Ovarian Cancer Spheroids Use Myosin-Generated Force to Clear the Mesothelium

Marcin P. Iwanicki, Rachel A. Davidowitz, Mei Rosa Ng, Achim Besser, Taru Muranen, Melissa Merritt, Gaudenz Danuser, Tan Ince, and Joan S. Brugge
INTRODUCTION

During the progression of ovarian cancer, tumor cells detach from the primary tumor site and form cell clusters, or spheroids, that can either remain unattached in the peritoneal cavity or implant onto peritoneal organs (1). Formation of implants depends on the ability of tumor cells to invade the mesothelial layer that covers peritoneal and pleural organs (2). Electron micrographs of mesothelial tissue sections with and without peritoneal metastases (3, 4) revealed that normal peritoneal mesothelial cells are flat and cover the entire surface of the peritoneum, such that cell–cell boundaries are difficult to discern, whereas mesothelial cells with peritoneal metastases are more rounded and separated from each other, revealing the submesothelial surface. These studies suggested that mesothelial cells retracted in the presence of the tumor. Furthermore, the cancer cells did not adhere to the mesothelial cells, but rather to connective tissue underneath the mesothelial cells. In addition, electron micrographs of excised human peritoneum–associated tumors revealed that mesothelial cells are not present directly under the tumor mass, suggesting mesothelial clearance from the area beneath the tumor mass (4). Early, in vitro experiments also provided evidence that mesothelial cells retract after coming in contact with tumor cells (5, 6). In these studies, ovarian cancer cell clusters disrupted mesothelial cell–cell junctions and penetrated under mesothelial cells, suggesting that the integrity of the mesothelial cell monolayer is altered by the attached tumor cells that bind with high affinity to the submesothelial matrix (6, 7). The cellular and molecular mechanisms of mesothelial clearance, however, are unknown.

We have used a live, image-based in vitro model in which interactions between tumor spheroids and mesothelial cells can be monitored in real time to provide spatial and temporal understanding of the process of mesothelial clearance. Using this model, we show that tumor spheroid attachment and spreading on a mesothelial monolayer promotes clearance of the mesothelial cells from the area underneath the spheroid. We provide evidence that force generation on the mesothelial cell–associated extracellular matrix (ECM) provokes mesothelial cells to migrate and clear from underneath the tumor spheroid. This mechanism might be relevant to processes involved in implantation of ovarian tumor aggregates into the submesothelial environment of the organs of the peritoneal and pleural cavities.

RESULTS

Interaction of Ovarian Cancer Spheroids with Mesothelial Monolayers Promotes Mesothelial Cell Clearance

To investigate the interaction between ovarian cancer spheroids (OVCAR3 ovarian cancer cell line) and green fluorescent protein (GFP)–expressing mesothelial cells (normal immortalized lung mesothelium), we used time-lapse microscopy to follow the dynamics of a mesothelial monolayer after cancer spheroid attachment, in real time. As the spheroid spread on the mesothelial monolayer, mesothelial cells were
displaced from the area directly underneath the spreading spheroid. This phenomenon will be referred to as mesothelial clearance. (Fig. 1A; and Supplementary Movie 1 and Video 1). The clearance area increased with time as the spheroid became more incorporated into the mesothelial monolayer (Fig. 1B). We also observed that primary tumor clusters isolated from the peritoneal fluid of ovarian cancer patients are able to attach to and clear the mesothelium (Fig. 1C, and Supplementary Movie 2). Overall, these data indicate that, following attachment to a mesothelial monolayer, clusters of ovarian cancer cells are able to induce clearance of the mesothelial cells directly underneath the tumor spheroid.

In vivo, mesothelial cells are separated from the underlying soft connective tissue by a layer of matrix (8). To examine whether mesothelial clearance can occur on more physiologically relevant substrates (of similar stiffness to connective tissue), mesothelial monolayers were plated on fibronectin-coated polyacrylamide (PAA) gels with elastic moduli of 0.3 kPa or 10 kPa. OVCA433 tumor spheroids were able to induce mesothelial clearance on both substrates (Supplementary Fig. 1A), indicating that mesothelial clearance can indeed occur on softer, more physiologically relevant substrates, and that the mesothelial clearance observed is not an artifact of cells grown on stiff glass surfaces.

To study the spheroid–mesothelial interaction more closely, we imaged a spheroid during the process of intercalation into a mesothelial monolayer in multiple focal planes and reconstructed the x–z planes to observe ovarian–mesothelial cell interactions at the ventral and dorsal cell surfaces. In the early stages of clearance (as shown in Fig. 1D and Supplementary Movie 3), cancer cells spread on top of the monolayer (as indicated by the arrows) and then penetrated under the mesothelium (as indicated by *). From these observations, we hypothesized that cancer spheroids adhere to the mesothelial monolayer and induce localized de-adhesion of the mesothelial cells to ultimately prompt movement of the mesothelial cells away from the spheroid.

