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Ovarian carcinoma spheroids disaggregate on type I collagen and invade live human mesothelial cell monolayers

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Abstract

Ovarian carcinoma patients frequently develop malignant ascites containing single and aggregated tumor cells, or spheroids. Spheroids have been shown to be resistant to many therapies, but their contribution to ovarian cancer dissemination remains undetermined. We have previously shown that ascites spheroids adhere to extracellular matrix (ECM) proteins and live human mesothelial cells via β1 integrin subunits. Here, we assessed the ability of spheroids that were generated from the human ovarian carcinoma cell line NIH:OVCAR5 to disseminate and invade *in vitro*. Spheroids were seeded on ECM proteins for 24 h. While laminin and type IV collagen stimulated some cell migration, spheroids completely disaggregated on type I collagen substrates. A monoclonal antibody against the β1 integrin subunit significantly inhibited disaggregation on all proteins tested. To test their invasive ability, spheroids were added to monolayers of live human LP9 mesothelial cells. Within 24 h, the spheroids adhered and disaggregated on top of the monolayers, and within a week had established foci of invasion encompassing a 200-fold larger surface area. Addition of a monoclonal antibody against the β1 integrin subunit drastically reduced spheroid invasion into the mesothelial cell monolayers. GM 6001, a broad-scale matrix metalloproteinase inhibitor, also significantly blocked spheroid invasion into the mesothelial cell monolayers. ε-amino-N-caproic acid, a serine protease inhibitor, partially inhibited spheroid invasion. Based on their ability to attach to, disaggregate on, and invade into live human mesothelial cell monolayers, spheroids should thus be regarded as potential contributors to the dissemination of ovarian cancer.

Abbreviations: BSA – bovine serum albumin; DMSO – dimethyl sulfoxide; ECM – extracellular matrix; EDTA – ethylenediaminetetraacetic acid; EHS – Engelbreth–Holm–Swarm; FBS – fetal bovine serum; mAb – monoclonal antibody; mIgG – normal mouse immunoglobulin; MMP – matrix metalloproteinase; PBS – phosphate buffered saline; εACA – ε-amino-n-caproic acid; uPA – urinary-type plasminogen activator

Introduction

Ovarian carcinoma is the most common cause of death from gynecologic malignancy of women in the United States. In 70% of the patients, extensive intraperitoneal dissemination has occurred by the time of initial diagnosis, leading to poor long-term survival [1]. In the current model of ovarian cancer metastasis, tumor cells are shed into the peritoneal cavity, where they seed the mesothelial surfaces and establish secondary tumors. Secretion of vascular permeability factors and lymphatic obstruction by tumor cells leads to an accumulation of ascites, which further facilitates tumor cell transport throughout the peritoneal cavity [2–5]. The mesothelial cells composing the serosal surfaces of the peritoneum normally provide a non-adhesive surface. In the ascites of ovarian cancer patients, tumor cells that do not adhere to the mesothelium often aggregate, forming spheroids. While spheroids have been used as models for tumor microenvironments and have been found to be resistant to some forms of chemotherapy and radiation, their adhesive and invasive properties have not been established, and their contribution to the dissemination of ovarian cancer remains undetermined [6–13].

Cancer metastasis occurs by a multi-step process involving alterations in tumor cell adhesion, migration, and invasion. It has been shown that ovarian carcinoma cells adhere to human mesothelial cells via β1 integrin interactions with extracellular matrix (ECM) proteins, and CD44 interactions with its ligand hyaluronan [14–19]. Our previous studies have shown that spheroids, either obtained from ascites of ovarian carcinoma patients or generated from the NIH:OVCAR5 cell line, are capable of β1 integrin-mediated adhesion

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to ECM proteins and to live human mesothelial cell monolayers [20, 21]. These results, demonstrating that spheroids are capable of the initial steps of metastasis, necessitate further study into their invasive potential.

A critical component of tumor invasion involves enzymatic degradation of the ECM, allowing penetration of the basement membrane and access to the vasculature. A complex balance of proteolytic enzymes mediates this process, including matrix metalloproteinases (MMPs) and serine proteases, among others. MMPs are zinc-dependent peptidases capable of degrading collagen, proteoglycans, gelatin, and fibronectin. MMP-2, MMP-9, and MTI-MMP have been implicated in the pathogenesis of ovarian cancer, and a variety of MMPs have been found to be expressed by tumor cells obtained from the ascites fluid [22, 23]. Plasminogen activators, which aid in the conversion of plasminogen to plasmin, allow further amplification of ECM degradation, and have also been observed in ovarian carcinoma [24–25].

In the present study, we determined the invasiveness of ovarian carcinoma spheroids generated from the NIH:OVCAR5 cell line by establishing their ability to disaggregate and invade into live mesothelial cell monolayers. Furthermore, we identified cell adhesion molecules and proteases thought to play a role in facilitating spheroid invasion. Our results suggest that ascites spheroids may present a previously unrecognized and untargeted source of secondary spread in ovarian carcinoma.

Materials and methods

Materials

Type TV collagen from mouse Engelbreth–Holm–Swarm (EHS) tumor was purchased from Trevigen (Gaithersburg, MD). Type I collagen from human placenta was purchased from Southern Biotech (Birmingham, AL). Vitrogen 100 (bovine type I collagen) was purchased from Collagen Biomaterials (Palo Alto, CA). Mouse EHS laminin was purchased from Invitrogen (Carlsbad, CA). Human plasma fibronectin, purified as described, was provided by Dr James McCarthy, University of Minnesota [26]. Bovine serum albumin (BSA), was purchased from Pierce Biotechnology (Rockford, IL).

Antibodies and inhibitors

Purified immunoglobulin of mouse monoclonal antibody (mAb) P5D2, which blocks the adhesive activity of the human β1 integrin subunit, was provided by Dr Leo Furcht (University of Minnesota). Normal mouse immunoglobulin (mIgG) was purchased from Sigma (St. Louis, MO). GM 6001 was purchased from Calbiochem (San Diego, CA). ε-amino-n-caproic acid (εACA) was purchased from Sigma.

Cell culture

The ovarian carcinoma cell line NIH:OVCAR5 was obtained from Dr Judah Folkman (Harvard Medical School, Boston, MA), and was chosen for its ability to mimic ovarian carcinoma progression *in vivo* when injected into mice [27, 28]. This cell line was maintained in RPMI 1640 media with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 0.2 U/ml insulin, and 50 U/ml penicillin G/streptomycin. The human peritoneal mesothelial cell line LP9 was purchased from the Coriell Cell Repository (Camden, NJ), and maintained in a 1:1 ratio of M199 and MCDB110 media, supplemented with 15% FBS, 2 mM glutamine, 5 ng/ml epidermal growth factor, 0.4 μg/ml hydrocortisone, and 50 U/ml penicillin/streptomycin. Both cell lines were cultured in 75 mm^2 tissue culture flasks in a 5% CO_2 humidified incubator at 37 °C.

