Production of Extracellular Matrix–Degrading Proteinases by Primary Cultures of Human Epithelial Ovarian Carcinoma Cells

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BACKGROUND. The authors analyzed the secretion of extracellular matrix–degrading proteinases, including urinary-type plasminogen activator (u-PA), matrix metalloproteinase-2 (MMP-2, gelatinase A), and MMP-9 (gelatinase B), by short term primary cultures of epithelial ovarian carcinoma cells derived from primary ovarian tumors, intraperitoneal metastases, or ascites. The presence of these enzymatic activities in samples of ascites was also evaluated. The effect of adhesive substratum on proteinase production was determined.

METHODS. A coupled spectrophotometric assay was utilized to evaluate the initial rate of plasminogen activation by u-PA in conditioned medium; this involved monitoring the activity of generated plasmin with a colorimetric substrate. MMP activity was evaluated by gelatin zymography.

RESULTS. Ascitic fluids from 18 patients contained u-PA, MMP-2, and MMP-9. However, short term primary cultures of cells derived from primary ovarian tumors (OVET), metastatic lesions (OVEM), or ascites (OVEA) produced very low levels of u-PA. Production of u-PA by OVET and OVEM cells was regulated by adhesive substratum. Conditioned media from OVET, OVEM, and OVEA cells contained high levels of both MMP-2 and MMP-9. MMP-9 levels decreased with increasing passage in culture, whereas MMP-2 activity was maintained. Production of neither MMP-2 nor MMP-9 was regulated by adhesive substratum.

CONCLUSIONS. These results demonstrate that primary cultures of epithelial ovarian carcinoma cells derived from three distinct anatomic locations produce MMP-2 and MMP-9, with low level secretion of u-PA. These data suggest that MMPs, particularly MMP-2, may play a significant role in the intraperitoneal invasion of ovarian carcinoma cells. Cancer 1997;80:1457–63.

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KEYWORDS: ovarian carcinoma, matrix metalloproteinases, plasminogen activators, extracellular matrix, proteinase, tumor metastasis.

Epithelial ovarian carcinoma is the leading cause of death from gynecologic malignancy and the fourth leading cause of cancer death among women in the U.S.¹ Due to the infiltrative nature of ovarian carcinoma and the current inability to detect early stage (Stage I or II) disease, approximately 75% of women are initially diagnosed after the disease has already spread throughout the peritoneal cavity (Stage III or IV). Epithelial ovarian carcinoma is thought to metastasize by surface shedding followed by peritoneal implantation, growth, and invasion. Exfoliated tumor cells also obstruct peritoneal lymphatics, leading to the accumulation of carcinomatous ascites, which further facilitates metastatic implantation. Although the invasion and spread of hematogenously metastasizing tumors is known...
to be mediated by the action of extracellular matrix-degrading proteinases, the role of proteolysis in intraperitoneal metastasis remains unclear. Proteolytic activity may be required for disruption of the mesothelial cell layer, during invasion of the implanted tumor through the submesothelial basement membrane into the visceral organ stroma, and for subsequent tumor-mediated angiogenesis.

Predominant among the proteinases produced by invading tumor cells are enzymes in the plasminogen activator (PA) and matrix metalloproteinase (MMP) families. PAs are serine proteinases that catalyze the conversion of the plasma zymogen plasminogen to the active proteinase plasmin. Plasmin is a broad-spectrum serine proteinase capable of degrading numerous extracellular matrix and matrix-associated proteins, including fibrin, laminin, fibronectin, and vitronectin. Because a large reservoir of potential proteolytic activity is available in the form of plasminogen, production of PAs provides a mechanism by which tumor cell degradation of the extracellular matrix is amplified. MMPs are a family of zinc-dependent metalloendopeptidases that function in the degradation of collagen, gelatin, and other extracellular matrix macromolecules. Expression of gelatinolytic MMPs, such as MMP-2 (gelatinase A, 72 kDa type IV collagenase) and MMP-9 (gelatinase B, 92 kDa type IV collagenase), has been linked to enhanced tumor invasion in numerous model systems. We have previously demonstrated that established cultures of epithelial ovarian carcinoma cells secrete elevated quantities of urinary-type PA (u-PA, urokinase), coexpress the cellular u-PA receptor, and overexpress gelatinolytic MMPs relative to normal ovarian epithelium. However, the production of matrix-degrading proteinases by short term primary cultures of epithelial ovarian carcinoma cells and the regulation of these proteinases has not been addressed. In this study, we have analyzed production of PAs and MMPs by primary cultures of epithelial ovarian carcinoma derived from the ovary, ascites, and metastatic implants. Our data indicate that, in contrast to established epithelial ovarian carcinoma cell lines, production of u-PA by cultured primary cells is negligible. However, gelatinolytic MMPs, including MMP-2 and MMP-9, were secreted in large amounts by primary cells, regardless of the original anatomic source of the culture. Furthermore, whereas u-PA production was regulated in part by cellular growth substrate, MMP secretion was not diminished by the cellular adhesive microenvironment. Together, these data suggest that initial production of MMP(s) may mediate the invasive behavior of epithelial ovarian carcinoma cells.

