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

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ABSTRACT

Dissemination of ovarian tumors involves the implantation of cancer spheroids into the mesothelial monolayer on the walls of peritoneal and pleural cavity organs. Biopsies of tumors attached to peritoneal organs show that mesothelial cells are not present under tumor masses. We have developed 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 mesothelial clearance. In this article, we provide evidence that ovarian cancer spheroids use integrin- and talin-dependent activation of myosin and traction force to promote displacement of mesothelial cells from underneath a tumor cell spheroid. These results suggest that ovarian tumor cell clusters gain access to the submesothelial environment by exerting force on the mesothelial cells lining target organs, driving migration and clearance of the mesothelial cells.

SIGNIFICANCE: This study uses time-lapse video microscopy to decipher cellular events associated with ovarian tumor cell intercalation of mesothelial cell layers. Ovarian cancer clusters were found to use actomyosin-generated force to physically displace mesothelial cells and gain access to the submesothelial environment. Blockade of force-conducting molecules, including αv integrin, talin I, and non-muscle myosin II, in cancer cells abrogated mesothelial displacement from underneath attached cancer spheroids. Cancer Discovery; 1(2):144–57. ©2011 AACR.

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 under 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 (OVCA433 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
Contractile Forces Generated by Ovarian Cancer Spheroids Promote Mesothelial Clearance

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 (GFP) 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

**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.
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 (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 1 to adhesion sites (10). We used shRNA to attenuate the expression of talin 1 in the spheroids (Fig. 3D). Attenuation of talin 1 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 1, 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 1 molecule in OVCA433, DOV13, and SKOV3 ovarian cancer cell lines (Supplementary Fig. 2D). These data indicate that talin 1 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.

αβ1 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 ovarian cancer (12) and increased invasion of ovarian cancer cells (13, 14), we addressed whether αβ1 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 Movie 8; Supplementary Fig. 3B). Blocking other adhesion receptors expressed by OVCA433 spheroids (data not shown), including CD44 and integrins α3 and αv, using function-blocking antibodies, did not have any significant effect on spheroid-induced mesothelial clearance (Supplementary Fig. 3C).

In parallel experiments, we overexpressed α5 integrin in ovarian cancer cells that have a low level of α5 integrin expression (OVCA5) and are unable to clear a 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 Fibrils 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 1 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 α5β1 integrin was inhibited by an α5 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 α5β1 integrin expressed by the cancer spheroids (Fig. 5C and D). These data suggest that ovarian cancer spheroids use α5β1 integrin to dissociate fibronectin from the mesothelial monolayer during clearance.

Cancer Cells Exert Force on a Fibronectin-Coated Substrate in an α5β1 Integrin-, Talin 1-, and Myosin-Dependent Manner

The data presented above show that α5β1 integrin, myosin, and talin are all required in ovarian cancer cells for mesothelial clearance and that α5β1 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 substratum by the spreading spheroids...
Figure 4. α5β1 integrin contributes to the activation of myosin in OVAR5 ovarian cancer cells and is required for OVAR5 spheroid-induced mesothelial clearance. A, Western blot analysis of α5 integrin expression levels in OVAR5 and OVCA433 ovarian cancer cells. Scale bar, 10 μm. B, images depict mesothelial clearance induced by OVCA433 cancer spheroids in the presence of control or α5 integrin–blocking antibody at 0- and 10-hour time points. Scale bar, 50 μm. C, quantification of mesothelial clearance from B. A total of 12 independent regions were analyzed per condition. D, Western blot analysis of α5 integrin, phospho-MLC, and MLC expression levels in control or α5-overexpressing OVAR5 ovarian cancer cells plated on fibronectin-coated glass. Images show organization of the actin cytoskeleton in control and α5 integrin-overexpressing OVAR5 ovarian cancer cells plated on fibronectin-coated glass. E, images represent mesothelial clearance induced by control or α5 integrin-overexpressing OVAR5 cells at 0- and 10-hour time points. Scale bar, 50 μm. F, quantification of mesothelial clearance from E. A total of 20 randomly chosen regions were analyzed per condition.