Spheroid culture

Spheroids were generated using a liquid overlay technique as we have previously described [20]. 24-well tissue culture plates were coated with 500 μl of 0.5% SeaKem LE agarose in complete media, and allowed to solidify for 30 min at room temperature. NIH:OVCAR5 cells were released from monolayer cultures with 0.5% trypsin/2 mM ethylenediaminetetraacetic acid (EDTA), and resuspended in complete media at 50,000 cells/ml. NIH:OVCAR5 cell suspensions were transferred on top of the agarose-coated wells at a volume of 1 ml/well, and incubated for 48 h at 37 °C. Prior to use in assays, NIH:OVCAR5 spheroids were centrifuged at $10 \times g$ for 3 min to remove single cells.

Spheroid disaggregation assays

96-well plates were coated overnight at 37 °C with 5 μg/ml laminin, fibronectin, monomeric human type I collagen, type IV collagen, and BSA. The wells were then blocked for 1 h with 2 mg/ml BSA and then rinsed twice with phosphate buffered saline (PBS). NIH:OVCAR5 spheroids were suspended in RPMI media and approximately five spheroids were added to each of the coated wells. Spheroids were digitally photographed at the time of plating, incubated at 37 °C for 24 h, and then rephotographed. The pixel area of the cells at both time points was determined using Adobe Photoshop by outlining the entire area of the spheroids or the dispersed cells with the lasso tool. The total area included the area of the disaggregated spheroid plus the area of any dispersed single cells in the near vicinity that were most likely to have come from the disaggregated spheroid. Spheroid disaggregation was determined as the fold change in pixel area of the spheroids from 0 to 24 h.

For inhibition assays, 50 μl suspensions of spheroids in RPMI were added to the coated wells and allowed to attach for 1 h at 37 °C. 50 μl solutions of

20 μg/ml mIgG or a mAb against the β1 integrin subunit in RPMI were then added to the wells, to obtain a final diluted concentration of 10 μg/ml in each well. Assays were then performed as described above. Values shown represent the average fold change in area of 30–50 spheroids ± standard error.

Collagen substrate disaggregation assays

Spheroid disaggregation was compared on polymerized bovine type I collagen gels versus monomeric human type I collagen film using the method described by Fassett et al. [29]. To make the polymerized type I collagen gels, 0.5 ml of 0.1 M NaOH was added to 4 ml of Vitrogen 100 to neutralize the acid in the collagen solution according to manufacturer's instructions. 0.5 ml of 5X RPMI media was mixed with the neutralized Vitrogen 100 to yield a gel solution of $\sim$ 2.4 mg/ml. Subsequent serial dilutions with 1X RPMI yielded 1.2, 0.6, and 0.3 mg/ml type I collagen solutions. The type I collagen gel solutions were added to the wells of a 24-well plate, and incubated at 37 °C in a drying oven for 1 h to polymerize. After polymerizing, the gels were rinsed twice with PBS, and blocked with 1% BSA for 20 min. To make the monomeric type I collagen films, wells were coated overnight at 37 °C with 5 μg/ml human type I collagen. The wells were then blocked for 1 h with 2 mg/ml BSA and then rinsed twice with PBS. Spheroids suspended in RPMI were added atop the polymerized gels or monomeric film at a density of 5–10 spheroids per well. 10 μg/ml of a mAb against the β1 integrin subunit or mIgG was added for the inhibition assays. Spheroids were digitally photographed at the time of plating, incubated at 37 °C for 24 h, and then re-photographed. The pixel area of the spheroids at both time points was determined using Adobe Photoshop. Spheroid disaggregation was determined as the fold change in pixel area of the spheroid from 0 to 24 h. Values shown represent the average fold change in area of 50–90 spheroids ± standard error.

Mesothelial monolayer invasion

To assess invasion, LP9 human mesothelial cells at 70,000 cells/well were added to 48-well plates and allowed to grow to confluence for 96 h, and then were gently rinsed with RPMI. Spheroids were resuspended in complete media to obtain approximately 5–10 spheroids/ml, and 1 ml of the suspension was added to each well atop the mesothelial cell monolayers. Spheroids were digitally photographed at the time of plating, and then were incubated at 37 °C. At time-points of 1, 4, and 7 days from the initial plating, spheroids were re-photographed. Invasion was quantified by calculating the fold change in area of the spheroids, determined by dividing the pixel area of invading foci at each time-point by the spheroid pixel area on the initial day of plating. The pixel area of the cells at all time points was determined using Adobe Photoshop by outlining the entire area of the spheroids or the dispersed cells with the lasso tool. Values shown represent the average fold change in area of 150–200 spheroids ± standard error.

Due to the size of the spheroids, they were readily distinguishable from the mesothelial cell monolayers. Spheroid invasion was defined as the disaggregation of the spheroid and its lateral growth within the same plane as the mesothelial cell monolayer. This involved mesothelial cell retraction and/or displacement by the cells of the spheroid, such that there were no mesothelial cells beneath the ovarian cancer cells. Visualization of invasion was achieved without the need for separate labeling of the cells, as the invading ovarian cancer cells were within the same plane of focus as the mesothelial cell monolayers, while cells overlying but not invading the monolayer appeared as a dense cell layer that was slightly out of focus.

For inhibition of invasion, 5–10 spheroids in 500 μl of complete media were added to each well and incubated at 37 °C for 1 h. 500 μl of a 2X inhibitor solution was then added to each well, at a final diluted concentration of 1X. Final concentrations of mIgG and the mAb against the β1 integrin subunit were 1 μg/ml; concentrations of GM 6001 ranged from 1–10 μM; and concentrations of εACA ranged from 1–10 mM. Since the GM 6001 was dissolved in DMSO, DMSO (10 μM) (Sigma) was used as a control for 10 μM GM 6001. Assays were then performed as described above. On each day of the assay, 500 μl of supernatant was removed from each well and was replenished with 500 μl of the appropriate fresh inhibitor solution. Values shown represent the average fold change in area of 30–100 spheroids ± standard error.

Statistical analysis

Student's *t*-test was performed as a test of significance with the use of Microsoft Excel 2000 (Microsoft, Redmond, WA). *P*-values of < 0.01 were considered to indicate statistically significant differences.

Results

Spheroids disaggregate on ECM molecules by use of β1 integrin subunits

Tumor cells can be stimulated to migrate in response to environmental cues such as ECM components and other chemotactic factors [30, 31]. Typically, quantification of tumor cell migration relies on assays that determine the ability of single tumor cells to migrate through a membrane toward a chemoattractant. Because such assays are designed for single cells, they do not sufficiently measure cell migration out of a more complex aggregate. Therefore, to assess spheroid cell migration, NIH:OVCAR5 spheroids were seeded into wells coated with 5 μg/ml of ECM components

and migration was determined by measuring the fold change in surface area of the spheroid over time. On type IV collagen and laminin, tumor cells migrated outward from the spheroid, resulting in a 2-fold change in area over 24 h when incubated with mIgG (Figure 1a). Little change was seen on fibronectin. However, on type I collagen the spheroid totally disaggregated. The cells migrated outward and spread to form a monolayer at the site of initial spheroid attachment, resulting in a 9-fold change in area compared to a BSA control, when incubated with mIgG (Figure 1a).

