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Expression of α V-Integrins in Uterine Serous Papillary Carcinomas; Implications for Targeted Therapy With Intetumumab (CNTO 95), a Fully Human Antagonist Anti- α V-Integrin Antibody

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Abstract

Objective—Uterine serous papillary carcinoma (USPC) is an aggressive variant of endometrial cancer characterized by an innate resistance to chemotherapy and poor prognosis. In this study, we evaluated the expression of α V-integrins in primary USPC cell lines and the *in vitro* ability of intetumumab (CNTO 95), a fully human monoclonal antibody against α V-integrins, to inhibit USPC cell adhesion and migration.

Materials and Methods—The surface expression of integrins belonging to the α V-family, including α V β 3, α V β 5, and α V β 6, was evaluated in 6 primary USPC cell lines using flow cytometry analysis. To test the ability of intetumumab to inhibit USPC cell adhesion and migration, adhesion assays in the presence of vitronectin and migration assays through an 8.0- μ m pore polycarbonate membrane also were performed.

Results—We found high expression of the α V-subunit on the cell surface of all 6 primary USPC cell lines tested (100% positive cells; mean fluorescence intensity range, 13.1–39.5). When the expression of single heterodimeric integrins was evaluated, α V β 3, α V β 5, and α V β 6 were expressed on 37.5%, 32.0%, and 16.3% of cells (mean fluorescence intensity range, 6.5–16.2, 9.2–32.5, and 6.2–11.5, respectively). Importantly, in functional assays, low doses of intetumumab were effective in inhibiting adhesion (0.15 μ g/mL, $P=0.003$) and migration (1.25 μ g/mL $P=0.02$) of primary USPC cell lines.

Conclusions—The α V-integrins are overexpressed on the cell surface of primary USPC cell lines. Intetumumab may significantly inhibit USPC cell adhesion and migration pathways and may therefore represent a novel treatment option for patients harboring this rare but highly aggressive variant of endometrial cancer.

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Keywords

Uterine serous papillary carcinoma; CNTO 95; Intetumumab; α V-integrin

Cancers of the uterine corpus are the most common gynecological cancers, with 43,470 estimated new cases and 7950 estimated deaths for 2010 in the United States.¹ Although the majority of endometrial tumors are diagnosed at early stage and are characterized by a low-grade endometrioid histology (type I tumors), their rare counterparts (i.e., type II tumors, including serous papillary, clear cell, and poorly differentiated endometrioid carcinomas) are responsible for the most relapses and deaths from uterine cancer.^{2,3} Uterine serous papillary carcinoma (USPC) accounts for up to 10% of endometrial cancer and is characterized by an aggressive biological behavior because of its propensity for early lymphatic and intraperitoneal spread and recurrence.^{4,5} Thus, development of novel, potentially effective targeted therapies against USPC metastatic pathways remains a high priority.

Integrins constitute a family of proteins involved in many physiological and pathological functions, including cell functioning, embryonic implantation and development, tissue morphogenesis, angiogenesis, blood clotting, wound healing, oncogenesis, metastasis, thrombosis, and inflammation.⁶ As transmembrane glycoproteins, they are expressed in different combinations by all cells, including platelets, leukocytes, mesenchymal tissues, and epithelial cells.⁷ Integrins achieve a diversity of functions by the formation of heterodimers, always consisting of one α -subunit and one β -chain, with 24 distinct combinations known so far.⁸ The α V subunit can associate with many different β -subunits, whereas other α -subunits dimerize with only one kind of β -chain.⁸ Both chains participate in the binding of the ligand, in cell-cell or cell-extracellular matrix (ECM) protein interactions. The intracellular portion of the integrin undergoes different associations with cytoskeletal elements and phosphorylation, in association with changes in cell morphology and movement, and provides signals via secondary messengers.⁷ Previous research has demonstrated that integrins play a role in multiple steps in tumorigenesis, including cell spreading, invasion, and survival of several types of solid tumors, such as melanoma, breast, prostate, and oral squamous cell carcinomas.^{9,10} Unfortunately, however, limited information is currently available on expression of α V-integrins in endometrial carcinoma.^{11,12}