contributes to mesothelial clearance. To determine if modulation of α5, talin 1, 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 OVAR5 cells, OVAR5 cells overexpressing α5 integrin, and α5-overexpressing cells treated with either talin 1 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 α5 integrin in cancer cells correlated with an increase in cellular contractility, as measured by strain energy exerted by the cells. The α5 integrin-induced force generation was dependent on myosin activation because OVCAR5 cells overexpressing α5 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 α5 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 α5 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

**Figure 5.** Cancer spheroids expressing functional α5β1 integrin dissociate fibronectin fibrils from the surface of the mesothelium. **A**, top, laser scanning confocal image showing the top view of an OVA433 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 OVA433 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 α5β1–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 α5β1 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.
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, α5β1 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 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 lymphogenous 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 Ovaca433 spheroids (αβ1, αβ6, αβ7, αβ12) did not significantly prevent Ovaca433 spheroid attachment to the mesothelium after 10 hours of coculture. Interestingly, however, our data indicated that interfering with the function of αβ1 integrin alone can significantly decrease Ovaca433, DOV13, and Skov3 spheroid-induced mesothelial clearance over a period of 10 hours (Supplementary Fig. 3A and C). Because αβ1 integrin is a fibronectin receptor, these results suggest that cancer spheroids can use αβ1 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 αβ1 integrin expressed by the cancer spheroids. In addition, we also observed that the expression level of αβ1 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 αβ integrin-independent mechanisms can mediate clearance as well.

Figure 6. Cancer cells exert forces on a fibronectin-coated PAA gel in an α5β1, integrin-, talin I-, and myosin-dependent manner. Ovaca5 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 substrate and a measure of the overall contractility of a cell (37). α5 integrin-expressing Ovaca5 cells have low endogenous α5 integrin (ITGA5) expression levels and are significantly less contractile than Ovaca5 cells overexpressing ITGA5 (P < 0.001). The high contractility of ITGA5-overexpressing Ovaca5 cells is attenuated by treatment with 1 μM blebbistatin (P < 0.001). B, contractility of ITGA5-overexpressing Ovaca5 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.
Integrins are the major molecules that can transmit traction forces to the outside environment (23). Although αv integrin binding to collagen I can induce fibril reorganization and transmit traction forces to the ECM in certain contexts (24), OVCA5 ovarian tumor spheroids that express high levels of αv, but not αv integrin, were unable to clear the mesothelium in our experiments (Supplementary Fig. 3C). In addition, blocking αv integrin in OVCA433 cells that express both αv 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 αv integrins promotes strong adhesiveness to the matrix; and second, recruitment of talin I stabilizes and reinforces formed αv integrin mediate adhesions (25), promoting the exertion of traction force on the matrix. Our study indicated that the interaction between fibronectin receptor αv 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 αv integrin-mediated formation of traction force and mesothelial clearance. We found that interfering with the function of another fibronectin receptor, αv 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 αv integrin, but not αv 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 αv integrin, myosin II, and talin I—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
accompanies migration of individual mesothelial cells from underneath the tumor spheroid. 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 breach 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 stochastic 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 mesothelial cells. In focal adhesion tracking experiments, LP9s, a peritoneum-derived mesothelial cell line (Cornell Cell Repositories), was used. Primary lung mesothelial cells were under passage. L9P cells were under passage 10. METSA 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**

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 stochastic 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 mesothelial cells. In focal adhesion tracking experiments, LP9s, a peritoneum-derived mesothelial cell line (Cornell Cell Repositories), was used. Primary lung mesothelial cells were under passage. L9P cells were under passage 10. METSA 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).