Integrins are key mediators of cell-matrix adhesion and contribute to ovarian cancer cell migration [19]. To determine the role that integrins play in spheroid disaggregation, NIH:OVCAR5 spheroids were allowed to adhere to ECM-coated wells for 30 min before the addition of a mAb against the $\beta 1$ integrin subunit

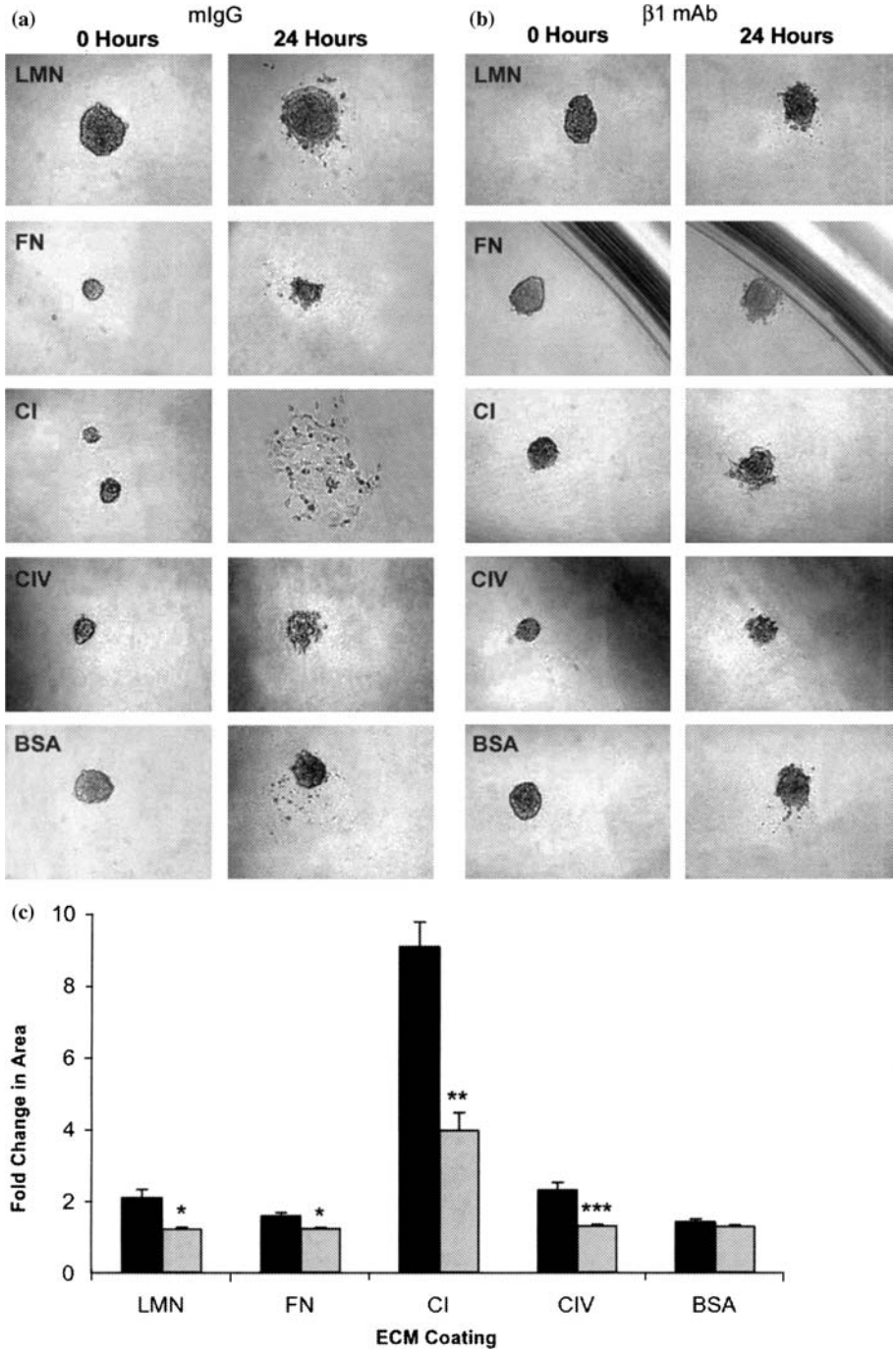

Figure 1. Spheroids spread on laminin and type IV collagen, and disaggregate on type I collagen. Spheroids were added to ECM protein-coated wells with 10 μg/ml of either mIgG or a blocking mAb against the $\beta 1$ integrin subunit. Photographs of spheroids on ECM proteins at 0 h and 24 h with mIgG (a) or a mAb against the $\beta 1$ integrin subunit (b), on laminin (LMN), fibronectin (FN), type I collagen (CI), type IV collagen (CIV) or bovine serum albumin (BSA). Spheroid disaggregation on ECM proteins was quantitated with mIgG (c, black bars) or a mAb against the $\beta 1$ integrin subunit (c, gray bars). Values shown represent the average fold change in pixel area of 30–50 spheroids over 24 h from quadruplicate experiments, $\pm$ standard error. Magnification 100×. Significance: $^{*}P = 0.001$, $^{**}P = 0.002$, $^{***}P = 0.00004$.

(Figure 1b). 10 μg/ml of the β1 integrin mAb almost completely eliminated cell migration out of the spheroids on laminin, fibronectin, and type IV collagen, and reduced spheroid disaggregation area on type I collagen by 50% compared to mIgG (Figures 1b, c). These data imply that the β1 integrin subunit is a key player in ovarian carcinoma spheroid disaggregation and tumor cell migration.

Spheroids disaggregate on polymerized type I collagen gels

Previous studies have shown that tumor cell adhesion, spreading, and signaling differ on monomeric type I collagen films *versus* polymerized type I collagen gels [29, 32, 33]. To determine whether ovarian carcinoma spheroids could be stimulated to disaggregate when placed on polymerized type I collagen gels, NIH:OVCAR5 spheroids were seeded on 0.3, 0.6, 1.2, and 2.4 mg/ml polymerized type I collagen gels for 24 h (Figure 2). On the less dense gels, the ability of cells to migrate outward from the spheroid was restricted, although spreading was still able to occur. For example, on 0.3 mg/ml gels, there was a 2-fold change in area, and on the 0.6 mg/ml gels, there was a 3-fold change in area (Figures 2a, c). As the density of the gels increased, spheroids began to disaggregate and spread, resulting in a 4-fold change in area on 1.2 mg/ml gels and a 5-fold change on 2.4 mg/ml gels over 24 h (Figures 2a, c). To determine the contribution of β1 integrins to disaggregation, 10 μg/ml of a mAb against the β1 integrin subunit was added to the wells (Figures 2b). The β1 integrin mAb inhibited disaggregation of the spheroids by approximately 50% on all concentrations of type I collagen gels compared to mIgG (Figure 2c).