To examine whether localized de-adhesion of mesothelial cell matrix adhesions indeed occurs upon contact with a tumor spheroid, we used total internal reflection fluorescent (TIRF) microscopy to monitor mesothelial cell adhesions labeled with paxillin-GFP (this protein localizes to
integrin–matrix engagement sites in multiple cell types). TIRF microscopy allows for visualization of fluorescent molecules present within 100 nm above the surface of the coverslip, thereby minimizing background intensity from the cytoplasm. We observed that cancer spheroids [labeled with red fluorescent protein (RFP)–actin] approached the mesothelial cell’s adhesions (GFPP) and promoted matrix adhesion disassembly (Fig. 2A; Supplementary Movie 4A and B). Furthermore, little adhesion assembly was observed within the area of contact. In contrast, mesothelial cell matrix adhesions that were not in contact with a tumor spheroid displayed spontaneous adhesion assembly and disassembly events (Fig. 2B; Supplementary Movie 5). In a separate experiment, we labeled approximately 1 in 500 mesothelial cells with GFP to track the movement of individual mesothelial cells and observed that cells contacting a cancer spheroid migrated significantly longer distances than did those cells not contacting a cancer spheroid (Fig. 2C and D; Supplementary Movie 6). Overall, our results are consistent with the hypothesis that ovarian cancer spheroids can attach to a mesothelial monolayer, intercalate into the monolayer, and trigger mesothelial cell matrix adhesion disassembly and migration, ultimately leading to mesothelial clearance.

**Coupling of Myosin Contractility to Integrins in Cancer Spheroids Is Required for Mesothelial Clearance**

Ovarian cancer cell adhesion to a mesothelial monolayer has been shown to involve integrins (9). Cells exert force on the ECM by coupling myosin contractility to integrins (10, 11). Therefore, we examined whether tumor spheroid expression of myosin II is required to promote mesothelial clearance. OVCA433 cancer spheroids express nonmuscle myosin isoforms IIA and IIB (Fig. 3A). Both myosin II isoforms were downregulated in OVCA433 cells, using small hairpin RNA (shRNA) and small interfering RNA (siRNA) targeting myosin IIA and IIB heavy chains, respectively (Fig. 3A). Myosin heavy chain IIA and IIB attenuation did not prevent the ovarian cancer cells from spreading on surfaces coated with fibronectin and collagen I, indicating that myosin IIA and IIB are not required for spheroid attachment and spreading on fibronectin- and collagen-coated glass surfaces (Supplementary Fig. 1B). However, OVCA433 spheroids with reduced levels of myosin IIA or IIB initiated but were unable to sustain mesothelial clearance (Fig. 3B and C; Supplementary Movie 7). Attenuation of myosin II in OVCA433 cells by shRNA or siRNA did not prevent adhesion of spheroids to the mesothelial monolayer.

**Figure 2.** Tumor spheroid induces mesothelial cell migration. **A**, temporal analysis of adhesion dynamics of paxillin-GFP-labeled LP9 human peritoneal mesothelial cells incubated with or without RFP-actin–expressing OVCA433 cancer spheroids. Events of adhesion assembly and disassembly were scored. Arrows indicate adhesion disassembly. Scale bar, 5 μm. **B**, bar graph shows the ratio of adhesion assembly and disassembly events within the same area. A total of 150 assembly and disassembly events were analyzed per condition. **C**, GFP-labeled lung mesothelial cells were mixed at 1:500 ratio with nonlabeled lung mesothelial cells. Images show temporal behavior of single mesothelial cells in the presence or absence of cancer spheroids. Arrows indicate mesothelial cell movement away from intercalating spheroid. Scale bar, 100 μm. **D**, migration distance of single mesothelial cells was measured in the presence and absence of OVCA433 cancer spheroids. A total of 50 GFP-labeled mesothelial cells were analyzed per condition.

Downloaded from cancerdiscovery.aacrjournals.org on July 3, 2017. © 2011 American Association for Cancer Research.
Coupling of myosin to integrins in cancer spheroids is required for mesothelial clearance. A, Western blot of nonmuscle myosin heavy chain A and B (NM IIA or NM IIB) expression levels in control or NM IIA/NM IIB shRNA-treated OVCA433 cells. B, images show the temporal behavior of mesothelial cells contacting control or myosin shRNA-expressing OVCA433 ovarian cancer cells. Scale bar, 50 μm. C, quantification of mesothelial clearance from B. A total of 20 spheroid attachment sites were analyzed per condition. D, Western blot of talin I expression levels in control or talin I shRNA-expressing OVCA433 cells. E, images show temporal behavior of mesothelial cells contacting control or talin I shRNA-expressing OVCA433 ovarian cancer cells. Scale bar, 50 μm. F, quantification of mesothelial clearance from E. A total of 15 randomly chosen regions were analyzed per condition.

monolayer (Supplementary Fig. 1C, left panel) or induce apoptosis (Supplementary Fig. 1C, right panel), suggesting that myosin II expression in these cells is dispensable for spheroid attachment. We validated these results with 2 additional independent shRNA sequences targeting myosin IIA combined with the same siRNA pool for myosin IIB molecules (Supplementary Fig. 1D). Taken together, these results suggest that OVCA433 cancer spheroids require myosin to induce mesothelial clearance.

