

# Characterization of Gelatinases Linked to Extracellular Matrix Invasion in Ovarian Adenocarcinoma: Purification of Matrix Metalloproteinase 2

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Substantial evidence indicates that proteolytic degradation of the extracellular matrix is necessary for invasion and metastasis by cancer cells. Our previous work has demonstrated elevated secretion by cultured ovarian adenocarcinoma cells of two gelatinolytic metalloproteinases, a 72-kDa enzyme resembling matrix metalloproteinase 2 (MMP-2) and a 92-kDa enzyme resembling MMP-9 (Moser *et al.*, *Int. J. Cancer* 56, 552-559, 1994). To assess the potential *in vivo* relevance of these enzymes, we have examined ovarian carcinoma ascites using gelatin substrate zymography. MMP species identical to those secreted from several well-characterized ovarian adenocarcinoma cell lines were found in the majority of ascites: MMP-2-like gelatinase (23 of 23 cases) and MMP-9-like gelatinase (18 of 23 cases), suggesting a prevalence of these species in the ovarian carcinoma microenvironment and their availability for tumor-associated proteolysis. The contribution of these proteinases to ovarian cancer invasion was further demonstrated by experiments measuring tumor cell-mediated proteolysis of native endothelial cell extracellular matrix (ECM) and tumor cell invasion of reconstituted basement membrane (Matrigel). These data showed that secretion of type IV collagenase activity by a series of independently isolated ovarian adenocarcinoma cell lines correlated well with the ability of these cells to proteolyze the ECM and invade the basement membrane. Furthermore, we have identified and characterized an ovarian carcinoma-associated gelatinase, the 72-kDa MMP found in conditioned media of the DOV 13 cell line, as MMP-2. This enzyme was identical to the previously described MMP-2 from other sources by Western blot, amino terminal sequence, and substrate specificity. Additionally, a large portion of the MMP-2 activity found in DOV 13 conditioned media is active without organomercurial treatment, suggesting that ovarian cancer cells have an endogenous activator of the zymogen. Together, these data suggest that ECM proteolysis mediated by tumor-associated proteinases plays an important role in the invasion and/or metastasis of ovarian carcinoma. © 1996

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## INTRODUCTION

Proteolytic degradation of the extracellular matrix (ECM) is hypothesized to be necessary for tissue invasion and metastasis by cancer cells [1]. The basement membrane, a specialized ECM containing large amounts of type IV collagen and laminin, serves a barrier function separating epithelial cells from the underlying stroma [2, 3]. For hematogenous metastasis to occur, a tumor cell must repeatedly cross this basement membrane barrier, a process for which proteolysis of ECM components is required. However, hematogenous metastasis occurs infrequently in the early stages of epithelial ovarian carcinomas which more commonly metastasize by surface shedding, followed by peritoneal dissemination, implantation, growth, and visceral invasion [4]. It is reasonable to hypothesize that proteolysis also plays a key role in intra-peritoneal metastasis, such as in the disruption of the mesothelial cell layer, during extension of the implanted tumor through the submesothelial basement membrane into the visceral organ stroma, and, importantly, in gaining access to the host vascular supply, a necessary step in progression of the implant [5].

The matrix metalloproteinase (MMP) family of proteolytic enzymes catalyzes the hydrolysis of numerous ECM molecules, most importantly the collagens, but also other ECM components [3, 6]. MMP-1, or interstitial collagenase, is specific for cleavage of native fibrillar collagens types I, II, and III found in interstitial ECM, while MMP-2 (gelatinase A, 72-kDa type IV collagenase) and MMP-9 (gelatinase B, 92-kDa type IV collagenase) rapidly degrade gelatins and basement membrane collagen types IV and V. MMP-3 (stromelysin 1) predominantly degrades noncollagenous ECM glycoproteins. Overexpression of MMPs, particularly the type IV collagenases, has been demonstrated in several tumor systems and has been linked to invasive potential of tumor cells [3, 7, 8]. Indeed, MMP-2 was first identi-

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fied based upon overexpression of type IV collagenase activity in tumor cells [9], and subsequently, its level of secretion was found to correlate with the metastatic potential of murine melanoma cell lines and other cell types [10]. Further studies have shown that type IV collagenase (either MMP-2 or MMP-9) secretion correlates with invasive or metastatic potential in tumor-tumor or tumor-normal cell fusion hybrids of varying metastatic potential [11], in nude mice using *H-ras* or *H-ras/E1A* oncogene-transfected rat embryo fibroblasts [12], and in other systems both *in vitro* and *in vivo* [13–17]. Type IV collagenase activity also correlates with malignant transformation, metastasis, and poor survival in many different cancers studied using clinical material [18–24]. Furthermore, the specific endogenous inhibitors of the MMPs, the tissue inhibitors of metalloproteinases (TIMPs), appear to function to suppress metastasis or invasion in animal models or reconstituted systems [25–30]. Together, these studies demonstrate that elevated proteinase secretion unchecked by an equivalent rise in inhibitor levels can enable a tumor cell to invade neighboring tissues and metastasize.

Substantial evidence indicates that proteolysis, catalyzed by both MMPs and serine proteinases such as urinary-type plasminogen activator (u-PA) and plasmin, also plays an important role in metastasis of ovarian cancer. Niedbala *et al.* [31] observed that ovarian cancer cells are capable of disrupting mesothelial cell layers and of fully degrading biosynthetically labeled extracellular matrix, suggesting the possibility that ovarian cancer cells possess both plasmin-like and MMP-like proteolytic activity. Furthermore, serine proteinase inhibitors, anticatalytic antibodies against u-PA, and TIMP inhibit the *in vitro* invasion of ovarian cancer cells on reconstituted basement membranes [32, 33]. Interestingly, a synthetic MMP inhibitor has been reported to retard progression of ovarian carcinoma xenografts in nude mice [34]. These studies did not elucidate the type of MMP species which were involved in the invasive mechanism. Previous studies from our laboratory comparing the normal ovarian epithelium with ovarian adenocarcinoma demonstrated that ovarian carcinoma cells *in vivo* and *in vitro* secrete elevated quantities of u-PA, coexpress the cell surface receptor for u-PA, and overexpress two gelatinolytic MMPs which resembled MMP-2 and MMP-9 [35, 36]. A number of additional studies provide further support for these findings of elevated plasminogen activator and metalloproteinase secretion in ovarian carcinoma [37–39].

In the present study we have sought to definitively characterize the gelatinolytic MMP secreted by the DOV 13 ovarian adenocarcinoma cell line. The enzyme was purified, identified as MMP-2 (gelatinase A, 72 kDa type IV collagenase) using Western blotting and N-terminal protein sequencing, and characterized regarding substrate specificity and zymogen activation properties. Furthermore, we have analyzed clinical samples of ovarian carcinoma ascites seeking *in vivo* evidence for MMP secretion in the ovarian carcinoma micro-

environment. Finally, studies were undertaken to examine the potential role of gelatinolytic MMPs secreted from five ovarian carcinoma cell lines in endothelial cell basement membrane proteolysis and in invasion of a reconstituted basement membrane.

## MATERIALS AND METHODS

**Material.** Polyclonal antibodies prepared against purified proMMP-2 (rabbit) and purified proMMP-9 (sheep) were the generous gift of Dr. Hideaki Nagase, University of Kansas Medical Center (Kansas City, KS) [40]. Collagen type I, collagen type V, alkaline phosphatase-conjugated anti-rabbit and anti-sheep IgG, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) alkaline phosphatase substrate tablets, cell culture reagents, and chromatography matrices were purchased from Sigma (St. Louis, MO). Collagen type IV and Matrigel were purchased from Collaborative Research (Bedford, MA). The synthetic collagenase substrate Dnp-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH<sub>2</sub> (Dnp-PLGLWAR-NH<sub>2</sub>) was purchased from Peptides International (Louisville, KY). Gelatin types I, IV, and V were prepared by denaturation of the corresponding collagens by heating at 60°C for 20 min. All other chemicals were reagent grade quality. A metalloproteinase reverse zymography kit was purchased from University Technologies International (Calgary, Alberta, Canada).

**Clinical specimens.** Twenty-three peritoneal ascites fluids from ovarian carcinoma patients were examined in the present study. The ascites were obtained at surgery from patients undergoing exploratory laparotomy for treatment of stage III or IV epithelial ovarian cancer at Duke University Medical Center. In all cases, informed consent of the patient was given prior to collection of fluids. The ascites were made cell-free by centrifugation and filtration and stored frozen at –70°C. The protein content of the fluids was measured using a BCA assay (Pierce, Rockford, IL).

