Proinvasive Properties of Ovarian Cancer Ascites-Derived Membrane Vesicles

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ABSTRACT

Malignant ovarian ascites are rich in cellular components, membrane-bound vesicles, and soluble proteins. This study focused on the structure of membrane-bound vesicles and their ability to promote invasion in cultured malignant ovarian epithelium. Membrane vesicles were derived from women with stage I–IV malignant ovarian ascites and from nonmalignant gynecologic ascites. Isolated vesicles were characterized by immunofluorescence and Western blot analysis. Using gel zymography for matrix metalloproteinase (MMP) detection and a colorimetric assay for urokinase-type plasminogen activator (uPA) analysis, we analyzed the proteinase activities of MMP-2, MMP-9, and uPA from the prepared vesicles, whole cells isolated from ascites, and the cell-free ultracentrifuged supernatant. The invasiveness of established cultured malignant ovarian epithelium on addition of ascites-derived vesicles was tested using a Matrigel-based invasion assay. Fractionation of malignant ascites revealed that extracellular matrix-degrading proteinases including MMPs and uPA are localized preferentially in membrane vesicles. All malignant vesicles tested, regardless of cancer stage, stimulated invasion. Furthermore, the combination of ovarian cancer cells and membrane vesicles resulted in greater uPA activation than that of cells or vesicles alone. Membrane vesicles from malignant ascites were also found to contain activated MMP-2, MMP-9, and uPA. Our data suggest that vesicle-stimulated proteinase activation leads to increased extracellular matrix degradation, which may facilitate tumor cell invasion and metastasis.

INTRODUCTION

In the United States, ovarian cancer is responsible for more morbidity and mortality than all other gynecologic malignancies combined (1). The pathogenesis of this disease involves widespread dissemination of malignant ovarian epithelium throughout the entire peritoneal cavity. Invasion of tumor cells through the extracellular matrix (ECM) is essential for hematogenous, lymphatic, and peritoneal metastases. ECM-degrading proteinases, including urokinase-type plasminogen activator (uPA) and matrix metalloproteinase (MMP)-2 and MMP-9, play a critical role in metastasis by facilitating invasion of epithelial ovarian cancer cells (2–7). MMPs degrade basement membrane collagens, whereas uPA catalyzes the conversion of plasminogen to plasmin, a broad spectrum serine protease that degrades numerous components of the ECM including fibrin, laminin, fibronectin, and vitronectin. Plasmin has also been implicated in activation of some MMP zymogens as well as in the conversion of pro-uPA (scuPA) to its two-chain active form. In addition to its proteolytic role, the NH_2-terminal fragment of uPA stimulates the proliferation of a variety of cell types including epithelial ovarian carcinoma cells (5).

It is well recognized that the presence of ascites is a poor prognostic indicator for survival in ovarian cancer. Development of ascites correlates with a significantly decreased 5-year survival rate [5% (with ascites) *versus* 45% (without ascites)] among women with stage III or IV epithelial ovarian carcinoma (8). Similarly, ascites has been found to be a poor prognostic factor in women with stage I disease (9). Malignant ovarian ascites is rich in proteolytic enzymes, including MMP-2, MMP-9, and uPA (10, 2). The presence of proteolytic enzymes in ascites and the correlation between ascites and poor prognosis suggest that this complex fluid may play an active role, rather than a passive role, in ovarian metastasis.

Shed membrane vesicles from ascites contain zymogen and activated MMP-2, MMP-9, and uPA (10). In an analysis of vesicles derived from ascites from 33 women with different gynecologic pathologies (19 benign ovarian lesions, 10 ovarian carcinomas, and 4 endometrial carcinomas), Ginestra *et al.* (11) found that vesicle content was generally higher in women with malignancy in comparison with those with benign disease. Vesicles from benign serous cysts contained minimal gelatinolytic activity, whereas vesicles from malignancies contained zymogen (pro-form) and active MMP-2 and MMP-9. Furthermore, a positive correlation was noted between malignant potential and vesicle-associated MMP-2 activity (11). These studies suggested that membrane vesicles in ascites may serve as stores of active proteinases and, as such, may aid in the metastatic process.

