Identification of Plasminogen in Matrigel™ and Its Activation by Reconstitution of This Basement Membrane Extract

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ABSTRACT

Matrigel™, a basement membrane (BM) extract of the Engelbreth-Holm-Swarm (EHS) sarcoma, used in tumor invasion assays, was found to contain plasminogen. Plasminogen was identified, using Western blot analysis and casein zymograms, by comparison with human plasminogen. Matrigel contained approximately 20–100 ng of plasminogen per 100 µg of protein as determined by these assays. Matrigel reconstitution and incubation at 37°C caused activation of plasminogen, which was serine protease dependent and involved tissue plasminogen activator (tPA) as an anti-tPA antibody which inhibited activation. This reconstitution and incubation also caused leupeptin-inhibitable degradation of Matrigel components as assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Degradation of the BM extract copolymerized in zymograms was caused by human plasminogen and plasminogen in the Matrigel. Maximal plasmin activity, following incubation of Matrigel at 37°C for 16 h, was equivalent to approximately 10 ng of purified plasmin using the plasmin substrate D-Val-Leu-Lys p-nitroanilide. Matrigel, therefore, contained all the components of the plasmin-generating system, including plasminogen. The plasmin generated degraded Matrigel components and exogenous substrates. Our data suggest that, since this tumor BM acts as a reservoir for enzymes of the plasmin-generating system, caution should be taken by investigators interpreting data concerning the effects of Matrigel on cell behavior and, in particular, cellular invasion.

INTRODUCTION

The Engelbreth-Holm-Swarm (EHS) sarcoma is a convenient model for studying basement membranes (BM), as its cells produce only BM as their matrix (28). Matrigel™ is an extract of the EHS tumor BM comprised largely of laminin, type IV collagen, heparan sulfate proteoglycan and entactin (10). In its reconstituted form, Matrigel has been widely used as a BM barrier to tumor cell invasion and for the study of BM effects on cellular behavior, including vascularogenesis, in vitro (1,12, 27,33,34). Tumor cell invasion across BM and tumor-associated angiogenesis are considered of central importance to metastatic progression (5,22,37). Therefore, the tumor origin of Matrigel makes its use of added significance in the study of these phenomena.

It has been proposed that a proteolytic cascade exists, involving enzymes of the plasmin system and metalloproteinases (MMP), that results in tumor cell invasion of BM (5,6,22,24, 30,37), and both enzyme systems would appear to be involved in vascularogenesis (27,33,34). Plasmin, generated by the action of cell-derived plasminogen activators (PAs) on exogenous plasminogen, can degrade the BM components laminin and type IV collagen (8,15,18) and activate MMPs (5,7,29, 30,37), and both enzyme systems may also result from the activation of proteases secreted by invading cells but may also result from the activation of enzymes sequestered within the BM. In this study we demonstrate that the EHS tumor BM extract Matrigel contains all the components of the plasmin-generating system including plasminogen and tissue plasminogen activator (tPA). We demonstrate the activation of this enzyme system following Matrigel reconstitution and incubation at 37°C, which generates plasmin that is active against BM components endogenous to Matrigel, itself, and against exogenous substrates.

MATERIALS AND METHODS

Reagents

Matrigel was purchased from Collaborative Research (Bedford, MA, USA). Bovine casein, purified human Glu-plasminogen, human plasmin, leupeptin, goat anti-human plasminogen antibody (P5276), anti-goat IgG-peroxidase conjugated secondary antibody, EDTA, N-ethylmaleimide (NEM) and the plasmin substrate D-Val-Leu-Lys p-nitroanilide were purchased from Sigma Chemical (St. Louis, MO, USA). Goat anti-human tPA antibody (No. 387) was purchased from American Diagnostica (Greenwich, CT, USA). Prestained molecular weight standards for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Bio-Rad (Hercules, CA, USA). Hybond™-C extra supported nitrocellulose hybridization membranes and chemiluminescence...
reagents for Western blotting were purchased from Amersham International (Bedford, England, UK).

**Plasmin Assay**

Plasmin substrate-degrading activity was assayed using the synthetic plasmin substrate D-Val-Leu-Lys p-nitroanilide as previously described (40). Briefly, Matrigel, plasmin and plasminogen plus tissue plasminogen activator (tPA) were assayed for substrate-degrading activity in reaction buffer containing 100 mM Tris and 0.5% Triton® X-100 (pH 8.8) in a final volume of 100 µL containing 10 µL of plasmin substrate (5 µg/µL). Matrigel was defrosted at 4°C and diluted 1:4 in phosphate-buffered saline (PBS). Samples were incubated either at 4°C or at 37°C for the times stipulated. Following incubation, Matrigel samples (100 µg) were mixed with reaction buffer containing plasmin substrate. Assays were performed in 96-well microplates and the samples were analyzed spectrophotometrically at a wavelength of 405 nm using a Titertek™ Multispec plate reader (Labsystems, Needham, MA, USA). Negative zymogram images provided better quality figures.

