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β1-Integrins Regulate the Formation and Adhesion of Ovarian Carcinoma Multicellular Spheroids

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Ovarian carcinoma multicellular spheroids are an *in vitro* model of micrometastasis whose adhesive abilities have not been elucidated. In this study, we identified adhesion molecules that mediate the formation of ovarian carcinoma spheroids and their subsequent adhesion to extracellular matrix proteins. The NIH:OVCAR5, but not the SKOV3, ovarian carcinoma cell line formed spheroids similar to multicellular aggregates isolated from patient ascitic fluid. NIH:OVCAR5 spheroid formation was augmented by a β1-integrin-stimulating monoclonal antibody or exogenous fibronectin, but was inhibited by blocking monoclonal antibodies against the α5- or β1-integrin subunits. By immunohistochemical staining, α2-, α3-, α5-, α6-, and β1-integrin subunits, CD44, and fibronectin were detected in NIH:OVCAR5 spheroids. NIH:OVCAR5 spheroids adhered to fibronectin, laminin, and type IV collagen, and this adhesion was partially inhibited by blocking antibodies against the α5-, α6-, and α2-integrin subunits, respectively. A blocking monoclonal antibody against the β1-integrin subunit completely inhibited adhesion of the spheroids to all three proteins. These results suggest that interactions between the α5β1-integrin and fibronectin mediate the formation of ovarian carcinoma spheroids and that their adhesion to extracellular matrix proteins at sites of secondary tumor growth may be mediated by a complex interaction between multiple integrins and their ligands. ***(Am J Pathol 2001, 159:2071–2080)***

Ovarian cancer is the leading cause of gynecological malignancy and the fifth leading cause of cancer death among women in the United States.[1] In ovarian carcinoma, cancer cells detach from the surface of the tumor into the peritoneal cavity. Subsequent peritoneal implants are characterized by the adhesion, migration, and invasion of the tumor cells into the peritoneum and underlying organs. Free-floating tumor cells are found in the peritoneal cavity both as both single cells and as multicellular aggregates.[2] However, because they are difficult to study and manipulate compared to single-cell suspensions, ovarian carcinoma multicellular aggregates have been primarily ignored in most studies.

Many human tumor cells and cell lines can be cultured as multicellular aggregates, which are spherical, mechanically stable, and viable.[3] Early *in vitro* studies indicated that ovarian carcinoma tumor cells and cell lines were unable to form spheroids, but remained exclusively as free-floating single cells or formed monolayers in tissue culture.[4] More recently, spheroids have been successfully generated from some ovarian cancer cells and cell lines, and used as three-dimensional *in vitro* models to study the efficacy of therapeutic strategies.[5–7] Cells in ovarian carcinoma spheroids exhibit changes in their position in the cell cycle and are protected from radiation-induced[5,6] and taxol-induced[8,9] apoptosis, compared to cells cultured as monolayers. However, the phenomena that mediate the formation of ovarian carcinoma multicellular spheroids and their subsequent abilities to adhere, migrate, invade, and proliferate at secondary growth sites have not been investigated, and their contributions to secondary tumor growth, if any, have not been assessed. It still remains unclear whether the floating multicellular aggregates found in patients' ascites fluid are capable of adhering to the extracellular matrix (ECM) of mesothelial cells or whether they are merely nonadhesive, and therefore noninvasive or benign, counterparts to the malignant ovarian carcinoma cells that adhere to the peritoneal lining.

Many cell-cell and cell-matrix interactions are regulated by integrins, a family of heterodimeric transmembrane receptors.[10] ECM proteins, which include fibronectin, type IV collagen, and laminin, affect the *in vitro* growth, morphology, survival, and differentiation of normal and malignant cells via their interactions with inte-

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grins.[11] In ovarian carcinoma, integrins have been shown to mediate the organization of ECM,[12] adhesion to ECM components,[13–15] and cell motility.[16–18] Integrins have also been shown to mediate interactions between ovarian carcinoma cells and the mesothelial cells that line the abdominal organs.[15] CD44, another cell surface receptor found on ovarian carcinoma cells,[15,18,19] binds the ECM glycosaminoglycan hyaluronan with high affinity[20] and also has a weak affinity for fibronectin, type IV collagen, and laminin.[21] Interactions between CD44 and hyaluronan affect cell adhesion,[15] migration,[18,19] and tumor growth[22] in ovarian carcinoma cells.

The purpose of this study was to elucidate the biological properties of ovarian carcinoma spheroids by developing an *in vitro* model from established ovarian carcinoma cell lines. We examined the roles of integrins, CD44, and ECM proteins in the formation of ovarian carcinoma spheroids. The proliferative ability and viability of ovarian carcinoma cells cultured as spheroids were also determined and compared to that of the same cell lines cultured as monolayers. We examined the expression of adhesion molecules in NIH:OVCAR5 spheroids. We assessed the ability of ovarian carcinoma spheroids to adhere to ECM molecules and identified integrin subunits that mediated these interactions. The results from this study identify adhesion molecules that participate in the formation of ovarian carcinoma spheroids and suggest that their subsequent adhesion to secondary sites of tumor growth may be integrin-dependent events. Our findings suggest that ovarian carcinoma cells present as free-floating multicellular aggregates may exhibit markedly different behavior than ovarian carcinoma single cells or monolayers. It is possible that these differences may then translate into different metastatic abilities *in vivo* and/or responses to treatments.

Materials and Methods

Unless otherwise stated, all standard reagents and materials were obtained from Sigma Chemical Company (St. Louis, MO), all pictures were photographed with a Nikon Coolpix 950 camera (Melville, NY), and all experiments were performed in triplicate and repeated a minimum of three times.

Cell Culture

The human ovarian carcinoma cell lines NIH:OVCAR5 and SKOV3 were chosen for their ability to mimic the progression of ovarian carcinoma when injected into *in vivo* mouse models.[23] These cells have also been shown to adhere to peritoneal mesothelial cells in *in vitro* models.[24,25] The ovarian carcinoma cell line SKOV3 was obtained from Dr. Robert Bast, Jr., M.D. Anderson Cancer Center, University of Texas, Houston, TX. These cells were maintained in McCoy's 5A medium supplemented with 15% fetal bovine serum, 2 mmol/L L-glutamine, and 50 U/ml penicillin G/streptomycin (Life Technologies, Grand Island, NY). The ovarian carcinoma cell line NIH:OVCAR5 was originally established by Dr. Thomas Hamilton (Fox Chase Cancer Center, Philadelphia, PA)[26] and obtained from Dr. Judah Folkman, Harvard Medical School, Boston, MA. This cell line was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 0.2 U/ml insulin, and 50 U/ml penicillin G/streptomycin. Both cell lines were maintained in 75-mm^2 tissue culture flasks in a humidified incubator with 5% CO_2 at 37°C.