The purpose of our study was to determine whether MMP-2, MMP-9, and uPA in the cellular, membrane vesicle, and soluble fractions of ascites impacted ovarian metastasis. Therefore, the effect of vesicles derived from women with early-stage and late-stage ovarian carcinoma on cellular invasion was evaluated. We report that ascites-derived membrane vesicles were found to contain activated MMP-2, MMP-9, and uPA and are potent inducers of proteinase-dependent invasion in cultured malignant ovarian epithelium. Additionally, on combination of ovarian cancer cells and membrane vesicles, uPA activity is enhanced via a mechanism independent of new protein synthesis. These data suggest that proteinase-rich vesicles may actively contribute to the metastatic dissemination of ovarian carcinoma.

MATERIALS AND METHODS

Cell Lines and Ascites Samples. Ovarian carcinoma cell lines OVCA 429 and DOV 13 were generously provided by Dr. R. Bast, Jr. (M. D. Anderson Cancer Center, Houston, TX). Cells were grown in 75-cm^2 culture flasks under standard conditions as described previously (6).

Ascites were collected from women undergoing surgical procedures for gynecological indications with institutional review board-approved consent. Preoperative and intraoperative ascites were collected under sterile conditions and frozen at $-20°C$.

Vesicle Purification. Thawed ascites were centrifuged at $500 \times g$ for 15 minutes, followed by centrifugation of the supernatant at $800 \times g$ for 10 minutes to remove cells and debris. This supernatant was then stratified on a Lymphoprep (Greiner Bio-One, Frickenhausen, Germany) gradient to remove any remaining red blood cells. Supernatants were centrifuged at $100,000 \times g$ for 1 hour at 4°C. Pelleted vesicles were washed and resuspended in PBS. Relative vesicle amounts were determined by analysis of protein concentration using the Bradford method (Bio-Rad, Hercules, CA; ref. 12). Vesicle and cell fractions were stained with the DNA dye 4′,6-diamidino-2-phenylindole

(Sigma, St. Louis, MO) and visualized with fluorescent microscopy to ensure that cells were not present in the vesicle preparations.

Vesicle Immunofluorescence. Vesicle preparations were allowed to attach to poly-L-lysine–coated glass slides overnight, followed by fixation in 4% paraformaldehyde in PBS for 10 minutes. Fixed vesicles were incubated with primary antibodies (1:1,000 dilution in 1% bovine serum albumin in PBS at 4°C overnight) including anti-membrane type 1 (MT1)-MMP hinge region (Sigma), anti-β_1 integrin (Chemicon International, Temecula, CA), anti-HGFR (Cell Signaling Technology, Beverley, MA), anti-uPA receptor (uPAR; American Diagnostica, Greenwich, CT), and normal IgG. Secondary antibodies included anti-species Cy3 (Sigma) at 1:5,000 dilution in 1% bovine serum albumin in PBS for 60 minutes at room temperature; washes were done in PBS. Vesicles were visualized in a Nikon Eclipse TE2000-U inverted fluorescent microscope.

Analysis of Proteinase Activities. MMP-2 and MMP-9 gelatinase activities were determined using SDS-PAGE gelatin zymography as described previously (13). Positive controls included samples treated for 1 hour at 37°C with 1.5 mmol/L aminophenyl mercuric acetate (Sigma) before electrophoresis.

Analysis of uPA activity was performed using a coupled assay as described previously (14). Overnight conditioned medium was incubated at 37°C in the presence of 0.3 μmol/L plasminogen and 0.3 mmol/L of the plasmin substrate D-Val-Leu-Lys 4-nitroanilide dihydrochloride (Sigma). Plasmin hydrolysis of D-Val-Leu-Lys 4-nitroanilide dihydrochloride was measured by monitoring absorbance at 405 nm in a Molecular Devices Thermomax microtiter plate reader. This assay detects both uPA and tissue-type plasminogen activator.

Western Analysis of Urokinase-Type Plasminogen Activator and Urokinase-Type Plasminogen Activator Receptor. The presence of uPA and uPAR in cells and vesicles was determined by Western analysis using Mab394 for uPA (American Diagnostica) and 399R (American Diagnostica) for uPAR. After lysis in Laemmli sample buffer, cells and vesicles were electrophoresed on 9% SDS-polyacrylamide gels and transferred to nitrocellulose. Membranes were then blocked for 1 hour in 5% milk and probed with the above antibodies at a 1:1,000 dilution in 5% milk. Horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were diluted to 1:5,000 in 5% milk, and immunoreactive bands were detected with enhanced chemiluminescence (Pierce, Rockford, IL).

