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Biochemical Characterization of Primary Peritoneal Carcinoma Cell Adhesion, Migration, and Proteinase Activity[1]

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Primary papillary serous carcinoma of the peritoneum (PPC) is clinically and histologically similar to advanced stage epithelial ovarian carcinoma. PPC classically presents with widespread intraperitoneal dissemination, superficial invasion, and minimal ovarian involvement. Surgical cytoreduction and combination chemotherapy utilized for patients with epithelial ovarian carcinoma have produced varying results for patients with PPC. These differences in response may be secondary to the stage of disease or due to biological differences in metastatic behavior between these carcinomas. In this study, short-term primary cultures of PPC and epithelial ovarian carcinoma (OVCA) were compared to enable biochemical comparison with respect to components of the metastatic cascade including adhesion, migration, and proteinase activity. These data demonstrated similar properties in adhesive profiles of PPC and OVCA, with preferential adhesion to type I collagen and vitronectin. Matrix-degrading proteinases including matrix metalloproteinases (MMP)-2, MMP-9, and urinary-type plasminogen activator were produced by both cell types. PPC migration was stimulated by multiple extracellular matrix proteins, whereas OVCA cells demonstrated maximal migration on type I collagen coated surfaces. Together our data suggest biochemical similarities between PPC and OVCA with respect to individual components of the metastatic cascade. © 1997 Academic Press

INTRODUCTION

Primary papillary serous carcinoma of the peritoneum (PPC) was first described by Swerdlow in 1959 [1]. This clinical and pathologic entity classically presents with little, if any, gross ovarian tumor involvement, a maximal ovarian diameter of 3–4 cm, minimal invasion into the ovarian stroma (<3 mm), and no other identifiable primary site [2–4]. Histologically, these tumors are identical to papillary serous ovarian carcinoma (OVCA) and present clinically with widespread peritoneal dissemination similar to advanced stage ovarian carcinomas. The diffuse presentation of these tumors is explained by the common coelomic epithelial origin of the peritoneal mesothelium and the müllerian duct epithelium. Several studies have shown that the histologic features and clinical behavior of peritoneal tumors are similar to advanced stage ovarian carcinomas [5–7]. PPC share identical microscopic features with serous ovarian carcinoma, yet are more widespread at initial diagnosis, and, therefore have a more guarded prognosis [2, 8–10]. Similarities between PPC and OVCA histology and presentation have led to the use of similar therapeutic approaches: surgical cytoreduction followed by multiagent chemotherapy. However, chemotherapeutic regimens utilized for OVCA yield varying results in patients with PPC. This difference in response may be secondary to the stage of disease or due to biological differences in the metastatic behavior between these carcinomas.

Metastatic tumor formation requires a coordinated cascade of events that includes tumor cell exfoliation, adhesion to a distant site, enzymatic degradation of the extracellular matrix (ECM), migration, proliferation, and ultimately, neovascularization [11]. Tumor cell adhesion, migration, and ECM degradation are active processes that are critical for metastasis formation [12]. Tumor cell adhesion employs cell surface molecules, such as integrins, to preferentially adhere to selective sites including liver, lung, and the peritoneal cavity [13, 14]. The ECM constituents that promote adhesion and motility, while varied depending on tissue site, include collagens and glycoproteins such as fibronectin (FN), laminin (LN), and vitronectin (VN) [15]. Enzymatic degradation of the ECM is mediated by proteinases such as matrix metalloproteinases (MMPs) and plasminogen activators (PAs) [16, 17]. MMPs are zinc-dependent endopeptidases with proteolytic activity directed against collagens and other components of the ECM [16, 18]. PAs are serine proteinases that catalyze the activation of plasminogen to plasmin, a broad specificity proteinase that is active in pericellular proteolysis of ECM proteins such as laminin and fibronectin [19, 20].