**Substrate Gel Electrophoresis**

The following substrates were incorporated into regular SDS-PAGE: 0.1% Matrigel for the analysis of enzymes capable of degrading this substrate; 0.1% bovine casein for caseinases (17); or 0.1% casein plus 5 µg/mL Glu-plasminogen for plasminogen activators (10). The electrophoretic separation of Matrigel, plasminogen, plasmin or tPA was performed under nonreducing conditions, and the gels were treated as previously described (17). Matrigel samples were solubilized in nonreducing sample buffer and loaded directly into zymograms. Gels containing plasminogen were incubated with 15 mM EDTA to inhibit endogenous metallo-proteinase activity. For inhibitor studies, gels were incubated in buffer (50 mM Tris/200 mM NaCl/5 mM CaCl₂ pH 8.0) containing leupeptin (100 µg/mL), EDTA (15 mM) or NEM (15 mM). The zymograms were stained with Coomassie blue, destained in a mixture of methanol:acetic acid:water (4:1:5) and dried using cellulose film (Gel Drying Kit; Promega, Madison, WI, USA). The dried gels were scanned using an Imaging Densitometer (Model GS670; Bio-Rad) and negative images were photographed using a Digital Palette (Model I-5000; Polaroid, Cambridge, MA, USA). Negative zymogram images provided better quality figures.

**Immunoblot Analysis**

Matrigel, human tPA, plasminogen and plasmin were separated by SDS-PAGE under nonreducing conditions using 10% polyacrylamide gels. The gels were run in the presence of pre-stained molecular weight standards. The proteins were transferred electrophoretically to nitrocellulose membranes in a buffer containing 25 mM Tris, 192 mM glycine and 20% methanol (pH 8.3). Western analysis was conducted as previously described (4). Briefly, nonspecific protein-binding sites were blocked for 2 h at room temperature using 5% non-fat dried milk in PBS. The blots were then incubated for 1 h at room temperature with an anti-human plasminogen antibody diluted 1:500 in blocking solution. The blots were washed and incubated for a further 1 h at room temperature with antigoat IgG horseradish peroxidase conjugated secondary antibody diluted 1:2000 in blocking solution. Antigen reactivity was demonstrated by chemiluminescence reaction (Amersham International). Immunoreactive bands were visualized on XAR-5 film (Eastman Kodak, Rochester, NY, USA). Molecular weights were approximated by comparison with pre-stained molecular weight markers using Molecular Analyst/PC software for the Bio-Rad Model GS-670 Imaging Densitometer.

**Statistical Analysis**

The Student’s t test was applied to detect significant differences following calculation of means and standard deviations of experimental data. By convention, it was taken that differences between sample means with a probability of P = 0.05 were significant.

**RESULTS AND DISCUSSION**

In this study, we demonstrate that plasminogen copurifies with the EHS tumor BM extract Matrigel and confirm previous reports that Matrigel also contains tPA (23,34). We demonstrate tPA-dependent activation of plasminogen following reconstitution of Matrigel and incubation at 37°C. The plasmin generated was available for the degradation of both the exogenous substrates and those endogenous to this tumor BM preparation.

Similar levels of plasminogen were observed in 3 different batches of regular Matrigel and in 2 different batches of growth factor-reduced Matrigel, assessed by zymogram (data not shown), suggesting that plasminogen is a standard component of Matrigel.

Figure 1 demonstrates the plasmin substrate-degrading activity of different concentrations of purified human plasmin and in Matrigel (100 µg) incubated for various times at 37°C, following thawing at 4°C, prior to the addition of plasmin substrate. Significant degradation of plasmin substrate, with respect to controls, was observed with 1, 10 and 100 ng of purified plasmin, with mean (± SD) values of 6 independent experiments of 0.31 ± 0.03 (P ≤ 0.05), 1.4 ± 0.25 (P ≤ 0.01) and 3.3 ± 0.32 (P ≤ 0.001) spectrophotometric units measured at a wavelength of 405 nm. Matrigel (100 µg), thawed at 4°C, but prior to incubation at 37°C, also degraded plasmin substrate when assayed under conditions identical to the purified plasmin standard. The mean (± SD) value obtained for 6 independent experiments of 0.32 ± 0.04 approximated that observed with 1 ng of purified plasmin and was significantly elevated over control values (P ≤ 0.05). Incubation of Matrigel at 37°C for 3, 6 and 16 h, prior to substrate addition, significantly increased plasmin substrate-degrading activity, with respect to that observed in unincubated Matrigel, to mean (± SD) values of 0.89 ± 0.18 (P ≤ 0.05), 1.15 ± 0.15 (P ≤ 0.01) and 1.53 ± 0.19 (P ≤ 0.01), respectively, for 6 independent experiments. The mean activity (1.53),
following 16 h of incubation at 37°C and prior to substrate addition, approximated the activity of 10 ng of purified plasmin standard (1.49). Enzyme activity in reconstituted, unincubated Matrigel and following incubation for 16 h at 37°C, prior to substrate addition, was completely inhibited by leupeptin (100 µg/mL) but not by EDTA (15 mM) or NEM (15 mM) (data not shown), confirming the serine protease nature of plasmin substrate-degrading activity.