**Cell culture.** The human ovarian adenocarcinoma cell lines DOV 13, OVCA 420, OVCA 429, OVCA 432, and OVCA 433 were maintained at 37°C under 5% CO<sub>2</sub> in supplemented minimal essential medium (MEM) as previously described [41]. Serum-free conditioned medium was collected for purification of MMP-2 as follows. Cells were propagated in 162-cm<sup>2</sup> flasks (Costar, Cambridge, MA) with serum-containing medium. At approximately 30 and 90% confluence, the cells were washed twice with phosphate-buffered saline and placed in serum-free medium for 24 hr. The serum-free conditioned medium was harvested and subsequently stored frozen at –20°C.

**Native basement membrane degradation assay.** Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics (San Diego, CA) and were cultured as previously described [42]. Metabolically labeled endothelial cell

basement membranes were produced by culture of HUVEC in the presence of [ $^3\text{H}$ ]proline as previously described [43]. DOV 13, OVCA 420, OVCA 429, OVCA 432, and OVCA 433 cells were harvested with EDTA, washed with PBS, resuspended in serum-free medium, enumerated, and plated at  $5 \times 10^5$  cells/well on wells containing [ $^3\text{H}$ ]proline-labeled HUVEC matrix. Control wells contained medium only or cells plus minocycline ( $50 \mu\text{g}/\text{ml}$ ), which inhibits MMP activity [44]. After a 24-hr incubation, the conditioned media were collected ( $500 \mu\text{l}$ ). The wells were rinsed twice with PBS and further incubated with  $500 \mu\text{l}$  CR-Dispase at  $37^\circ\text{C}$  for 48 hr to remove nonsolubilized matrix. The conditioned media and CR-Dispase digests were clarified by centrifugation ( $10,000g \times 5 \text{ min}$ ) and radioactivity was determined by scintillation counting as described above. Cellular ECM degradation was calculated as the percentage of counts in the conditioned medium relative to the total counts in the well (conditioned medium + Dispase digest). Assays were performed in triplicate.

*Matrigel invasion assay.* Transwell invasion chambers containing polycarbonate filters ( $8\text{-}\mu\text{m}$ , Costar) were coated on the upper surface with Matrigel. Conditioned medium from NIH 3t3 cells was added to the lower chamber as a chemoattractant. Ovarian cancer cells ( $3 \times 10^5$ ) were placed in the upper chamber in a  $200\text{-}\mu\text{l}$  total volume and incubated at  $37^\circ\text{C}$  for 18 hr. The filter was then removed, fixed, and stained with hematoxylin and eosin. Noninvading cells adherent to the upper surface of the filter were removed with a cotton swab. Cell invasion was assessed by counting a  $5 \times 5\text{-mm}$  square using an inverted phase microscope with a  $20\times$  objective. Assays were performed in triplicate.

*Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) zymography.* Samples of ovarian cancer ascites fluids were analyzed by SDS-PAGE zymography according to the method of Heussen and Dowdle [45] to determine the molecular weights and relative abundance of the gelatinases present. Samples containing  $70 \mu\text{g}$  of protein were activated by incubation with  $1.0 \text{ mM}$  aminophenylmercuric acetate (APMA) for 1 hr at  $37^\circ\text{C}$  and were electrophoresed according to the method of Laemmli [46] without reduction on 9% SDS-polyacrylamide gels copolymerized with 0.1% gelatin. Gels included a conditioned media standard from DOV 13 and OVCA 432 cells as positive controls and for identification of the gelatinases in ascites relative to the known 72- and 92-kDa secretory products of these cell lines, respectively [36]. Gelatinolytic bands were assessed for semiquantitative analysis using an arbitrary graded scale. Scale categories were defined as follows: 0, no band detected; +/-, faint band detected,  $<1.0 \text{ mm}$  width; 1+, clear band detected,  $1.0\text{--}1.5 \text{ mm}$  width; 2+, intense band detected,  $1.5\text{--}3.0 \text{ mm}$  width; 3+, very intense band detected,  $>3.0 \text{ mm}$  width.

Metalloproteinase reverse zymography was used to ana-

lyze purified DOV 13 MMP-2 and was performed using a kit according to the manufacturer's specifications [47].

*Purification of MMP-2.* The  $M_r$  72,000 gelatinase from DOV 13 cells [36] was purified using a modification of previously published procedures [48, 49]. Serum-free conditioned medium (2.5 liters) from DOV 13 cells was concentrated 50-fold by ultrafiltration using a YM-30 membrane (Amicon, Beverly, MA) and applied to a gelatin agarose column ( $12 \times 2 \text{ cm}$ ) equilibrated with  $50 \text{ mM}$  Tris-HCl,  $5 \text{ mM}$   $\text{CaCl}_2$ ,  $0.5 \text{ M}$  NaCl,  $0.02\%$  Brij 35, pH 7.6 (TCS buffer). After elution with TCS buffer containing 10% dimethyl sulfoxide, fractions containing gelatinase activity as determined by zymography were dialysed against  $50 \text{ mM}$  Hepes,  $10 \text{ mM}$   $\text{CaCl}_2$ ,  $0.05\%$  Brij 35, pH 7.2 (HBC buffer). The dialysate was applied to a DEAE-Sephacel column ( $10 \times 2 \text{ cm}$ ) equilibrated with HBC buffer and eluted with HBC buffer containing  $1.0 \text{ M}$  NaCl. The eluate was concentrated 50-fold by ultrafiltration (Centriprep-30, Amicon) and desalted into  $50 \text{ mM}$  Tris-HCl,  $0.02\%$  Chaps, pH 7.5, using a NAP-5 column (Pharmacia, Uppsala, Sweden). Aliquots of material at each purification step were reserved for analysis by SDS-PAGE and collagenase assay.

*Collagenase activity assays.* The collagenase activity of conditioned media and purified enzyme samples was assayed quantitatively using the synthetic collagenase substrate Dnp-PLGLWAR-NH $_2$  as previously described [50]. Dnp-PLGLWAR-NH $_2$  ( $20 \mu\text{M}$ ) was incubated with  $50\times$  concentrated serum-free conditioned medium ( $4.5 \mu\text{g}$ ) or purified DOV 13 MMP-2 ( $0.48 \mu\text{g}$ ) in  $0.05 \text{ M}$  Tris-HCl,  $5 \text{ mM}$   $\text{CaCl}_2$ ,  $0.2 \text{ M}$  NaCl, pH 7.7, at  $37^\circ\text{C}$  for 16 hr. In some cases, the samples also contained  $0.6 \text{ mM}$  APMA alone or with  $2 \text{ mM}$  *o*-phenanthroline. The reactions were stopped by addition of  $2 \text{ mM}$  *o*-phenanthroline and the cleavage products were analyzed by reverse-phase HPLC using a C18 column ( $4.6 \text{ mm} \times 25 \text{ cm}$ ) with a guard column ( $4.6 \text{ mm} \times 1.5 \text{ cm}$ ) on a Rainin Dynamax HPLC and quantitated by monitoring the column effluent at  $370 \text{ nm}$ .

*Western blotting.* The immunoreactivity of the purified DOV 13 MMP with antibodies raised against either MMP-2 or MMP-9 was determined by Western blotting. Purified DOV 13 MMP ( $1 \mu\text{g}$ ) was electrophoresed on a 9% polyacrylamide gel and electroblotted to an Immobilon membrane. The blots were probed with either rabbit anti-MMP-2 polyclonal antibody (1:500) or sheep anti-MMP-9 polyclonal antibody (1:1000). Subsequently, the blots were developed by probing with the corresponding species-specific alkaline phosphatase-conjugated secondary antibodies (1:2000), followed by colorimetric development employing the BCIP/NBT alkaline phosphatase substrate.

*NH $_2$ -terminal sequence analysis.* The amino terminal sequence of the purified, APMA-activated DOV 13 metalloproteinase was determined by Edman degradation as previously described [51]. Purified zymogen of the DOV 13

MMP (30  $\mu$ g) was activated with 0.5 mM APMA in buffer containing 20 mM Tris-HCl, pH 7.4, 0.02% Chaps at 37°C for 60 min. The reaction was stopped by addition of 2 mM *o*-phenanthroline and dilution in reducing SDS-PAGE sample buffer. The reactants were subsequently separated by SDS-PAGE (8% acrylamide), electroblotted to an Immobilon membrane, and stained with Coomassie blue. The bands corresponding to the fully activated enzyme were excised, placed on Porton sample support disks, and subjected to automated Edman degradation using an Applied Biosystems 477A liquid-phase sequencer with "on-line" 120A phenylthiohydantoin analysis. The amino acid sequence was determined for 15 cycles.