In this study, short-term primary cultures of PPC and

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OVCA were established to enable biochemical comparison and characterization of this unusual tumor with respect to individual components of the metastatic cascade (adhesion, migration, and proteinase activity). Our data demonstrate similarities in adhesive profiles and secretion of matrix-degrading proteinases. The common clinical, biochemical, and histological findings observed between PPC and OVCA suggest that our current utilization of similar chemotherapeutic agents for patients with these malignancies is justified.

MATERIALS AND METHODS

Materials. Bovine serum albumin (BSA), alkaline phosphatase-conjugated secondary antibodies, fibronectin, laminin, human collagen types I (CI) and IV (CIV), 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) tablets, D-Val-Leu-Lys–*p*-nitroanilide (VLKpNA), and cell culture reagents were purchased from Sigma (St. Louis, MO). Vitronectin and plasminogen were purified from human plasma as previously described [21]. Murine anti-integrin subunit antibodies were purchased from Gibco BRL (Grand Island, NY). High MW uPA was purchased from Calbiochem (San Diego, CA).

Cell culture. Two PPC and ten papillary serous OVCA short-term primary cell cultures were generated from patients with advanced stage carcinoma (III or IV). Tissue samples and ascites were obtained with Institutional Review Board approval from patients undergoing surgical intervention. Cell cultures derived from solid tumors and/or ascites were established and maintained in supplemented minimal essential medium (MEM) containing 20% fetal calf serum as previously described [22, 23]. Immunohistochemical staining with anticytokeratin was used as a positive control to verify the epithelial origin of the cells [22, 24]. Murine IgG2a was used as a negative immunohistochemical control. Cells remained viable for an average of eight passages, with the majority of experiments performed using cells from passages 3–6. Before determining proteinase activity, cells were washed with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS) and incubated with 1 m*M* EDTA in Ca^{2+}/Mg^{2+}-free PBS to release cells from the culture flask without adding exogenous proteinases. Cells were resuspended in serum-free MEM, replated at a constant density of 1×10^5 cells/ml, and incubated for 18 hr at 37°C. Unless otherwise specified, unconcentrated samples were used for all experiments. Ascites was centrifuged to remove cells and debris, analyzed for protein concentration using the BCA method (Sigma Chemical Co.), aliquoted, and stored at −20°C.

Cell attachment assay. Cells were maintained in short-term cultures and adhesive profiles were determined using immobilized types I and IV collagen, fibronectin, laminin, and vitronectin. Bovine serum albumin was used as a nonspecific control. Flat-bottom 24-well culture plates were coated by passive adsorption thin layer coating as previously described [25]. Cells (1.0×10^5) were added to the coated wells, incubated for 2 hr at 37°C, washed to remove unbound cells, fixed, stained, and enumerated [23]. Adhesion studies were repeated in triplicate.

Immunohistochemical staining. For immunohistochemical staining with anti-integrin antibodies, preconfluent cells were harvested and subcultured into 60-mm plastic dishes at a density of 1×10^5 cells/ml. After 24 hr the cells were rinsed with PBS and fixed with a −20°C solution of acetone:methanol (1:1, vol:vol) for 2 min. Fixed cells were washed twice for 5 min with PBS and blocked for 30 min at room temperature with 20 m*M* TRIS (pH 7.5)/0.15 *M* NaCl/1% NP40/3% BSA (buffer 1). Primary antibodies were added to fixed cells at a dilution of 1:50 in buffer 1, incubated overnight at room temperature in a humid chamber with gentle rotation, and washed twice with buffer 1 and twice with 20 m*M* Tris/0.15 *M* NaCl/1% NP40, pH 7.5 (buffer 2). The corresponding alkaline phosphatase-conjugated secondary antibody was added (1:200 dilution in buffer 1), incubated for 1 hr at room temperature, and washed as described above. The alkaline phosphatase substrate BCIP/NBT was added and plates were developed for 20 min before stopping with 10 m*M* Tris, pH 8.5, containing 5 m*M* EDTA. Control plates utilized an isotype-matched IgG as a negative control. Immunohistochemical staining was performed in triplicate. Positive integrin expression was defined as >80% cells staining positively with anti-integrin antibodies.

