Melanoma-mediated Dissolution of Extracellular Matrix: Contribution of Urokinase-dependent and Metalloproteinase-dependent Proteolytic Pathways

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ABSTRACT

Constitutive expression of both urokinase and matrix metalloproteinase (MMP) activity is frequently observed in individual malignant tumors. In this study we describe the combined contribution of these distinct enzyme systems to the invasive phenotype of a highly metastatic human melanoma cell line (M24met).

M24met cells were found to secrete a spectrum of MMPs, including interstitial collagenase, type IV collagenases (M, 92,000 and 72,000 progelatinases), and stromelysin. Urokinase, but not tissue-type plasminogen activator, was detected in M24met-conditioned media and on cell surfaces. The contribution of these enzymes to extracellular matrix dissolution was determined by exploiting specific inhibitors, namely tissue inhibitor of the metalloproteinases-2 and plasminogen activator inhibitor-2. Due to the coexpression of urokinase and MMP-dependent activity, M24met cells were observed to degrade multiple components of the extracellular matrix and to significantly degrade both interstitial and basement membrane matrices. Urokinase-dependent removal of matrix glycoprotein was observed to precede MMP-dependent collagenolysis as a prerequisite rate-limiting step. We present evidence which suggests that this temporal relationship is imposed by the structural architecture of the matrix such that matrix glycoprotein serves to protect associated collagen from MMP-dependent degradation. In addition to mediating significant collagenolysis, MMP activity was further implicated in the dissolution of matrix tropoelastin. Urokinase/plasmin activity was not found to be required for MMP-zymogen activation.

INTRODUCTION

Degradation of occluding ECM is a prerequisite step in a multistage process that culminates in distant metastatic disease. The ability of tumor cells to compromise the structural integrity of the ECM has been attributed to the activity of a spectrum of hydrolases, including the serine, cysteine and carboxyl peptidases, the metalloproteinases, and the glycosidases (1). While tumor heterogeneity dictates that each tumor may use its own unique spectrum of hydrolases to break down subtumor ECM, a large body of in vitro evidence now suggests a pivotal role for tumor-associated MMPs or PAs (1-3). The relevance of these neutral proteases is self-evident, given their ability to directly or indirectly initiate the degradation of key structural elements of the ECM.

Plasminogen activators of either the uPA or tissue type are neutral serine proteases whose main proteolytic activity is restricted to the conversion of the abundant zymogen plasminogen into plasmin. In contrast to its activators, plasmin is a nondiscriminate protease that has been reported to degrade multiple elements of the ECM, including fibronectin, laminin, and type IV collagen (4, 5). This broad degradative capability and the reported ability of plasmin to activate certain MMPzymogens (6) suggest that the PAs may have a pivotal role at the top of a potent hydrolytic cascade. The matrix metalloproteinases constitute a family of zinc-dependent endopeptidases whose members are able, by definition, to degrade at least one element of the ECM (7, 8). On the basis of ECM specificity, MMPs are categorized into three subclasses which include the interstitial collagenases (MMP-1 and -8), the type IV collagenases/gelatinases (MMP-2 and -9), and the stromelysin/transins (MMP-3, -10, and -11). Interstitial collagenase activity is restricted to the cleavage of fibrillar types I, II, and III collagen. Documented substrates for the type IV, M, 72,000 and 92,000 progelatinases include types IV, V, VII, and XII collagens, gelatin, and elastin (8, 9). Type IV, M, 72,000 progelatinase has further been shown to digest fibronectin and laminin (8). Members of the stromelycin subclass exhibit a broad substrate specificity. Stromelysin-1, for example is known to degrade proteoglycans, laminin, fibronectin, and types III, IV, and V collagen (10, 11).

Ubiquitous natural inhibitors of MMP activity include a2-macroglobulin and two specific tissue inhibitors of the metalloproteinases (TIMP-1 and -2) (12, 13). PA activity is regulated by a family of serine-protease inhibitors (serpins), that includes a2-antiplaxmin, protease nexin-1, and the specific PA/plasmin inhibitors PAI-1 and PAI-2 (14). In accordance with the metastatic relevance of PA/MMP activity, epigenetic or genetic manipulation of PAI or TIMP levels has resulted in significant alteration of invasiveness and metastatic potential (15-19).

Examination of reports independently associating MMP or PA activity with metastatic potential reveals numerous examples of individual tumor variants or cell lines that constitutively express both MMP and PA activity. Coexpression of type IV collagenases and PAs has, for example, been independently reported for the B16-F10 melanoma, the Lewis lung carcinoma, the HT1080 fibrosarcoma, and the T10 sarcoma (1). Furthermore, it has been suggested that coexpression of MMP and PA activity is a prerequisite for successful invasion and metastasis (20, 21). Despite these observations there has been little effort to study the joint contribution or interaction of PA- and MMP-dependent pathways with respect to ECM degradation and invasion by individual metastatic tumors.