**Substrate specificity studies.** The substrate cleavage specificity of the purified DOV 13 MMP was determined using SDS-PAGE separation of degradation products. Purified DOV 13 MMP zymogen was activated with 1.5 mM APMA for 2 hr at 37°C. Activated MMP at a concentration of 12.5 ng/ml (gelatins) or 25 ng/ml (collagens) was incubated with 16  $\mu$ g collagen types I, IV, or V or the corresponding gelatins for various time periods at 33°C (collagens) or 37°C (gelatins) in 0.05 M Tris-HCl, 0.2 M NaCl, 0.01 M CaCl<sub>2</sub>, 0.25 M glucose, pH 7.7. Additional experiments included the matrix glycoproteins fibronectin (10  $\mu$ g) or laminin (10  $\mu$ g) as the substrate. Experiments incubated in parallel contained the metalloproteinase inhibitor *o*-phenanthroline (4 mM). The reactions were stopped by addition of 2.5 mM *o*-phenanthroline and boiling in reducing SDS sample buffer. Following SDS-PAGE on 7.5% polyacrylamide gels, substrate and cleavage products were visualized by staining with Coomassie blue.

**APMA activation of pro-MMP-2.** To further investigate the mode of activation of DOV 13 MMP-2 with APMA, aliquots of the purified zymogen (1  $\mu$ g) were treated with 0.5 mM APMA for various time periods at 37°C in 20 mM Tris-HCl, pH 7.5. As a control experiment APMA was omitted from one aliquot and the sample was incubated in parallel with the last time point. The reactions were stopped by addition of 2.5 mM *o*-phenanthroline and boiling in reducing SDS sample buffer. Following SDS-PAGE on an 8.0% polyacrylamide gel, the zymogen and activation products were visualized by silver staining (Gelcode, Pierce).

## RESULTS

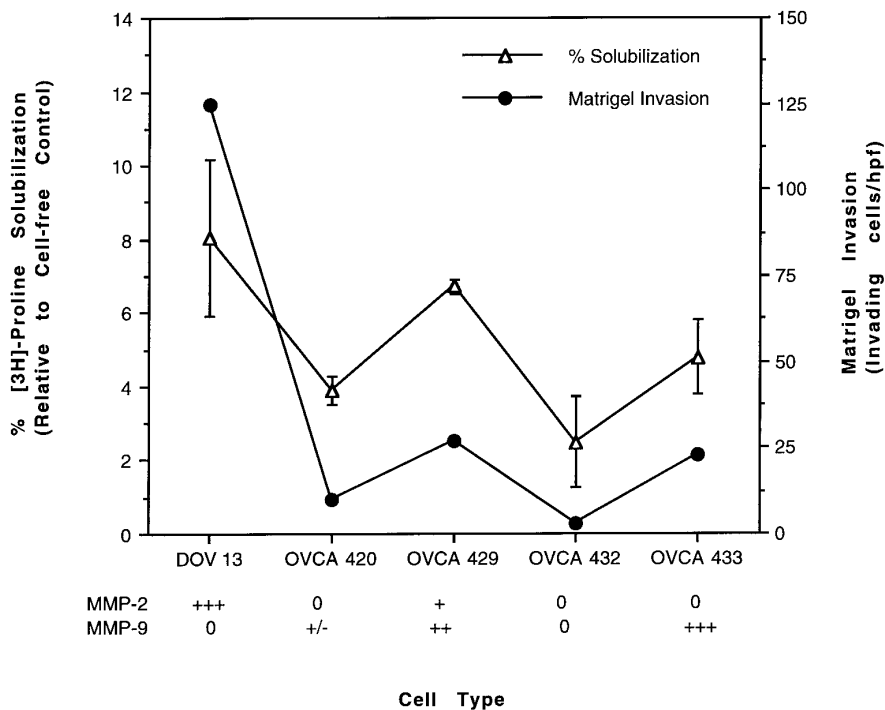
### *Characterization of Tumor Cell Proteolysis and ECM Invasion*

Tumor cell-mediated proteolysis of ECM components is a postulated mechanism for invasion and metastasis [1]. Previous studies have shown that MMP activity is necessary for tumor invasion or metastasis [17, 25, 28, 52, 53]. Furthermore, work from our laboratory employed zymography to demonstrate that several ovarian carcinoma cell lines secrete

elevated levels of gelatinolytic MMPs relative to normal ovarian epithelial cells [36]. As tabulated semiquantitatively in Fig. 1, DOV 13 and OVCA 429 cells secrete significant levels of a 72-kDa *o*-phenanthroline-inhibitible gelatinase resembling MMP-2, and OVCA 429 and OVCA 433 cells secrete high levels of a 92-kDa *o*-phenanthroline-inhibitible gelatinase resembling MMP-9. OVCA 432 cells secrete much lower levels of the 92-kDa gelatinase apparent only after concentration of the conditioned medium, while OVCA 420 cells do not produce detectable gelatinase. These data demonstrate heterogeneous secretion by ovarian adenocarcinoma cells of gelatin- and type IV collagen-degrading MMPs which have been implicated in tumor invasion.

However, since the activity of secreted proteinases may be regulated extracellularly by zymogen activation, cofactor modulation, or inhibition, it is not possible to predict the biological effects of this observed overexpression of gelatinases. For this reason, the invasive potential of the ovarian carcinoma cells on extracellular matrix was determined using two types of cellular assays and compared with the gelatinase expression of the cells. To demonstrate directly the activity of cellular type IV collagenases on native basement membrane, an assay was employed measuring the solubilization of HUVEC basement membranes metabolically labeled with [<sup>3</sup>H]proline [31]. Proline release from ECM is strongly linked to collagenolysis due to the very high proline content of collagens relative to other proteins and the marked predominance of collagen in ECM. When plated on a substrate of [<sup>3</sup>H]proline-labeled HUVEC ECM, DOV 13, OVCA 429, and OVCA 433 cells displayed significantly higher solubilization of [<sup>3</sup>H]proline relative to OVCA 420 and OVCA 432 cells (Fig. 1, open triangles). This degradation was inhibited fully by minocycline (data not shown), previously shown to act as a metalloproteinase inhibitor [44], suggesting that the [<sup>3</sup>H]proline solubilization was MMP-mediated. These data demonstrate that [<sup>3</sup>H]proline solubilization and hence proteolysis of type IV collagen in a native basement membrane correlates with the secretion of 72-kDa gelatinase and/or 92-kDa gelatinase by these cell types. Furthermore, these data also indicate that the secreted proMMP zymogens become activated and that the elevated secretion of MMPs is not offset by an equivalent increase in secretion of inhibitors.

To assess whether this elevated type IV collagenase activity plays a role in tumor cell invasion of the ECM, two-chamber invasion assays using reconstituted Englebreth-Holm-Swarm cell basement membranes (Matrigel) [54, 55] were employed. Heterogeneity of invasive potential was found in the 5 cells lines, with DOV 13 cells 5-fold more invasive than OVCA 429 and OVCA 433 cells, which were in turn 2.5- to 8-fold more invasive than the poorly invasive cell lines, OVCA 420 and OVCA 432 (Fig. 1, filled circles). This pattern of invasivity correlates well with the type IV collagenolytic activity measured by [<sup>3</sup>H]proline solubilization and the measured secretion of gelatinolytic MMPs, par-



**FIG. 1.** Comparison of Matrigel invasion and [ $^3\text{H}$ ]proline release from HUVEC ECM by ovarian carcinoma cells. ECM invasion was determined in five ovarian adenocarcinoma cell lines by two methods. Matrigel invasion assays (filled circles) were performed in transwell chambers containing a Matrigel-coated ( $12.5 \mu\text{g}/\text{filter}$ ) polycarbonate filter ( $8 \mu\text{m}$  pore size). Cells ( $2 \times 10^5$ ) were incubated 18 hr in the upper chamber under serum-free conditions, and cell penetration of the Matrigel layer was assessed by staining and counting. Solubilization of metabolically labeled HUVEC ECM was quantitated by cell-mediated [ $^3\text{H}$ ]proline release (open triangles). ECM produced by HUVEC maintained in the presence of [ $^3\text{H}$ ]proline were harvested by removal of cells with NaOH and air-dried. Cells ( $5 \times 10^5/\text{well}$ ) were plated on the HUVEC ECM in serum-free medium (0.5 ml) and incubated 24 hr in the presence or absence of minocycline ( $50 \mu\text{g}/\text{ml}$ ). Control wells contained media only. Solubilized radioactivity was determined in the conditioned media and nonsolubilized radioactivity was measured following digestion with CR-Dispase. Specific ECM degradation was determined relative to cell-free controls and expressed as percentage solubilization of total counts. Note that lines connecting the points of the histogram are included only as a visual aid and do not imply that the independent cell lines represent a continuous variable.

ticularly the 72-kDa gelatinase, which is secreted preferentially by the highly invasive DOV 13 cells. Also, no correlation was seen with the secretion of u-PA, which is the principal plasminogen activator produced by these cell types, or with the cell surface association of u-PA [36]. Taken together, these data suggest that type IV collagenase secretion and subsequent type IV collagenase activity is rate limiting for ECM invasion in these cell types.

