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 were 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 activation of some MMP zymogens as well as in the conversion of pro-uPA (scuPA) to its two-chain active form. In addition, MMPs and uPA can be secreted in an inactive or zymogen form (pro-uPA and pro-MMP), and these proteinases are potent inducers of proteinase-dependent invasion in cultured malignant epithelial ovarian cell lines. In addition, they may also be stored in membrane vesicles of 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 were 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.

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-cm² tissue 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 × g for 15 minutes, followed by centrifugation of the supernatant at 800 × g for 10 minutes to remove cells and debris. This supernatant was then centrifuged at 10,000 × 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

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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.
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-β1 integrin (Chemicon International, Temecula, CA), anti-HGFR (Cell Signaling Technology, Beverly, 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 fluorescence 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 in 1.5 mmol/L aminophenyl mercapturic acid (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 DevicesThermomax 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 Berhingt). Experiments were performed in triplicate, and at least three filters per well were counted.

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 on 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, β1 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 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-β1 integrin (Chemicon International, Temecula, CA), anti-HGFR (Cell Signaling Technology, Beverly, 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 fluorescence microscope.
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. 3B).

**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. 4A; 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. 5C). 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

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**Fig. 1.** Immunofluorescent staining of ascites-derived membrane vesicles. Membrane vesicles purified from a stage III ascites stain positive for (A) MT1-MMP, (B) β1 integrin, (C) epidermal growth factor receptor, and (D) uPAR. Bars, 50 μm. Appropriate controls for normal IgG and nuclear staining are not shown.

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**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.

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**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.

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**Table 1**

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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.
acellular 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...
VESICLES PROMOTE INVASION IN OVARIAN CANCER

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 contributes 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 kallikrein (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 ultrastructured supernatant of ascites. Interestingly, the combination of cells and vesicles enhanced uPA activation, thus supporting a role for proteinases 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.

REFERENCES


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