It has been proposed that a tumor expressing multiple enzymatic activities will display enhanced degradation of occluding ECM, and will have a concomitantly high metastatic potential (1). Thus, the range of susceptible substrates will be large with overlapping substrate specificities resulting in the enhanced degradation of individual components. Proteolytic degradation of a given substrate by a particular enzymatic pathway may also be expected to increase the susceptibility of this substrate or associated substrates to other enzymatic pathways. Finally, the activity of a given pathway may facilitate the activation of unrelatedzymogens, resulting in the initiation of a proteolytic cascade. In this study we have addressed these issues with respect to a highly metastatic human melanoma cell line (M24met) which elaborates both uPA and a spectrum of metalloproteinases. The contribution of these enzymes to ECM dissolution and invasion was assessed by...
using specific high affinity inhibitors, namely, TIMP-2 and PAI-2. It has long been proposed that such protease inhibitors may be used for therapeutic intervention in the metastatic process.

**MATERIALS AND METHODS**

**Materials.** Recombinant tissue inhibitor of the metalloproteinases, TIMP-2, was obtained from the culture medium of CHO cells transfected with TIMP-2 complementary DNA (22). Specific inhibitor of the uPA/plasmin system, PAI-2, was a kind gift of Dr. Gregor Schulz (Behringwerke AG, Marburg, Germany). Plasminogen was obtained from Sigma (St. Louis, MO). 125I-Fibronectin was purchased from ICN (Costa Mesa, CA). N[proline-2,3,3-H]Laminin, and type I and IV collagen were purchased from NEN (Boston, MA). 1-[2,3,4,5-3H]Proline was purchased from Amersham (Arlington Heights, IL). Recombinant high molecular weight uPA was obtained from Calbiochem (La Jolla, CA), and single chain recombinant tissue-type PA was from American Diagnostica (Greenwich, CT). Antibiotic specific for M, 72,000 type IV collagenase was kindly provided by Dr. Gillian Murphy (Strangeways Research Laboratory, Cambridge, United Kingdom). Antibiotic specific for rat transin and cross-reactive with human stromelysin was kindly provided by Dr. Lynn Matrisian (Vanderbilt University, Nashville, TN). Anti-human uPA monoclonal antibody 394 was purchased from American Diagnostica.

**Tumor Cell Line.** Human melanoma cell line M24 was kindly supplied by Dr. D. L. Morton (UCLA, Los Angeles, CA). M24met cells were derived from the lymph node of a nude mouse given injections s.c. of M24 cells (23). M24met cells were routinely cultured in RPMI 1640 supplemented with human FBS (Whittaker Bioproducts). Cells were passaged by using 0.5 mM EDTA-0.15 M NaCl, 0.02 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Versene). Culture supernatant was harvested and filtered to remove residual cells. Adherent monolayers were treated with 0.2 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid containing 0.5 mM EDTA-0.15 M NaCl, for counting. Proteases bound to M24met cell membranes were eluted by treating the cells for 3 min at room temperature with 50 mM glycine-HCl buffer containing 100 mM NaCl at pH 3.0 (24). The acid eluate was neutralized by using 0.5 M Tris-HCl at pH 7.8.

Culture supernatant was subsequently dialyzed against a collagenase buffer [Tris-HCl (50 mM), CaCl2 (10 mM), and NaCl (200 mM) pH 7.5] or against a Tris-HCl plasmin buffer [Tris-HCl (50 mM) pH 8.1]. Dialyzed conditioned media or cellular eluate was concentrated (50- to 80-fold) by ultrafiltration by using Diaflo YM10 (M, 10,000 cutoff) ultrafiltration membranes (Amicon, Lexington, MA). Aliquots of concentrated conditioned media were stored at 80°C.

**Zymography.** Matrix metalloproteinases in tumor-conditioned media were detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the presence of 0.2% type I collagen (Sigma) or 1% k-elasticin (Elastin Products, Owensville, MO) as previously described (9, 13). Plasminogen activators were detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the presence of 0.2% collagen and plasminogen at 10 μg/ml (25). The appearance of clear bands in these zymograms correspond to zones of gelatinolytic activity resulting from the conversion of plasminogen to plasmin by plasminogen activators.