The generation of plasmin substrate-degrading activity following Matrigel incubation for 16 h at 37°C was significantly inhibited by an antibody against human tPA at a concentration of 5 µg of IgG per 100 µg of Matrigel. Mean percentage inhibition for 6 independent experiments was 55 ± 6.4% (P ≤0.01) (Figure 1C). This inhibition was overcome by the simultaneous addition of 100 ng of urokinase plasminogen activator (uPA) with antibody (Figure 1C, lane 3), which significantly increased mean activity above values observed in Matrigel incubated alone for 16 h, by 35.4 ± 3.2% (P = 0.001). The addition of uPA (100 ng) alone had a similar effect, increasing activity by 37.2 ± 4.2% (P ≤0.001). These data suggest a role for tPA in the generation of plasmin activity in Matrigel, that the precursor to plasmin substrate-degrading activity in Matrigel can also be activated by exogenous PAs and that endogenous PA levels limit the extent of plasmin activity generated. It has previously been reported that Matrigel contains tPA (23,34). We confirmed this by zymograms, which showed that Matrigel contained PA activity, similar in molecular weight to purified human tPA, of equivalent activity to between 1 and 10 ng of purified human tPA per 100 µg of protein (Figure 2). Neither purified tPA nor uPA alone degraded the plasmin substrates used in this study (data not shown).

To characterize further the plasmin substrate-degrading activity in Matrigel, substrate gel electrophoresis was performed. This represents a sensitive technique for detecting proteolytic activity of individual enzymes without requiring extensive purification. However, it does not distinguish between active and pro-form enzyme, because of the activating power of SDS (3). Four leupeptin (but not EDTA or NEM) inhibitable bands of caseinolytic activity ranging in approximate molecular weight from 70 to 100 kDa were identified in reconstituted Matrigel that had not been incubated at 37°C (Figure 3A). Incubation of reconstituted Matrigel at 37°C for 3, 6 and 16 h resulted in the gradual disappearance of higher molecular weight bands and the appearance of additional lower molecular weight caseinolytic species ranging from 75–80 kDa to 45 kDa. The change in the caseinolytic profile upon Matrigel reconstitution and incubation at

Figure 1. Plasmin substrate (β-Val-Leu-Lys-pNA) degrading activity. Panel A: With different concentrations of purified human plasmin, and Panel B: in 100 µg of reconstituted Matrigel incubated for 0, 3, 6 and 16 h at 37°C prior to assay. Results expressed as mean ± SD spectrophotometric units at 405 nm wavelength of 6 independent experiments. Panel C: Percentage change in plasmin substrate-degrading activity with respect to (wrt): (1) 100 µg of Matrigel incubated alone at 37°C for 16 h (100%); (2) presence of 5 µg of anti-tPA antibody; (3) presence of 5 µg of anti-tPA antibody plus 100 ng of urokinase; or (4) presence of 100 ng of urokinase alone. Results expressed as mean ± SD percentage change in activity in 6 independent experiments. (* refers to significant differences with respect to controls, evaluated by Student’s t test.)

Figure 2. Substrate gel electrophoresis demonstrating plasminogen activator activity in 50 µg of Matrigel (lane 1), and in 10 ng (lane 2) and 1 ng (lane 3) of purified human single-chain tPA. A negative image of the zymogram is displayed.