Purification of Primary Ovarian Carcinoma Cells

Primary ascites fluid samples from six patients diagnosed with serous ovarian adenocarcinoma were obtained with the approval of the University of Minnesota Institutional Review Board from the University of Minnesota Cancer Center Tissue Procurement Facility. Tumor cells were collected by centrifugation (1000 $\times$ *g*, 10 minutes). To lyse erythrocytes, the cells were resuspended in 10 mmol/L potassium bicarbonate, 155 mmol/L ammonium chloride, 0.1 mmol/L ethylenediaminetetraacetic acid, pH 7.4, for 5 minutes. The remaining cells were collected by centrifugation (1000 $\times$ *g*, 10 minutes). The tumor cells were layered on Ficoll-Paque Plus (Pharmacia Biotech, Uppsala, Sweden) and centrifuged at 2000 $\times$ *g* for 15 minutes. The tumor cells were removed from the top of the Ficoll layer and washed with RPMI 1640 medium.

Spheroid Culture

The method used to generate spheroids was based on the liquid overlay technique.[27] To prohibit cell adhesion to a substratum, the wells of 24-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ) were coated with 200 μl of 0.5% SeaKem LE agarose (BioWittaker Molecular Applications, Rockland, ME) in serum-free media, and allowed to solidify for 30 minutes at 20°C. NIH:OVCAR5 or SKOV3 cells were grown in monolayer cultures, released with 0.5% trypsin, 2 mmol/L ethylenediaminetetraacetic acid as described previously,[28] and resuspended in complete cell culture media at 5000 to 50,000 cells/ml. Cell suspensions were layered onto the top of the solid agarose-coated plates at a volume of 1 ml/well, and then incubated for 48 hours at 37°C. By this technique, cells remained afloat in the media, and did not become incorporated or implanted in the solidified agarose that coats the wells of the 24-well plates. After 48 hours, the resulting spheroids were gently removed from the surface of the solidified agarose and centrifuged at 300 $\times$ *g* for 3 minutes to remove single cells before use in subsequent experiments.

Antibodies

Purified monoclonal antibodies (mAbs) that block the adhesive activity of human integrin subunits α1 (clone FB12), α2 (clone P1E6), α3 (clone P1B5), α4 (clone P1H4), α5 (clone P1D6), α6 (clone GoH3), and $\alpha v\beta$3 (clone LM609) were purchased from Chemicon International (Temecula, CA). A purified mAb against the human β1-integrin subunit that stimulates cell adhesion to ECM

proteins (clone 21C8) was also purchased from Chemicon International. Purified immunoglobulin (IgG) of mouse mAb P5D2, which blocks the adhesive activity of human β1-integrin subunits, was provided by Dr. Leo Furcht (University of Minnesota). Affinity-purified IgG of mAb IM7, which blocks the hyaluronan-binding site of CD44, was purchased from Pharmingen (San Diego, CA). Polyclonal rabbit IgG against human fibronectin was purchased from Calbiochem-Novabiochem Corporation (San Diego, CA). Normal mouse IgG and normal goat serum were purchased from Sigma.

ECM Molecules

Type IV collagen, isolated from mouse Engelbreth-Holm-Swarm tumor, was purchased from Life Technologies. Mouse Engelbreth-Holm-Swarm laminin, prepared as previously described,[29] was provided by Dr. Leo Furcht, University of Minnesota. Human plasma fibronectin, purified as described,[30] was provided by Dr. James McCarthy, University of Minnesota.

Proliferation Assays

Single-cell suspensions of NIH:OVCAR5 or SKOV3 cells were added to 24-well tissue culture plates to form monolayers, or to agarose-coated 24-well plates to form spheroids, at a density of 500 cells/200 μl. The cells were cultured in complete cell culture media for up to 21 days. At various time points, 2 mg/ml WST-1 (Boehringer-Mannheim Corporation, Indianapolis, IN) was added to each well and incubated for 2 hours. WST-1 is a tetrazolium salt that is reduced by mitochondrial dehydrogenases to form a formazan dye. The formazan product was quantitated by a SpectaMax 250 scanning multiwell spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA) by measuring absorbance at 450 nm. These experiments were performed in quadruplicate.

Role of Adhesion Molecules in Multicellular Aggregation

To examine the role of cell surface receptors in ovarian carcinoma spheroid formation, single-cell suspensions of NIH:OVCAR5 or SKOV3 cells in serum-free media were added to agarose-coated 24-well plates at a density of 5000 cells/200 μl in the presence of 10 μg/ml of normal mouse IgG or mAbs against integrin subunits or CD44. To examine the effect ECM proteins on spheroid formation, the cells were incubated in the presence of 25 μg/ml of fibronectin, laminin, type IV collagen, or ovalbumin. The cells were incubated at 37°C for up to 24 hours in a humidified incubator, examined under a light microscope, and photographed.

Expression of Cell Surface Receptors and ECM Molecules in Spheroids

NIH:OVCAR5 spheroids were collected by centrifugation at 300 × *g* for 3 minutes and resuspended in 80 μl of expired human plasma (American Red Cross, Minneapolis, MN). To suspend the spheroids in a semisolid clot suitable for embedding, 40 μl of 10 U/ml of human thrombin was added to the suspension. Alternately, NIH:OVCAR5 spheroids were suspended in 1% agarose. After polymerization, the clots were embedded in OCT frozen embedding material (Fisher Scientific, Pittsburgh, PA) on dry-ice. Six μm-thick sections were cut on a cryostat and collected on poly-L-lysine-coated slides. The sections were washed with phosphate-buffered saline (PBS), pH 7.6, and blocked with normal goat serum for 30 minutes. The samples were then incubated with 1 μg/ml of the primary mouse IgG for 1 hour, followed by incubation with the secondary goat anti-mouse biotinylated antibody for 30 minutes. Endogenous peroxidase was quenched by incubating the sections in 0.3% H_2O_2 in PBS for 30 minutes. The sections were incubated for 30 minutes with Vectastain ABC reagent (Vector Laboratories, Burlingame, CA) and developed with the peroxidase substrate solution to obtain optimal color. The enzymatic reaction was quenched with excess PBS, and the sections were fixed in 2% formaldehyde, followed by mounting with Cytoseal (Fisher Scientific), and examination under a light microscope. In some cases, the samples were counterstained with methyl green stain (Vector Laboratories) according to the manufacturer's instructions.