**Metalloproteinase-mediated Substrate Degradation Assays.** Concentrated, serum-free conditioned media in collagenase buffer were tested for proteolytic activity against 125I-labeled fibronectin and N[proline-2,3,3-H]labeled laminin and collagen (types I and IV). Aliquots (50 μl) containing 20 μg of types I or IV collagen (1000 cpn/μg) or 20 μg of laminin (1500 cpn/μg) were placed into each well of a 96-well microtiter plate (Corning, NY). Microtiter plates were left overnight in a laminar flow cabinet to allow the substrate solutions to dry to films. Fibronectin (20 μg; 4000 cpn/μg) in 0.05 M carbonate buffer (pH 9.6) was coated to microtiter plates overnight at 4°C. Plates were washed repeatedly with phosphate-buffered saline until unbound fibronectin was removed. From 40 to 50% of the radioactivity retained was counted in the wells. Fifty-μl samples of tumor-conditioned media equivalent to 1 × 10⁵ cells were added to each well containing radiolabeled substrate. APMA was added to conditioned media at a final concentration of 1 μM. Samples containing APMA were preincubated for 1 h at 37°C prior to adding to the wells. TIMP-2 and plasminogen were added as indicated in the text. Samples were incubated on the radiolabeled substrates at 37°C for 24 h.

**Urokinase-based Substrate Degradation Assays.** Tumor-conditioned media and cellular eluate in 100 μl Tris-HCl (pH 8.1) were tested for proteolytic activity against 125I-labeled fibrin, 125I-fibronectin, and N[proline-2,3,3-H]labeled laminin. 125I-Fibrin plate assays for plasminogen activator activity were performed as described (26). Fibrinogen was iodinated with the use of Iodo-Gen (Pierce, Rockford, IL). Fibrinogen (50 μg/well; 4000 cpn/μg) and 3H-laminin (25 μg/well; 1500 cpn/μg) were coated onto 12-well polystyrene plates as described above. Tumor-conditioned media equivalent to 25,000 cells were added to each well containing radiolabeled substrate. Final test volumes were 1 ml. Plasminogen, PAI-2, and anti-uPA monoclonal antibody 394 were added as indicated in the text. Samples were incubated on the radiolabeled substrates at 37°C for 4 h (fibrin) or 24 h (laminin and fibronectin) prior to determining radioactive release.

**Intersitial Matrix Degradation Assays.** 1-[3,4,5-3H]Proline-labeled rat smooth muscle cell matrices were prepared in individual 35-mm plastic dishes (Falcon, Oxnard, CA) as previously described (27). To test for proteolytic activity, 2 × 10⁴ M24met cells were seeded into each dish in 2 ml of RPMI-1640 supplemented with acid-treated FBS. Optimal levels of recombinant TIMP-2 and/or PAI-2 (20 and 45 μg/ml, respectively) were added to the media as indicated in the text; 200 μl of media were harvested every 2 days for scintillation counting and the media were replaced. After 14 days tumor cells were lysed with 25 mM NH₂OH and the matrices were assessed for glycoprotein, elastin, and collagen content by sequential overnight treatment with trypsin, elastase, and bacterial collagenase (27). Glycoprotein-depleted matrices were prepared by overnight treatment with trypsin at 10 μg/ml. The trypsin was subsequently neutralized with a 5-fold excess of soybean trypsin inhibitor (Sigma) and the matrices were washed extensively with RPMI prior to the addition of cells.

**Basement Membrane Degradation Assay.** Endothelial cells derived from human umbilical veins were seeded onto gelatin-coated 24-well plates and grown to confluence in 1 ml of endothelial growth medium-umbilical vein (Clonetics, San Diego, CA). At confluence the media were replaced with proline- or serine-free endothelial growth medium-umbilical vein containing 5 μCi/ml of [1-3,4,5-3H]Proline or 5 μCi/ml of L-[G-3H]Serine (Amersham). Labeling was maintained for 12 days, with media and label replaced every 3 days. Twenty μg/ml of fresh ascorbate was added daily. Human umbilical vein endothelial cell monolayers were subsequently lysed with 25 mM NH₂OH, and underlying matrices were washed repeatedly with phosphate-buffered saline.

M24met cells (1 × 10⁴) were seeded directly onto the matrices in 1 ml of RPMI containing 10% acid-treated FCS and PAI-2 or TIMP-2 as indicated in the text. Cells were maintained in culture for 6 days with the media and inhibitors being replaced every 2 days. Treatments were performed in triplicate; 100-μl aliquots were counted every 24 h. Where indicated, matrices were depleted of noncollagenous components by overnight pretreatment with human plasmin (Sigma) at 10 μg/ml in Tris-HCl (50 mM) pH 8.1. Matrices were extensively washed in RPMI prior to the addition of cells.