Analysis of Cellular Invasion. *In vitro* invasion assays were performed using Matrigel-coated wells (11 μg per filter; 8 μm, pore size). After overnight starvation in serum-free media, approximately 1.5×10^5 cells were plated onto each well and incubated with or without 30 μg of vesicle proteins at 37°C for 24 hours (DOV 13) or 3 days (OVCA 429). After mechanical removal of noninvading cells, the filters were fixed and stained with the Diff-Quick staining kit (Dade Berhring). Experiments were performed in triplicate, and at least three fields were counted per well.

To assess the effects of vesicles on the expression of MMP-2, MMP-9, and uPA in cultured ovarian epithelia, cells were plated on Matrigel-coated dishes with or without vesicle treatment and lysed after the 24-hour incubation. For experiments containing cycloheximide, a concentration of 10 μg/mL was used, and it was added at the time that cells were plated onto Matrigel. Cell lysis was achieved by incubation in Laemmli sample buffer and subjection to three freeze/thaw cycles. Spun lysates or cell-free conditioned media were analyzed by gelatin zymography or the colorimetric uPA assay as described above.

For analysis of the effects of MMP and uPA inhibition on vesicle-stimulated invasion, invasion assays were performed as described above with the addition of the uPA blocking antibody Mab394 (American Diagnostica) or the broad spectrum MMP inhibitor GM6001 (Chemicon International).

Statistical Analysis. Statistical analysis using the Mann-Whitney test was performed on protein concentration data of ascites-derived vesicles samples. The data were pooled into two groups for the analysis. The first group contained noncancer and disease stage I and II samples; the second group incorporated samples from stage III and IV. This grouping was necessary due to the small number of samples in the noncancer, stage I, and stage II categories.

RESULTS

Ascites-Derived Membrane Vesicles Are Rich in Membrane Type 1–Matrix Metalloproteinase, β_1 Integrins, Epidermal Growth Factor Receptor, and Urokinase-Type Plasminogen Activator Receptor. To visualize ascites-derived membrane vesicles, immunofluorescent staining was performed on vesicle preparations

from stage III and IV ascites, representing the most malignant stages of disease. Vesicles were allowed to adhere to poly-L-lysine–coated slides overnight and then fixed and stained for antigens previously associated with invasive ovarian cancer cells including MT1-MMP (5), β_1 integrin (15), epidermal growth factor receptor (16), and uPAR (ref. 6; Fig. 1). Vesicles exhibited a heterogeneous size distribution and were visualized as clusters at the light microscopy level. Isolated vesicles did not stain positively for the nuclear marker proliferating cell nuclear antigen, indicating lack of contamination of the purified vesicles with whole cells or nuclei (data not shown).

Matrix Metalloproteinase-2 and -9 and Urokinase-Type Plasminogen Activator Activities Are Predominately Vesicle Associated. Fractionation of ascites was performed to evaluate the localization and content of proteolytic activities. Membrane vesicles were prepared from ascites obtained from women with all FIGO (International Federation of Gynecologists and Obstetricians) stages of epithelial ovarian cancer and from women with nonmalignant ascites associated with benign gynecologic disease (Meig's syndrome, ovarian hyperstimulation syndrome). Using gelatin zymography for MMP detection and a colorimetric assay for uPA analysis, the proteinase activities of MMP-2, MMP-9, and uPA in the prepared vesicles, whole cells from ascites, and the cell-free ultracentrifuged supernatant from which the vesicles had been removed were analyzed. As reported previously (3) and shown in Fig. 2A, ascites contain both pro-MMP-2 and pro-MMP-9. Interestingly, some samples contained activated MMP-2 and MMP-9, and these active enzymes are predominantly associated with the purified vesicles. Specifically, 16 of 20 vesicle preparations tested had activated MMP-9, and 18 of 20 had activated MMP-2. A small amount of these active enzymes was detected in the cell fraction; however, very little active MMP-2 and no active MMP-9 were detected in the cell- and vesicle-free supernatant.