Adhesion Assays

Glass chamber slides (Nalge Nunc International, Naperville, IL) were coated with 50 μg/ml of fibronectin, laminin, type IV collagen, or ovalbumin in PBS for 16 hours at 37°C. The slides were blocked with 2 mg/ml ovalbumin in PBS for 1 hour at 37°C. Approximately 50 to 80 NIH:OVCAR5 spheroids in 200 μl of serum-free medium were added to the slides and incubated for up to 4 hours at 37°C. The total number of spheroids in each sample was manually counted, and then nonadherent cells were gently rinsed off with PBS. Adherent cells were fixed with 2% formaldehyde in PBS, stained with Diff-Quik (Dade Behring Inc., Newark, DE), sealed with Cytoseal, and manually counted under a light microscope.

Inhibition of Spheroid Adhesion

NIH:OVCAR5 spheroids were allowed to adhere to glass chamber slides as described above, except that the spheroids were incubated in the presence of 10 μg/ml of normal mouse IgG or mAbs against the α2-, α5-, α6-, or β1-integrin subunits, or CD44. After a 2-hour incubation, the total number of spheroids that were present in each chamber was manually counted before the nonadherent cells were removed as described above. The adherent spheroids were fixed, stained, manually counted, and expressed as a percentage of the total spheroids initially added to each chamber.

Statistical Analysis

Student's *t*-test was performed as a test of significance with the use of Microsoft Excel 1997 (Microsoft Co., Red-

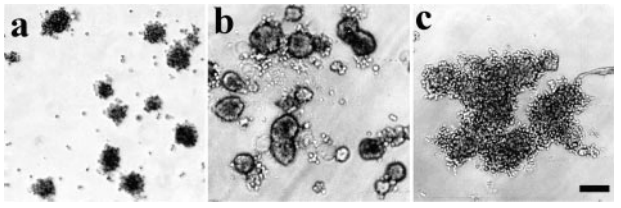

Figure 1. Ovarian carcinoma spheroid formation. Ovarian carcinoma cells obtained from patients' ascitic fluid, the NIH:OVCAR5 ovarian carcinoma cell line, or the SKOV3 ovarian carcinoma cell line were cultured in 0.5% agarose-coated 24-well plates at a density of 20,000 cells/well for 48 hours then photographed. These pictures were representatives of ovarian carcinoma cells obtained from one of the six different patients diagnosed with serous ovarian carcinoma (**a**), the NIH:OVCAR5 cell line (**b**), and the SKOV3 cell line (**c**). Scale bar, 250 μm.

mond, WA). *P* values of <0.01 were considered to indicate statistically significant differences.

Results

Formation of Ovarian Carcinoma Spheroids

Multicellular aggregates of ovarian carcinoma cells were observed in the ascitic fluid obtained from six different patients diagnosed with stage III or stage IV ovarian carcinoma. The sizes of the multicellular aggregates in the patient samples ranged from 50 to 750 μm in diameter; a representative sample is shown in Figure 1a. All of the patients' samples contained spheroids of varying sizes, and the range of sizes of the spheroids varied from patient to patient. Typically, however, the spheroids ranged in size from a score of − to ++ (see Table 1), with the majority of the spheroids scoring as ++. It was not possible to disaggregate these spheroids into a single-cell suspension, even when the spheroids were physically manipulated (via repetitive pipetting) and treated with trypsin (not shown).

Ovarian carcinoma cell lines were cultured in agarose-coated plates to determine whether spheroid phenotypes could be generated in the absence of an adhesive substratum. After 48 hours, the NIH:OVCAR5 ovarian carcinoma cell line formed spheroids (Figure 1b) that appeared similar to the multicellular aggregates found in ovarian carcinoma patients' ascites samples (Figure 1a) and were similarly resistant to physical manipulation and trypsin treatment. In addition, like the multicellular aggregates obtained from the patients' ascites samples, NIH:OVCAR cells formed spheroids of varying sizes that ranged from 60 to 400 μm in diameter, with a score of ++ in size.

In contrast, the SKOV3 ovarian carcinoma cell line formed irregular multicellular aggregates (Figure 1c) that dispersed when subjected to mild agitation. These SKOV3 cell aggregates were significantly larger than those found in patients' ascites samples; SKOV3 spheroids contained hundreds of cells and scored as +++ by our criteria (Table 1). In all cases, the cells were viable, as determined by trypan blue staining (data not shown).

Table 1. Quantitation of Spheroid Formation

Number of cells in spheroid	Score	Description of spheroid
1	−	None
2 to 5	+	Small
6 to 20	++	Medium
>20	+++	Large

In order to maintain the ovarian carcinoma cells in suspension and prohibit cell adhesion to the bottom of tissue culture plates, the wells of 24-well tissue culture plates were coated with agarose and allowed to solidify as described in the Materials and Methods section. Cell suspensions were layered onto the top of the solid agarose, and then incubated for 48 hours at 37°C. By this technique, cells remained afloat in the media, and did not become incorporated or implanted in the solidified agarose that coated the wells. After 48 hours, the resulting spheroids were gently removed from the surface of the solidified agarose and centrifuged to remove single cells before use in subsequent experiments. The sizes of the spheroids formed in the experiments were quantified based on the above scoring system.

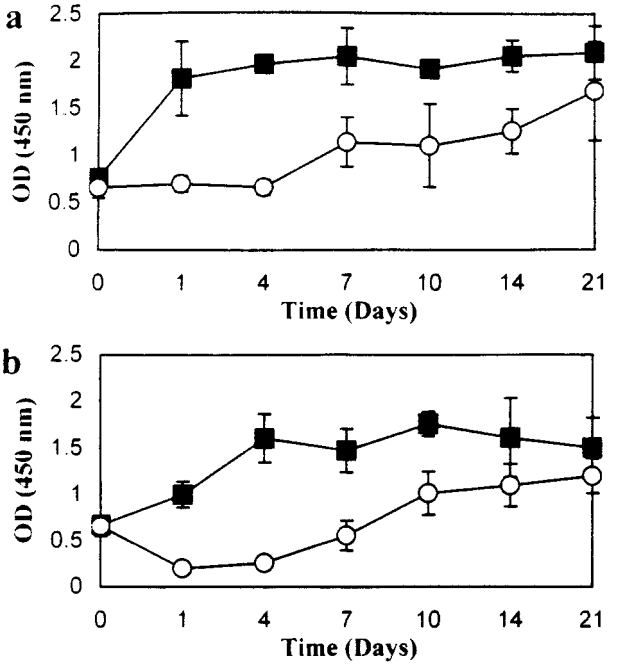

Figure 2. Proliferative index of ovarian carcinoma cells grown as monolayers or spheroids. NIH:OVCAR5 (**a**) and SKOV3 cells (**b**) were added to agarose-coated wells at a concentration of 500 cells/well and cultured as spheroids (**open circles**) or as monolayers (**filled squares**) for up to 21 days. The level of proliferation was quantitated as described in the Material and Methods. Data are expressed are mean ± SD.