The presence of integrins on the cell surface renders them accessible to antibody-based therapies. Intetumumab (formerly CNTO 95; Centocor, Inc, Malvern, PA) is a human monoclonal antibody (mAb) that recognizes α V-integrin with high affinity ($K_d = 200$ pM for purified α V-chain and $K_d = 1-24$ nM for α V-integrins on expressing cells¹³). Previous studies have shown that this antibody binds α V β 1, α V β 3, α V β 5, and α V β 6; blocks α V β 3 and α V β 5; and inhibits integrin-mediated tumor growth and angiogenesis in vitro and in vivo on different animal models (mice,¹³ rats,^{13,14} and monkeys¹⁵) and in several different types of human tumors, including melanoma,¹³ breast,¹⁶ and small cell lung cancers.¹⁴ Phase I clinical trials have been conducted, showing that it is well tolerated in patients with advanced solid tumors¹⁷ and in hormone-refractory prostate cancer patients when administered with docetaxel and prednisone.¹⁸

To our knowledge, no studies have specifically described the expression of α V-integrins in USPCs. In this report, in an attempt to fill this gap in knowledge, we carefully investigated the surface expression of multiple α V-containing integrins in primary USPC cell lines and evaluated for the first time the in vitro potential of intetumumab as a novel targeted agent against this biologically aggressive and chemotherapy-resistant variant of endometrial cancer.

MATERIALS AND METHODS

Establishment of USPC Cell Lines

Six primary USPC tumor cell lines were established, after sterile processing, from fresh tumor biopsy samples collected at the time of surgery after approval from the institutional review board, as described previously.^{19,20} Briefly, tissue was mechanically minced to less than 3 mm³ in an enzyme solution made of 0.07% collagenase type I (Sigma, St Louis, MO) and 0.005% DNase 2000 KU/mg (Sigma) in RPMI 1640 and incubated in the same solution in a magnetic stirring apparatus for 1 hour at room temperature. Enzymatically dissociated cells were then washed twice in RPMI 1640 with 10% fetal bovine serum (FBS; Gemini Bio-products, Calabasas, CA) and maintained in RPMI 1640 supplemented with 10% FBS, 50 IU/mL of penicillin, 50 µg/mL of streptomycin (Cellgro; Mediatech, Manassas, VA), and 0.75 µg/mL Fungizone (GIBCO Cell Culture; Invitrogen Corporation, Grand Island, NY) at 37°C, 5% CO₂. Purity and homogeneity of fresh tumor cultures were tested by morphology, immunohistochemical staining, and/or flow cytometry with antibodies against cytokeratins. Only primary cultures that had at least 95% viability and contained greater than 99% epithelial cells were used in the experiments described below. Briefly, after seeding on plastic ware (48–72 hours), nonadherent cells and contaminant inflammatory cells were gently removed from the culture by multiple washings with phosphate buffered saline. Table 1 shows the characteristics of the patients from which the cell lines have been obtained. Tumors were staged using the International Federation of Obstetrics and Gynecology 1988 endometrial cancer staging system.

Flow Cytometry Analysis

Flow cytometry studies were performed using intetumumab, a fully humanized anti- α V-integrin IgG₁ κ mAb (Centocor, Inc). As isotype control antibody, the chimeric anti-CD20 IgG₁ mAb rituximab (Rituxan; Genentech, San Francisco, CA) was used. A fluorescein isothiocyanate-conjugated goat anti-human F(ab')₂ immunoglobulin (BioSource International, Camarillo, CA) was used as a secondary reagent. Other mouse antihuman mAbs used to test the expression of α V-integrin family were as follows: mAb 1953Z (clone P3G8, anti- α V subunit), mAb 1951Z (clone P4G11, anti- β 1 subunit), mAb 1976Z (clone LM609, anti- α V β 3), mAb 1961 (clone P1F6, anti- α V β 5), and mAb 2077Z (clone 10D5, anti- α V β 6) (all Chemicon International, Temecula, CA). A mouse IgG_{2a} κ isotype control (BD Biosciences, San Diego, CA) and a fluorescein isothiocyanate-conjugated polyclonal goat antimouse IgG (BD Biosciences) were used for the staining. In dose titration studies, we found 2.5 µg/mL to be the optimal intetumumab concentration to stain USPC cells (data not shown). Therefore, all further analyses were performed using intetumumab, as well as all other primary antibodies, at a concentration of 2.5 µg/mL. Analysis was conducted with a FACSCalibur, using CellQuest software (BD Biosciences). Data were collected for 10,000 cells gated per sample and displayed as histograms plotting the number of cells counted versus the intensity of fluorescence.