These results demonstrated that the majority of active MMP-2 and MMP-9 are vesicle or cell associated, whereas soluble pro-MMP-2 and pro-MMP-9 are found in the cell-free, vesicle-free fraction of the fluid. In addition to active MMPs, membrane vesicles contain active uPA (Fig. 2B). This activity is largely confined to vesicles because minimal uPA activity is detected in the cell or cell-free fractions. All 20 vesicle samples tested had activated uPA. However, inhibition of uPA activity with a blocking antibody resulted in a near-complete depletion of enzyme activity in the assay (data not shown), suggesting that uPA rather than tissue-type plasminogen activator is the chief plasminogen activator prevalent in vesicles. This was confirmed by Western analysis, demonstrating the presence of both uPA and uPAR in ascites vesicles as well in the cell fraction (Fig. 2C).

The Amount and Content of Vesicles Differ between Early-Stage and Late-Stage Cancers. Comparison of the abundance and content of vesicles with different stages of epithelial ovarian carcinoma demonstrated that ascites from women with late-stage cancers contained more vesicles than that from women with early-stage cancer or nonmalignant ascites. Table 1 shows the protein content of all vesicle preparations used in this study separated by stage of disease. Ascites-derived vesicle preparations from stages III and IV contained significantly higher amounts of protein than those derived from stages I and II or noncancer specimens.

Comparison of the proteolytic profiles of vesicles from early- and late-stage ascites samples showed variable amounts of active MMP-2 and MMP-9 in ascites from stage II, III, and IV cancers (Fig. 3A). The presence of activated MMP-2 was minimal in the nonmalignant ascites-derived vesicles and all of the stage I, II, and III samples tested. However, four of six stage IV samples contained high levels of activated MMP-2. In contrast, activated MMP-9 was present in most of the ovarian cancer specimens. Activity was observed in 2 of 2 stage II samples, 9 of 10 stage III samples, and 5 of 6 stage IV samples. Activated MMP-9 was not observed in the noncancer specimens or
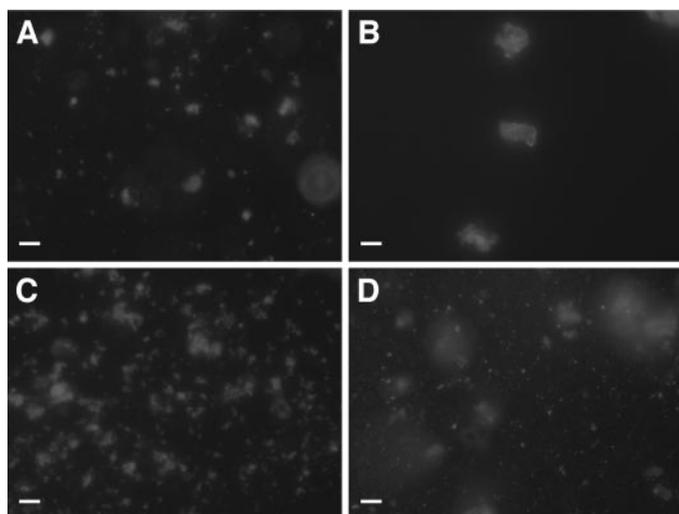
Fig. 1. Immunofluorescent staining of ascites-derived membrane vesicles. Membrane vesicles purified from a stage III ascites stain positive for (*A*) MT1-MMP, (*B*) β_1 integrin, (*C*) epidermal growth factor receptor, and (*D*) uPAR. *Bars*, 50 μm. Appropriate controls for normal IgG and nuclear staining are not shown.
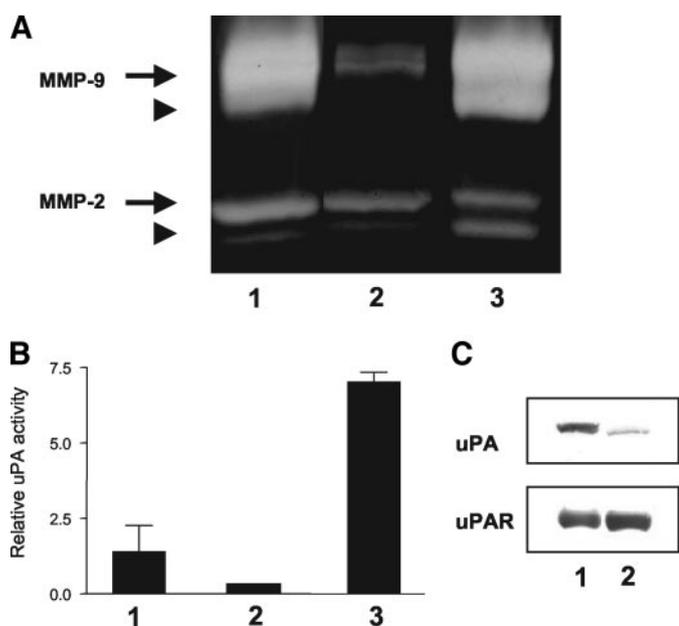