**RESULTS**

**Tumor-associated Synthesis of Matrix Metalloproteinases.** Gel substrate zymography was used to confirm the presence of gelatinolytic metalloproteinases in serum-free tumor-conditioned media. A major band of gelatinolysis was observed at an estimated molecular weight of 72,000 (Fig. 1, Lanes 1 and 7). The identity of this band was confirmed by using an antibody specific for M, 72,000 type IV collagenase (Fig. 1, Lane 5). A lesser band of gelatinolysis migrating at a molecular weight of 62,000 (Fig. 1, Lanes 1 and 7) is due to the presence of activated M, 72,000 type IV collagenase. In accordance with this, organomercurial treatment of the tumor-conditioned media resulted in a significant increase in gelatinolytic activity.
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Fig. 1. Detection of metalloproteinases in serum-free tumor-conditioned media. Conditioned media derived from $2 \times 10^6$ M24met cells were analyzed by gelatin-based zymography on a 10% sodium dodecyl sulfate-polyacrylamide gel containing 0.2% gelatin. Clear bands correspond to zones of gelatinolytic activity in untreated conditioned media (Lane 1) or in conditioned media preincubated in 1 nM APMA for 1 h at 37°C (Lane 2). Conditioned media derived from $1 \times 10^6$ M24met cells were subject to casein-based zymography (Lane 3) or elastin-based zymography (Lane 4). Conditioned media derived from $5 \times 10^5$ M24met cells were analyzed by gelatin-based zymography with overnight incubation in the presence of buffer alone (Lane 7), buffer and 20 μg/ml of TIMP-2 (Lane 8), or buffer and 20 μM EDTA (Lane 9).

migrating at this molecular weight (Fig. 1, Lane 2). Further bands of gelatinolysis are apparent at estimated molecular weights of 92,000 and 82,000 (Fig. 1, Lanes 1 and 7). The molecular weight of this activity is consistent with the presence of latent and active M, 92,000 type IV collagenase.

Multiple bands of weak gelatinolytic activity are also apparent between molecular weights of 48,000 and 52,000 (Fig. 1, Lanes 1 and 7). Activity associated with these weak bands also resulted in significant casein degradation (Fig. 1, Lane 3). The most prominent of these bands, migrating at an estimated molecular weight of 48,000, appears as a strong protein band on Coomassie-stained gels (data not shown). Elution and sequencing of this band with the use of a 475A protein sequencer (Applied Biosystems, Foster City, CA) gave an NH₂-terminal amino acid sequence of FPATLETQEQD. This sequence is identical to that of human interstitial procollagenase (28). The presence of stromelysin in tumor-conditioned media was confirmed by Western blotting with a specific antibody (Fig. 1, Lane 6). Bands staining at molecular weights of 46,000 and 28,000 indicate the presence of both high and low molecular mass-activated species.

The gelatinolytic activity evident on zymography (Fig. 1, Lane 7) was significantly inhibited by incubation of gels in the presence of either TIMP-2 (Fig. 1, Lane 8) or EDTA (Fig. 1, Lane 9). This inhibition confirms membership of the matrix metalloproteinase family.

Elastolytic activity in M24met-conditioned media was assessed by using elastin-based zymography. Application of this technique resulted in a single band of elastinolytic activity at an estimated molecular weight of 72,000 (Fig. 1, Lane 4). Following APMA activation this activity showed a characteristic drop in molecular weight of 10,000 (data not shown).

Tumor-associated Synthesis of Urokinase. Gelatin-based zymography, performed in the presence of plasminogen, was used to confirm the presence of uPA both in cellular eluate and in serum-free tumor-conditioned media.

Zymography reveals a single broad band of gelatinolysis both in the cellular eluate (Fig. 2, Lane 4) and in serum-free tumor-conditioned media (Fig. 2, Lane 3). The molecular mass of the proteolytic activity observed is consistent with that of uPA (Fig. 2, Lane 2) but not of tissue type PA (Lane 1). The level of uPA activity found in cellular eluate indicates that roughly 50% of the PA activity secreted by M24met melanoma cells is bound to cell surface receptors. The PA activity observed was inhibited by incubating zymograms with PAl-2 or monoclonal antibodies specifically directed against human uPA (data not shown).

Characterization of MMP-dependent Proteolysis. Minimal degradation of N-[propionate-2,3-3H]labeled type I collagen was observed following incubation in the presence of untreated tumor-conditioned media (Table 1). Significant activity was, however, evident after organomercurial (APMA) activation. This pattern of proteolysis suggests the presence of a large pool of interstitial collagenase which is primarily in a latent form. This is in agreement with the detection of interstitial procollagenase following zymography. Assuming complete APMA activation of available procollagenase, the latent:active enzyme ratio is in the order of 30:1. In the presence of TIMP-2, APMA-dependent type I collagenase activity was inhibited by over 95%.

Addition of exogenous plasminogen to the serum-free (plasminogen-free) tumor-conditioned media failed to increase in type I collagenolysis (Table 1). This observation militates against a uPA/plasmin-dependent mechanism for procollagenase activation. It is unlikely that the results obtained are due to failure to generate plasmin, since under similar experimental conditions, tumor-associated uPA was found to generate significant plasmin-dependent fibrinolysis (Table 2).