The ability of cells to exert force on the outside environment depends on linkage of the actin and myosin network to integrins through recruitment of talin I to adhesion sites (10). We used shRNA to attenuate the expression of talin I in the spheroids (Fig. 3D). Attenuation of talin I expression in OVCA433 spheroids did not induce apoptosis (Supplementary Fig. 1C, right panel) and did not affect spreading on glass surfaces coated with fibronectin and collagen I (Supplementary Fig. 2A). However, decreased expression of talin I, but not talin II, significantly reduced mesothelial clearance, even though spheroid adherence to the monolayer was unaffected (Fig. 3C; Supplementary Movie 8; and Supplementary Fig. 2B and C). We validated these results with 2 additional independent shRNA sequences targeting the talin I molecule in OVCA433, DOV13, and SKOV3 ovarian cancer cell lines (Supplementary Fig. 2D). These data indicate that talin I is required for tumor cell intercalation into the mesothelium and suggest that the linkage of integrins to the actomyosin network in ovarian spheroids contributes to mesothelial clearance.

α5β1 Integrin Is Required for Spheroid-Induced Mesothelial Clearance and Contributes to the Activation of Myosin in Cancer Cells

Because expression of the α5 integrin fibronectin receptor has been shown to correlate with the development of myosin-driven contractility (12) and increased invasion of ovarian cancer cells (13, 14), we addressed whether α5β1 integrin-mediated activation of contractility contributes to ovarian spheroid-induced mesothelial clearance. First, we blocked the function of α5 integrin in cells that express high levels (OVCA433 ovarian cancer cells; Fig. 4A). Treatment of
OVCA433 cell spheroids with α5 integrin–blocking antibody significantly decreased spheroid-induced mesothelial clearance (Fig. 4B and C; Supplementary Movie 9). Blocking α5 integrin in DOV13 and SKOV3 spheroids also significantly decreased mesothelial clearance (Supplementary Fig. 3A). The α5 integrin–blocking antibodies did not, however, prevent the OVCA433 spheroids from adhering to the mesothelial monolayer (Supplementary Fig. 3D). Ectopic expression of α5 integrin in OVCA5 cells increased activation of myosin, promoted cell spreading, increased stress fibers and other cortical actin contractile structures, and increased mesothelial clearance (Fig. 4D,E, and F; Supplementary Movie 10). These results support the hypothesis that α5β1-dependent activation of myosin in ovarian cancer spheroids is required for spheroid-mediated mesothelial clearance.

Cancer Spheroids Expressing Functional α5β1 Integrin Detach Fibronectin Fibris from the Surface of the Mesothelium

Our data suggest that engagement of the fibronectin receptor α5β1 integrin is an important step in spheroid-induced mesothelial clearance. Fibronectin has been found to be present on the surface of murine mesothelial cells (15).
Thus, we addressed whether ovarian cancer spheroids reorganize the fibronectin matrix presented on the dorsal surface of the mesothelial monolayer. To determine if fibronectin is organized on the dorsal surface of the mesothelial monolayer, the monolayer was immunostained with an antibody that recognized human fibronectin. We observed that fibronectin fibrils were present on top of the mesothelial monolayer (Fig. 5A; Supplementary Movie 11). We also detected organized collagen fibers on top of the mesothelium (data not shown); however, blocking the αβ₁ integrin collagen receptor did not affect mesothelial clearance (Supplementary Fig. 3C). Because fibronectin fibrils contacting the periphery of an intercalated spheroid appeared to be preferentially associated with spheroid, but not mesothelial cells (Supplementary Movie 11 and Video 1), we investigated whether cancer spheroids detach fibronectin from the mesothelial monolayer. We followed the dynamics of rhodamine-labeled fibronectin organized on top of the mesothelial monolayer in the presence of OVCA433 cancer spheroids. As shown in Fig. 5B and Supplementary Movie 12, cancer spheroids induced detachment of fibronectin fibrils from the mesothelial cells. As time progressed, some of the fibronectin fibrils organized around the spheroid. In addition, when αβ₁ integrin was inhibited by an α₁ function-blocking antibody, fibronectin did not dissociate from the mesothelial cells, suggesting that dissociation of fibronectin from the top of the mesothelial monolayer was dependent on functional αβ₁ integrin expressed by the cancer spheroids (Fig. 5C and D). These data suggest that ovarian cancer spheroids use αβ₁ integrin to dissociate fibronectin from the mesothelial monolayer during clearance.