Fig. 2. Ascites-derived vesicles contain activated proteinases. *A*, Ascites vesicles contain both zymogen and activated MMP-2 and MMP-9 (*Lane 3*). *Top bands* represent MMP-9 (pro-MMP-9, *top band*; activated MMP-9, *second band from top*), and *bottom bands* represent MMP-2 (pro-MMP-2, *third band from top*; activated MMP-2, *bottom band*). Cells in ascites predominately contain pro-MMP-9 (92 kDa) and pro-MMP-2 (72 kDa), with a small amount of activated enzymes often observed as well (*Lane 1*). Activated MMP-9 (86 kDa) and MMP-2 (66 kDa) are rarely observed in cell- and vesicle-free supernatants; however, we do observe some pro-MMP-9 and pro-MMP-2 (*Lane 2*). *B*, uPA activity is predominately vesicle associated (*3*), although some activity is detected in cells (*1*). Very little activity is present in the cell- and vesicle-free supernatant (*2*). *Numbers* on the *Y* axis represent uPA activity as assayed by a colorimetric assay. *Error bars* represent SD, and experiments were performed in triplicate. *C*, Western analysis shows that both cells (*Lane 1*) and vesicles (*Lane 2*) in ascites contain uPA and uPAR.

stage I samples. Only one sample (stage IV) lacked both activated MMP-2 and MMP-9. Relative uPA activity does not appear to differ between the vesicles from early-stage ovarian cancer ascites and those from late-stage ascites (Fig. 3*B*).

Purified Ascites-Derived Vesicles Promote Invasion of Cultured Ovarian Cancer Cells. The ability of the malignant ascites-derived vesicles to stimulate invasion in cultured ovarian cancer cells was evaluated. As shown in Fig. 4, the addition of purified vesicles resulted in a

dose-dependent increase in cellular invasion by both DOV 13 and OVCA 429 ovarian carcinoma cells (Fig. 4*A*; data not shown). This increase in invasion was similar for all vesicles, regardless of cancer stage. The stimulation of invasion was consistent yet more pronounced in OVCA 429 than DOV 13 cells for vesicles corresponding to the same disease stage (see Figs. 4 and 5). To determine whether ascites-stimulated invasion is MMP or uPA dependent, invasion was assayed in the presence of vesicles with either the general MMP inhibitor GM6001 or a uPA blocking antibody. As shown in Fig. 5, vesicle-stimulated invasion is largely inhibited by either treatment and thus depends on both MMP and uPA activities. A subtle increase in invasion was observed in DOV 13 cells treated with vesicles in the presence of GM6001 as compared with those with inhibitor treatment alone (Fig. 5*C*). This result suggests that at least one component of the vesicle invasion-stimulating capacity is not MMP or uPA dependent. These results were similar for all vesicles tested.

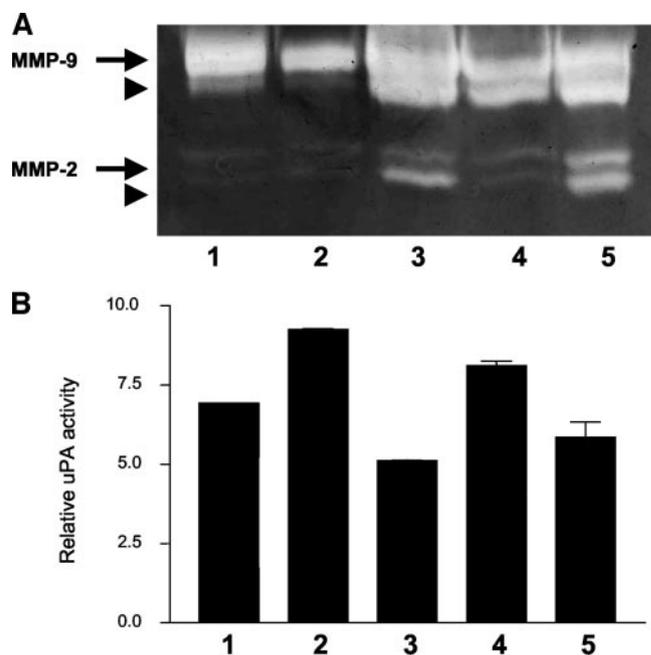Finally, we addressed the possibility that vesicles could affect