Zymographic analysis. Zymograms demonstrating gelatinolytic activity in nonreduced samples were prepared by incorporating 0.1% gelatin into 9% polyacrylamide gels according to the method of Heussen and Dowdle [26]. Prior to electrophoresis, samples (20 μl) were incubated with nonreducing Laemmli sample dilution buffer containing 2.5% SDS for 30 min at room temperature. Latent metalloproteinases were activated by incubating the sample with 1.5 m*M* aminophenylmercuric acetate (APMA) for 1 hr at 37°C prior to electrophoresis. Additionally, control samples contained 100 μM *o*-phenanthroline during incubation to inhibit metalloproteinase activity. Following electrophoresis, gels were washed with 2.5% Triton X-100 for 30 min at room temperature to remove SDS and incubated in 0.1 *M* glycine, 10 m*M* $CaCl_2$, 1 μM $ZnCl_2$, pH 8.3, at 37°C overnight. In the inhibition experiments, *o*-phenanthroline (100 μM) was included in the overnight incubation buffer. After staining with Coomassie blue, regions of proteolytic activity were visualized as clear zones against a blue background.

Analysis of proteinase activity. Total plasminogen activator activity was quantitated by incubating serum-free conditioned medium with 0.3 μM plasminogen and the plasmin substrate VLKpNA (0.3 m*M* final concentration). The amidolytic activity of generated plasmin was detected at 37°C by monitoring the absorbance at 405 nm using a Molecular Devices Thermomax plate reader (Menlo Park, CA).

Cell migration assay. In order to directly assess tumor cell motility without the confounding problem of cell division, a short-term cell migration assay was used according to the method of Albrecht-Buehler [27] as modified by Woodley *et al.* [28]. Briefly, coverslips were coated with colloidal gold particles and placed in polystyrene 24-well flat-bottom plates. Extracellular matrix molecules were added at a concentration of 10–50 μg/ml in 300 μl of Hanks' balanced salt solution with calcium and magnesium and incubated for 2 hr at 37°C. Migration was evaluated on coverslips coated with type I collagen, type IV collagen, fibronectin, laminin, and vitronectin. Bovine serum albumin was included as a nonspecific protein control. Previous studies with radiolabeled matrix components demonstrated that approximately 50–87% of the added matrices were adsorbed to the coverslips [28]. Tumor cells (2×10^3) were plated in each well in serum-free growth medium. Cultures were incubated at 37°C for 15 hr, washed, and fixed in 3% formaldehyde in PBS. To quantify migration, 100 cells were examined (in triplicate) with an Olympus inverted microscope under dark field optics, and the percentage of area of each field occupied by phagokinetic tracks was calculated using computer-assisted image analysis as previously described [28].

Statistical analysis. All statistical analysis was performed using the SAS statistical analysis software (SAS Institute Inc., Cary, NC). Statistical tests included one-way analysis of variance, one sample *t* test, and independent sample *t* test. A probability level of <0.05 was chosen to represent statistical significance.

RESULTS

Short-term primary cell cultures of PPC and OVCA were compared with respect to adhesion, integrin expression, migration, and proteinase activity. Specifically, 2 cultures of papillary serous PPC were compared to 10 cultures of papillary serous OVCA. For comparison, all data shown below utilize 2 specific cultures designated as PPC1 and OVEA6. The behavior of these individual cultures is representative of results obtained from all cultures tested (2 PPC and 10 OVCA short-term primary cell cultures, respectively).

Cell matrix adhesion. Immunohistochemical staining of short-term primary cultures of PPC and OVCA cells showed positive cytokeratin reactivity (data not shown), verifying the epithelial origin of the cells as previously reported by other investigators [29]. Since adhesion represents an initial step in tumor invasion and metastasis, the adhesive profile of short-term cultures of PPC was determined and compared to primary OVCA cells. Data from a representative experiment using PPC1 are shown in Fig. 1. Early passage PPC cells exhibited preferential adhesion to type I collagen, type IV collagen, and vitronectin (Fig. 1A), whereas short-term primary OVCA cells adhered predominantly to type I collagen and vitronectin (Fig. 1B). Although the adhesive profiles were similar, the magnitude of PPC adhesion was greater than that of OVCA cells.