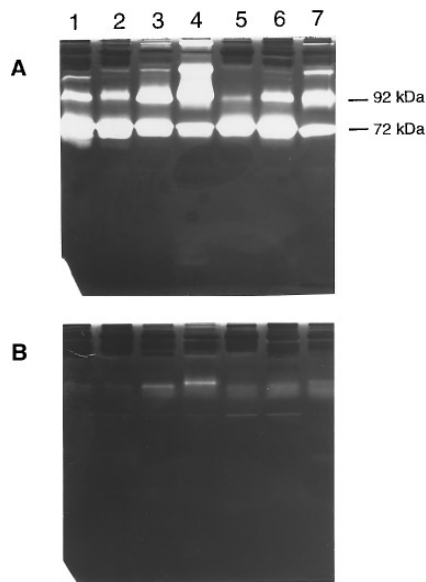
#### Characterization of Gelatinase Activity in Ascitic Fluids

Since tissue culture experiments suggested a role of MMP gelatinases in ovarian carcinoma tumor invasion, we sought to characterize the nature of MMPs present in the ovarian cancer microenvironment *in vivo*. For this purpose, ascites fluids collected from ovarian carcinoma patients were examined using SDS-PAGE gelatin-substrate zymography (Fig. 2). Two gelatinolytic proteinases were observed in the ascites (Fig. 2A), including a 72-kDa band resembling MMP-2 and a 92-kDa band resembling MMP-9. The gelatinases were identified as metalloproteinases based upon inhibition by

*o*-phenanthroline (Fig. 2B) and had affinity for gelatin (data not shown) consistent with their presumed identification as MMP-2 and MMP-9. The 72-kDa gelatinase was detected in 100% of ascites ( $n = 23$ ), in high amounts (2–3+) in 96%; and the 92-kDa MMP was found in 78%, with a bimodal distribution, absent or scarce (0 or +/-) in 52% and abundant (2–3+) in 43% (Table 1). These gelatinases also comigrate with the corresponding species observed in tissue culture of ovarian carcinoma cells [36], suggesting the possibility that these species are identical and that the ascites gelatinases may (although do not necessarily) originate from peritoneal tumor implants. Furthermore, these data demonstrate that gelatinolytic MMPs are readily available in the ovarian carcinoma microenvironment.

#### Purification and Characterization of Metalloproteinase Activity

Because secretion of a 72-kDa gelatinase resembling MMP-2 correlated with a highly invasive phenotype in the DOV 13 cell line, we sought to purify, identify, and further



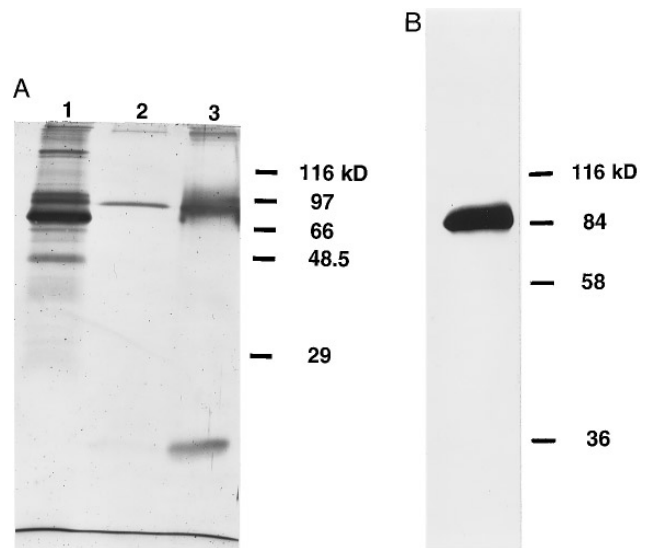
**FIG. 2.** Metalloproteinases from ovarian carcinoma ascites demonstrated by gelatin zymography. Samples were electrophoresed under nonreducing conditions on an SDS-PAGE gel containing 9% acrylamide and 0.1% gelatin. Samples were activated (1.3 mM APMA) for 60 min (37°C) prior to electrophoresis. Following removal of SDS with 2.5% Triton X-100, the gels were incubated overnight in 20 mM glycine, 10 mM CaCl<sub>2</sub>, 1 μM ZnCl<sub>2</sub>, pH 8.3, at 37°C. (A) Lanes 1–7, randomly selected ovarian carcinoma ascites samples (70 μg protein/lane). (B) Lanes 1–7, the identical samples, except with addition of 2.5 mM *o*-phenanthroline prior to electrophoresis and during incubation. The positions of MMP-2 (72 kDa gelatinase) and MMP-9 (92 kDa gelatinase) standards are indicated. Two major *o*-phenanthroline-inhibitable gelatinases are present in ovarian carcinoma ascites which comigrate with MMP-2 and MMP-9.

characterize this enzyme. The 72-kDa gelatinase was purified from serum-free DOV 13 cell conditioned medium by a combination of gelatin affinity chromatography and DEAE-Sephacel anion exchange chromatography (Fig. 3A). The enzyme purified predominantly as an 82-kDa zymogen. A

**TABLE 1**  
**Summary of Matrix Metalloproteinase Zymographic Analysis of Ovarian Carcinoma Ascites**

Activity <sup>a</sup>	Number of cases ( <i>n</i> = 23)				
	0	+/-	1+	2+	3+
72-kDa MMP	0	0	1	20	2
92-kDa MMP	5	7	1	8	2

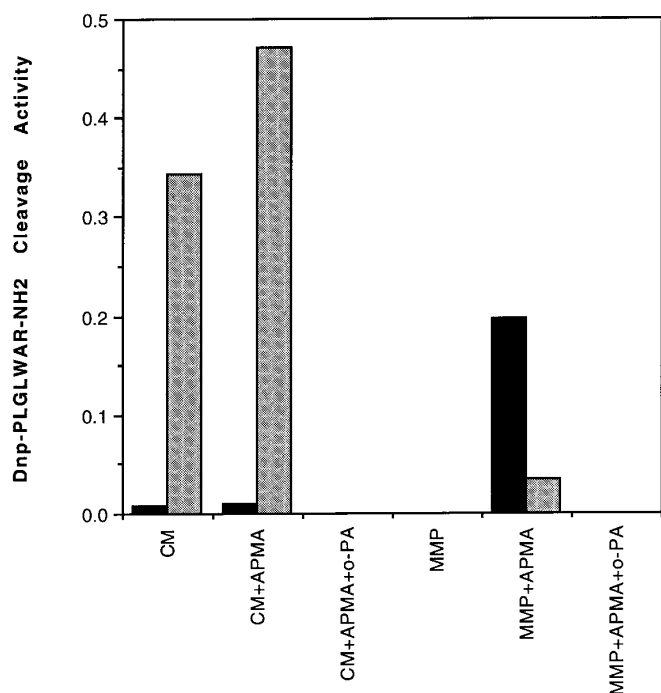
<sup>a</sup> Ascites fluids were normalized for protein content and analyzed by SDS-PAGE gelatin substrate zymography as described under Materials and Methods. Activity score represents an arbitrary graded scale analysis of bands representing MMP activity on zymograms. Scale categories are defined as follows: 0, no band detected; +/-, faint band detected, <1.0 mm width; 1+, clear band detected, 1.0–1.5 mm width; 2+, intense band detected, 1.5–3.0 mm width; 3+, very intense band detected, >3.0 mm width.