Type IV collagenase activity present in M24met-conditioned media (Fig. 1) was characterized with N-[propionate-2,3-3H]labeled type IV collagen. Both latent and active type IV collagenase activity was
detected in tumor-conditioned media (Table 1). In the presence of TIMP-2, APMA-dependent type IV collagenase activity was inhibited by 75%. As observed with interstitial collagenase, addition of extrinsic plasminogen failed to activate or induce further type IV collagen digestion (Table 1).

Minimal MMP-dependent degradation of both fibronectin and laminin was observed. This activity may be attributed to the presence of stromelysin or the M₇ 72,000 type I collagenase (8).

Characterization of Urokinase-dependent Proteolysis. The contribution of secreted or cell-associated uPA-dependent activity to ECM degradation was characterized by the use of radiolabeled fibrin, fibronectin, and laminin (Table 2). Urokinase was not found to have any intrinsic proteolytic activity toward these substrates. Significant proteolysis of all these substrates was, however, evident following the addition of exogenous plasminogen. These data confirm a role for uPA-dependent plasmin generation. Comparable levels of proteolysis were mediated by conditioned media and by material eluted from the surface of M24met cells. Plasminogen activation was inhibited up to 95% by PAI-2 and 80% by a uPA-specific antibody.

Urokinase- and MMP-dependent Degradation of Interstitial Matrix. Degradation of interstitial matrix by M24met cells was examined with L-[3,4-³H]proline-labeled matrices laid down by rat smooth muscle cells (R22CID). In these matrices approximately 49% of the labeled proline was incorporated into glycoprotein, 27% into elastin, and 29% into collagen.

The kinetics of interstitial matrix degradation by M24met cells in the presence or absence of specific inhibitors is shown in Fig. 3. Untreated M24met cells caused significant accumulated degradation over the 14-day culture period. In the presence of TIMP-2 or PAI-2, the total accumulated degradation was inhibited by 30 and 50%, respectively.

During the first 5 days of culture there was a relatively rapid hydrolysis of the matrix by untreated M24met cells. During this early phase less than 10% of the observed hydrolysis was inhibited by TIMP-2. By contrast, up to 50% of this early activity was inhibited by PAI-2. These findings suggest an early and rapid uPA-dependent hydrolysis, with a relatively minor contribution from a metalloproteinase-dependent pathway. After day 5, untreated M24met cells were observed to degrade the matrix at a slower but constant rate. During this late phase, TIMP-2 inhibition increased. Thus, TIMP-2 was observed to inhibit approximately 80% of the degradation occurring between days 9 and 14. These data suggest the delayed expression of a metalloproteinase-dependent proteolytic pathway that predominates during the later stages of matrix hydrolysis.

Sequential enzymatic digestion (27) was exploited to determine the extent of matrix component degradation following incubation in the presence of M24met cells. After 14 days of culture approximately 55% of the glycoprotein, 18% of the elastin, and 33% of the collagen had been digested (Fig. 4A). Similar analysis performed 7 days after the initiation of culture (Fig. 4B) clearly demonstrates that the majority of glycoprotein is digested during the first 7 days (40% at day 7, versus 55% at day 14). In contrast, collagen degradation mainly takes place between days 7 and 14 (9% at day 7 versus 33% at day 14). This delayed collagenolysis coincides with a period of relatively slow hydrolysis and increasing inhibition by TIMP-2 (Fig. 3). Pre-treatment of the matrices with trypsin to deplete glycoprotein was, however, observed to significantly potentiate subsequent collagenolysis.
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Fig. 4. Tumor-mediated degradation of interstitial matrix components. M24met cells in RPMI and 10% acid-treated FBS were plated on complete or glycoprotein-depleted smooth muscle cell matrices. Culture media were replaced every 2 days. After 7 or 14 days of culture M24met cells were lysed by using 25 mM NH₄OH, and the extent of component degradation was determined by sequential digestion with trypsin, elastase, and bacterial collagenase.

ysis by M24met cells (Fig. 4C). Thus, 62% of the collagen was degraded by day 7. These data suggest that the interstitial glycoprotein(s) may function to protect the fibrillar collagen from proteolytic degradation. Trypsin activity associated with glycoprotein-depleted matrices was neutralized with excess soybean trypsin inhibitor and the matrices were extensively washed to prevent procollagenase activation by residual trypsin.