Figure 3. Substrate gel electrophoresis showing caseinolytic activity. Panel A: Caseinolytic activity in 100 µg of Matrigel incubated at 37°C prior to analysis for 0 h (lane 1), 3 h (lane 2), 6 h (lane 3) and 16 h (lane 4). Panel B: Caseinolytic activity in 100 µg of Matrigel incubated for 16 h at 37°C with 5 µg of anti-tPA antibody (lane 1); without antibody (lane 2); and with leupeptin (100 µg/mL) (lane 3). Lane 4: Unincubated Matrigel (100 µg). Panel C: Caseinolytic activity in 1 µg of human Glu-plasminogen incubated for 16 h at 37°C alone (lane 1), with 10 ng of purified human uPA (lane 2) and with 10 ng of purified human tPA (lane 3). Negative images of zymograms are displayed.
37°C was completely inhibited by leupeptin (100 µg/mL) and partially inhibited by anti-tPA antibody (5 µg/100 µg Matrigel) (Figure 3B). Neither EDTA (15 mM) nor NEM (15 mM) inhibited this change (data not shown). All caseinolytic species in unincubated Matrigel and new species generated by incubation at 37°C were inhibited, in zymograms, by leupeptin (100 µg/mL), but not by EDTA (15 mM) or NEM (15 mM) (Figure 4). These data confirm the serine protease nature of caseinolytic activity in Matrigel and suggest that the change in the caseinolytic profile was serine protease(s) and, in part, tPA dependent. We compared the caseinolytic bands produced by tPA and uPA activation of purified Glu-plasminogen with those produced by incubation of Matrigel at 37°C (Figure 3C). Plasminogen activator-dependent activation of purified Glu-plasminogen also produced numerous additional bands of caseinolytic activity, several of which were of similar molecular size to those observed in Matrigel following incubation (Figure 3, A and B). These data suggest a high degree of identity between enzyme activity in Matrigel and plasminogen. The presence of plasminogen in Matrigel preparations was confirmed by Western blot analysis using an anti-human plasminogen antibody. This antibody recognized protein species in unincubated Matrigel (94–100 kDa) of similar molecular weight to purified human plasminogen, but not of purified human plasmin (Figure 5), and were of identical molecular weight to caseinase activity in Matrigel. It was estimated, by zymogram, that 100 µg of Matrigel contained caseinolytic activity equivalent to 100 ng of purified human plasminogen (Figure 6) and, by Western blots, the equivalent of approximately 20 ng of human plasminogen per 100 µg of Matrigel (Figure 5).

Purified plasminogen (1 µg), plasmin (1 µg) and enzymes in Matrigel (100 µg) degraded Matrigel copolymerized as a substrate in zymograms (Figure 7A). The Matrigel-degrading activity in Matrigel, itself, was of identical molecular weight to caseinolytic activity and was inhibited by leupeptin (100 µg/mL) (Figure 7A, lane 4). Examination of Matrigel components by regular SDS-PAGE revealed leupeptin-inhibitable degradation of a high molecular weight component (ca. 200 kDa) following incubation at 37°C for 16 h (Figure 7B). This confirmed the ability of serine protease activity, generated following Matrigel incubation, to degrade components endogenous to this extract. Matrigel contains both laminin and type IV collagen (13), which can be degraded by plasmin (8,15,18).

Unincubated Matrigel contained plasminogen in zymogen form, suggesting its separation from PAs in vivo. The preservation of zymogen during purification most likely reflected the temperature constraints of the extraction procedure (4°C), as it was readily activated upon incubation at 37°C. The generation of plasmin activity in Matrigel following incubation suggested that both PAs and plasmin were present in excess of inhibitors.
The presence of plasminogen in Matrigel adds to the list of enzymes already reported in this BM extract (19, 20, 23, 33, 34) and confirms that Matrigel acts as a reservoir for enzymes in addition to cytokines and growth factors (38). A recent report localizing plasminogen and plasmin to the extracellular matrix of normal mouse aorta, in vivo (7), would also suggest that BMs, in vivo, may also sequester plasminogen. We are investigating this possibility further.

The source of plasminogen within Matrigel remains to be elucidated. Tumor cells generally secrete elevated levels of proteases, which may be incorporated in BM structures (5, 6, 17, 19, 22, 23, 37). However, there are no reports that EHS tumor cells express plasminogen. It is more likely that plasminogen, within Matrigel, is of extra-tumor origin, and copurification is the result of its binding affinity for BM and laminin (14, 25). Indeed, we have previously reported that a caseinolytic serine protease of similar molecular weight to plasmin copurifies with EHS tumor laminin (19).

Plasmin degrades BM glycoprotein and collagenous components (6, 15, 18) and can activate growth factors such as TGFbeta (16) and proteases (5, 6, 9, 23, 26, 31). Therefore, the ability of BM to sequester this protease would, no doubt, be of considerable biological significance and adds to the increasing body of evidence that BM may play a central role in the regulation of matrix-degrading protease activity. In this respect, matrices have been reported to effect enzyme configuration, as both laminin and fibronectin stimulate tPA activity (35, 36) and matrix components have been reported to stimulate enzyme expression in a variety of cell lines (11, 20, 39).

Our results may, in part, explain some of the observations reported using Matrigel, such as the requirement for plasminogen activators in endothelial cell tube formation (27, 34) and for tumor cell invasion (1, 5, 30); the presence of active TGFbeta in Matrigel (16, 38); and the ability of Matrigel to activate MMP-9 (2, 32). Our data should caution investigators when interpreting data concerning the effects of Matrigel on cell behavior and, in particular, assays involving cellular invasion.

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