**Figure 5.** Cancer spheroids expressing functional αβ₁ integrin dissociate fibronectin fibrils from the surface of the mesothelium. A, top, laser scanning confocal image showing the top view of an OVCA433 cancer spheroid (blue) inserted into a mesothelial monolayer (green) stained with an antibody directed against human fibronectin (red). Scale bar, 10 μm; bottom, a side view reconstruction of multiple Z planes of the same image. B, images show temporal acquisition of mesothelium (green)–associated fibronectin (red) by OVCA433 cancer spheroids (blue). Scale bar, 10 μm. C, Dissociation of fibronectin (red) from mesothelial monolayer (green) after 7 hours in response to spheroids (blue) pretreated with either control or αβ₁-blocking antibody. Scale bar, 10 μm. D, Quantification of fibronectin dissociation from a mesothelial monolayer in response to an attached cancer spheroid pretreated with either control or αβ₁ antibody. A total of 8 randomly chosen regions were analyzed in the control group, and 20 randomly chosen regions were analyzed in the ITGAS group.
**Cancer Cells Exert Force on a Fibronectin-Coated Substrate in an α5β1 Integrin-, Talin I- and Myosin-Dependent Manner**

The data presented above show that αβ integrin, myosin, and talin are all required in ovarian cancer cells for mesothelial clearance and that αβ integrin-dependent binding of the ovarian cancer spheroids to fibronectin organized by the mesothelium is important for clearance processes. These findings would suggest that the traction force exerted on the substrate by the spreading spheroids contributes to mesothelial clearance. To determine if modulation of α, talin I, or myosin affects force generation in ovarian cancer cells, we used traction force microscopy (TFM; refs. 16–18). This microscopy technique involves tracking fluorescent beads embedded in the substrate on which cells spread or migrate. Displacement of these beads is used to measure traction force exerted by cells on substrate during spreading/migration. Control OVCAR5 cells, OVCAR5 cells overexpressing α integrin, and α5-overexpressing cells treated with either talin I siRNA or blebbistatin were allowed to spread on fibronectin-coated PAA substrates embedded with fluorescently labeled beads. Spreading caused deformation of the substrate, as indicated by movement of the embedded beads. Tracking bead displacement and reconstructing the cellular traction stresses allowed us to measure the strain energy invested by the cells to deform the elastic substrate (17). The strain energy can be used to measure the contractile strength of cells. As shown in Fig. 6A, increased expression of α integrin in cancer cells correlated with an increase in cellular contractility, as measured by strain energy exerted by the cells. The α integrin-induced force generation was dependent on myosin activation because OVCAR5 cells overexpressing α integrin that were treated with blebbistatin significantly decreased elastic energy exerted on the matrix (Fig. 5A). We also observed that downregulation of talin I, but not talin II, in OVCAR5 cells overexpressing α integrin decreased force generation on fibronectin substrates (Fig. 6B). These results are consistent with a model in which talin I and myosin act downstream of α integrin to generate force as ovarian cancer cells interact with fibronectin matrix.

**DISCUSSION**

In summary, these studies provide new insights into the mechanism whereby ovarian tumor spheroids induce mesothelial cell clearance. Clearance-competent tumor spheroids were found to adhere to the dorsal surface of the mesothelial cells and initiate spreading. Protrusions from the spreading cells penetrated underneath the mesothelial cells, causing localized breakdown of the mesothelial cell matrix adhesions, and provoked migration of the cells. In tumor spheroids, αβ integrin, talin I, and myosin II were found to be required for spheroid-induced mesothelial clearance. These experiments suggest that ovarian cancer spheroids use actomyosin contractility to exert force via matrix adhesion to the fibronectin organized on the mesothelial monolayer, ultimately leading to mesothelial clearance (see model, Fig. 7). The mesothelial cell clearance mechanism whereby ovarian tumor spheroids induce mesothelial cell clearance.

---

**Figure 6.** Cancer cells exert forces on a fibronectin-coated PAA gel in an αβ integrin-, talin I-, and myosin-dependent manner. OVCAR5 ovarian cancer cells were plated on a PAA gel substrate coated with fibronectin (10 μg/mL). Cells were allowed to adhere to and spread on the PAA substrate, and displacement of fluorescently labeled beads embedded in the substrate was monitored. The strain energy was calculated from measured bead displacement data and used to reconstruct traction stresses. The strain energy is the energy invested by the cells to deform the substrate and a measure of the overall contractility of a cell (37). A, OVCAR5 cells have low endogenous α integrin (ITGA5) expression levels and are significantly less contractile than OVCAR5 cells overexpressing ITGA5 (P < 0.001). The high contractility of ITGA5-overexpressing OVCAR5 cells is attenuated by treatment with 1 μM blebbistatin (P < 0.001). B, contractility of ITGA5-overexpressing OVCAR5 cells can also be significantly attenuated by siRNA-mediated knockdown of talin I, but only slightly attenuated by knockdown of talin II. Scale bar, 20 μm.
clearance we observe in vitro may be relevant in human tumors because it has been shown that mesothelial cells are not present under ovarian tumor masses found attached to the peritoneal tissues.