Preferential adhesion to type I collagen and vitronectin suggested that PPC cells may express integrins known to mediate adhesion to collagen ($\alpha2\beta1$) and/or vitronectin ($\alpha v\beta3$, $\alpha v\beta5$) [15]. Immunohistochemical staining of fixed and permeabilized adherent cells demonstrated the presence of the integrins $\alpha2\beta1$, $\alpha v\beta3$ in the PPC cells and the $\alpha2\beta1$, $\alpha v\beta5$ integrins in the OVCA cells (Table 1).

Analysis of proteinase activity. Tumor cell-mediated proteolysis of ECM components is required for invasion and metastasis. Previous studies have found that MMP and/or PA activity are necessary for tumor invasion and metastatic spread and we have previously reported that OVCA cells secrete both PAs and MMPs [22, 24, 30]. Ascites derived from patients with PPC and OVCA were evaluated to determine which proteinases are present in the *in vivo* microenvironment. Both MMP and PA activity were detected in the ascites derived from both PPC and ovarian cancer patients. Using SDS–PAGE gelatin-substrate zymography, two gelatinolytic proteinases were observed in the ascitic fluids, migrating at 72 and 92 kDa (Fig. 2A, lanes 1 and 2). These proteinases were activatable with aminophenylmercuric acetate and inhibited by 1,10-phenanthroline (data not shown), confirming their identity as MMP-2 (gelatinase A) and MMP-9 (gelatinase B), respectively. PA activity was evaluated by zymography and revealed a band which comigrated with the urinary-type PA (uPA) standard (data not shown). Total uPA activity in ascites was quantitated using a coupled colorimetric assay to monitor the amidolytic activity of generated plasmin. Ascites derived from both PPC and ovarian carcinoma patients contained significant uPA activity (Fig. 2B).

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 tumor cells were the source of these ECM degrading proteinases, short-term primary cell cultures of OVCA and PPC cells were established and analyzed for proteinase production. Tumor cell conditioned medium from both carcinomas was found to have low uPA activity (Figs. 3A and 3B) that increased somewhat with successive passage. Although uPA production by primary OVCA cells was 10-fold greater than that of PPC cells, conditioned medium from both cell types contained low uPA activity relative to established cultures of OVCA [30]. However, uPA activity increased with passage number, suggesting that uPA production may be subject to negative regulatory influences *in vivo.* Analysis of MMP activity by zymography demonstrated both MMP-2 and