**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 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 NaHPO4; 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 1 polyclonal antibody (1:1000; Cell Signaling), anti-actin monoclonal antibody (1:1000; Sigma), anti-myois heavy chain IIA polyclonal antibody (1:1000; Covance), anti-myosin heavy chain IIIB 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-α1 integrin polyclonal antibody (1:1000; Cell Signaling), anti-γ-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 incubated and washed with PBS (140 mM NaCl; 0.27 mM KCl) for 10 minutes. Western blots 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 (OPTI-MEM) with the following cell adhesion-blocking antibodies: anti-α1, integrin (5 μg/mL; BD Biosciences), anti-α1, β1 integrin (5 μg/mL; BD Biosciences), and anti-CD44 (5 μg/mL; Sigma). We used nonspecific serum 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.
To attenuate the expression level of nonmuscle myosin IIB in OVCAR43 cells or talin 1 in OVCARS cells, we also used a pool of siRNA oligonucleotides against nonmuscle myosin IIB or talin 1 (Dharmacon). To ectopically express α5 integrin in OVCARS cells, we used a retroviral vector (pLZRS) encoding the human α5 integrin gene (generous given by Dr. Erik Danen, The Netherlands Cancer Institute, Amsterdam, The Netherlands). Infected cells were selected in growth medium containing 600 μg/mL G418 (Dulbecco). Staurosporin was purchased from CalBiochem.

**Live Cell Imaging**

**Spheroid-induced mesothelial clearance assay** Imaging was performed using a Nikon Ti-E Inverted Motorized Widefield Fluorescence Microscope with integrated Perfect Focus System and low [20·×·0.75 numerical aperture (NA)] magnification/NA differential interference contrast (DIC) optics, a Nikon halogen transilluminator with 0.52 NA long working distance (LWD) condenser, Nikon fast (-100-milliseconds switching time) excitation and emission filter wheels, Sutter fast transmitted and epifluorescence light path Smart Shutters, a Nikon linear-encoded motorized stage, a Hamamatsu ORCA-AG cooled charge-coupled device (CCD) camera, a custom-built microscope incubation chamber with temperature and CO2 control, Nikon NIS-Elements AR software version 3, and a TMC vibration isolation table.

**TIRF analysis** Mesothelial cell adhesion dynamics were visualized using a Nikon Ti-E inverted microscope with integrated Perfect Focus System; Nikon 1.4 NA TIRF DIC optics (60×); a Nikon halogen transilluminator with 0.52 NA LWD and 0.85 NA Dry condenser; a Nikon dual-port TIRF/Epi-Illuminator with motorized incident angle adjustment and motorized switching between TIRF and epi-illumination; a Solamere laser launch with 100-mW/491-nm, 75-mW/561-nm, and 30-mW/640-nm solid-state lasers with fiber-optic delivery system and 4-channel acousto-optic tunable filters (AOTF); a Prior controller; Prior fast excitation and emission filter wheels, Prior fast transmitted and epifluorescence light path shutters; a Prior linear-encoded motorized stage; a Hamamatsu ImageM 512×512 back-thinned electron-multiplying cooled CCD camera; a 20/20 Technologies Bioinomc Controller/Stage heater insert; Molecular Devices MetaMorph version 7.7; and a TMC vibration isolation table.

**Analysis of the dynamics of rhodamine-labeled fibronectin** Imaging was performed using a spinning disk confocal microscope: Nikon Ti-E inverted motorized microscope equipped with integrated Perfect Focus System; Nikon Plan Apo 1.4 NA DIC optics (60×); Nikon halogen transilluminator with 0.52 NA LWD and 0.85 NA Dry condenser; 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; a Spectral Applied Research custom laser merge module (LMM-7) with AOTF and 100- to 200-mW solid-state 442-nm, 491-nm, 515-nm, 561-nm, and 642-nm lasers; Semrock 405/488/561/647 and 442/514/647 dichroic mirrors; a Prior ProScan II controller; a Prior NanoScan piezo Z stage insert for high-speed z-series; Prior fast transmitted and epifluorescence light path shutters; a Hamamatsu ORCA-AG cooled CCD camera; a custom-built 37°C microscope incubator enclosure with 5% CO2 delivery; Molecular Devices MetaMorph version 7.7; and a TMC vibration isolation table.