In contrast to other epithelial tumors that use hematogenous or lymphatogenous routes to metastasize, ovarian cancer cells predominantly move within the ascites fluid to reach new sites within the peritoneal cavity (8). The mesothelial monolayer surface provides a variety of ligands to support the attachment of ovarian cancer cells (1). These ligands include hyaluronic acid, mesothelin, and ECM molecules that are able to engage integrins (19–21). Both CD44 and β1-containing integrin dimers have been implicated as receptors that can mediate adherence of ovarian cancer cells to the mesothelium. However, function-blocking antibodies directed against β1 integrin or CD44 only partially block adherence of ovarian cancer cells to the mesothelial monolayer in short-term, in vitro adhesion assays (9, 19, 22). This finding suggests that multiple ligands and receptors can support ovarian tumor cell adhesion to the mesothelial monolayer and that targeting a single molecule will not abrogate cancer cell interaction with mesothelial cells. Consistent with this idea, we found that blocking CD44 or selected β1-containing integrin heterodimers expressed by OVCA433 spheroids (αvβ3, αvβ6, αvβ5) did not significantly prevent OVCA433 spheroid attachment to the mesothelium after 10 hours of coculture. Interestingly, however, our data indicated that interfering with the function of αvβ5 integrin alone can significantly decrease OVCA433, DOV13, and SKOV3 spheroid-induced mesothelial clearance over a period of 10 hours (Supplementary Fig. 3A and C). Because αvβ5 integrin is a fibronectin receptor, these results suggest that cancer spheroids can use αvβ5 to bind to the fibronectin surrounding the mesothelial cells as a means of mediating mesothelial clearance. In support of this idea, we found that, as a spheroid clears a space in a mesothelial monolayer, the fibronectin fibrils organized on top of the mesothelial cells are redistributed away from the mesothelial cells and under the spheroid. This process was dependent on functional αvβ5 integrin expressed by the cancer spheroids. In addition, we also observed that the expression level of αvβ5 integrin in various ovarian cancer cell lines correlated with the ability of these cells to clear the mesothelium (data not shown). However, it is likely that other, αvβ5 integrin-independent mechanisms can mediate clearance as well.

Integrins are the major molecules that can transmit traction forces to the outside environment (23). Although α5 integrin binding to collagen I can induce fibril reorganization and transmit traction forces to the ECM in certain contexts (24), OVCAR5 ovarian tumor spheroids that express high levels of αv, but not α5, integrin, were unable to clear the mesothelium in our experiments (Supplementary Fig. 3C). In addition, blocking αv integrin in OVCA433 cells that express both α5 and αv integrin did not prevent mesothelial clearance (Supplementary Fig. 3C). It is possible that αv integrin does not transmit sufficient traction force under conditions of adherence to mesothelial cells to induce clearance of the mesothelial cells. The generation of traction force on fibronectin has been shown to involve 2 steps: First, clustering of αvβ5 integrins promotes strong adhesiveness to the matrix; and second, recruitment of talin I stabilizes and reinforces formed αvβ5 adhesions (25), promoting the exertion of traction force on the matrix. Our study indicated that the interaction between fibronectin receptor αvβ5 integrin expressed by tumor cells and mesothelial-associated fibronectin is a molecular event that contributes to the clearance process. In addition, we show that expression of talin I by tumor spheroids is required for αvβ5-mediated formation of traction force and mesothelial clearance. We found that interfering with the function of another fibronectin receptor, αvβ3 integrin, did not affect spheroid-induced mesothelial clearance (Supplementary Fig. 3C), suggesting that these receptors do not contribute to development of myosin contractility by OVCA433 spheroids that adhered to the mesothelial monolayer. This observation is consistent with
previous experiments implicating α5β1, but not α6β4, integrins in the development of contractility (26–28). Our data, as well as earlier findings (3), show that the mesothelial cells retract in response to cancer cluster attachment. This finding raises an interesting question: How does the tumor induce retraction in the mesothelial cells? One possibility is that the spreading tumor cells induce retraction by pulling on the mesothelial cells’ associated ECM and provoking the migration of mesothelial cells away from the spheroid. Alternatively, the retraction of mesothelial cells could be provoked by a repulsive ligand presented on the tumor cells. In this study, we have shown that force produced by a spreading ovarian cancer cell cluster—via α5β1 integrin, myosin II, and talin 1—is important for mesothelial clearance. The evidence that ovarian cancer spheroids deficient in non-muscle myosin II were unable to sustain mesothelial clearance suggests that mere contact between tumor cells and mesothelial cells is not sufficient to induce retraction and migration of mesothelial cells and that a repulsive ligand presented by the spheroid does not trigger retraction of the mesothelial cells. However, it is possible that interfering with myosin function also perturbs expression or activity of repulsive ligands present on tumor cell plasma membrane. Spheroid-induced mesothelial clearance was accompanied by disassembly of mesothelial cell matrix adhesion sites, indicating that force exerted on the mesothelium by cancer spheroids initiates a migratory response in individual mesothelial cells. In spheroid-induced matrix adhesion turnover experiments, mesothelial cells that originated from peritoneal wall (LP9) exhibited much more dynamic integrin adhesion when compared with mesothelium isolated from lungs (MET5A; compare Supplementary Movie 4A and B). This observation suggests that mesothelial cells covering different organs might elicit distinct migratory responses when contacting tumor spheroids.