**FIG. 3.** Purification of *M<sub>r</sub>* 72,000 MMP from DOV 13 epithelial ovarian carcinoma. The *M<sub>r</sub>* 72,000 gelatinase from DOV 13 cells was purified using a two-step chromatography procedure. Serum-free conditioned medium was applied to a gelatin agarose column equilibrated with TCS buffer. After elution with TCS/10% dimethyl sulfoxide, fractions containing gelatinase activity were dialyzed against HBC buffer. The dialysate was applied to a DEAE-Sephacel column and eluted with HBC/1.0 M NaCl. The eluate was concentrated by ultrafiltration and desalted. Aliquots of each step were analyzed by SDS-PAGE (9% acrylamide) followed by silver staining (A) or Western blotting (B): A contains the following: lane 1, serum-free conditioned medium (10 μg protein); lane 2, gelatin agarose eluate (1 μg protein); lane 3, DEAE-Sephacel eluate (1 μg protein). (B) Blots with purified enzyme (1 μg protein) were immunostained with rabbit anti-MMP-2 polyclonal antibody (1:500) or sheep anti-MMP-9 polyclonal antibody (1:1000) (data not shown). The DOV 13 metalloproteinase reacts vigorously with anti-MMP-2 antibody. No staining was observed with anti-MMP-9 antibody.

prominent 21-kDa band copurifying with the enzyme migrates with the molecular weight described for TIMP-2, a specific inhibitor of MMP-2, previously described to copurify with this enzyme as a noncovalent enzyme-inhibitor complex [56, 57]. Additionally, the 21-kDa band was observed to exhibit metalloproteinase inhibitory activity using reverse zymography and comigrates with TIMP-2 (data not shown), commensurate with its presumed identification as TIMP-2.

The purification was monitored using the synthetic collagenase substrate, Dnp-PLGLWAR-NH<sub>2</sub> (50) in a reverse-phase HPLC based assay (Fig. 4). The purification results in a 70-fold purification of the zymogen form of the enzyme with approximately 28% yield (relative to the zymogen component of the starting material). The purified zymogen is inactive against Dnp-PLGLWAR-NH<sub>2</sub>, gains proteolytic activity following treatment with APMA, and is inhibited by the zinc chelator *o*-phenanthroline. A large component of the activity in the starting material is active prior to APMA treatment; however, this material did not copurify with the zymogen. Since endogenously activated type IV collagenase



**FIG. 4.** Hydrolysis of the synthetic collagenase substrate Dnp-PLG-LWAR-NH<sub>2</sub> by DOV 13 MMP-2. Dnp-PLGLWAR-NH<sub>2</sub> cleavage activity of DOV 13 conditioned medium and the purified DOV 13 MMP-2 with or without APMA activation and *o*-phenanthroline inhibition. Dnp-PLGLWAR-NH<sub>2</sub> (20 μM) was incubated with concentrated serum-free conditioned medium (4.5 μg) or purified DOV 13 MMP-2 (0.48 μg) in 0.05 M Tris-HCl, 5 mM CaCl<sub>2</sub>, 0.2 M NaCl, pH 7.7, at 37°C for 16 hr. In some cases, the samples also contained 0.6 mM APMA alone or with 2 mM *o*-phenanthroline. Reactions were stopped by addition of 2 mM *o*-phenanthroline, and the products were analyzed by reverse-phase HPLC and quantitated by monitoring the column effluent at 370 nm. Substrate hydrolytic activity (nmole/hr) was obtained by integrating area-under-the-curve of the cleavage product compared to a known standard. Shaded bars represent total (the calculated activity of the entire pool of purification material) substrate hydrolytic activity (nmole/hr); solid bars represent specific (relative to the amount of protein in the purification material) substrate hydrolytic activity (pmole/hr/μg protein).

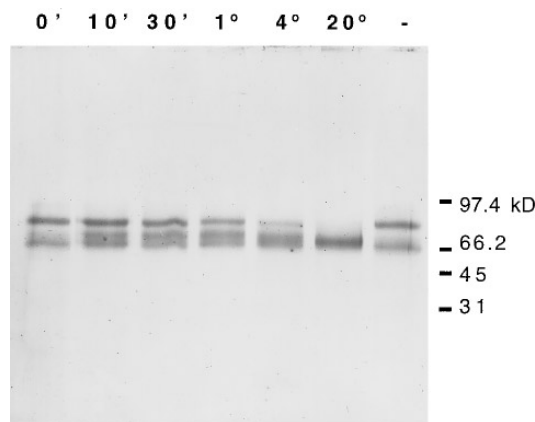
was observed in experiments employing cell-mediated [<sup>3</sup>H]-proline solubilization (Fig. 1), whereas purified proMMP-2 required organomercurial activation, together these data suggest that endogenously activated proteinase may arise via a cellular activator molecule. The purified MMP reacts with antiserum against MMP-2 but not MMP-9 on Western blots (Fig. 3B) and N-terminal sequence analysis of the activated form gave a sequence of NH<sub>2</sub>-YNFFPRKPKWQDKNQL, which is identical with the previously published N-terminal sequence of MMP-2 [58]. Upon treatment of the 82-kDa zymogen with APMA, the active 66-kDa enzyme was produced via an intermediate of approximately 72 kDa (Fig. 5). The substrate specificity was similar to that previously reported for MMP-2 [40, 58], cleaving all tested gelatins, native collagens IV and V, but not native collagen I (Fig. 6). In contrast to earlier studies, one recent report indicates

that MMP-2 free of TIMP-2 in fact is capable of hydrolyzing collagen I [59]. Our data do not refute these findings since a proMMP-2/TIMP-2 complex was obtained in the current purification and was used in the substrate specificity studies. Additionally, the enzyme did not degrade fibronectin and laminin under these conditions (data not shown). Based on these studies, the 72-kDa MMP appears to be identical to the previously characterized MMP-2.

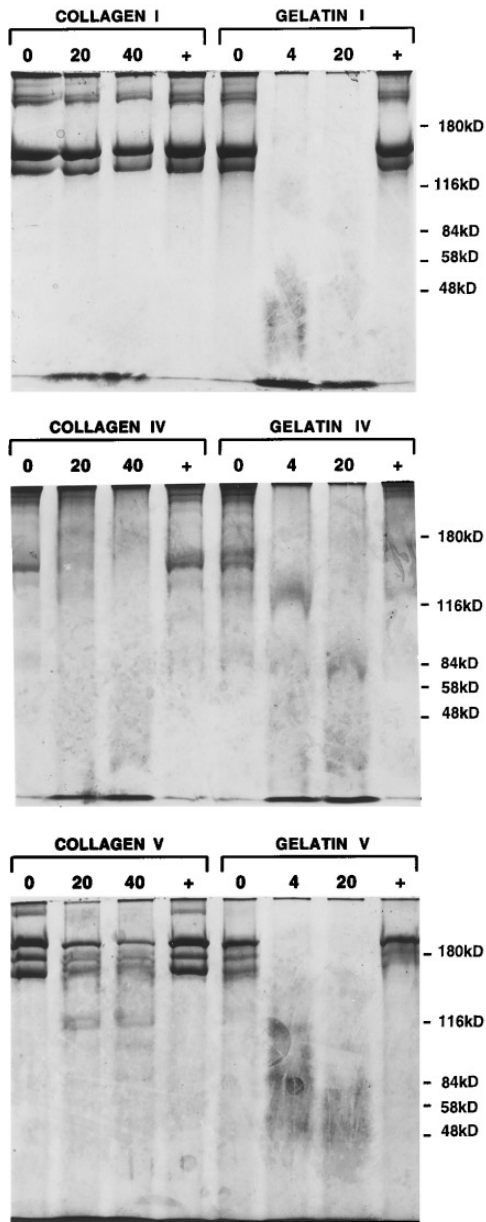
## DISCUSSION

Ovarian cancer is the leading cause of gynecologic cancer death and the fifth leading cause of cancer death overall in women. Each year 20,000 new cases are diagnosed in the United States and 12,500 deaths occur [60], owing to the advanced metastatic stage of disease usually present at diagnosis. Clearly, there is great need to gain an understanding of the mechanisms which underlie the intraperitoneal invasion of ovarian carcinoma cells.

In the present study, we have directly demonstrated that ovarian carcinoma cells have the capacity to degrade native and reconstituted basement membranes. Degradation is inhibited by minocycline, an MMP inhibitor, suggesting that an MMP is responsible for basement membrane collagen hydrolysis by these cells. Good correlation was observed between the levels of gelatinase secretion by ovarian cancer cells and both their invasive potential on Matrigel and their ability to solubilize HUVEC ECM metabolically labeled with [<sup>3</sup>H]proline. These correlations suggest that gelatinase



**FIG. 5.** Activation of DOV 13 MMP-2 with 4-aminophenylmercuric acetate (APMA). Aliquots of purified DOV 13 proMMP-2 (1 μg) were treated with 0.5 mM APMA for various time periods (indicated in min' and hr° above lanes) at 37°C in 20 mM Tris-HCl, pH 7.5. In a control experiment (designated “-” above the lane) APMA was omitted, and the sample was incubated in parallel with the last time point. Reactions were stopped by addition of 2.5 mM *o*-phenanthroline and reducing SDS sample buffer. Following SDS-PAGE (8.0% acrylamide), the reactants were visualized by silver staining. ProMMP-2 is activated by APMA from an inactive 82-kDa precursor to a 66-kDa active form via a 72-kDa intermediate species.