Fig. 3. Vesicle-associated proteinase content does not clearly correlate with disease stage. *A*, representative results. MMP-2 and MMP-9 were detected by gelatin zymography. *Top bands* represent MMP-9 (pro-MMP-9, *top band*; activated MMP-9, *second band from top*), and *bottom bands* represent MMP-2 (pro-MMP-2, *third band from top*; activated MMP-2, *bottom band*). *B*, uPA activity is similar among vesicles from different stages of disease. *Numbers* on the *Y* axis represent uPA activity as assayed by a colorimetric assay. *Error bars* represent SD, and experiments were performed in triplicate. *1*, noncancer; *2*, stage I; *3*, stage II; *4*, stage III; *5*, stage IV epithelial ovarian cancer.

Table 1 *Ascites vesicle content (μg protein) by disease stage*

Noncancer, Stages I and II	Stage III*	Stage IV*
4,923	2,438	14,470
510	5,136	11,168
500 (stage I)	3,443	7,107
2,382 (stage II)	1,243	1,271
2,535 (stage II)	6,680	10,581
	495	2,484
	10,529	1,007
	6,634	
	6,462	
	1,441	
	6,723	
	6,840	

NOTE. Median ascites vesicle content (μg protein): noncancer and stages I and II, 2,382; stages III and IV, 6,462. Mann-Whitney test considered these two groups to differ significantly at one-tailed *P* of 0.0375.

*These two stages were pooled into one group for statistical analysis.

Fig. 4. Ascites vesicles stimulate invasion through Matrigel in cultured ovarian cancer cells. *A*, Vesicles stimulate invasion in a dose-dependent fashion. As shown here, increasing amounts (micrograms of protein) of stage III vesicles as indicated were added to OVCA 429 cells. *B* and *C*, Invasion-stimulating capabilities of ascites vesicles do not correlate with disease stage. *Pairs of bars* represent individual experiments (□, control invasion; ■, invasion in the presence of vesicles). *B* represents experiments with DOV 13 cells, and *C* represents experiments with OVCA 429 cells. *Numbers* on the *Y* axis represent numbers of cells invaded. *Error bars* represent SD, and all experiments were performed in triplicate.
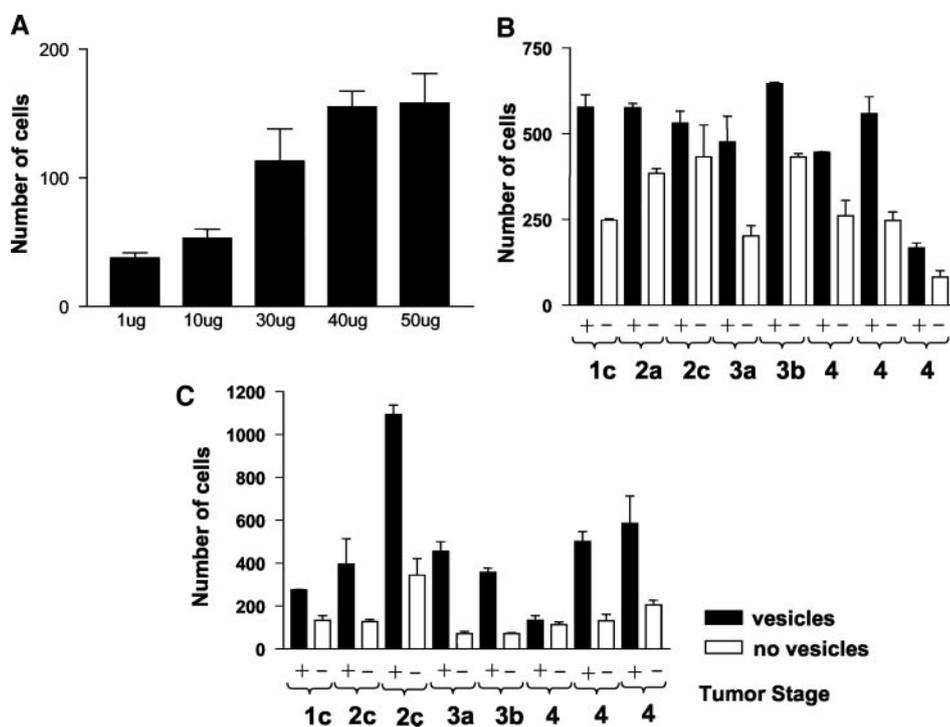
cellular proteinase expression. A simulated invasion assay was performed in which cells were plated on Matrigel with or without vesicles and compared with vesicles plated on Matrigel alone. The vesicles used in this study were from stage IV ascites and contained activated MMP-2, MMP-9, and uPA. After an incubation period identical to that used for the invasion analysis, the cells and cell-conditioned media were analyzed for uPA and MMP activities. As shown in Fig. 5E, the combination of cells and vesicles resulted in a 2-fold increase in uPA activity in OVCA 429 cells as compared with cells alone. The uPA activity observed with the combination is greater than the additive activity of the two components individually, suggesting that the combination of cells and vesicles resulted in pro-uPA activation. This is supported by the observation that the increase in uPA activity is not inhibited by cycloheximide and is therefore not dependent on new protein synthesis (Fig. 5F).