Earlier studies implicated mesothelial apoptosis as a mechanism of clearance resulting from tumor cluster attachment (29). In our assays, clearance of the mesothelium started about 30 minutes after spheroid attachment and was accompanied by migration of individual mesothelial cells from underneath the tumor. This observation indicates that, in our assay, mesothelial cells respond to contacting tumor cells by activating migratory, but not apoptotic, pathways. However, it is possible that mesothelial cells that are “stuck” underneath the spheroid, and cannot escape, undergo apoptosis.

In patients with advanced disease, ovarian tumor clusters predominantly implant into the mesothelial lining of peritoneal cavity–associated organs. Invasive tumor implants are able to cross the mesothelial layer and gain access to stroma beneath the mesothelium (30). These observations suggest that the mesothelium presents a functional barrier to the spread and progression of ovarian tumors. Hence, one would expect progression toward invasive disease to be associated with alterations that enable tumor cells to adhere to the mesothelium and break the mesothelial barrier by provoking mesothelial clearance. Our studies indicate that integrin-dependent activation of myosin contractility in tumor cells is required to perturb the mesothelial barrier. Therefore, our results may show that acquisition of contractile phenotypes in ovarian tumor cells represents a step toward malignant progression.

METHODS

Cell Culture

All cells used in this study were cultured in a 1:1 ratio of Medium 199 (GIBCO) and MCDB 105 (Cell Applications, Inc.) supplemented with 10% fetal bovine serum (GIBCO). Normal lung mesothelial cells were obtained from a benign pleural effusion in a patient with pneumothorax. These cells were immortalized by simultaneous inactivation of p53 and Rb through ectopic expression of SV40 T antigen and overexpression of human telomerase (fused to GFP), as described previously (31, 32). In experiments involving a mixture of labeled and unlabeled mesothelial cells, MET5A (human mesothelioma cells, American Type Culture Collection) were used as a source of unlabeled cells. In focal adhesion tracking experiments, LP9s, a peritoneum-derived mesothelial cell line (Coriell Cell Repositories), was used. Primary lung mesothelial cells were under passage 20. LP9 cells were used under passage 10. MET5A mesothelial cells were under passage 10 and these cells morphologically resembled primary lung mesothelial cells that we used during the course of this experiment. OVCA433 and OVCA5 ovarian cancer cell lines were a generous gift from Dr. Dennis Slamon (University of California, Los Angeles, California).

Spheroid-induced mesothelial clearance assay The mesothelial cells were plated on glass-bottom dishes (Mat-TEK Corporation) coated with 5 μg/mL of fibronectin (Sigma) and/or collagen I (Sigma). Cells were maintained in culture until confluent (48 hours after plating). To generate spheroids, cells were dissociated by trypsinization, labeled with CMTX–red membrane dye (Molecular Probes), washed 2 times with PBS, resuspended in culture medium, and plated on Poly-Hema–coated culture dishes (33). Spheroids were collected for experiments 36 to 48 hours later. The number of cells per spheroid varied from 100 to 500. OVCAR5 spheroids were generated in the presence of 10 μg/mL of soluble bovine fibronectin to increase spheroid cohesion (34, 35). In coculture experiments, spheroids were added to a confluent mesothelial monolayer, allowed to attach for 60 minutes, and imaged for the indicated time. Only spheroids that remained attached during the experiment were used for quantification.

Fibronectin labeling of mesothelial cells and quantification of fibronectin dissociation Twenty micrograms of rhodamine-conjugated fibronectin (Cytoskeleton) was added for a period of 24 hours to a confluent monolayer of human lung mesothelial cells expressing GFP. To quantify fibronectin dissociation from the top of the mesothelial monolayer in the presence of cancer spheroids, we divided total fluorescent intensity of the fibronectin present beneath the cancer spheroid by the total intensity of GFP-labeled mesothelial cells.