The susceptibility of interstitial glycoprotein, elastin, or collagen to either metalloproteinase- or uPA-dependent proteolytic pathways was determined by sequential enzymatic digestion of matrices preincubated with tumor cells and specific inhibitors. Tumor-mediated glycoprotein digestion was inhibited by approximately 45% in the presence of PAI-2 and by only 10% in the presence of TIMP-2 (Fig. 5A). These data are consistent with a relatively rapid uPA-dependent removal of the interstitial glycoprotein as previously shown. The relatively low levels of inhibition seen with TIMP-2 indicate limited metalloproteinase-dependent glycoprotein digestion. Limited MMP-dependent degradation of purified glycoproteins has previously been shown (Table 1).

In contrast to the glycoprotein digestion, elastinolytic activity was strongly inhibited by TIMP-2 but remained unaffected by PAI-2 (Fig. 5B). A role for M24met-associated M₇₂,₀₀₀ type IV collagenase in the elastinolysis observed was indicated following elastin-based zymography (Fig. 1, Lane 4).

Interstitial collagen digestion was inhibited up to 80% by TIMP-2 (Fig. 5C). Interestingly, PAI-2 was also observed to significantly inhibit collagenolysis. This inhibition was, however, completely abrogated on matrices previously depleted of glycoprotein. These data indicate that PAI-2 protects against MMP-dependent collagenolysis by virtue of its ability to inhibit the digestion of glycoprotein. This

Fig. 5. Contribution of tumor-associated uPA- and MMP-dependent proteolysis to the degradation of interstitial matrix components. M24met cells in RPMI and 10% acid-treated FBS were plated on complete or glycoprotein-depleted smooth muscle cell matrices. TIMP-2 or PAI-2 were added to this media at 20 or 45 μg/ml, respectively. Culture media and inhibitors were replaced every 2 days. After 14 days of culture M24met cells were lysed by using 25 mM NH₄OH, and the extent of component degradation was determined by sequential digestion with trypsin, elastase, and bacterial collagenase. Results are expressed as percentage of inhibition of component degradation observed in the absence of inhibitors.
glycoprotein in turn serves to protect associated collagen from MMP-dependent proteolysis. These data further suggest that collagenase activation is independent of the uPA/plasmin system.

At the concentrations used neither PAI-2 nor TIMP-2 were observed to significantly alter M24met adhesion or growth (data not shown).

**Urokinase- and MMP-dependent Degradation of Basement Membrane.** The ability of M24met cells to degrade isotopically labeled basement membrane was examined in the presence of TIMP-2 and PAI-2. M24met cells were observed to cause rapid and significant degradation of isotopically labeled matrices. After 6 days the total degradation of [³H]proline- and [³H]serine-labeled matrices was 84 and 85%, respectively (Fig. 6).

In the presence of TIMP-2 or PAI-2, degradation of [³H]proline-labeled matrices was inhibited by 79 and 89%, respectively (Fig. 6A). Such levels of inhibition indicate a significant role for both uPA- and MMP-dependent pathways in the dissolution observed. Since [³H]proline is preferentially incorporated into collagen at a higher molecular ratio than other matrix components, it is probable that the TIMP-2 inhibition is due to the abrogation of MMP-mediated collagenolysis. In accordance with this, we have previously demonstrated the presence of type IV collagenase activity in M24met-conditioned media (Fig. 1, Lane 1; Table 1). The dramatic inhibition mediated by PAI-2 was significantly reduced following the pretreatment of matrices to remove noncollagenous components (Fig. 6A, Inset). These data suggest that inhibition by PAI-2 may be due to the protection of proteoglycans and/or glycoproteins which shield basement membrane collagen from MMP-dependent proteolysis. A limited role for uPA/plasmin-mediated MMP zymogen activation cannot, however, be ruled out, since removal of noncollagenous components did not completely abrogate PAI-2 inhibition.

The degradation of [³H]serine-labeled matrices was examined since this radioisotope is more widely incorporated into component proteins of the subendothelial basement membrane. In the presence of TIMP-2 or PAI-2, degradation of [³H]serine-labeled matrices was inhibited by 35 and 60%, respectively (Fig. 6B). The relatively limited TIMP-2 inhibition observed is predictable given a greater distribution of isotope into noncollagenous components of the matrix. Incomplete inhibition in the presence of both TIMP-2 and PAI-2 may indicate the presence of other uncharacterized enzyme systems capable of digesting serine-labeled components.

**DISCUSSION**

M24met cells rapidly and consistently form spontaneous metastatic deposits in the lymph nodes and lungs of SCID mice (23). In keeping with many highly metastatic tumors M24met cells were found to coexpress both plasminogen activator and MMP activity. In this study we have described the combined contribution of these distinct enzyme systems to the invasive phenotype of this tumor.