**FIG. 6.** Determination of substrate cleavage specificity of purified DOV 13 MMP-2. Purified DOV 13 proMMP-2 was activated with 1.5 mM APMA for 2 hr at 37°C. Activated MMP at a concentration of 12.5 ng/ml (gelatins) or 25 ng/ml (collagens) was incubated with collagen type I, IV, or V (16  $\mu$ g), or the corresponding gelatins for various time periods (indicated in hours) at 33°C (collagens) or 37°C (gelatins) in 0.05 M Tris-HCl, 0.2 M NaCl, 0.01 M CaCl<sub>2</sub>, 0.25 M glucose, pH 7.7. Some experiments (indicated +) incubated in parallel with the longest time point contained the metalloproteinase inhibitor *o*-phenanthroline (4 mM). Reactions were stopped by addition of 2.5 mM *o*-phenanthroline and reducing SDS sample buffer. Following SDS-PAGE (7.5% acrylamide), reactants were visualized by Coomassie blue staining. DOV 13 MMP-2 degrades collagen type IV and to a lesser extent collagen type V, but does not cleave collagen type I. All corresponding gelatins are efficiently hydrolyzed.

production may be a rate-limiting step in invasion and that secreted gelatinases are endogenously activated and not counterbalanced by inhibitors.

Because cells expressing the highest levels of an MMP-2-like 72-kDa gelatinase (DOV 13) were also most invasive, the MMP secreted by DOV 13 cells was purified and characterized. Based on immunologic cross-reactivity, NH<sub>2</sub>-terminal sequence analysis, and substrate specificity, the enzyme purified from DOV 13 cells is identical to MMP-2 purified from other sources [40, 58]. As further discussed below, this enzyme appears also to be identical with the major gelatinase found in ovarian carcinoma ascites *in vivo*. This observation is particularly interesting in light of the fact that normal ovarian epithelium does not produce any detectable MMP [36] and provides further evidence correlating the expression of a type IV collagen-degrading metalloproteinase with the malignant phenotype [1].

Since members of the MMP family of proteinases are secreted as inactive zymogens, requiring cleavage of the amino terminal propeptide to gain full activity, zymogen activation is postulated to be an important factor in the post-translational regulation of metalloproteinase activity. Our studies of ovarian carcinoma cell-mediated degradation of HUVEC ECM indicate that the secreted proMMPs become activated by an endogenous cellular mechanism. Our purification studies of proMMP-2 further demonstrate that significant amounts of activated metalloproteinase capable of specifically cleaving a collagenase peptide substrate are present in freshly isolated DOV 13 conditioned medium. This endogenously activated material likely represents MMP-2, since no other gelatinolytic or caseinolytic MMP activity can be detected zymographically in the DOV 13 conditioned medium [36; M. S. Stack, unpublished observations]. The failure of the purification procedure to yield any of the active MMP-2 protein is not surprising given the known instability of the active proteinase. ProMMP-2 activated in the absence of TIMP-2 is known to undergo rapid autolysis [40] and would not be expected to survive a purification procedure requiring a number of days, including membrane dialysis steps which would remove proteolytic fragments. However, the possibility that an additional nongelatinolytic, non-caseinolytic metalloproteinase may be present in the conditioned medium cannot be excluded completely. Regardless of the identity of the active MMPs present, further studies will be necessary to demonstrate the nature of the MMP activator(s) present in ovarian cancer. The recently described MT-MMPs, MMP family members containing a transmembrane domain [61, 62], and pump-1 (MMP-7) [63] have been reported to activate proMMP-2 and are potential physiologic activators. ProMMP-9 may also be proteolytically activated by MMP-3, cathepsin G, and trypsin [64, 65]. Neither progelatinase is directly activated via the plasminogen activator/plasmin cascade; however, this may occur indirectly via plasmin activation of proMMP-3 [66]. Our previous work has shown elevated plasminogen activator expression in the cell lines used in the current study [35, 36]. Preliminary immunochemical studies in our laboratory demonstrate that

MT-MMP also is expressed in these cell lines [M. S. Stack, unpublished observations], potentially explaining the high levels of APMA-independent metalloproteinase activity observed in DOV 13 cell conditioned medium.

MMP-2, unlike other members of the MMP family, has been reported to undergo activation by a one-step mechanism in the presence of APMA, an autolytic cleavage of Asn80–Tyr81 [48]. Other MMPs are activated by a two-step mechanism involving a partially activated intermediate resulting from an upstream cleavage (either autolytic or mediated by an extrinsic proteinase) of the propeptide [67]. In contrast to previous studies, we observed that activation of the 82-kDa zymogen with APMA produced the active 66-kDa enzyme via an intermediate of approximately 72 kDa. An autolytic activation intermediate of MMP-2 was also recently reported by Murphy *et al.* [68], and an intermediate occurring during membrane-associated activation has been described [69]. Together, these data suggest that MMP-2 appears to undergo activation by a two-step mechanism typical of other MMPs.

In addition to production by ovarian cancer cells in culture, we find that gelatinolytic MMPs resembling MMP-2 and MMP-9 are detectable in ovarian carcinoma ascites at relatively high levels. While ovarian carcinoma cell lines [36] and primary culture isolates of ovarian carcinoma ascitic cells [M. S. Stack, unpublished observations] secrete the identical MMP species found in ascites, it cannot be unequivocally established that the observed MMP activities originate from tumor cells rather than from a variety of nontumor cell types present in this milieu. In light of this observation, we have previously demonstrated that MMP-2 binds to the plasma membranes of DOV 13 and OVCA 432 cells, and, as a consequence of binding, the gelatinolytic activity of the enzyme is stimulated [70]. The ability of individual cell types to bind MMP-2 was independent of the secretion of MMPs, as the OVCA 432 cell line was observed to bind MMP-2 in the absence of MMP secretion. These data suggest that the source of MMP-2 activity in the ovarian cancer microenvironment need not necessarily be the tumor cells. Rather, it is interesting to speculate that cell surface binding represents an important mechanism whereby the invading ovarian cancer cell may recruit MMP-2 that is secreted either in an autocrine manner by the tumor cell or in a paracrine fashion from host cells.

In summary, the present study has identified and characterized a major ovarian carcinoma-associated gelatinase as MMP-2. This enzyme, purified from a cultured cell line, was identified as the predominant gelatinase present in ovarian carcinoma ascites (along with an MMP-9-like activity), suggesting a potential relevance of this proteinase in tumor-associated proteolysis *in vivo*. In support of this observation, we found that secretion of type IV collagenase activity by a series of independently isolated ovarian adenocarcinoma cell lines correlated well with the ability of these cells to

proteolyze and invade the basement membrane. Together, these data suggest that extracellular matrix proteolysis mediated by tumor-associated proteinases is associated with peritoneal implantation and/or metastasis of ovarian carcinoma.

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## REFERENCES

1. Liotta, L. A., Rao, C. N., and Wewer, U. M. Biochemical interactions of tumor cells with the basement membrane, *Annu. Rev. Biochem.* **55**, 1037–1057 (1986).
2. Yurchenco, P. D., and Schittny, J. C. Molecular architecture of basement membranes, *FASEB J.* **4**, 1577–1590 (1990).
3. Mignatti, P., and Rifkin, D. B. Biology and biochemistry of proteinases in tumor invasion, *Physiol. Rev.* **73**, 161–195 (1993).
4. Moore, D. H. Primary surgical management of early epithelial ovarian carcinoma, In *Ovarian Cancer* (S. C. Rubin and G. P. Sutton, Eds.), McGraw–Hill, New York (1993).
5. Folkman, J. Tumor angiogenesis, *Adv. Cancer Res.* **19**, 331–358 (1974).
6. Docherty, A. J. P., and Murphy, G. The tissue metalloproteinase family and the inhibitor TIMP: a study using cDNAs and recombinant proteins, *Ann. Rheum. Dis.* **49**, 469–479 (1990).
7. Liotta, L. A., and Stetler-Stevenson, W. G. Metalloproteinases and cancer invasion, *Semin. Cancer Biol.* **1**, 99–106 (1990).
8. Tryggvason, K., Hoyhtya, M., and Pyke, C. Type IV collagenases in invasive tumors, *Breast Cancer Res. Treat.* **24**, 209–218 (1993).
9. Liotta, L. A., Abe, S., Robey, P. G., and Martin, G. R. Preferential digestion of basement membrane collagen by an enzyme derived from a metastatic murine tumor, *Proc. Natl. Acad. Sci. USA* **76**, 2268–2272 (1979).
10. Liotta, L. A., Tryggvason, K., Garbisa, S., Hart, I., Foltz, C. M., and Shafie, S. Metastatic potential correlates with enzymatic degradation of basement membrane collagen, *Nature* **284**, 67–68 (1980).
11. Turpeenniemi-Hujanen, T., Thorgeirsson, U. P., Hart, I. R., Grant, S., and Liotta, L. A. Expression of collagenase IV (basement membrane collagenase) activity in murine tumor cell hybrids that differ in metastatic potential, *J. Natl. Cancer Inst.* **75**, 99–108 (1985).
12. Garbisa, S., Pozzati, R., Muschel, R. J., Saffiotti, U., Ballin, M., Goldfarb, R. H., Koury, G., and Liotta, L. Secretion of type IV collagenolytic protease and metastatic phenotype: Induction by transfection with c-Ha-ras but not c-Ha-ras plus Ad2-E1a., *Cancer Res.* **47**, 1523–1528 (1987).
13. Nakajima, M., Welch, D., Belloni, P. N., and Nicolson, G. L. Degradation of basement membrane type IV collagen and lung subendothelial matrix by rat mammary adenocarcinoma cell clones of differing metastatic potentials, *Cancer Res.* **47**, 4869–4876 (1987).
14. Nakajima, M., Lotan, D., Baig, M. M., Carralero, R. M., Wood, W. R., Hendrix, M. J. C., and Lotan, R. Inhibition by retinoic acid of type IV collagenolysis and invasion through reconstituted basement membrane by metastatic rat mammary adenocarcinoma cells, *Cancer Res.* **49**, 1698–1706 (1989).