Western blots and antibodies Cells were lysed in 100 μl of radioimmunoprecipitation assay buffer (50 mM HEPES, pH 7.4; 1% Triton X-100; 1% sodium deoxycholate; 0.1% SDS; 0.1 M NaCl; 1 mM sodium orthovanadate; 0.1 M sodium pyrophosphate; 100 mM NaF; and 1 mM phenylmethylsulfonyl fluoride). Lysates were clarified by centrifugation at 13,000 g for 10 minutes. Clarified lysates were boiled in 1X sample buffer (0.04 M Tris-HCl, pH 6.8; 1% SDS; 1% β-mercaptoethanol; and 10% glycerol) for 10 minutes and resolved by SDS-PAGE. Proteins were transferred to Immobilon membranes (Whatman) and blocked with 5% bovine serum albumin in PBS (140 mM NaCl, 0.27 mM KCl; 0.43 mM Na2HPO4, 7H2O; 0.14 mM KH2PO4, pH 7.3), 0.1% Tween 20, pH 7.2, for 1 hour at room temperature. Membranes were incubated overnight at 4°C with one of the following antibodies: anti–talin I monoclonal antibody (1:1000; Cell Signaling), antiaxin monoclonal antibody (1:1000; Sigma), anti–myosin heavy chain IIA polyclonal antibody (1:1000; Covance), anti–myosin heavy chain IIB polyclonal antibody (1:1000; Covance), and anti–myosin heavy chain IIB polyclonal antibody (1:1000; Covance).
anti-filamin A polyclonal antibody (1:1000; Cell Signaling), anti-cleaved caspase-3 polyclonal antibody (1:1000; Cell Signaling) anti-α5 integrin polyclonal antibody (1:1000; Cell Signaling), anti-phosphorylated myosin light chain serine 18 polyclonal antibody (1:1000; Cell Signaling), or anti-myosin light chain polyclonal antibody (1:1000; Cell Signaling). Membranes were subsequently probed with secondary antibodies linked to horseradish peroxidase (HRP; Santa Cruz Biotechnology). Western blot membranes were developed using an enhanced chemiluminescent substrate for detection of HRP (VWR). Western blot results were visualized using Kodak film developer and an Epson 3000 scanner. OVCA433 spheroids were treated for 45 minutes in the presence of low serum medium (OPTIMEM) with the following cell adhesion-blocking antibodies: anti-αβ1 integrin (5 μg/mL; BD Biosciences), anti-α5 integrin (5 μg/mL; BD Biosciences), and anti-CD44 (5 μg/mL; Sigma). We used nonspecific isotope IgG (10 μg/mL; Sigma) in function-blocking experiments. Treated spheroids were washed twice with PBS (CellGrow), resuspended in culture medium, and added to the mesothelial monolayer.

**shRNAs, siRNAs, cDNA plasmids, and reagents**

To attenuate the expression level of talin 1 and nonmuscle myosin heavy chain IIA, OVCA433 cells were infected with lentiviruses lacking an shRNA sequence (pLKO) as a control or with plasmid containing talin 1 (Open Biosystems; sequence 1: 5′-CAGGCGACACCTGATGACAGTTGGCTCTTCTCTCTCTCTCAGGTTTACACATGTCATACTCGACGTCGTTTTG3′; and sequence 3: 5′-CCGGCGCATCAACTTTGAGACATCCATCTCGAGATGATTGGTGATGACATC AAAGTTGATGCGTTTTTG-3′ sequences (Open Biosystems; sequence 1: 5′-ATGCCAATGCGTTTTTG-3′-CCGGCGCATCAACTTTGAGACATCCATCTCGAGATGATTGGTGATGACATC AAAGTTGATGCGTTTTTG-3′-CCGGGCCTCAGATAATCTGGTGAAACTC; sequence 2: 5′TTG-3′AGATGTAGCCAAACTCGAGTTTGGCTACATCTTTCACTGCTTT-3′. Lacking an shRNA sequence (pLKO) as a control or with plasmid containing talin 1 (Open Biosystems; sequence 1: 5′-ATGCCAATGCGTTTTTG-3′-CCGGCGCATCAACTTTGAGACATCCATCTCGAGATGATTGGTGATGACATC AAAGTTGATGCGTTTTTG-3′-CCGGGCCTCAGATAATCTGGTGAAACTC; sequence 2: 5′TTG-3′AGATGTAGCCAAACTCGAGTTTGGCTACATCTTTCACTGCTTT-3′). Treated spheroids were incubated with 0.1 N NaOH and air dried. The surfaces were previously (Wang and Pelham, 1998). In brief, the glass surfaces (Invitrogen) were prepared on glass-bottomed dishes, as described previously (Wang and Pelham, 1998). In brief, the glass surfaces were incubated with 0.1 N NaOH and air dried. The surfaces were then subsequently incubated with 3-aminopropyltrimethoxysilane...
(Sigma) and 0.5% glutaraldehyde (Sigma) and were washed in distilled H2O between incubations. After drying, a drop of acrylamide/bis-acrylamide solution containing ammonium persulfate (BioRad), tetramethylethylene diamine (TEMED; Sigma), and 0.2-μm fluorescent microspheres was pipetted onto the modified glass surface. A coverslip was then placed over the droplets to ensure a flat gel surface after polymerization. Fibronectin was coupled to the PAA substrates via the bifunctional crosslinker sulfosuccinimidyl hexanoate (sulfo-SANPAH; Pierce). For traction force measurements of OvCar5 and OvCar5 cells overexpressing integrins, gels with elastic moduli of approximately 10 to 20 kPa were used.