Aggregation into Multicellular Spheroids Decreases the Proliferative Abilities of Ovarian Carcinoma Cell Lines

To more fully examine the effects of multicellular aggregation on the viability and proliferative ability of the cells, NIH:OVCAR5 and SKOV3 cells were cultured as monolayers or multicellular aggregates for up to 21 days (Figure 2). When cultured in tissue culture-treated plates as monolayers, the proliferation rates of the NIH:OVCAR5 cells (Figure 2a, filled squares) and SKOV3 cells (Figure 2b, filled squares) increased until 4 days, when confluence was achieved. In striking contrast, the proliferative rates of both NIH:OVCAR5 cells (Figure 2a, open circles) and SKOV3 cells (Figure 2b, open circles) cultured in

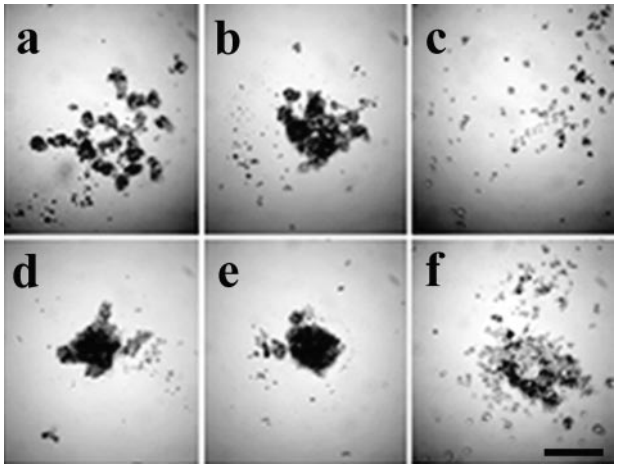

Figure 3. Formation of ovarian carcinoma spheroids is mediated by β1-integrins. Single-cell suspensions of NIH:OVCAR5 cells at a density of 5000 cells/200 μl were added to agarose-coated wells for 8 hours (**a–c**) or 24 hours (**d–f**). The cells were incubated in serum-free medium (**a** and **d**) or in the presence of 10 μg/ml of a mAb that stimulates β1-integrin subunits (**b** and **e**), or 10 μg/ml of a mAb that blocks β1-integrin subunits (**c** and **f**). Scale bar, 250 μm.

agarose-treated 24-well plates, which prohibited the adhesion of the cells to a substratum, were initially much lower. The proliferative rates of the NIH:OVCAR5 spheroids and the SKOV3 multicellular aggregates slowly increased until they approached that of the monolayers by 21 days. The multicellular aggregates and monolayers that formed were viable, as determined by trypan blue and propidium iodide staining, which identify dead cells, and immunohistochemical staining for annexin V, which identifies apoptotic cells (data not shown). Furthermore, samples were inspected under a light microscope to ensure nearly complete incorporation of cells into spheroids and that monolayers had not formed either beneath or atop the agarose applied to the wells (not shown).

Spheroid Formation Is Mediated by α5- and β1-Integrin Subunits

We have previously shown that β1-integrin subunits mediate the adhesion of single-cell suspensions of ovarian carcinoma cells to ECM molecules and mesothelial cells.[15] We therefore hypothesized that β1-integrins also play a role in ovarian carcinoma spheroid formation. Single-cell suspensions of NIH:OVCAR5 cells were incubated in serum-free medium in the presence or absence of mAbs that blocked or stimulated the β1-integrin subunit for up to 24 hours (Figure 3). At 8 hours, spheroids began to form in serum-free medium (Figure 3a), scored as ++. Spheroid formation was accelerated by a mAb that stimulates the adhesive abilities of human β1-integrin subunits (Figure 3b), scored as +++. Spheroid formation was inhibited by a mAb that blocks the binding site of the β1-integrin subunit (Figure 3c), scored as +/−. At 24 hours, large spheroids, scored as +++, had formed in serum-free medium (Figure 3d) and in the presence of the β1-integrin-stimulating mAb (Figure 3e). The β1-integrin-blocking mAb continued to partially retard spheroid formation (Figure 3f), scored as ++/+++. These mAbs had similar effects on the aggregation of SKOV3 cells into multicellular aggregates (not shown). These data suggest that β1-integrin subunits may mediate the initial formation of ovarian carcinoma spheroids.

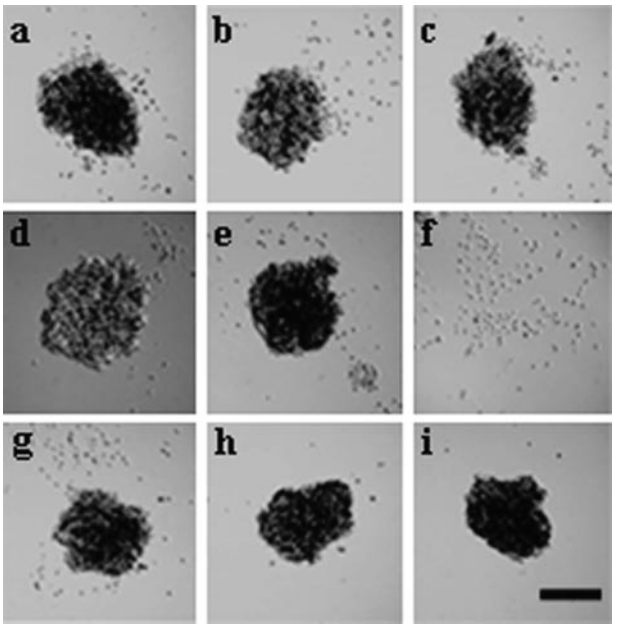

Figure 4. Formation of ovarian carcinoma spheroids is mediated by the α5-integrin subunit. NIH:OVCAR5 cells at a density of 5000 cells/200 μl were added to agarose-coated wells in serum-free medium in the presence of 10 μg/ml of mouse IgG (**a**) or 10 μg/ml of blocking mAbs against the α1-integrin subunit (**b**), α2-integrin subunit (**c**), α3-integrin subunit (**d**), α4-integrin subunit (**e**), α5-integrin subunit (**f**), α6-integrin subunit (**g**), integrin αvβ3 (**h**), or CD44 (**i**) for 16 hours. Scale bar, 500 μm.