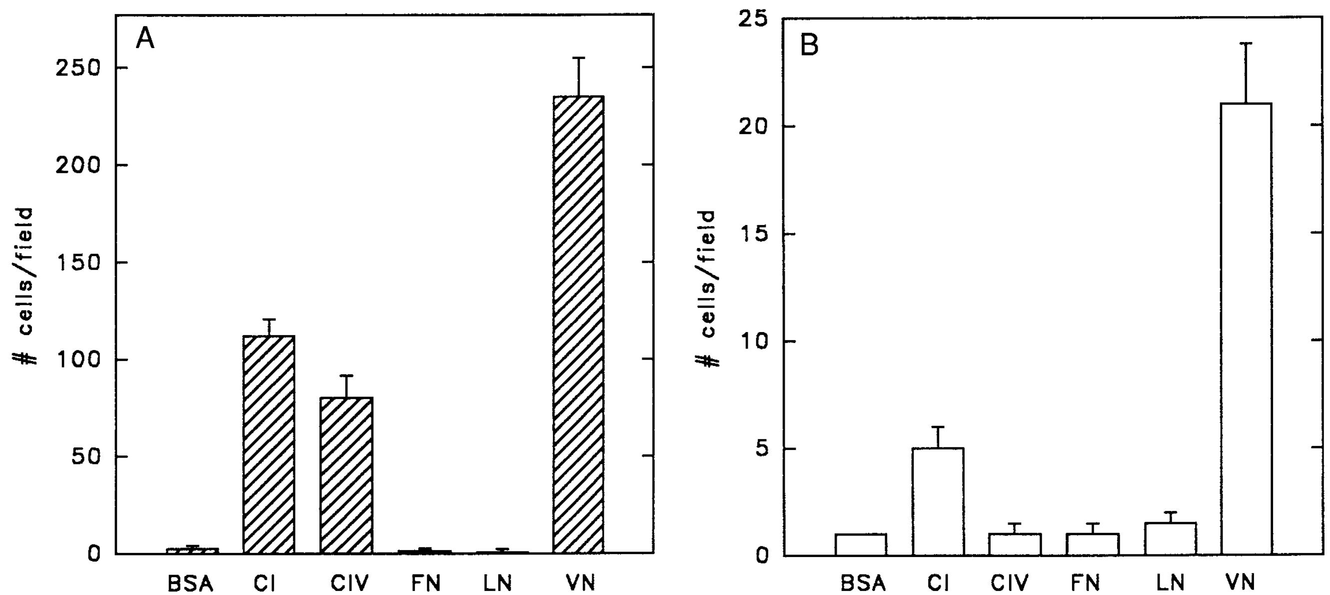

FIG. 1. Adhesion of PPC and OVCA cells to extracellular matrix proteins. Tissue culture wells were coated with albumin (BSA), type I collagen (CI), type IV collagen (CIV), fibronectin (FN), laminin (LN) or vitronectin (VN), seeded with 1×10^5 cells, and adherent cells were quantitated as described under Materials and Methods. (A) Adhesion of PPC1 cells; (B) Adhesion of OVEA6 cells.

MMP-9 in conditioned medium from the OVCA and PPC cell cultures (Fig. 2A, lanes 3 and 4).

Cellular migration. Enhanced tumor cell migration is associated with increased invasive potential. Analysis of cell migration on colloidal gold coated coverslips overlaid with ECM proteins indicated that PPC migration was increased on all matrix proteins whereas OVCA cells demonstrated enhanced migration primarily on interstitial type I collagen (Fig. 4). PPC displayed significantly increased migration on CI, CIV, FN, LN, and VN compared to BSA ($P < 0.05$) (Table 2). However the increase in PPC migration between the ECM components is not statistically significant ($P = 0.07$). OVCA cells also demonstrated significantly increased migration on CI, CIV, and VN compared to BSA ($P = 0.001$). However, OVCA migration was not enhanced when exposed to FN or LN in contrast to PPC. Significant differences in cellular migration were observed between PPC and OVCA on all ECM components except CIV ($P = 0.14$). Maximal migration was observed on CI for both PPC and OVCA ($P < 0.05$).

TABLE 1
Integrin Staining of Integrin Subunits in OVCA and PPC Cells

Integrin	PPC	OVCA
$\alpha 2\beta 1$	+	+
$\alpha v\beta 3$	+	−
$\alpha v\beta 5$	−	+

Note. Cells were fixed and immunostained with alkaline phosphatase as described under Materials and Methods.

DISCUSSION

Histologically and clinically, PPC resembles metastatic OVCA with widespread peritoneal dissemination, yet PPC has normal or minimally involved ovaries [6, 7]. While the metastatic spread of OVCA appears to be primarily mediated by direct exfoliation of surface cells resulting in diffuse peritoneal implantation, the etiology for PPC is uncertain. It has been hypothesized that PPC may originate from the ovarian surface epithelium with subsequent intraperitoneal spread or alternatively may involve multifocal malignant transformation of the abdominopelvic mesothelium [31–33]. PPC has an epithelial phenotype which is almost identical to OVCA, suggesting that field carcinogenesis occurs with a common response to an unknown oncogenic stimulus [6]. The observation that abdominopelvic mesothelium can produce serous tumors similar to those from ovarian surface epithelium is expected since both share the same embryologic derivation from coelomic epithelium. The multifocal origin of this tumor was further supported by Muto *et al.,* who demonstrated allelic loss on chromosomes 1, 3, 4, and 17 [34]. Also, the mutational pattern of the p53 gene varied at tumor sites within the same patient in four of six cases of PPC. While PPC is similar to OVCA based on similar light and electron microscopic, histochemical, and immunohistochemical features, the biological behavior of PPC cells has not been previously analyzed. In this study we have analyzed the properties of tumor cell adhesion, migration, and proteinase production in short-term primary cell cultures of PPC and OVCA cells in an attempt to enhance our understanding of these malignancies.