M24met-associated MMP and uPA-dependent activity were initially, characterized by using purified ECM components. These components, namely, fibronectin, laminin, and types I and IV collagen represent significant structural elements of host ECM. Due to the coexpression of both uPA- or MMP-dependent activity M24met cells were able to significantly degrade all of these major components. A dichotomy was evident wherein uPA-dependent activity was restricted to the dissolution of interstitial or basement membrane glycoproteins (i.e., fibronectin and laminin), while MMP activity was primarily limited to interstitial or basement membrane collagen (types I and IV). The uPA-dependent activity observed was absolutely dependent upon the presence of plasminogen, implicating plasmin as the final proteolytic mediator. Some overlap in substrate specificity was observed due to limited MMP-dependent dissolution of purified fibronectin and laminin, and this may be due to the presence of tumor-associatedstromelysin and/or the M, 72,000 type IV collagenase. The addition of plasminogen to serum-free M24met-conditioned media failed to result in significant plasmin-mediated collagenolysis. The resistance of type I collagen to plasmin is well documented (8), while the capacity of plasmin to digest native type IV collagen under physiological conditions remains to be established (4, 5). Since expression of optimal MMP activity required organomercurial activation, the ultimate contribution of the MMPs to host ECM degradation will depend upon the availability of exogenous physiological activators of pro-MMPs.

It has been postulated that uPA may play a pivotal role in ECM dissolution by virtue of being at the apex of a potent proteolytic
cascade. This cascade incorporates the conversion of plasminogen to plasmin with plasmin in its turn expressing broad proteolytic activity that includes the activation of procollagenases (6). This said, it has been demonstrated that tumors coexpressing both PAs and procollagenases are able to independently activate their own collagenase activity through the generation of plasmin (29). We present evidence that in our tumor system uPA is not involved, as large pools of latent MMP activity in serum-free tumor-conditioned media could not be activated by the addition of exogenous plasminogen. This finding is unlikely to be due to a failure to generate plasmin, since the addition of plasminogen to M24met-conditioned media did result in significant plasmin-mediated fibrinolysis and fibronectin digestion under similar experimental conditions. Furthermore, PAI-2 was not observed to inhibit MMP-dependent degradation of elastin or MMP-mediated dissolution of interstitial collagen in the absence of glycoprotein. In support of our findings, O’Grady et al. (30), describe the production of both active interstitial collagenase and plasminogen activator by a rat mammary carcinoma cell line. In this tumor, activation of interstitial procollagenase was not affected by the PA inhibitor tranexamic acid. PA-independent mechanisms for procollagenase activation may include the cathepsins, trypsin-like serine proteases, or some other as yet unidentified physiological activator(s) (8).

The contribution of tumor-associated uPA or MMP-dependent proteolytic pathways to the degradation of multistructure ECM was examined by using smooth muscle cell matrices which are considered to be representative of complex host interstitial tissue matrix (27). These matrices contain a mixture of glycoprotein, elastin, and interstitial collagen (types I and III) in a native form. The glycoprotein component has been described as having properties consistent with the presence of microfibrillar protein and fibronectin (27). We found that because of the coexpression of both uPA- or MMP-dependent activity, M24met cells are able to significantly degrade all the structural components of these matrices. Consistent with data derived from studies making use of purified components, MMP-dependent activity was primarily associated with the dissolution of interstitial collagen, while uPA-dependent proteolysis was restricted to the dissolution of the glycoprotein. The limited MMP-dependent dissolution of glycoprotein found may be due to the presence of stromelysin and/or M, 72,000 type IV collagenase. Significantly, we also observed some MMP-dependent elastinolysis. In this regard the ability of the type IV collagenases to degrade purified elastin has recently been documented (9). A role for M24met-associated M, 72,000 type IV collagenase was confirmed in this study by using elastin-based zymography.

Most interestingly, we observed a temporal relationship between uPA-dependent glycoprotein dissolution and MMP-dependent collagenolysis. Thus, a relatively rapid uPA-dependent removal of interstitial glycoprotein was observed to precede a relatively slow dissolution of the interstitial collagen. We present evidence which suggests that this temporal relationship is imposed by the structural architecture of the matrix such that the interstitial glycoprotein serves to protect associated collagen from MMP degradation. Accordingly uPA-dependent glycoprotein digestion functions as a prerequisite rate-limiting step required for subsequent MMP-mediated collagenolysis. We present two major pieces of evidence to support this conclusion. First, prestripping matrices of their glycoprotein component was observed to dramatically potentiate MMP-dependent collagenolysis. Second, the ability of PAI-2 to inhibit collagenolysis was entirely abrogated on glycoprotein-depleted matrices. Jones and De Clerck (31) first proposed the possibility of such a mechanism while studying the proteolysis of interstitial matrices by a human fibrosarcoma (HT 1080). This study, however, lacked the benefit of specific protease inhibitors, so the precise mechanism and enzymatic pathways involved were not elucidated. In this study we have clearly delineated a mechanism based on the operation of two defined proteolytic systems. Since these same proteolytic systems are commonly coexpressed in a variety of tumor types (1), the temporal mechanism described is likely to be of general relevance to metastatic spread. An alternative mechanism, independent of matrix architecture, may be proposed if the urokinase-dependent proteolysis observed or the experimental trypsinization used results in the generation of bioactive matrix-associated fragments. These fragments may then have an inductive effect on the tumor cells, resulting in enhanced MMP secretion and concomitant type I collagen degradation. It has recently been shown, for example, that fibronectin-derived fragments are able to induce MMP activity in fibroblasts (32). However, this mechanism presupposes that insufficient amounts of MMP are available for proteolysis during the early phase of matrix degradation. We did, however, detect high levels of interstitial collagenase when cells were cultured on interstitial matrix for as little as 48 h. The presence of this collagenase was evident following casein-based zymography (data not shown). Furthermore, significant collagenolysis was observed as early as day 2 when M24met cells were seeded on radioactive interstitial matrices depleted of glycoprotein (data not shown). Despite these observations, it is conceivable that the generation of bioactive fragments may be required for the induction of undefined proteases required for pro-MMP activation.