### Table 1: Patient Data

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### Materials and Methods

#### Materials

Cell culture reagents, laminin, fibronectin, type I collagen, type IV collagen, bacterial collagenase, D-Val-Leu-Lys-p-nitroanilide (VLKpNA), Lowry total protein determination kit, aminophenylmercuric acetate (APMA), 1,10-phenanthroline (o-PA), and 2,4-dichloroisocoumarin (DCI) were purchased from Sigma Chemical Co., St. Louis, Missouri. The u-PA was the product of American Diagnostica, Greenwich, Connecticut. Vitronectin and plasminogen were purified from outdated human plasma as previously described. The established epithelial ovarian carcinoma cell line DOV 13 was generously provided by Dr. Robert C. Bast, Jr., of the M. D. Anderson Cancer Center, Houston, Texas. MMP-2 was purified from the serum free conditioned medium of DOV 13 cells as described previously.

#### Cell Culture

Patient materials, including ascites, primary tumor, and metastatic lesions, were obtained from 18 patients undergoing primary surgery for Stage III or IV ovarian carcinoma after informed patient consent and with approval of the Northwestern University Institutional Review Board. Details of the patient data are given in Table 1. Solid tissue was immediately dissected (to separate tumor macroscopically from stromal tissue), minced, and disrupted using bacterial collagenase; cultures of ascites-derived cells were established as
FIGURE 1. Proteinase activity in ovarian carcinoma ascites fluids is shown. (A) Spectrophotometric determination of plasminogen activator (PA) activity is shown. Total PA activity in eight randomly selected ascites fluids was determined using a coupled assay to measure the amidolytic activity of generated plasmin, as described in “Materials and Methods.” Values were normalized for protein concentration. (B) Determination of matrix metalloproteinase (MMP) activity by gelatin zymography is represented. Eight randomly selected ascites fluids (50 μg protein) were preactivated with aminophenylmercuric acetate and analyzed by gelatin zymography as described in “Materials and Methods.” The migration position of MMP-2 and MMP-9 standards (Lane S) are designated by arrowheads.

FIGURE 2. Analysis of changes in matrix metalloproteinase (MMP) production by primary cells with increasing passage in culture. Cells (1 × 10⁵) were grown for 18 hours in serum free medium; conditioned medium was activated with aminophenylmercuric acetate and analyzed for matrix metalloproteinase activity by gelatin zymography as described in “Materials and Methods.” Passage number (P) is indicated above each sample. (A) ovarian epithelial tumor cells; (B) ovarian epithelial metastasis cells; (C) ovarian epithelial ascites cells.

described by Rodriguez et al.⁹ Cells were maintained in supplemented minimal essential medium (MEM) containing 20% fetal bovine serum as previously described.¹⁰ Immunohistochemical staining with anticytokeratin (Dako, Carpinteria, CA) was used to verify the epithelial origin of the cells. Cells remained viable for an average of 8 passages, with the majority of experiments performed using cells from passages 3–6. Cells obtained from primary epithelial tumor tissue or peritoneal metastases were designated OVET (ovarian epithelial tumor) and OVEM (ovarian epithelial metastasis), whereas ascites-derived cells were designated OVEA (ovarian epithelial ascites). At least six cultures from each anatomic location were established and analyzed as described below.