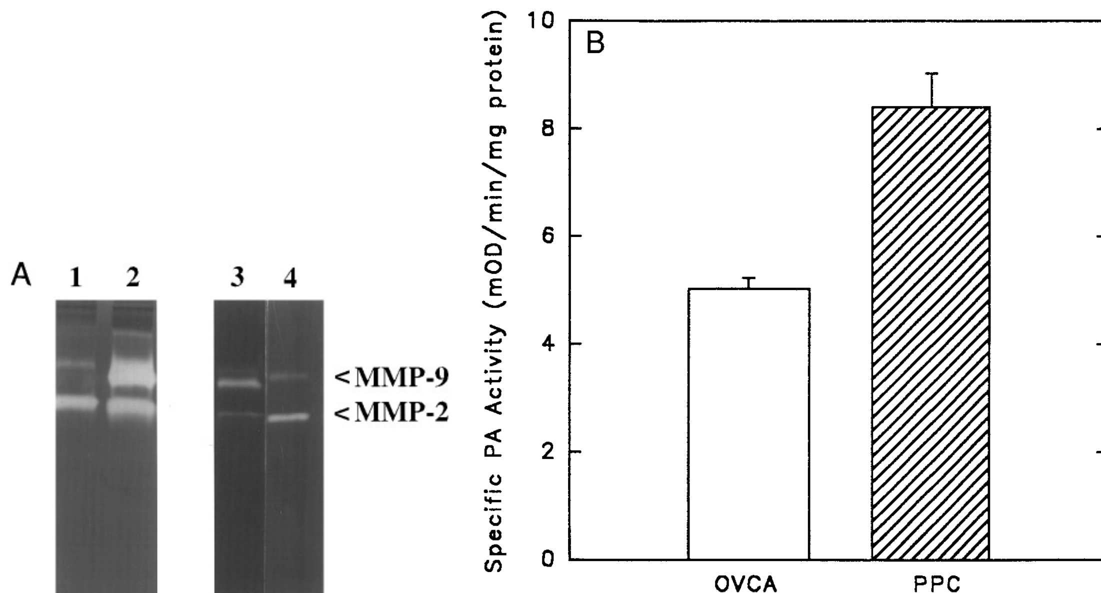

FIG. 2. Analysis of proteinase activity in ascites and conditioned medium. (A) Determination of MMP activity by zymography. Ascites fluid (50 μg protein) obtained from patients with (1) PPC or (2) OVCA and serum-free tumor conditioned medium from short-term primary cultures of (3) PPC1 cells or (4) OVEA6 cells were activated with APMA and analyzed by gelatin zymography as described under Materials and Methods. The migration position of MMP-2 and MMP-9 standards are indicated by arrowheads. (B) Plasminogen activator activity in ascites. Total plasminogen activator activity in ascites fluid (50 μg) obtained from patients with PPC1 (hatched bar) or OVEA6 (open bar) were analyzed using a coupled spectrophotometric assay as described under Materials and Methods.

Metastatic spread of solid tumors is dependent upon a critical cascade of events that includes tumor cell adhesion to a distant site, ECM degradation, migration, proliferation, and ultimately, neovascularization [11]. OVCA cells preferentially adhere to interstitial type I collagen, a protein which is abundant in the mesothelial-lined peritoneal cavity [23]. Results from this study demonstrate that PPC cells also preferentially adhered to type I collagen and displayed positive $\alpha 2\beta 1$ immunoreactivity. Furthermore OVCA and PPC cells also adhered to vitronectin and ex-