DISCUSSION

Proteolytic enzymes such as MMP-2, MMP-9, and uPA play an active role in ovarian cancer metastasis. Previous reports have demonstrated the presence of these molecules in shed membrane vesicles. The mechanism of vesicle formation remains unclear; however, this process has been associated with the release of growth factors, dissemination of tumor antigens, increased cellular invasiveness, metastasis, and suppression of immune surveillance (17–23). In this report we investigated the potential role of proteinase-rich vesicles on cellular invasion and describe a novel function of ascites that may account for the extremely aggressive metastatic behavior exhibited by epithelial ovarian carcinoma cells.

Our analysis of the cellular, membrane vesicle, and membrane-free fractions of ovarian carcinoma-stimulated ascites demonstrated that

Fig. 5. Inhibition of MMPs or uPA inhibits vesicle-stimulated invasion in DOV 13 (*A* and *C*) and OVCA 429 (*B* and *D*) cells. The addition of a blocking antibody to uPA (*A* and *B*) or the broad spectrum MMP inhibitor GM6001 (*C* and *D*) inhibited vesicle-stimulated invasion to near-basal levels. Vesicles from stage I disease were used in the experiment depicted here. *1*, control (no vesicles or inhibitor); *2*, inhibitor (uPA antibody or GM6001) alone; *3*, vesicles alone; *4*, vesicles and inhibitor. UPA activity is enhanced on the combination of ovarian cancer cells and ascites vesicles; this enhancement is not inhibited by cycloheximide (*E*). *1*, uPA activity of vesicles alone (from stage IV ascites); *2*, cellular uPA activity alone; *3*, uPA activity on the combination of cells and vesicles. In *F*, the experiment was repeated with the addition of cycloheximide to all three samples. *Error bars* represent SD, and all experiments were performed in triplicate.
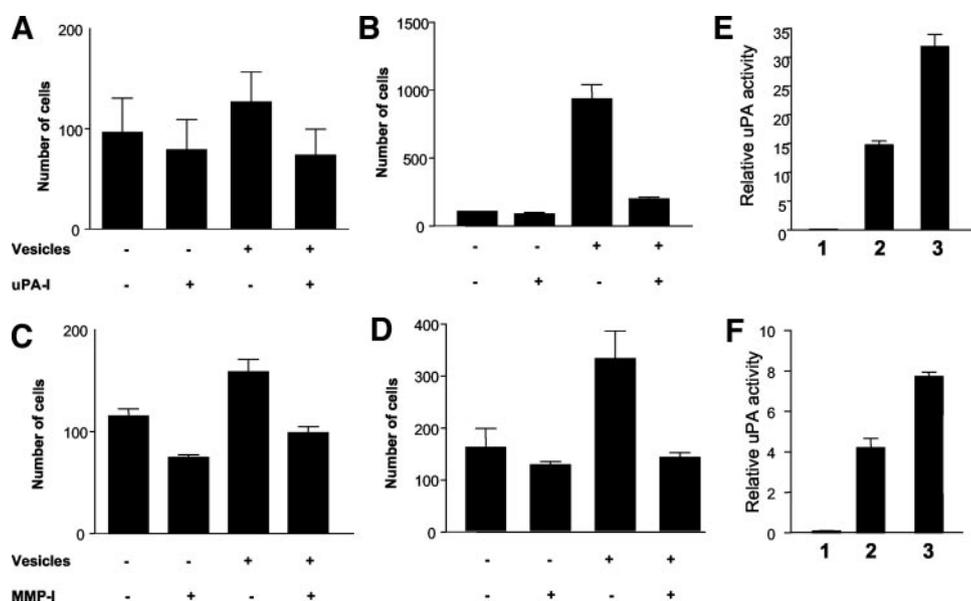
MMP-2, MMP-9, and uPA activities are primarily concentrated in vesicles. These proteinases degrade components of the ECM, an essential process in the migration, invasion, and dissemination of tumor cells. Furthermore, activated MMPs and uPA may serve to directly and indirectly activate other proteinases and signaling cascades to modulate cellular growth and survival. The interaction of MMP and uPA-rich vesicles with tumor and stromal cells could therefore potentially activate numerous enzymatic processes and signaling cascades that would facilitate metastasis, making vesicles active modulators of the tumor microenvironment.