We have previously shown that α-integrin subunits and CD44 also mediate the adhesion of ovarian carcinoma cells to ECM molecules and mesothelial cells.[15] To determine the role of these cell surface receptors in ovarian carcinoma spheroid formation, single-cell suspensions of

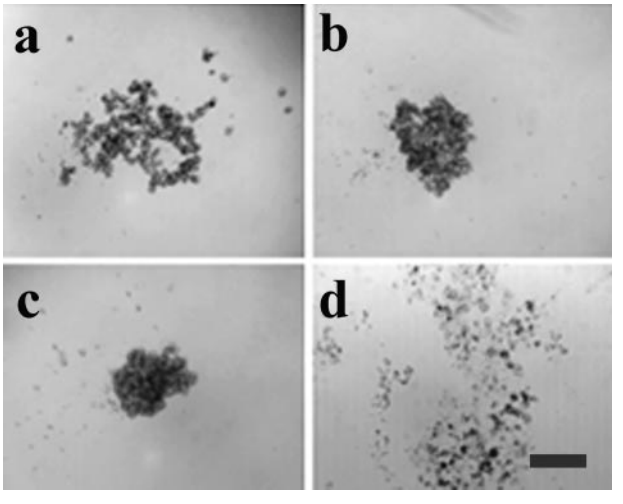

Figure 5. Addition of exogenous ECM proteins alters the formation of ovarian carcinoma spheroids. A single-cell suspension of NIH:OVCAR5 cells at a density of 5000 cells/200 μl was cultured in agarose-coated plates in serum-free medium with 25 μg/ml of ovalbumin (**a**), fibronectin (**b**), laminin (**c**), or type IV collagen (**d**) for 16 hours and then photographed. Scale bar, 250 μm.

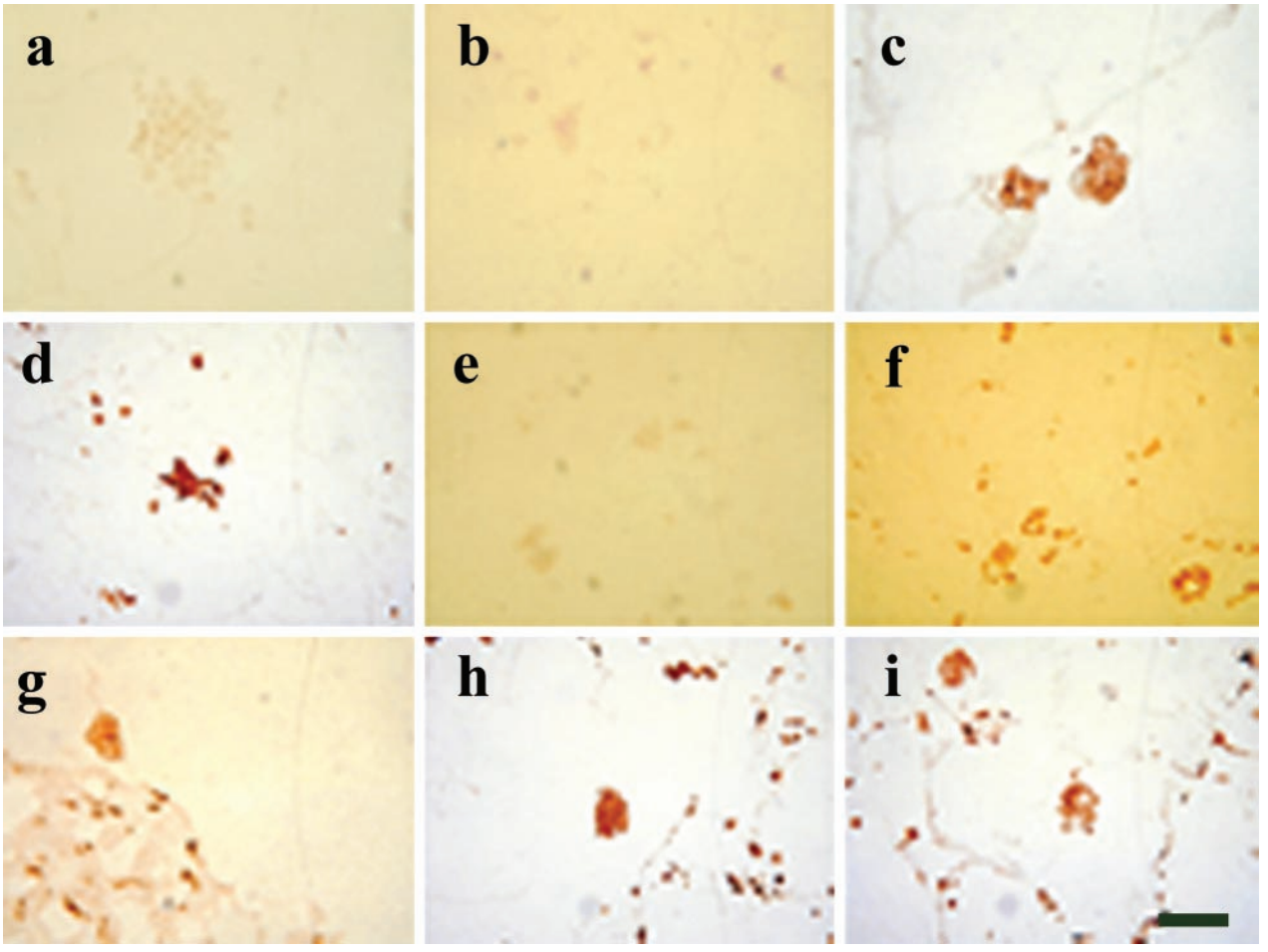

Figure 6. Spheroids express adhesion molecules. NIH:OVCAR5 spheroids were suspended in thrombin clots, embedded in OCT, sectioned at 6 μm thick, and stained with mouse IgG (**a**) or mAbs against the α1-integrin subunit (**b**), α2-integrin subunit (**c**), α3-integrin subunit (**d**), α4-integrin subunit (**e**), α5-integrin subunit (**f**), α6-integrin subunit (**g**), β1-integrin subunit (**h**), or CD44 (**i**). Scale bar, 1 mm.

NIH:OVCAR5 cells were incubated in serum-free medium in the presence of normal mouse IgG or blocking mAbs against α-integrin subunits, integrin $\alpha v\beta 3$, or CD44 (Figure 4). Spheroid formation was inhibited by a mAb against the α5-integrin subunit (Figure 4f) and scored as +/−. In contrast, large spheroids, scored as +++, formed in the presence of normal mouse IgG (Figure 4a) or mAbs against the α1-integrin subunit (Figure 4b), α2-integrin subunit (Figure 4c), α3-integrin subunit (Figure 4d), α4-integrin subunit (Figure 4e), α6-integrin subunit (Figure 4g), integrin $\alpha v\beta 3$ (Figure 4h), or CD44 (Figure 4i). These mAbs had similar effects on the aggregation of SKOV3 cells into multicellular aggregates (not shown). This suggests that the $\alpha 5\beta 1$-integrin may mediate ovarian carcinoma spheroid formation.