Spheroids invade human mesothelial cell monolayers

The mechanism of ovarian carcinoma dissemination relies on ovarian tumor cell adhesion to and invasion of the peritoneal mesothelium, followed by proliferation at the site of invasion. The conventional invasion assays that quantitate single tumor cell migration through a cell monolayer grown on a filter fail to allow quantitation of invasion by spheroids. We have previously shown that both NIH:OVCAR5 and patient ascites spheroids can adhere to live monolayers of human mesothelial cells via β1 integrin interactions [20, 21]. Here, we use a novel assay to assess the ability of spheroids to invade by measuring their disaggregation and proliferation within a live monolayer of human mesothelial cells. NIH:OVCAR5 spheroids were placed on confluent monolayers of human LP9 mesothelial cells for 7 days. The spheroid area was determined at the time of the initial plating and again at days 1, 4, and 7 to calculate the fold change in area over time. After 24 h, the spheroids appeared to disaggregate and spread across the top of the mesothelial cell monolayers (Figure 3a). However, by day 4, the spheroid cells had invaded the mesothelial cell monolayer and had rapidly established foci of invasion (Figure 3a). Spheroid invasion resulted in the establishment of a monolayer of ovarian cancer cells that replaced the mesothelial cell monolayer as the spheroid cells invaded. As the mesothelial cells retracted, it was clear that invading NIH:OVCAR5 cells were in the same plane as the mesothelial cell monolayers, while NIH:OVCAR5 cells that were unable to invade were slightly out of focus on top of the mesothelial cell monolayers. By day 7, the area occupied by the original spheroids had increased in size nearly 200-fold, as the mesothelial cells receded and the ovarian tumor cells proliferated on the exposed tissue culture surface (Figures 3a, b). In some instances when an excess of spheroids was added, the multiple invading foci merged and were able to completely overtake the well, leaving little of the mesothelial cell monolayer intact. In most cases, the foci appeared to grow laterally as the cells proliferated, rather than dispersing as individual cells throughout the monolayers. Similar experiments performed using methanol-fixed or irradiated mesothelial cell monolayers yielded comparable results (data not shown), indicating that proliferation of, or signaling from, the mesothelial cells did not hamper the invasive ability of the tumor cells. From experiments with fixed mesothelial cell monolayers, the border between the invading ovarian cancer cells and the mesothelial cells was markedly clear, as the foci of invasion grew and dispersed the mesothelial cells.

Spheroid invasion is partially mediated by β1 integrin subunits

To determine the role of β1 integrins in the invasion process, a mAb against the β1 integrin subunit or mIgG was added to wells containing spheroids and mesothelial cell monolayers (Figure 3). Media was replenished daily to maintain the concentration of mIgG and β1 integrin mAb throughout the assay. After 24 h in the presence of the mAb against the β1 integrin subunit, there was a slight decrease in area compared to mIgG as the spheroids disaggregated and spread across the top of the mesothelial cell monolayers (Figure 3b; $P < 0.0001$). By day 4, a marked inhibition was seen in the presence of the β1 integrin mAb, with a 70% reduction in the area of invasion compared to the mIgG control (Figure 3b; $P < 0.0001$). Notably, by day 7, spheroid invasion into the mesothelial cell monolayer was blocked approximately 90% by the β1 integrin mAb compared to the mIgG control (Figure 3b; $P < 0.0001$). While a constant concentration of the β1 integrin mAb slightly altered the confluence of the mesothelial monolayer by day 7, both the mesothelial cells and tumor cells present in the assay were alive as determined by trypan blue staining. These data suggest a major role for the β1 integrin subunit in mediating the invasion process of ovarian carcinoma spheroids.