M24met cells were further observed to efficiently hydrolize isotopically labeled basement membrane matrices. This hydrolysis required coexpression of both uPA- and MMP-dependent pathways. Interestingly, PAI-2 was observed to almost completely abrogate the degradation of proline-labeled matrices despite the susceptibility of these matrices to MMP-dependent proteolysis. Modulation of MMP activity by PAI-2 may be attributed to a requirement for plasmin-mediated activation of pro-MMPs. However, type IV collagenase activity was detected in M24met-conditioned media generated in the absence of plasminogen, and this activity was not increased by the addition of exogenous plasminogen. Indeed, the ability of plasmin to effectively activate type IV collagenase activity has been contested (5, 8). On the basis of the data presented it cannot, however, be ruled out that plasmin may play a limited role in maximizing MMP-zymogen activation during the degradation of basement membranes. In a process reminiscent of that observed with the interstitial matrices, we propose that PAI-2 may function to protect glycoprotein or proteoglycans that are able to shield associated basement membrane collagen from MMP-dependent proteolysis. In support of this concept, pretreatment of matrices to remove noncollagenous components was observed to reduce the ability of PAI-2 to inhibit matrix degradation. Following initial observations that plasmin is able to remove noncollagenous components of basement membranes (4), a number of investigators have suggested a role for plasmin in exposing basement membrane collagens to specific collagenases (4, 5). Given the significant abrogation of hydrolysis by TIMP-2, it is unlikely that plasmin per se caused appreciable degradation of basement membrane collagen. The ability of PAI-1 and PAI-2 to abrogate the degradation of proline-labeled basement membranes has recently been reported for fibrosarcoma and colon carcinoma cell lines (18, 19).

The MMP- and uPA-dependent degradation of both interstitial and basement membrane matrices described above occurred despite limited constitutive expression of both TIMP-1 and TIMP-2 and PAI-1 and PAI-2 by M24met cells (data not shown). While the presence of these inhibitors may be expected to reduce net proteolytic activity it is clear that the enzyme to inhibitor ratio is still such as to favor significant proteolysis.

Degradation of noncollagenous components of both interstitial matrix and basement membranes was significantly but incompletely inhibited by PAI-2. Plasminogen-independent basal degradation of...
ECM has been described in a number of tumor types, including melanomas, and has been attributed to the presence of other proteolytic systems, including cathepsins (1) and tumor-associated trypsinogens (33). The elaboration of other enzyme systems by M24met cells cannot be ruled out. Alternatively, incomplete inhibition by PAI-2 may be attributed to inhibitor inactivation or limited enzyme inhibitor accessibility.

In conclusion, we have shown the contribution of uPA-dependent and MMP-dependent proteolytic pathways to the invasive phenotype of a highly metastatic human melanoma. Due to the coexpression of these enzyme systems this melanoma was observed to degrade multiple elements of the ECM, including fibronectin, laminin, elastin, and both interstitial and basement membrane collagens. Urokinase-dependent activity was required for the efficient dissolution of glycoproteins, but not for the activation of procollagenase activity. MMP-dependent activity was implicated in collagenolysis, elastinolysis, and in limited glycoprotein degradation. We present evidence that uPA-dependent glycoprotein digestion is a prerequisite rate-limiting step required for subsequent MMP-dependent collagenolysis. Data presented suggest that both TIMP-2 and PAI-2 may serve to limit tumor invasion and concomitant metastasis. The question remains, however, whether inhibition of one or both of these pathways is required to abrogate metastasis.

REFERENCES


Melanoma-mediated Dissolution of Extracellular Matrix: Contribution of Urokinase-dependent and Metalloproteinase-dependent Proteolytic Pathways


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