To examine the effect of ECM proteins, the ligands of integrins, on spheroid formation, single-cell suspensions of NIH:OVCAR5 cells were cultured at a density of 5000 cells/well in agarose-coated wells in serum-free media in the presence of 25 μg/ml fibronectin, laminin, or type IV collagen (Figure 5). The addition of exogenous fibronectin enhanced spheroid formation (Figure 5b), scored as +++, compared to the ovalbumin control (Figure 5a), scored as +/++. Enhanced spheroid formation was also observed in the presence of laminin (Figure 5c), scored as +++, but not in the presence of type IV collagen (Figure 5d), scored as +/++. These results suggest that ECM proteins affect ovarian carcinoma spheroid formation.

Immunolocalization of Adhesion Molecules in Spheroids

The expression of integrin subunits and CD44 on NIH:OVCAR5 spheroids was analyzed by immunohistochemistry (Figure 6). The ovarian carcinoma spheroids, which were embedded in thrombin clots, stained positively for integrin subunits α2 (Figure 6c), α3 (Figure 6d), α5 (Figure 6f), α6 (Figure 6g), and β1 (Figure 6h). In addition, the ovarian carcinoma spheroids stained positively for CD44 (Figure 6i). In contrast, the integrin subunits α1 (Figure 6b) and α4 (Figure 6e) were not detected in the spheroids.

The potential interaction of the $\alpha 5\beta 1$-integrin and its ligand, fibronectin, in fully formed NIH:OVCAR5 ovarian carcinoma spheroids was also assessed by immunohistochemical staining (Figure 7). Because the human plasma used to make thrombin clots contains fibronectin, the spheroids in this set of experiments were embedded

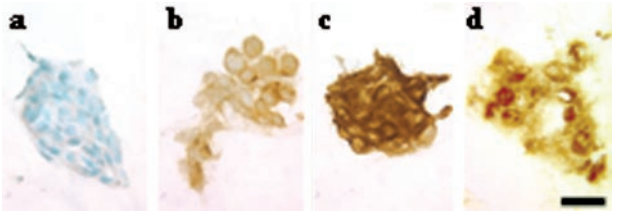

Figure 7. Localization of the α5- and β1-integrin subunits and fibronectin in spheroids. NIH:OVCAR5 spheroids were suspended in agarose clots, embedded in OCT, sectioned at 6 μm thick, and stained with normal mouse IgG (**a**), a mAb against the α5-integrin subunit (**b**), a mAb against the β1-integrin subunit (**c**), or a polyclonal antibody against fibronectin (**d**). The slides were then counterstained with methylene green. The α5- and β1-integrin subunits, as well as fibronectin, localized to the cell surface and were concentrated at points of cellular contact. Scale bar, 100 μm.

in agarose clots. Also, the samples were counterstained with methyl green stain after immunostaining. The α5- and β1-integrin subunits were detected on the surface of individual NIH:OVCAR5 cells in the spheroids (Figure 7, b and c, respectively). Fibronectin was also detected on the surface of the cells and in the ECM surrounding them (Figure 7d). This suggests that interactions between the α5β1-integrin and fibronectin may continue to mediate early adhesion events in ovarian carcinoma spheroids. No staining was observed on the surface of cells incubated in the presence of normal mouse IgG (Figure 7a).

Ovarian Carcinoma Spheroid Adhesion to ECM Proteins Is Mediated by Integrins

The ability of NIH:OVCAR5 spheroids to adhere to ECM components was assessed as a model to determine whether spheroids are inherently unable to adhere, which would explain the free-floating multicellular aggregates found in ovarian carcinoma patients' ascites fluid. Only NIH:OVCAR5 cells were used in the following series of experiments, because of their phenotypic resemblance to patient ascites cells, in that the NIH:OVCAR5 spheroids remained intact after manipulations, unlike the SKOV3 multicellular aggregates, which dispersed with even minimal manipulation. To determine the ability of ovarian carcinoma spheroids to adhere to ECM proteins, NIH:OVCAR5 spheroids were allowed to adhere to glass chamber slides coated with 50 μg/ml of fibronectin, laminin, or type IV collagen for up to 4 hours (Figure 8). Spheroid adhesion to all three ECM proteins occurred in a time-dependent manner, with maximum adhesion attained by 4 hours. Approximately 90% of the spheroids adhered to fibronectin (Figure 8, squares) and laminin (Figure 8, triangles) at 4 hours, and ~80% of the spheroids adhered to type IV collagen (Figure 8, circles) at 3 hours. The spheroids failed to adhere to chamber slides coated with ovalbumin (Figure 8, diamonds). These data suggest that ovarian carcinoma spheroids have the ability to adhere to ECM glycoproteins, despite the fact that many ovarian carcinoma multicellular aggregates are found floating in the ascitic fluid of patients and seemingly fail to adhere *in vivo*.

The role of integrin subunits in NIH:OVCAR5 spheroid adhesion toward fibronectin, laminin, and type IV collagen was investigated (Table 2). Spheroids were allowed to adhere to the ECM proteins or ovalbumin for 2 hours in the presence of blocking mAbs against integrin subunits. Spheroid adhesion to all three ECM proteins was almost completely inhibited in the presence of a blocking mAb against the β1-integrin subunit ($P < 0.001$). In addition, a mAb against the α5-integrin subunit inhibited spheroid adhesion to fibronectin by 60% ($P < 0.005$), a mAb against the α6-integrin subunit decreased spheroid adhesion to laminin by 40% ($P < 0.01$), and a mAb against the α2-integrin subunit decreased spheroid adhesion to type IV collagen by 55% ($P < 0.01$). Spheroid adhesion to all three ECM proteins was slightly increased in the presence of a β1-integrin-stimulating mAb, but was not significantly affected by blocking mAbs against the α3- or α4-integrin subunits, integrin αvβ3, or CD44 (data not shown). These results suggest that spheroid adhesion to secondary growth sites is a complex, multivalent phe-