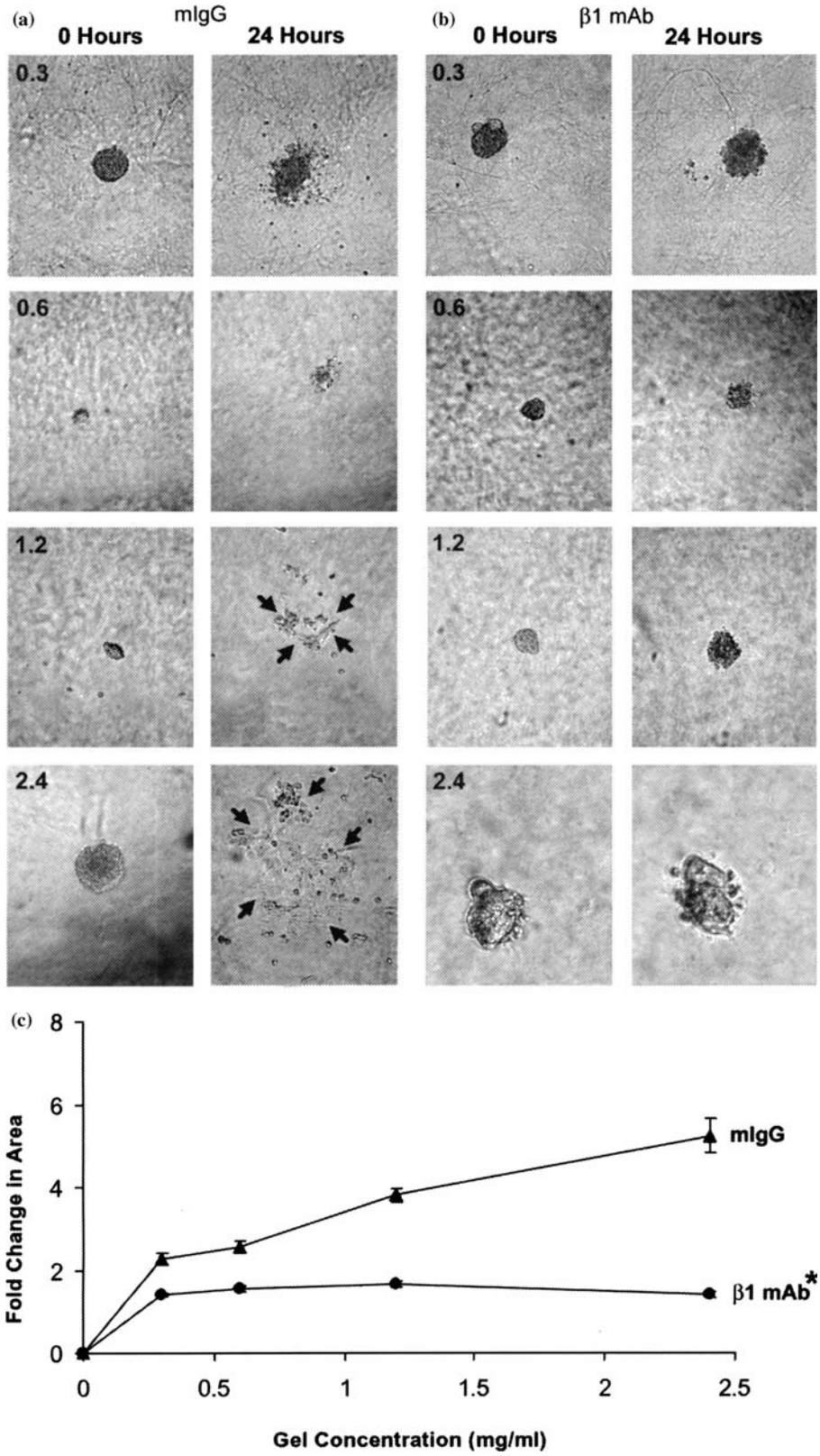

Figure 2. Spheroids disaggregate on polymerized type I collagen. Spheroids were plated on polymerized type I collagen gels with 10 μg/ml of either mIgG or a mAb against the β1 integrin subunit. Photographs of spheroids at 0 and 24 h with mIgG (a) or a mAb against the β1 integrin subunit (b) on 0.3, 0.6, 1.2, and 2.4 mg/ml polymerized type I collagen gels. Arrows delineate the perimeter of the disseminating spheroids. The fold change in spheroid area was determined with mIgG (c, triangles) or a mAb against the β1 integrin subunit (c, circles.) Results are expressed as the average fold change in pixel area of 50–100 spheroids over 24 h from quadruplicate experiments, ± standard error. Magnification 100×. Significance: $^{*}P < 0.0001$ for all data points.

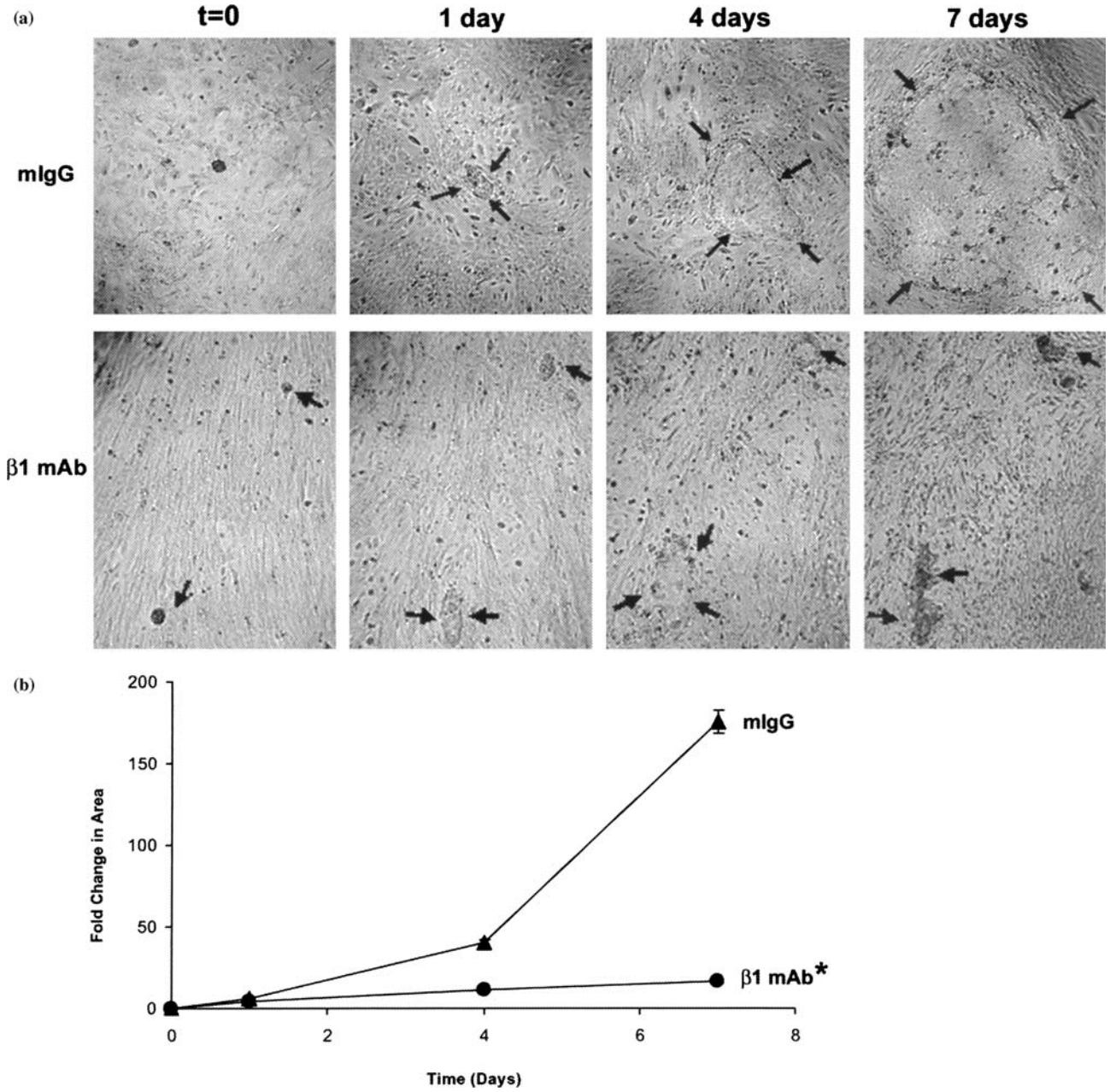

Figure 3. A mAb against the β1 integrin subunit inhibits spheroid invasion into live human mesothelial cell monolayers. Spheroids were added to monolayers of mesothelial cells with 10 μg/ml of mIgG (b, triangles) or a mAb against the β1 integrin subunit (b, circles), and were incubated for 7 days. Digital photography was used to capture images of the spheroids at the day of plating ($t = 0$) and again at 1, 4, and 7 days (a). Arrows delineate the perimeter of the disseminating spheroids. The fold change in pixel area of the spheroid and subsequent invading foci was calculated for each time-point (b). Data points represent an average fold change in area for 150–200 spheroids from quadruplicate experiments, ± standard error. Magnification 40×. Significance: $^{*}P < 0.0001$ for all data points.