Analysis of Proteinase Activity
Prior to use in experiments, cells were washed twice with Ca²⁺/Mg²⁺–free phosphate buffered saline (PBS) and incubated with 1 mM EDTA in Ca²⁺/Mg²⁺–free PBS to release cells from the culture flask without the addition of exogenous proteinases. Cells were resuspended in serum free medium and plated at a density of 2 × 10⁵ cells/mL in 24-well plates. In some experiments, cells were plated onto wells coated with albumin, type I collagen, type IV collagen, fibronectin, lam-
PAs and MMPs to assess the prevalence and identity of matrix-degrading proteinases in the ovarian carcinoma in vivo microenvironment. As indicated in the eight representative fluid samples shown in Figure 1A, PA activity was detectable in all samples. Zymographic analysis demonstrated that the PA activity comigrated with a u-PA standard and was inhibitable by DCI (data not shown), confirming its identity as u-PA. Two gelatinolytic proteinases were also present at various levels in all samples, as indicated in the same eight representative examples shown in Figure 1B, which were activated by incubation with APMA, inhibited by o-PA, and comigrated with MMP-2 and MMP-9 standards. These data indicate that both PAs and MMPs are available in the tumor cell microenvironment in vivo and may thereby contribute to the pathology of ovarian carcinoma. However, the presence of PAs and MMPs in ascitic fluid does not verify that these enzymes are tumor cell–derived, as numerous other cell types, including inflammatory cells, are also present in ascites.

To determine whether the epithelial tumor cells were a potential source of these extracellular matrix-degrading proteinases, short term primary cultures were established using cells derived from ovarian tumor tissue (OVET), peritoneal metastatic lesions (OVEM), or ascitic fluid (OVEA), and conditioned media from each passage were analyzed for proteinase activity. As shown in the representative examples in Figure 2, tumor cells derived from all three anatomic locations initially produce both MMP-9 and MMP-2. However, with increasing passage in culture, MMP-9 production decreases significantly, whereas MMP-2 activity is maintained, suggesting that a component (or components) of the ovarian carcinoma microenvironment not present in vitro may regulate MMP-9 production. It is noteworthy that u-PA production by primary cells was negligible (Fig. 3). This is in marked contrast to data previously reported by our laboratory.

RESULTS
Ascites obtained from 18 patients with Stage III or IV ovarian carcinoma were analyzed for the activity of
2 and MMP-9 (Fig. 4A, OVEA), predominantly MMP-2 (Fig. 4B, OVEM) or predominantly MMP-9 (Fig. 4C, OVET). In contrast, u-PA levels were regulated to some extent by matrix interaction. Secretion of u-PA was slightly enhanced in the presence of collagen, fibronectin, and laminin in OVET cells (Fig. 5A) and inhibited significantly by collagen, laminin, and vitronectin in OVEM cells (Fig. 5B). Similar to data previously reported using established ascites-derived epithelial ovarian carcinoma cells,\(^6\) the u-PA activity of OVEA cells was unaltered by growth substratum (Fig. 5C).

**DISCUSSION**

The activity of extracellular matrix–degrading proteinases has been correlated with invasive activity in a number of hematogenously metastasizing tumors\(^2,3\); however, the role of proteolysis in the intraperitoneal metastasis of epithelial ovarian carcinoma is unclear. It is reasonable to hypothesize that proteolytic activity may be required for disruption of the mesothelial cell monolayer and invasion of the submesothelial basement membrane. Indeed, previous immunohistochemical studies have demonstrated colocalization of staining for u-PA and its receptor in ovarian carcinomas, with the most prominent staining at the invasive edge between cancer cells and normal tissues.\(^6\) Studies utilizing whole tumor extracts have also shown that u-PA antigen levels are higher in malignant ovarian epithelial tumors than in benign counterparts and are higher in metastatic tissue than in primary tumor.\(^11\)–\(^14\)

We have previously demonstrated that established cultures of ascites-derived ovarian carcinoma cells over-express u-PA relative to normal ovarian epithelium\(^5\); however, our current data demonstrate minimal production of u-PA by primary ovarian carcinoma cells.