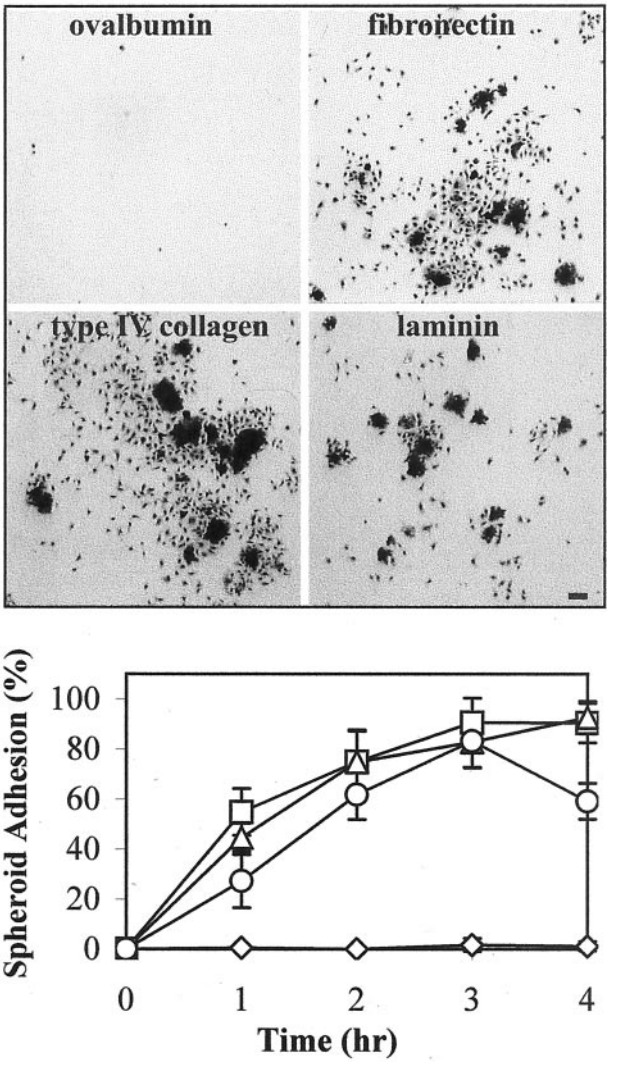

Figure 8. Adhesion of NIH:OVCAR5 spheroids to ECM proteins. NIH:OVCAR5 spheroids were incubated on glass chamber slides coated with 50 μg/ml of ovalbumin (**diamonds**), fibronectin (**squares**), laminin (**triangles**), or type IV collagen (**circles**) for up to 4 hours. Nonadherent cells were washed away, and the remaining adherent cells were fixed, stained, and photographed. Scale bar, 250 μm. Data are expressed as mean ±SD.

Table 2. Spheroid Adhesion to ECM Proteins Is Mediated by Integrins

mAb	Fibronectin	Laminin	Type IV collagen
IgG	69 ± 12	56 ± 8	55 ± 4
α2	71 ± 9	71 ± 9	24 ± 10‡
α5	25 ± 13†	50 ± 3	31 ± 8
α6	64 ± 18	35 ± 9‡	38 ± 7
β1	2 ± 9*	3 ± 5*	0 ± 0*

Glass chamber slides were coated with 50 μg/ml of fibronectin, laminin, type IV collagen, or ovalbumin. NIH:OVCAR5 spheroids were incubated on the coated slides in serum-free medium for 2 hours in the presence of 10 μg/ml of mouse IgG or 10 μg/ml of blocking mAbs against the α2-, α5-, α6-, or β1-integrin subunits. Values are expressed as a percentage of the total number of spheroids that adhered to each substrate. Fewer than 5% of cells adhered to ovalbumin under any conditions. Data are expressed as mean ± SD.

*$P < 0.001$.

†$P < 0.005$.

‡$P < 0.01$, compared to the normal mouse IgG control.

nomenon that is mediated by multiple cell-matrix interactions between integrins and ECM components.

Discussion

In ovarian carcinoma, both single cells and multicellular aggregates are found in patients' ascitic fluid.[2] Extensive research has been performed using single-cell suspensions of cell lines derived from primary ovarian carcinoma tumors and ascites cells. However, because multicellular aggregates of ovarian carcinoma cells are not suited for assays that require single-cell suspensions, they have been primarily overlooked by the scientific community. In this study, we generated ovarian carcinoma cell spheroids, which are intermediate in complexity between monolayers and solid tumors, and more closely approximate the *in vivo* conditions of ovarian carcinoma patients than single-cell suspensions or monolayers. We used this model to identify adhesion molecules that mediate the formation of ovarian carcinoma spheroids and their subsequent adhesion to ECM proteins.

Early attempts to create spheroids from ovarian cancer cells were unsuccessful.[4] Ovarian carcinoma spheroids were eventually created; however, some primary tumor cells and cell lines required weeks to form stable spheroids, whereas others did not form multicellular aggregates at all.[2,4–9] In this study, we used the liquid overlay method[27] to show that the NIH:OVCAR5 ovarian carcinoma cell line formed stable spheroids within 48 hours. These spheroids appeared similar to those present *in vivo* in patients' ascites samples. In contrast, the SKOV3 ovarian carcinoma cell line failed to form stable spheroids unless incubated for more than 14 days (not shown). Another group was able to generate SKOV3 spheroids in 10 days using an alternate method.[8] This disparity may reflect the heterogeneity of ovarian carcinoma cells, even within well-defined cell lines. It is also important to note that cell lines, which are selected for their ability to proliferate and adhere, may form spheroids at a much faster rate than ovarian carcinoma cells *in vivo*.

Condensation into spheroids or multicellular aggregates decreased the proliferative abilities of the NIH:OVCAR5 and SKOV3 ovarian carcinoma cell lines when compared to the same cells cultured as monolayers. Both cell lines underwent a marked decrease in proliferation in the absence of adhesion to a substratum. However, the proliferative rates of the spheroids eventually approached those of the confluent monolayers. The cells in the spheroids and monolayers remained viable, as determined by staining for markers of cell death and apoptosis. Previous reports show that increased percentages of ovarian carcinoma monolayer cells accumulate in G_2/M phase compared to spheroid cells when exposed to Taxol.[9] The spheroid cells may be arrested at an earlier step of the cell cycle, which may inhibit both cell proliferation and apoptosis. The slower proliferative rate of ovarian carcinoma spheroids may protect them from therapies directed against fast-growing tumor cells.

Although several groups have generated spheroids from ovarian carcinoma cells,[5–9] the biological mechanisms by which the spheroids formed have not been defined. The NIH:OVCAR5 and SKOV3 ovarian carcinoma cell lines express a variety of adhesion molecules on their surfaces, including integrins, ICAM-1, and CD44.[15,18] In this study, we report that a functional blocking mAb against the β1-integrin subunit inhibited the formation of spheroids by NIH:OVCAR5 cells at an 8-hour time point, whereas a mAb that stimulates β1-integrin-mediated cell adhesion hastened the phenomenon. These data suggest that β1-integrin subunits mediate the initial formation of ovarian carcinoma spheroids. The incomplete inhibition of the β1-integrin subunits at the 24-hour time point suggests that if the β1-integrin subunits are inactivated, the ovarian carcinoma cells may possess a compensatory mechanism to facilitate spheroid condensation. However, it is also possible that the mAbs against the β1-integrin subunits may simply be internalized by 24 hours, eventually allowing the ovarian carcinoma cells to condense into spheroids. We also report that a functional blocking mAb against the α5-integrin subunit inhibited the formation of spheroids by NIH:OVCAR5 cells. Monoclonal antibodies that blocked the functional sites of the α1-, α2-, α3-, α4-, and α6-integrin subunits, integrin $\alpha v\beta 3$, or CD44 had no effect on spheroid formation. This suggests that ovarian carcinoma spheroid formation may be regulated by the integrin $\alpha 5\beta 1$.