Spheroid invasion is mediated by MMPs

Many studies have demonstrated the importance of proteases in the invasion process of a variety of tumors [34]. In ovarian cancer, matrix metalloproteinases have been shown to be involved in facilitating tumor cell invasion [23, 35, 36]. However, no study has yet addressed the contribution of proteases to ovarian carcinoma spheroid invasion. Therefore, a broad-scale MMP inhibitor, GM 6001, was added to wells containing spheroids and mesothelial cell monolayers to determine the effect that blocking proteases has on the invasion process (Figure 4a). At 24 h, 10 μM GM 6001 (Figure 4b, squares) had no significant effect on the initial spheroid disaggregation and spreading on top of the mesothelial cell monolayers compared to a DMSO control (Figure 4b, diamonds). Increasing the concentration of GM 6001 up to 25 μM had no additional effect (Figure 4b, circles.) By day 4, however, 10 μM GM 6001 inhibited spheroid invasion into the monolayers by 50%, while increasing the concentration of GM 6001 up to 25 μM resulted in a 60% reduction in invasion (Figure 4b; $P < 0.0001$). By day 7, 10 μM GM 6001 blocked mesothelial cell monolayer

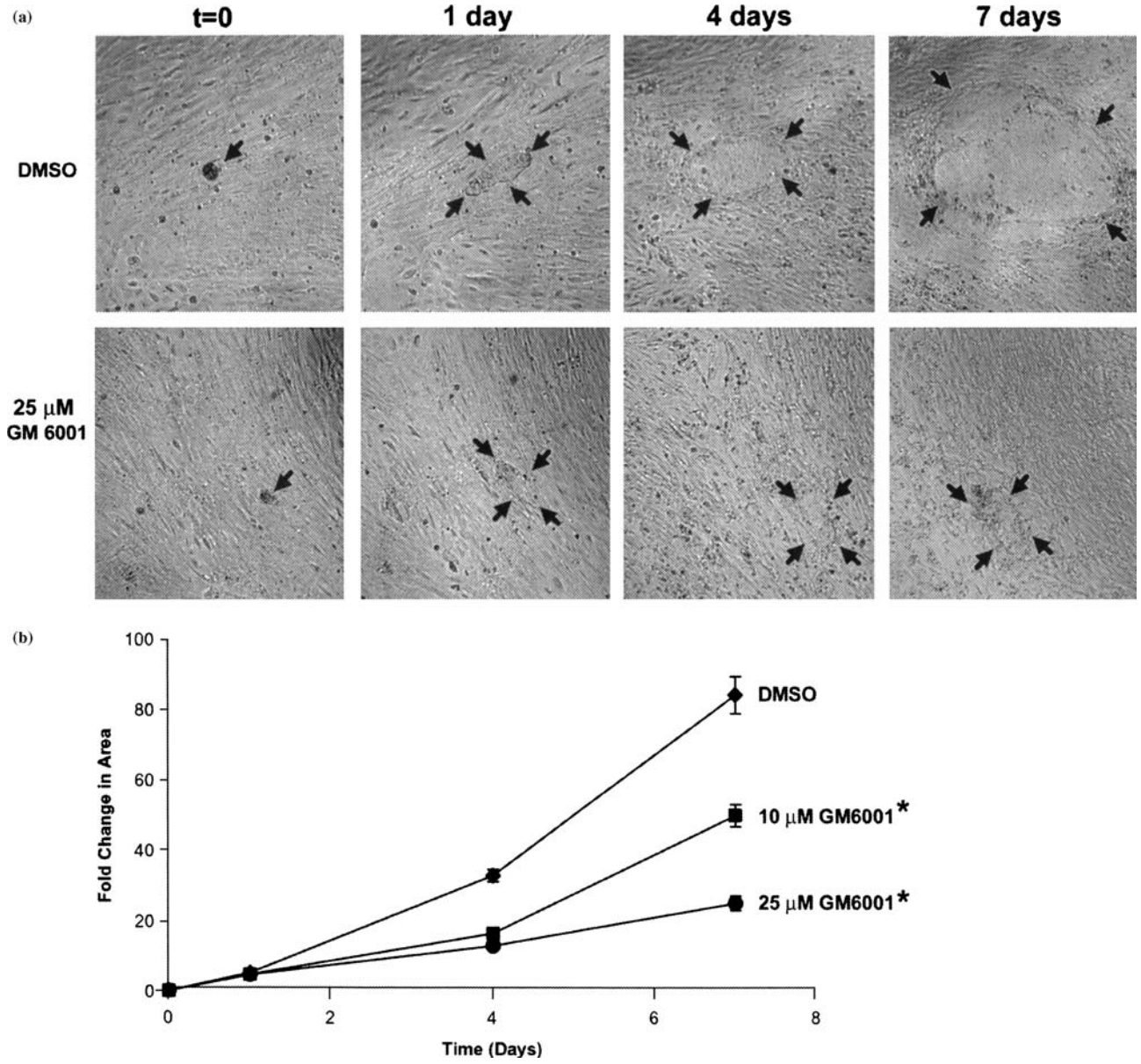

Figure 4. GM 6001 inhibits spheroid invasion into live human mesothelial cell monolayers. Spheroids were added to monolayers of mesothelial cell with DMSO (b, diamonds), 10 μM GM 6001 (b, squares), or 25 μM GM6001 (b, circles), and were incubated for 7 days. Digital photography was used to capture images of the spheroids at the day of plating ($t = 0$) and again at 1, 4, and 7 days. (The photos in panel (a) represent spheroids in the presence of 25 μM GM 6001.) Arrows delineate the perimeter of the disseminating spheroids. The fold change in pixel area of the spheroid and subsequent invading foci was calculated for each time-point (b). Data points represent an average fold change in area for 50–100 spheroids from quadruplicate experiments, $\pm$ standard error. Magnification 40×. Significance: $^{*}P < 0.0001$ for days 4 and 7.

invasion by 50% and 25 μM GM 6001 inhibited invasion by 70% (Figure 4b; $P < 0.0001$). Use of DMSO as a control did not affect mesothelial cell monolayer viability as indicated by trypan blue staining, although there was a slight inhibitory effect on invasion compared to mIgG. These results suggest that MMPs contribute substantially to spheroid invasion.

Spheroid invasion is partially mediated by serine proteases

Serine proteases have previously been implicated in the pathogenesis of ovarian cancer [24, 25]. To determine whether serine proteases contribute to spheroid invasion, ε-amino-n-caproic acid (εACA), a serine protease inhibitor which also inhibits plasmin, was added to wells containing spheroids and mesothelial cell monolayers (Figure 5a). By 24 h, 1 mM εACA (Figure 5b, squares; $P < 0.0001$) inhibited spheroid invasion into the mesothelial cell monolayers by 15% compared to mIgG (Figure 5b, triangles). By 4 days, invasion in the presence of 1 mM εACA was decreased by 30% compared to mIgG (Figure 5b; $P < 0.0001$). At day 7, spheroid invasion into the mesothelial cell monolayers was reduced by about 55% in the presence of 1 mM εACA compared to a mIgG control (Figure 5b; $P < 0.0001$). Increasing the concentration of εACA up to 10 mM did not further inhibit invasion.