It is interesting to speculate that the u-PA antigen measured in prior studies, which utilized ovarian carcinoma tissues, may be secreted by adjacent stromal cells and subsequently bound to tumor cell receptors. Alternatively, a component (or components) of the in vivo microenvironment may be necessary to stimulate cellular u-PA production. Nevertheless, the results of this and other studies\(^6,15\) demonstrating high levels of u-PA activity and antigen in ascites suggest that u-PA contributes to the pathology of ovarian carcinoma, regardless of its cellular origin.

Relatively little information is available regarding the production and regulation of MMPs by ovarian carcinoma cells. In studies using human epithelial ovarian tumor tissues, in situ hybridization, immunohistochemical analyses, and gelatin zymography have demonstrated the presence of both MMP-2 and MMP-9, with enhanced MMP-2 immunoreactivity observed at the invasive front.\(^16\)–\(^18\) The importance of MMPs in ovarian carcinoma pathology in vivo was clearly demonstrated in studies examining the effect of a synthetic MMP inhibitor BB94 (Batimastat) on human ovarian carcinoma xenografts growing in nude mice.\(^19\) Animals treated with this inhibitor showed a dramatically reduced tumor burden with stromal encapsulation of the solid tumor, indicative of decreased matrix turnover. Concomitant with the decreased tumor burden was a five- to six-fold increase in survival. Our current data show high levels of both MMP-2 and MMP-9 in very early passage cultures (passages 1–3), with a subsequent decrease in MMP-9 levels while MMP-2 secretion is maintained. It has been suggested that the MMP-9 observed in tissue specimens may be contributed by tumor associated macrophages,\(^17\) and it remains formally possible that undetected macro-
phages may be the source of the MMP-9 observed in the current study. However, we have previously demonstrated two established ovarian carcinoma cell lines (OVCA 429 and OVCA 433) also produce MMP-9. Regardless of the cellular source, the presence of both MMP-2 and MMP-9 in ascites and tumor cell–conditioned medium suggest that these proteinases also contribute to the pathology of ovarian carcinoma.

In light of the current findings, it is interesting to note that ovarian carcinoma cells, regardless of the cellular capacity for MMP production, can nevertheless bind MMP-2 to the cell surface. As a consequence of tumor cell surface binding, MMP-2 catalytic activity is enhanced, leading to an increased potential for ECM degradation and enhanced cellular invasive activity. Furthermore, ovarian carcinoma cells have the capacity to activate proMMP-2 via a membrane type 1 MMP (MT1-MMP)–dependent mechanism. Together, these data suggest that MMP-2 production by either tumor or stromal cells would result in active, tumor cell–bound MMP-2 with which to mediate proteolysis associated with invasion and metastasis.

Previous studies have demonstrated that adhesion of both normal and neoplastic cells to various extracellular matrix proteins can regulate the secretion of proteolytic enzymes. To determine whether the adhesive substratum altered proteinase production by primary ovarian carcinoma cells, conditioned media from cells grown on a variety of matrix proteins were assayed for u-PA and MMP activity. Similar to data obtained using established ovarian carcinoma cell lines, MMP production was independent of adhesive substratum.
Substratum-induced changes in u-PA activity were observed in the conditioned media of cells derived from the primary tumor (OVET) or metastatic lesion (OVEM). However, due to the inherently low level of u-PA production by the primary tumor cells, the potential biologic significance of this observation is difficult to evaluate. These results indicate that proteinase activity is regulated by a mechanism other than cell:matrix adhesion in ovarian carcinoma cells. In addition, proteinase inhibitors, including PA-inhibitors (PAIs) and tissue inhibitors of metalloproteinases (TIMPs), may also function in the regulation of proteinase activity in vivo.

In summary, we have shown that extracellular matrix-degrading proteinases, including u-PA, MMP-2, and MMP-9, are abundant in ascites obtained from ovarian carcinoma patients. Furthermore, conditioned media from primary cultures of epithelial ovarian carcinoma cells derived from the primary tumor, metastatic lesion, and ascites contain both MMP-2 and MMP-9, whereas u-PA production by primary cells is minimal. MMP-9 levels are diminished by extended cell culture and secretion of neither MMP-2 nor MMP-9 are regulated by the adhesive substratum. These data suggest that tumor cell production of MMPs, particularly MMP-2, may play a role in the peritoneal invasion of ovarian carcinoma cells.

REFERENCES