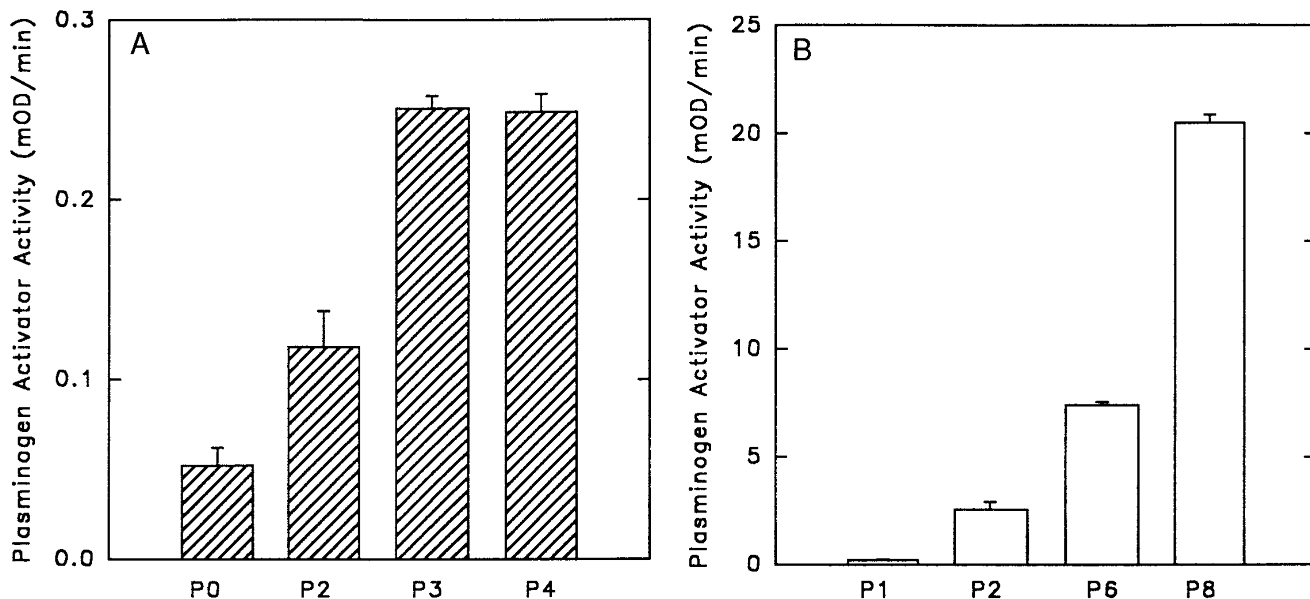

FIG. 3. Plasminogen activator activity in PPC and OVCA cell conditioned medium. Cells (1×10^5) were grown for 18 hr in serum-free medium and total plasminogen activator activity in conditioned medium was analyzed using a coupled spectrophotometric assay. The passage number is indicated beneath each bar. (A) PPC1 cell conditioned medium; (B) OVEA6 cell conditioned medium.

pressed integrins with known affinities for vitronectin ($\alpha v\beta 5$ and $\alpha v\beta 3$, respectively). The function of these different αv receptor complexes may be dependent on cell type. Cell adhesion is associated with the formation of focal adhesion contacts, the clustering of integrin receptors, and organization of the actin filaments in the cytoskeleton into bundled structures [13, 35]. Therefore, although several integrins can interact with one ligand, distribution and cytoskeletal association may differ.

Cell motility is a biological parameter distinct from cell matrix attachment. Some tumor cells can invade tissue parenchyma passively, through cell growth and expansion. However, active motility enhances metastatic potential and is a behavior displayed by most metastatic cells. Individual ECM proteins have been found to induce a motility response in tumor cells through integrin receptors [15]. In this study, cellular migration of both PPC and OVCA cells was significantly stimulated by interstitial type I collagen, a protein which is predominant in the peritoneal ECM and to which both cell types displayed preferential adhesion. OVCA migration was maximally stimulated (4.2-fold) when exposed to interstitial type I collagen while PPC migration was stimulated in a similar manner by all ECM components (twofold). This large variety of stimuli could provide PPC cells with a physiologic advantage for traversal through different microenvironments during the metastatic process. OVCA motility differs from PPC in that migration was not stimulated by fibronectin or laminin. These observed differences in stimulated motility may in part explain the widespread and superficial nature of PPC as compared to the deeply invasive metastatic lesions of OVCA.