**TFM and Calculations of Traction Forces**

Cells on PAA substrates were imaged with a multispectral multimode spinning disk confocal microscope consisting of a Nikon Ti-E inverted motorized microscope equipped with a custom-built 37°C microscope incubator enclosure with 5% CO2 delivery, an integrated Perfect Focus System, a 40× 0.95 NA Plan Appl Obj ective, a Yokogawa CSU-X1 spinning disk confocal head with internal motorized high-speed emission filter wheel and Spectral Applied Research Borealis modification for increased light throughput and illumination homogeneity, and a Hamamatsu ORCA-AG cooled CCD camera. Images were acquired using MetaMorph software (MDS Analytical Technologies). After imaging, cells were trypsinized with 0.25% trypsin to obtain an image of unstrained bead positions as reference frames for analyses. Positions of fluorescent beads were extracted from image series, and time-integrated cross-correlation tracking was used, as described previously. Traction forces generated by the cells were determined using custom MATLAB programs following the boundary element and Fourier transform traction microscopy (FTTC) methods described by Sabass et al. (17).

Correlation tracking was used, as described previously. Traction force measurements of OvCar5 and OvCar5 cells overexpressing α6 integrin, gels with elastic moduli of approximately 10 to 20 kPa were used.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We thank Ghassan Mouneimne and Cheuk Leung for lab meeting discussions and valued input into the direction of this project; Benedikt Sabass and Ulrich Schwarz for providing the regularized FTTC algorithm; and the Nikon Imaging Center at Harvard Medical School for help with light microscopy, especially Jennifer Waters, Wendy Salmon, Lara Petrak, and Cassandra Rogers.

**REFERENCES**


Correction: Ovarian Cancer Spheroids Use Myosin-Generated Force to Clear the Mesothelium

In this article (Cancer Discovery 2011;1:144–57), which was published in the July 2011 issue of Cancer Discovery (1), the name and affiliation of the eighth author is incorrect. The correct name and affiliation are Tan A. Ince, Department of Pathology, Braman Family Breast Cancer Institute and Interdisciplinary Stem Cell Institute, University of Miami Miller School of Medicine, Miami, Florida.

In addition, there are several textual errors in the Results and Methods sections. These changes do not affect any of the data presented in the manuscript or the conclusions. The applicable passages including the correct reference callouts are provided below:

Results, page 152, Figure 6 legend, 4th sentence:
The strain energy was calculated as the product of measured bead displacement and reconstructed traction stresses, integrated over the cell footprint.

Methods, page 155, second column, first paragraph:
We used Student t test to calculate statistical significance.

Methods, page 156, first column, first paragraph:
The regularization parameter $\lambda^2 = 10^{-6}$ has been determined using the L-curve method.

Methods, page 156, first column, first paragraph:
Traction forces were reconstructed from the measured bead displacements using an implementation of the regularized Fourier Transform Traction Cytometry method (37) provided in Ref. 17.

Methods, page 156, first column, second paragraph:
Here, $T$ and $\delta$ denote the reconstructed traction stress and measured bead displacement, respectively.

Methods, page 156, first column, second paragraph:
We used Student t test to calculate statistical significance.

The online version of the article has been corrected and therefore no longer matches the print version. The authors regret these errors.

REFERENCE

Published Online First November 30, 2011.
doi: 10.1158/2159-8290.CD-11-0279
© 2011 American Association for Cancer Research
# Ovarian Cancer Spheroids Use Myosin-Generated Force to Clear the Mesothelium

Marcin P. Iwanicki, Rachel A. Davidowitz, Mei Rosa Ng, et al.

*Cancer Discovery* Published OnlineFirst June 14, 2011.

<table>
<thead>
<tr>
<th>Updated version</th>
<th>Access the most recent version of this article at: doi:10.1158/2159-8274.CD-11-0010</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplementary Material</td>
<td>Access the most recent supplemental material at: <a href="http://cancerdiscovery.aacrjournals.org/content/suppl/2011/06/13/2159-8274.CD-11-0010.DC1">http://cancerdiscovery.aacrjournals.org/content/suppl/2011/06/13/2159-8274.CD-11-0010.DC1</a> <a href="http://cancerdiscovery.aacrjournals.org/content/suppl/2011/06/13/2159-8274.CD-11-0010.DC2">http://cancerdiscovery.aacrjournals.org/content/suppl/2011/06/13/2159-8274.CD-11-0010.DC2</a></td>
</tr>
</tbody>
</table>

| E-mail alerts | Sign up to receive free email-alerts related to this article or journal. |
| Reprints and Subscriptions | To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at [pubs@aacr.org](mailto:pubs@aacr.org). |
| Permissions | To request permission to re-use all or part of this article, contact the AACR Publications Department at [permissions@aacr.org](mailto:permissions@aacr.org). |