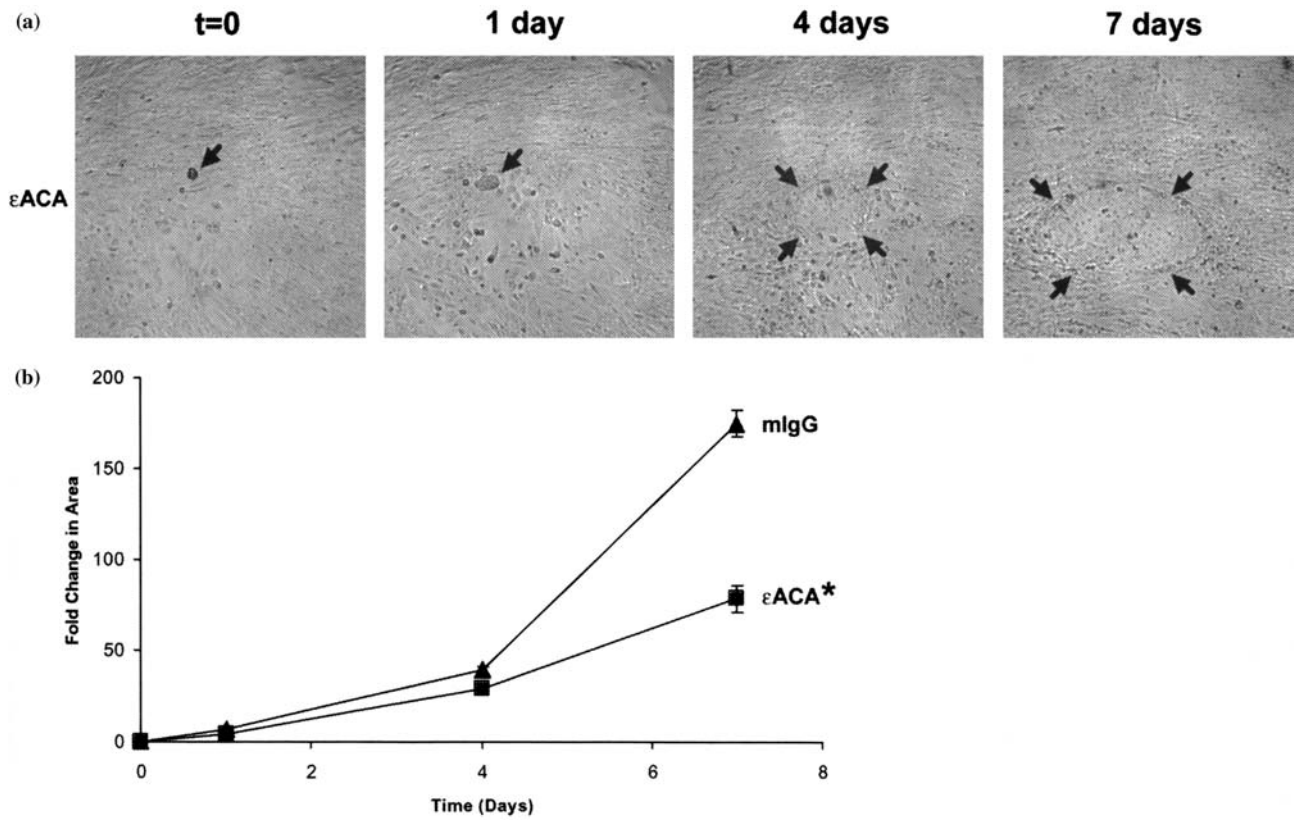

Figure 5. εACA partially inhibits spheroid invasion into live human mesothelial cell monolayers. Spheroids were added to monolayers of mesothelial cells with 10 μg/ml of either mIgG (b, triangles) or 1 mM εACA (b, squares) and were incubated for 7 days. Digital photography was used to capture images of the spheroids (a) at the day of plating ($t = 0$) and again at 1, 4, and 7 days. Arrows delineate the perimeter of the disseminating spheroids. The fold change in pixel area of the spheroid and subsequent invading foci was calculated for each time-point (b). Data points represent an average fold change in area for 30–50 spheroids from quadruplicate experiments, ± standard error. Magnification 40×. Significance: $^{*}P < 0.0001$ for all data points.

These data suggest a partial role for plasmin or other serine proteases in facilitating ovarian carcinoma spheroid invasion.

Discussion

In ovarian carcinoma, dissemination occurs as tumor cells are shed into the peritoneal cavity and follow the natural flow of the ascites, establishing secondary tumors often without the need to enter the vasculature [37]. This atypical method of metastasis necessitates an understanding of the role that ascites and its cellular contents play in secondary tumor growth.

Ovarian cancer research has mostly focused on single tumor cells. However, many studies have demonstrated that spheroids are more resistant to chemotherapy and radiation than their single cell counterparts [8, 9, 11, 38, 39]. Still, studies regarding the adhesive or invasive properties of spheroids are scarce, as spheroids are largely considered to be non-adhesive. Recent studies by our group have refuted this paradigm by demonstrating that spheroids isolated from the ascites of ovarian carcinoma patients or generated from the NIH:OVCAR5 cell line can adhere to ECM components and mesothelial cells [20, 21]. The logical extension of these studies was to investigate the invasive properties of ovarian carcinoma spheroids.

Tumor cell migration has classically been measured in Boyden chambers by counting the number of tumor cells that migrate through a filter toward a chemoattractant [19]. While this method is suitable for single cells, it is not sufficient for quantitating cell migration out of a spheroid. Studies in glioma spheroid models have addressed this problem by determining spheroid migration on ECM-coated substrates as the change in the orthogonal diameter of the spheroids over time, and have found these data consistent with results from single cells in Boyden chambers [40–44]. Kawano et al. made this method more amenable for irregularly shaped spheroids by calculating the change in pixel area [45]. Our study is the first to introduce this system of quantitating spheroid migration in ovarian carcinoma. We report here that spheroids generated from the NIH:OVCAR5 cell line can be stimulated to spread on type IV collagen and laminin, and can completely disaggregate on monomeric films of type I collagen. Blocking the β1 integrin subunit reduced spheroid disaggregation on type I collagen by 50%, and almost completely inhibited this migration on all other ECM proteins. These data suggest that interactions between β1 integrin subunits and type I collagen

play a key role in stimulating ovarian carcinoma spheroid dissemination.

Because of its abundance in the interstitial matrix, type I collagen is a substrate ovarian cancer cells inevitably encounter upon exposure to the submesothelial ECM during invasion. Ovarian carcinoma cells have shown preferential adhesion to and migration on type I collagen-coated surfaces [21 32, 33, 46]. However, while type I collagen in its monomeric form permits adhesion and migration, the interaction of ovarian cancer cells with polymerized collagen gels can result in striking increases in protease activation and production [32, 33]. In this study, we examined ovarian carcinoma spheroids on a variety of type I collagen substrates. We found that on low density polymerized type I collagen gels, spheroids were restricted in their ability to spread. As the density of the gels increased, the spheroids were able to more fully spread and disaggregate. However, tissue culture plates coated with a monomeric film of type I collagen typically resulted in the most dramatic spheroid cell dispersal, suggesting that spheroids need a rigid type I collagen surface for significant disaggregation. One reason for this may be differences in engagement and clustering of cell receptors, depending upon the denseness or rigidity of the substrates. Further studies into the mechanisms of spheroid disaggregation are warranted.