15. Ura, H., Bonfil, R. D., Reich, R., Reddel, R., Pfeifer, A., Harris, C. C., and Klein-Szanto, A. J. P. Expression of type IV collagenase and procollagen genes and its correlation with the tumorigenic, invasive, and metastatic abilities of oncogene-transformed human bronchial epithelial cells, *Cancer Res.* **49**, 4615–4621 (1989).
16. Frisch, S. M., Reich, R., Collier, I. E., Genrich, L. T., Martin, G., and Goldberg, G. I. Adenovirus E1A represses protease gene expression and inhibits metastasis of human tumor cells, *Oncogene* **5**, 75–83 (1990).
17. Bernhard, E. J., Gruber, S. B., and Muschel, R. J. Direct evidence linking expression of matrix metalloproteinase 9 (92-kDa gelatinase/collagenase) to the metastatic phenotype in transformed rat embryo cells, *Proc. Natl. Acad. Sci. USA* **91**, 4293–4297 (1994).
18. Monteagudo, R., Merino, M., San-Juan, J., Liotta, L. A., and Stetler-Stevenson, W. Immunohistochemical distribution of type IV collagenase in normal, benign, and malignant breast tissue, *Am. J. Pathol.* **136**, 585–592 (1990).
19. Levy, A. T., Cioce, V., Sobel, M. E., Garbisa, S., Grigioni, W. F., Liotta, L. A., and Stetler-Stevenson, W. G. Increased expression of the  $M_r$  72,000 type IV collagenase in human colonic adenocarcinoma, *Cancer Res.* **51**, 439–444 (1991).
20. Campo, E., Merino, M. J., Liotta, L., Neumann, R., and Stetler-Stevenson, W. Distribution of the 72-kd type IV collagenase in nonneoplastic and neoplastic thyroid tissue, *Hum. Pathol.* **23**, 1395–1401 (1992).
21. Brown, P. D., Bloxidge, R. E., Stuart, N. S. A., Gatter, K. C., and Carmichael, J. Association between expression of activated 72-kilodalton gelatinase and tumor spread in non-small-cell lung carcinoma, *J. Natl. Cancer Inst.* **85**, 574–578 (1993).
22. Stearns, M. E., and Wang, M. Type IV collagenase ( $M_r$  72,000) expression in human prostate: benign and malignant tissue, *Cancer Res.* **53**, 878–883 (1993).
23. Davies, B., Miles, D. W., Happerfield, L. C., Naylor, M. S., Bobrow, L. G., Rubens, R. D., and Balkwill, F. R. Activity of type IV collagenases in benign and malignant breast disease, *Br. J. Cancer* **67**, 1126–1131 (1993).
24. Rao, J. S., Steck, P. A., Mohanam, S., Stetler-Stevenson, W. G., Liotta, L. A., and Sawaya, R. Elevated levels of  $M_r$  92,000 type IV collagenase in human brain tumors, *Cancer Res.* **53**, 2208–2211 (1993).
25. Schultz, R. M., Silberman, S., Persky, B., Bajowski, A. S., and Carmichael, D. F. Inhibition by human recombinant tissue inhibitor of metalloproteinases of human amnion invasion and lung colonization by murine B16-F10 melanoma cells, *Cancer Res.* **48**, 5539–5545 (1988).
26. Khokha, R., Waterhouse, P., Yagel, S., Lala, P. K., Overall, C. M., Norton, G., and Denhardt, D. T. Antisense RNA-induced reduction in murine TIMP levels confers oncogenicity on Swiss 3T3 cells, *Science* **243**, 947–950 (1989).
27. Alvarez, O. A., Carmichael, D. F., and DeClerck, Y. A. Inhibition of collagenolytic activity and metastasis of tumor cells by a recombinant human tissue inhibitor of metalloproteinases, *J. Natl. Cancer Inst.* **82**, 589–595 (1990).
28. Albin, A., Melchiorri, A., Santi, L., Liotta, L. A., Brown, P. D., and Stetler-Stevenson, W. G. Tumor cell invasion inhibited by TIMP-2, *J. Natl. Cancer Inst.* **83**, 775–779 (1991).
29. Alexander, C. M., and Werb, Z. Targeted disruption of the tissue inhibitor of metalloproteinases gene increases the invasive behavior of primitive mesenchymal cells derived from embryonic stem cells in vitro, *J. Cell Biol.* **118**, 727–739 (1992).
30. Testa, J. Loss of the metastatic phenotype by a human epidermoid carcinoma cell line, HEp-3, is accompanied by increased expression of tissue inhibitor of metalloproteinases-2, *Cancer Res.* **52**, 5597–5603 (1992).
31. Niedbala, M. J., Crickard, K., and Bernacki, R. J. In vitro degradation of extracellular matrix by human ovarian carcinoma cells, *Clin. Exp. Metastasis* **5**, 181–197 (1987).
32. Kanemoto, T., Martin, G. R., Hamilton, T. C., and Fridman, R. Effects of synthetic peptides and protease inhibitors on the interaction of a human ovarian cancer cell line (NIH:OVCAR-3) with a reconstituted basement membrane (Matrigel), *Invasion Metastasis* **11**, 84–92 (1991).
33. Kobayashi, H., Ohi, H., Sugimura, M., Shinohara, H., Fujii, T., and Terao, T. Inhibition of in vitro ovarian cancer cell invasion by modulation of urokinase-type plasminogen activator and cathepsin B, *Cancer Res.* **52**, 3610–3614 (1992).
34. Davies, B., Brown, P. D., East, N., Crimmin, M. J., and Balkwill, F. R. A synthetic matrix metalloproteinase inhibitor decreases tumor burden and prolongs survival of mice bearing human ovarian carcinoma xenografts, *Cancer Res.* **53**, 2087–2091 (1993).
35. Young, T. N., Rodriguez, G. C., Moser, T. L., Bast, R. C., Jr., Pizzo, S. V., and Stack, M. S. Coordinate expression of urinary-type plasminogen activator and its receptor accompanies malignant transformation of the ovarian surface epithelium, *Am. J. Obstet. Gynecol.* **170**, 1285–1296 (1994).
36. Moser, T. L., Young, T. N., Rodriguez, G. C., Pizzo, S. V., Bast, R. C., Jr., and Stack, M. S. Secretion of extracellular matrix-degrading proteinases is increased in epithelial ovarian carcinoma, *Int. J. Cancer* **56**, 552–559 (1994).
37. Karlan, B. Y., Amin, W., Band, V., Zurawski, V. R., Jr., and Littlefield, B. A. Plasminogen activator secretion by established lines of human ovarian carcinoma cells *in vitro*, *Gynecol. Oncol.* **31**, 103–112 (1988).
38. Autio-Harmanen, H., Karttunen, T., Hurskainen, T., Hoyhtya, M., Kauppila, A., and Tryggvason, K. Expression of 72 kilodalton type IV collagenase (gelatinase A) in benign and malignant ovarian tumors, *Lab. Invest.* **69**, 312–321 (1993).
39. Miyagi, E., Yasumitsu, H., Hirahara, F., Minaguchi, H., Koshikawa, N., Miyazaki, K., and Umeda, M. Characterization of matrix-degrading proteinases and their inhibitors secreted by human gynecological carcinoma cells, *Jpn. J. Cancer Res.* **86**, 568–576 (1995).
40. Okada, Y., Morodomi, T., Enghild, J. J., Suzuki, K., Yasui, A., Nakaniishi, I., Salvesen, G., and Nagase, H. Matrix metalloproteinase 2 from human rheumatoid synovial fibroblasts: Purification and activation of the precursor and enzymic properties, *Eur. J. Biochem.* **194**, 721–730 (1990).
41. Rodriguez, G. C., Berchuck, A., Whitaker, R. S., Schlossman, D., Clarke-Pearson, D., and Bast, R. C., Jr. Epidermal growth factor receptor expression in normal ovarian epithelium and ovarian cancer: Relationship between receptor expression and response to epidermal growth factor, *Am. J. Obstet. Gynecol.* **164**, 745–750 (1991).
42. Stack, M. S., Rinehart, A. R., and Pizzo, S. V. Comparison of plasminogen binding and activation on extracellular matrices produced by vascular smooth muscle and endothelial cells, *Eur. J. Biochem.* **226**, 937–943 (1994).
43. Rinehart, A. R., Mallya, S., and Simon, S. R. Human  $\alpha_1$ -proteinase inhibitor binds to extracellular matrix in vitro, *Am. J. Respir. Cell Mol. Biol.* **9**, 666–679 (1993).
44. Golub, L. M., Ramamurthy, N. S., McNamara, T. F., Greenwald, R. A., and Rifkin, B. R. Tetracyclines inhibit connective tissue breakdown: New therapeutic implications for an old family of drugs, *Crit. Rev. Oral Biol. Med.* **2**, 297–322 (1991).
45. Heussen, C., and Dowdle, E. B. Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates, *Anal. Biochem.* **102**, 196–202 (1980).
46. Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* **227**, 680–685 (1970).