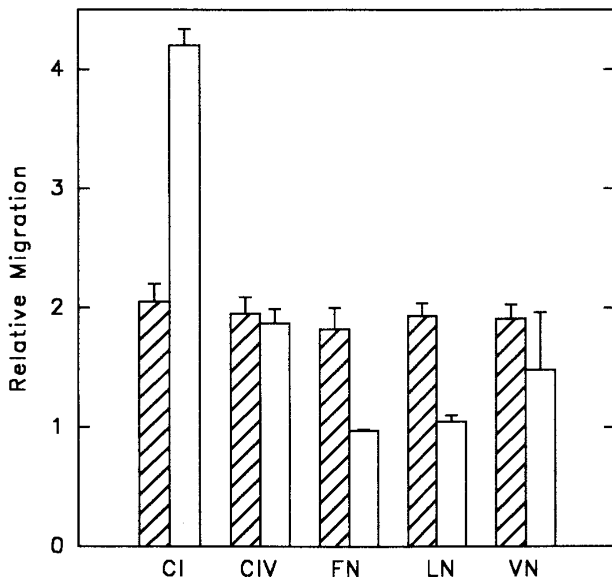

FIG. 4. Analysis of PPC and OVCA cell migration. Cells (2×10^3) were plated onto coverslips containing colloidal gold particles overlaid with the extracellular matrix proteins type I collagen (CI), type IV collagen (CIV), fibronectin (FN), laminin (LN) or vitronectin (VN). Coverslips coated with bovine serum albumin were included as a control. Migration was quantified as described under Materials and Methods and is expressed relative to the bovine serum albumin control. PPC1 cell migration (hatched bars); OVEA6 cell migration (open bars).

TABLE 2
Comparison of PPC and OVCA Migration on ECM Components Relative to BSA

Cell line	CI	CIV	FN	LN	VN
PPC					
(mean)	2.05*	1.95*	1.82*	1.93*	1.91*
OVEA6					
(mean)	4.20*	1.87*	0.97	1.05	1.48*

* Indicates $P < 0.05$.

Following adhesion, metastatic tumor cells elaborate a variety of proteolytic enzymes which initiate degradation of ECM macromolecules and facilitate invasion into the proteolytically modified tissue. We have previously shown that OVCA cells overexpress MMPs and PAs relative to normal ovarian tissue, catalyze the degradation of multiple ECM proteins, and invade a synthetic basement membrane in an MMP-2-dependent manner [22–24, 30]. Enhanced production of uPA and its receptor has also been associated with ovarian cancer progression [20, 24]. The current study demonstrates that PPC cells also produce matrix-degrading proteinases including MMP-2, MMP-9, and uPA.

The biochemical similarities in adhesive substratum preferences and proteinase secretion profiles observed between PPC and OVCA suggest a common phenotypic behavior for these carcinomas. It is due to the observed histologic and clinical similarities between both malignancies that current chemotherapeutic options for PPC have mimicked those for OVCA. Survival rates for patients with PPC are quite varied; some authors have noted worse outcomes than those for OVCA, while recent studies have demonstrated equivalent outcomes with modern chemotherapy [36–38]. Since the recent introduction of platinum-based chemotherapy regimens, including platinum–paclitaxel, long-term survival data is unavailable [37, 40–42]. It is unclear whether the poor prognosis of PPC is due to the advanced clinical stage or to inherent biological factor(s) which renders PPC more aggressive than stage-matched OVCA. While a potential limitation of our study is that only 2 PPC and 10 OVCA short-term primary cell lines were evaluated, our data suggest that based on the biochemical similarities between PPC and OVCA, the utilization of chemotherapeutic regimens as for epithelial ovarian carcinoma is warranted.

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