The mesothelial surface of the peritoneal cavity is the most frequent site of secondary tumor development in ovarian carcinoma. Based on our previous data that showed spheroids isolated from patients' ascites or generated from the NIH:OVCAR5 cell line could adhere to live monolayers of mesothelial cells, we next determined whether spheroids were capable of invasion [21]. While single tumor cell invasion is often determined by quantitating the number of tumor cells that migrate through a cell monolayer grown on a filter, this assay is not suitable for quantitative measurement of spheroid invasion. Therefore, we designed a novel invasion assay based on our spheroid migration assays, as described above. Spheroid invasion was defined as the ability of ovarian carcinoma cells from a spheroid to disaggregate and displace a live mesothelial cell monolayer, replacing them with a rapidly proliferating monolayer of ovarian cancer cells. Here, we show that within 24 h, NIH:OVCAR5 spheroids disaggregated and spread across the top of a mesothelial cell monolayer. By day 4, the ECM molecules underlying the monolayer became exposed as the mesothelial cells retracted, possibly due to proteases secreted by the spheroids and/or the mesothelial cells. The spheroids then invaded the monolayer, where they rapidly proliferated, and formed large, confluent foci of ovarian cancer cells by day 7. We also performed these assays using methanol-fixed or irradiated mesothelial cell monolayers to determine whether mesothelial cell proliferation or signaling affected invasion. We found that the spheroids invaded live, fixed, or irradiated mesothelial cell monolayers to the same extent. In contrast to earlier studies whereby single NIH:OVCAR5 cells invaded fixed mesothelial cell monolayers within a week, the NIH:OVCAR5 spheroids in this study formed invading foci much more rapidly and to a much greater extent [47]. Most notably, the area of invasion of the spheroids at day 4 was comparable to the area of the invading single cells at day 7. The speed of the invasion is likely due to the large number of tumor cells present in the spheroid that can proliferate at the site of attachment, rather than a change in the proliferative rate of the cells comprising the spheroid. It is interesting to speculate that a similar event might occur *in vivo*, whereby a spheroid may attach, disaggregate, and rapidly invade the mesothelial lining of the peritoneal cavity. In contrast, a single tumor cell may take a much longer period of time to proliferate and invade to the same extent.

Considering the major role that $\beta 1$ integrins play in mediating spheroid adhesion, we investigated its contribution to the invasion process [20, 21]. Blocking the $\beta 1$ integrin subunit inhibited spheroid invasion of mesothelial cell monolayers by 90%. The inhibitory effect of the mAb did not appear to be due to prevention of initial spheroid attachment, since the ability of spheroids to disaggregate and spread across the monolayers remained unaffected at 24 h. Initial invasion appeared to occur, although by day 4 there was a clear decrease in the area of the invading foci. By day 7, the area of invasion was dramatically reduced in the presence of the mAb. Frequently, the areas of invasion shrank, as some of the tumor cells detached from the monolayers and underlying ECM. The remaining invading ovarian cancer cells were somewhat rounded in appearance and not fully spread. While the presence of the $\beta 1$ integrin mAb sometimes altered the confluence of the mesothelial cell monolayers by day 7, both the mesothelial cells and the invading cancer cells were alive as indicated by trypan blue staining. These observations suggest that $\beta 1$ integrins play a significant role in mediating a sustained invasion of mesothelial cells by ovarian carcinoma spheroids.

A number of studies have addressed the importance of proteases in the invasion process. In particular, ovarian cancer cells have been shown to express MMP-1, -2, -3, and -9, MT1-MMP and MT-2-MMP [22, 23, 35, 48]. To investigate the role of MMPs in ovarian carcinoma spheroid invasion, we used the broad-scale MMP inhibitor GM 6001, which blocks MMP-1, -2, -3, -8, and -9. Within one week, spheroid invasion of live mesothelial cell monolayers was inhibited 50% with 10 μM GM 6001 and 70% with 25 μM GM 6001. In fixed mesothelial cell monolayers, the inhibitory effect of GM 6001 on spheroid invasion was minimal (data not shown). These data suggest that the mesothelial cells themselves may produce low levels of MMPs, such that the addition of GM 6001 blocks proteases secreted by both spheroids and mesothelial cells. Indeed, mesothelial cells have been shown to produce MMPs, and cultured media from mesothelial cells can

induce MMP expression by ovarian cancer cells [49–51]. Future studies are planned to identify the individual MMPs involved in ovarian carcinoma spheroid invasion.

Serine proteases have also been implicated in the pathogenesis of ovarian cancer. Several studies have shown that ovarian tumor cells secrete plasminogen activator, the urinary-type plasminogen (uPA) being more common than the heterogeneously expressed tissue-type plasminogen [24, 25, 35, 48]. Here, we inhibited serine proteases with εACA to study their effect on spheroid invasion. Invasion was reduced in the presence of 10 mM εACA by approximately 55% by day 7. Increasing the concentration of εACA up to 10 mM or using εACA in combination with 10 μM GM 6001 failed to elicit further inhibition (data not shown.) These data suggest that plasmin or other serine proteases may play a lesser role than MMPs in facilitating spheroid invasion.

The ascites fluid itself may enhance ovarian carcinoma spheroid invasion. Rieppi et al showed that cell free supernatant from cultured mesothelial cells could induce ovarian cancer cells to migrate [52]. uPA, MMP-2, MMP-9, and MMP-13 have been detected in the ascites fluid from ovarian carcinoma patients [24, 35, 53]. Ascites fluid has also been shown to contain a variety of factors that increase carcinoma cell adhesion and invasion, including lysophosphatidic acid, colony-stimulating factor, tumor necrosis factor α, interleukin-1β, interleukin-6, and vascular endothelial growth factor [54–57]. Considering the abundance of stimulatory factors in the ovarian carcinoma spheroid milieu, *in vivo* dissemination and invasion may be further enhanced compared to our *in vitro* model system.

The β1 integrin subunit has been found to be associated with a number of signaling molecules in ovarian cancer cells, including serine protein kinases [58]. Ellerbroek et al demonstrated that the stimulation and aggregation of β1 integrins on ovarian carcinoma cells upon binding to type I collagen matrices results in the activation of both MMP-2 and MT-1 MMP [59]. Furthermore, Davidson et al showed that ovarian carcinoma cells isolated from ascites fluid express β1 integrin subunits and MMPs, and also demonstrated a significant co-localization between integrins and MMPs in ovarian cancer tissues [22, 60–62]. Taking these data into consideration, we can thus construct an *in vivo* model in which spheroids adhere to the peritoneal mesothelium, activating β1 integrin subunits and subsequently, downstream proteases. Protease production exposes the underlying type I collagen matrix, upon which the spheroids attach, disaggregate, proliferate, and further invade the mesothelial cell monolayers, leading to secondary tumor formation.

Based on the data presented here, ovarian carcinoma spheroids are capable of adhering to, disaggregating on, and invading into a live human mesothelial cell monolayer, indicating that they present a metastatic potential. As the current treatments for ovarian cancer often fail to target tumor cell aggregates, it is clear that a better understanding of their contribution to the spread of the disease is necessary. We further suggest that the migration and invasion assays presented in this study have applications in analyzing cell aggregates in other diseases and systems, with the hopes of advancing knowledge of the biology of spheroids.

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