The chief ligand of the $\alpha 5\beta 1$-integrin is fibronectin.[31] Fibronectin has been reported to promote the adhesion of ovarian carcinoma cells[32,33] and may crosslink these cells via their $\alpha 5\beta 1$-integrin receptors. In our hands, the addition of exogenous fibronectin promoted ovarian carcinoma spheroid formation, which supports our theory that ovarian carcinoma spheroid formation may be mediated by the $\alpha 5\beta 1$-integrin. Interestingly, the addition of exogenous laminin also enhanced spheroid formation, whereas type IV collagen inhibited spheroid formation. It is possible that these adhesion molecules may indirectly alter spheroid formation, perhaps by signal transduction mechanisms.

By immunohistochemistry, we detected α5-integrin subunits, β1-integrin subunits, and fibronectin in regions of cell-cell contact on the surface of NIH:OVCAR5 spheroids generated after 48 hours in tissue culture. These

results are consistent with our finding that the α5β1-integrin mediates NIH:OVCAR5 spheroid formation. Although we observed fibronectin on the surface of ovarian carcinoma spheroids, we did not determine whether the fibronectin was secreted by the ovarian carcinoma cells themselves or whether it was incorporated into their pericellular matrix from the complete medium in which they were initially cultured. Fibronectin has been detected in peritoneal fluids obtained from both normal and ovarian cancer patients, and elevated expression of fibronectin has been measured by others in malignant ascites fluid.[34] Therefore, we decided to perform the spheroid formation portion of this assay in the presence of sera, because this would more closely approximate *in vivo* conditions. Interestingly, NIH:OVCAR5 cells grown in fibronectin-free serum substitute media for 48 hours did form spheroids when cultured in agarose-coated wells (data not shown). Altered ECM composition has been reported in glioma cell spheroids, which were found to contain fibronectin and a small proteoglycan not detected when the cells were cultured in monolayers.[35] Such alterations may facilitate spheroid formation, suggesting that the cells themselves can manufacture the additional ECM needed or incorporate it from exogenous sources. These results suggest that cell surface α5β1-integrins may mediate ovarian carcinoma cell aggregation via interactions with fibronectin that the cells have synthesized and retained on their surfaces or sequestered from their environment.

The α5β1-fibronectin interaction may later be augmented or replaced by other cell-cell interactions, including the gap junctions, tight junctions, and desmosomes detected in mature spheroids.[36–38] Squamous epithelial cells grown as spheroids express significantly less epidermal growth factor receptors than squamous cell monolayers.[39] Altered levels of expression of ICAM-1, CD44, and LFA-1 have been reported in cancer cells grown as spheroids compared to monolayers.[40] By flow cytometric analysis, we previously detected α1-, α2-, α3-, α5-, α6-, and β1-integrin subunits on the surface of single-cell suspensions of the NIH:OVCAR5 cell line.[18] In this study, we report that the α2-, α3-, α5-, α6-, and β1-integrin subunits, but not α1-integrin subunits, were detected on NIH:OVCAR5 spheroids by immunohistochemistry. Taken together, these data suggest that condensation into multicellular aggregates resulted in decreased expression of the α1-integrin subunit, which in turn may result in a similarly decreased ability to adhere to a substratum. The condensation of ovarian carcinoma cells into spheroids or multicellular aggregates may induce other alterations in adhesion molecule expression or ECM composition, with concurrent effects on their adhesive abilities.

An earlier study of single-cell suspensions of NIH:OVCAR5 cells reported >90% adhesion of these cells to fibronectin, laminin, and type IV collagen within 30 minutes[15] However, in this study we found that NIH:OVCAR5 spheroids required 4 hours to achieve maximum adhesion. Factors that may contribute to the longer time period required for spheroid adhesion to various substrates include: decreased expression of receptors, decreased avidity of receptors, competing cell-cell and cell-matrix interactions within the spheroids, physical constraints that limit the cells' ability to spread onto the anchoring surfaces, and the effects of mechanical forces on a structure with a greater surface area/volume ratio. Any of these factors may contribute to the presence of free-floating multicellular aggregates found in ovarian carcinoma patient ascites.[2]

In this study, we report that the adhesion of ovarian carcinoma spheroids to ECM components is a β1-integrin-mediated event. The nearly complete inhibition of adhesion by a blocking mAb against the β1-integrin subunit, coupled with partial inhibition in the presence of blocking mAbs against α-integrin subunits, suggest that multiple integrin-ECM interactions are involved in the process. Our results suggest that the interactions of the α5β1-integrin with fibronectin, the α6β1-integrin with laminin, and the α2β1-integrin with type IV collagen mediate ovarian carcinoma spheroid adhesion. Recently, Kawano and colleagues,[41] reported that the α2β1-, α6β1-, α3β1-integrins mediated the adhesion of squamous epithelial multicellular aggregates to type I collagen, laminin 1, and laminin 5, respectively. Taken together, these data suggest that spheroid adhesion is an integrin-dependent event, although the precise integrin-ligand interactions involved may be tissue-specific. However, additional cell-matrix and cell-cell interactions, which have been reported in spheroids generated from other cell types,[36–40] may mediate the initial formation and continued maintenance of spheroid morphology as they progress with time.

The examination of multicellular aggregates in ovarian carcinoma may be of clinical importance. This study demonstrates that ovarian carcinoma cells cultured as spheroids exhibit decreased proliferative and adhesive properties compared to ovarian carcinoma cells cultured as monolayers. Ovarian carcinoma spheroids are less sensitive than monolayers to cancer drugs and ionizing radiation,[4,5,9,42] which may be partly because of the low proliferative rates we report in this study. Also, the kinetics of drug absorption are altered in ovarian carcinoma spheroids, compared to ovarian carcinoma monolayers.[43] Taken together, these data suggest that spheroids may represent a tenacious, long-term source of secondary tumor growth in ovarian carcinoma that is not addressed by current therapeutic strategies, which target highly proliferative cancer cells. The biological properties of ovarian carcinoma spheroids require further study to better understand their significance in secondary tumor growth and to effectively eradicate them during the treatment of the disease. The model of ovarian carcinoma spheroid formation and adhesion presented in this study will also lay the groundwork for future studies in which ovarian carcinoma spheroids isolated from patient ascites fluid can now be monitored for their cell surface receptors and adhesive properties.

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