The presence of vesicles in ovarian ascites varies according to disease stage. Late-stage ascites contain statistically significant higher amounts of vesicles than those with early-stage disease. We also found that vesicles from early- *versus* late-stage epithelial ovarian carcinomas are equally effective at stimulating invasion of ovarian cancer cells. This invasion stimulation was largely MMP and uPA dependent because blockage of these enzymes inhibited vesicle-stimulated invasion for all vesicles tested. Most vesicles contained activated MMP-2 or MMP-9, and these proteinases may facilitate cellular invasion through cleavage of ECM components and activation of other enzymes. In addition, all vesicles tested contained active uPA, which could lead to additional uPA activation and could also promote MMP activation. The MMP and uPA dependence of vesicle-stimulated invasion may reflect activation of these proteinases on vesicle addition or may be due simply to a requirement of endogenous cellular levels of these enzyme activities for invasion to occur. Proteinase activation likely does contribute to vesicle-stimulated invasion, however, because the combination of vesicles and cultured cells resulted in enhanced uPA activation. Enhanced uPA activation was not inhibited by cycloheximide and therefore likely involves activation of preexisting molecules on the cell or vesicle membranes. uPA converts plasminogen to plasmin, which activates a number of metalloproteinases as well as pro-uPA molecules. This notion may explain the finding that the addition of vesicles and cells leads to uPA activation. Because both cells and vesicles from ascites contain uPA and uPAR, the activation could be due to cellular activation of vesicle-associated uPA, vesicle activation of cell-associated uPA, or both. Furthermore, in addition to the enzymes analyzed here, other proteinases may be present in vesicles that could contribute synergistically to their invasion-stimulating properties. One such group of proteinases is the kallikreins; several members of this family are overexpressed in ovarian cancer, including human kallirein (hK) 5, hK6, hK7, hK8, hK11, and hK14 (24). The proposed potential biomarkers of ovarian cancer, hK5, hK6, hK10, and hK14 (25–29), appear to be the most likely candidates involved in determining the active proteolytic phenotype of vesicles.

Although just as effective in stimulating invasion, vesicles from early-stage cancers are less numerous than those from late-stage cancers. An increase in vesicle shedding with associated tumor volume may facilitate metastasis by providing larger quantities of invasion-stimulating membrane vesicles. Such vesicles were found to stimulate invasion of cultured ovarian cancer cells in a dose-dependent fashion, and therefore more vesicles would likely lead to greater invasive potential. Additional studies are necessary to determine more precisely how vesicles promote cellular invasion and to identify which vesicle-associated molecules are responsible for their invasion-stimulating abilities. In addition, quantitative analyses would be required to determine if there are statistically significant differences in MMP or uPA levels between vesicles from different cancer histologies, grades, and stages of disease. However, the results of this study suggest that vesicle-associated MMP and uPA are involved in promoting cellular invasion, and the activities of these molecules are largely associated with vesicles and not the cellular or ultracentrifuged supernatant of ascites. Interestingly, the combination of cells and vesicles enhanced uPA activation, thus supporting a role for protein-ases in vesicle-stimulated invasion. In summary, ovarian carcinoma-derived vesicles are powerful stimulators of cellular invasion, and shedding of proteinase-rich vesicles may serve to facilitate ECM breakdown and metastasis of epithelial ovarian cancer cells.

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