47. Leco, K. J., Khokha, R., Pavloff, N., Hawkes, S. P., and Edwards, D. R. Tissue inhibitor of metalloproteinases-3 (TIMP-3) is an extracellular matrix-associated protein with a distinctive pattern of expression in mouse cells and tissues, *J. Biol. Chem.* **269**, 9352–9360 (1994).
48. Stetler-Stevenson, W. G., Krutzsch, H. C., Wachter, M. P., Margulies, I. M. K., and Liotta, L. A. The activation of human type IV collagenase proenzyme: Sequence identification of the major conversion product following organomercurial activation, *J. Biol. Chem.* **264**, 1353–1356 (1989).
49. Stack, M. S., Emberts, C., and Gray, R. D. Application of *N*-carboxylated peptides to the inhibition and affinity purification of the porcine matrix metalloproteinases collagenase, gelatinase, and stromelysin, *Arch. Biochem. Biophys.* **287**, 240–249 (1991).
50. Stack, M. S., and Gray, R. D. Comparison of vertebrate collagenase and gelatinase using a new fluorogenic substrate peptide, *J. Biol. Chem.* **264**, 4277 (1989).
51. Matsudaira, P. Sequence from picomole quantities of proteins electrophoretically transferred onto polyvinylidene difluoride membranes, *J. Biol. Chem.* **262**, 10035 (1987).
52. Mignatti, P., Robbins, E., and Rifkin, D. B. Tumor invasion through the human amniotic membrane: requirement for a proteinase cascade, *Cell* **47**, 487–498 (1986).
53. Ossowski, L. Invasion of connective tissue by human carcinoma cell lines: Requirement for urokinase, urokinase receptor, and interstitial collagenase, *Cancer Res.* **52**, 6754–6760 (1992).
54. Kleinman, H. K., McGarvey, M. L., Hassel, J. R., Star, V. L., Cannon, F. B., Laurie, G. W., and Martin, G. R. Basement membrane complexes with biological activity, *Biochemistry* **25**, 312–318 (1986).
55. Albin, A., Iwamoto, Y., Kleinman, H. K., Martin, G. R., Aaronson, S. A., Kozlowski, J. M., and McEwan, R. N. A rapid in vitro assay for quantitating the invasive potential of tumor cells, *Cancer Res.* **47**, 3239–3245 (1987).
56. Stetler-Stevenson, W. G., Krutzsch, H. C., and Liotta, L. A. Tissue inhibitor of metalloproteinase (TIMP-2): A new member of the metalloproteinase inhibitor family, *J. Biol. Chem.* **264**, 17374–17378 (1989).
57. Goldberg, G. I., Marmer, B. L., Grant, G. A., Eisen, A. Z., Wilhelm, S., and He, C. Human 72-kilodalton type IV collagenase forms a complex with a tissue inhibitor of metalloproteinase designated TIMP-2, *Proc. Natl. Acad. Sci. USA* **86**, 8207–8211 (1989).
58. Collier, I. E., Wilhelm, S. M., Eisen, A. Z., Marmer, B. L., Grant, G. A., Seltzer, J. L., Kronberger, A., He, C., Bauer, E. A., and Goldberg, G. I. H-ras oncogene-transformed human bronchial epithelial cells (TBE-1) secrete a single metalloproteinase capable of degrading basement membrane collagen, *J. Biol. Chem.* **263**, 6579–6587 (1988).
59. Aimes, R. T., and Quigley, J. P. Matrix metalloproteinase-2 is an interstitial collagenase: Inhibitor-free enzyme catalyzes the cleavage of collagen fibrils and soluble native type I collagen generating the specific  $\frac{3}{4}$ - and  $\frac{1}{4}$ -length fragments, *J. Biol. Chem.* **270**, 5872–5876 (1995).
60. Silverberg, E., Boring, C., and Squires, T. Cancer statistics, *CA* **40**, 9 (1990).
61. Sato, H., Takino, T., Okada, Y., Cao, J., Shinagawa, A., Yamamoto, E., and Seiki, M. A matrix metalloproteinase expressed on the surface of invasive tumor cells, *Nature* **370**, 61–65 (1994).
62. Takino, T., Sato, H., Shinagawa, A., and Seiki, M. Identification of the second membrane-type matrix metalloproteinase (MT-MMP-2) from a human placental cDNA library: MT-MMP's form a unique membrane-type subclass in the MMP family, *J. Biol. Chem.* **270**, 23013–23020 (1995).
63. Crabbe, T., Smith, B., O'Connell, J. P., and Docherty, A. J. P. Human progelatinase A can be activated by matrilysin, *FEBS Lett.* **345**, 14–16 (1994).
64. Okada, Y., Gonoji, Y., Naka, K., Tomita, K., Nakanishi, I., Iwata, K., Yamashita, K., and Hayakawa, T. Matrix metalloproteinase 9 (92-kDa gelatinase/type IV collagenase) from HT 1080 human fibrosarcoma cells: Purification and activation of the precursor and enzymic properties, *J. Biol. Chem.* **267**, 21712–21719 (1992).
65. Ogata, Y., Enghild, J. J., and Nagase, H. Matrix metalloproteinase 3 (stromelysin) activates the precursor for the human matrix metalloproteinase 9, *J. Biol. Chem.* **267**, 3581–3584 (1992).
66. Okada, Y., Harris, E. D., Jr., and Nagase, H. The precursor of a metalloendopeptidase from human rheumatoid synovial fibroblasts: Purification and mechanisms of activation by endopeptidases and 4-aminophenylmercuric acetate, *Biochem. J.* **254**, 731–741 (1988).
67. Nagase, H., Suzuki, K., Morodomi, T., Enghild, J. J., and Salvesen, G. Activation mechanisms of the precursors of matrix metalloproteinases 1, 2, and 3, *Matrix (Suppl. 1)*, 237–244 (1992).
68. Murphy, G., Nguyen, Q., Cockett, M. I., Atkinson, S. J., Allan, J. A., Knight, C. G., Willenbrock, F., and Docherty, A. J. P. Assessment of the role of the fibronectin-like domain of gelatinase A by analysis of a deletion mutant, *J. Biol. Chem.* **269**, 6632–6636 (1994).
69. Strongin, A. Y., Marmer, B. L., Grant, G. A., and Goldberg, G. I. Plasma membrane-dependent activation of the 72-kDa type IV collagenase is prevented by complex formation with TIMP-2, *J. Biol. Chem.* **268**, 14033–14039 (1993).
70. Young, T. N., Pizzo, S. V., and Stack, M. S. A plasma membrane-associated component of ovarian adenocarcinoma cells enhances the catalytic efficiency of matrix metalloproteinase 2, *J. Biol. Chem.* **270**, 999–1002 (1995).