**Quantification of mesothelial clearance and dissociation of fibronectin** To quantify mesothelial clearance, the nonfluorescent area in the GFP mesothelial monolayer was measured over time and divided by the initial area of the cancer spheroid. To quantify the dissociation of fibronectin from mesothelial cells, the total fluorescence intensity of rhodamine-labeled fibronectin (enclosed within the area of the spheroid) was divided by the total fluorescent intensity of GFP-labeled mesothelial cells beneath the spheroid. Data were plotted as a point distribution using JMPB statistical software. We used the nonparametric Mann–Whitney t test to calculate statistical significance (*, P = 0.05; **, P = 0.01; and ***, P < 0.001).

**Cancer spheroid adhesion assay** OvCA43 spheroids were co-cultured with mesothelial monolayers for 5 hours. Spheroids that did not adhere to the monolayer within this time were removed and replated on fibronectin- and collagen I-coated glass-bottomed dishes for 60 minutes, and the spheroids were counted. All spheroids that adhered to the mesothelial monolayer were counted. The number of spheroids adhered to the mesothelial monolayer plus the number of spheroids attached to the matrix-coated dish represented the total number of spheroids. The percentage of spheroids adhered to the mesothelium was calculated by dividing the number of spheroids attached to the mesothelium by the total number of spheroids (mesothelium + culture dish).

**Preparation of PAA gel substrates for TFM** Fibronectin-coated PAA gels containing 0.2-μm fluorescent microspheres (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), tetramethylethylenediamine (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 sulfo-succinimidyl hexanoate (sulfo-SANPAH; Pierce). For traction force measurements of OvCar5 and OvCar5 cells overexpressing α5 integrin, 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 Apo objective, 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 with 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 cymometry (FTTC) methods described by Sabass et al. (17). Square image blocks with a template size of 25 pixels (4.1 μm) were...
centered on each reference bead position, identified as intensity maxima in the reference frame. Bead displacements were defined as the x-y shift that maximizes the cross-correlation score of these image blocks in a corresponding region of the deformed bead image. To minimize false-positive template matching, bead displacements with insignificant maxima in the cross-correlation score function are rejected (36). Traction forces were reconstructed from the measured bead displacements, using an implementation of the regularized fast FTTC method (37) provided in ref. 17. For this study we regarded the PAA substrate as isotropic, linear elastic, infinite half-space and thus applied the Boussinesq Green function. To solve the ill-posed inversion problem, we applied zero-order Tikhonov regularization (38). The regularization parameter $\lambda=2 \times 10^{-6}$ has been determined using the L-curve method, and we used the strain energy $U$, invested by the cell to deform the substrate, as a measure of cellular contractility (37): $U = \frac{1}{2} \int (T(\mathbf{r}) \cdot \nabla T(\mathbf{r})) d\mathbf{r}$. Here, $\vec{T}$ and $\vec{u}$ denote the measured displacement and reconstructed traction stress, respectively. To avoid possible boundary artifacts introduced by the FTTC algorithm (37), integration was performed over a hand-drawn elliptical domain $\Omega$ that just covered the whole footprint of the cell. Data were plotted as a point distribution with JMP8 statistical software. We used the nonparametric Mann-Whitney $t$ test to calculate statistical significance (*, $P = 0.05$; **, $P = 0.01$; and ***, $P = 0.001$).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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26. Moyano JV, Maqueda A, Casanova B, Garcia-Pardo A. Alpha4beta1 integrin/ligand interaction inhibits alpha6beta1-induced stress
Contractile Forces Generated by Ovarian Cancer Spheroids Promote Mesothelial Clearance


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

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

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