 blocked PMA-induced pro-MMP-9 and active MMP-9 when compared with control. However, both PMA and B428 had no effect on activity of MMP-2 (Fig. 4C). To further explore the role of uPA-activated MMP-9 in *in vitro* invasion of U1242 cells, fibronectin invasion assay was used, because uPA-activated MMP-9 degraded fibronectin more efficiently (Fig. 2C). Fibronectin is one of the ECM proteins found in GBM patient specimens [14]. The results showed that B428 significantly inhibited basal invasion (20.1%, $p < 0.05$) and PMA-stimulated invasion (55.6%, $p < 0.001$) in U1242 cells as compared with DMSO (Fig. 4D). In addition, a combination of functional neutralizing antibodies of MMP-9 and uPA synergically inhibited U1242 cell invasion by 63.74% ($P < 0.001$) compared with uPA or MMP-9 antibody alone. Treatment of U-1242 cells with uPA or MMP-9