Adhesion Assay

Microtest 96-well plates (BD Falcon, Franklin Lakes, NJ) were coated at 37°C for 2 hours with vitronectin (1 µg/mL; Promega, Madison, WI) and blocked for 1 hour with 2 mg/mL of bovine serum albumin in phosphate buffered saline. Cells were incubated at 2×10^5 /mL density with various concentrations of intetumumab or rituximab at 37°C for 15 minutes. Approximately 100 µL of the cell-antibody mixture was added to each well and incubated for 20 minutes at 37°C. Non-adherent cells were removed by rinsing the plate with serum-free RPMI 1640, and adherent cells were fixed with 10% buffered formalin phosphate (Fisher Scientific, Fair Lawn, NJ) and stained with crystal violet (0.1% weight/volume [w/v] in ddH₂O; Sigma) for 25 minutes at room temperature. After rinsing the plate in ddH₂O,

Triton X-100 (0.5% in ddH₂O; Acros Organics, Fair Lawn, NJ) was added to the wells to allow solubilization of the dye overnight at room temperature, before reading the absorbance at 595 nm in a microtiter plate reader (VersaMax; Molecular Devices, Silicon Valley, CA).

Migration Assay

Cell migration assays were performed in 24-transwell chambers with polycarbonate membrane (6.5 mm diameter, 8.0- μ m pore size; Corning Incorporated, Corning, NY). Cells were kept in starvation for 24 hours before performing the assay. Cells (100,000/200 μ L) were added to the upper chambers in the presence or absence of antibodies, in serum-free RPMI 1640. Approximately 800 μ L of RPMI 1640 with 10% FBS was added to the lower chambers as a chemoattractant. The plates were incubated at 37°C for 48 hours. Migration was terminated by fixing the membranes with 10% buffered formalin phosphate and staining with crystal violet (0.1% w/v in ddH₂O) for 25 minutes at room temperature. After rinsing the filters in ddH₂O, the interior of each insert was scrubbed with a cotton swab to remove unemigrated cells. The stained cells were lysed with Triton X-100 (0.5% in ddH₂O) overnight at room temperature. The resulting colored mixture was transferred to a 96-well plate, and optical densities were read in triplicate at 595 nm in a VersaMax microtiter plate reader.

Statistical Analysis

Mann-Whitney *U* test was used to compare the behavior of cells in the presence versus absence of antibody in both adhesion and migration assays. Statistical analysis was performed using PASW Statistics version 18 (SPSS, Chicago, IL). A *P* < 0.05 was considered statistically significant.

RESULTS

α V-Integrin Is Expressed on the Surface of USPC Cell Lines

We evaluated the surface expression of α V-integrin by FACS analysis on 6 primary USPC cell lines using intetumumab, a fully human mAb that recognizes α V-integrin with high affinity. We found all USPC cell lines tested (ie, 6 of 6 [100%]) to be strongly positive (average positive gated cells, 100%) for α V-integrin expression on the cell surface, with a mean fluorescence intensity (MFI) of 28.4 (SD, \pm 9.55; range, 13.1–39.5) when using intetumumab (Fig. 1). The mAb 1953Z, the murine mAb anti- α V, demonstrated a similar positivity with an average (98.5%) of positive gated cells and an MFI of 32.2 (SD, \pm 8.27; range, 24.7–48.4).

Then, we checked the expression of multiple β -subunits to which the α V-chain can bind and heterodimerize in different combinations. Specifically, we tested the expression of β 1-integrin alone and α V β 3, α V β 5, and α V β 6 heterodimers (Table 2). We found the β 1-subunit to be expressed in 12.5% of the cells, with an MFI of 11.6 (SD, \pm 5.73; range, 7.3–22.1) (Table 2). The α V β 3 and α V β 5 showed a higher expression, with 37.5% and 32.0% of positive cells and an MFI of 12.3 (SD, \pm 4.02; range, 6.5–16.2) and 17.5 (SD, \pm 9.23; range, 9.2–32.5), respectively (Table 2). Finally, we found a lower expression of α V β 6 with only 16.3% of cells being positive and an MFI of 7.8 (SD, \pm 2.01; range, 6.2–11.5) (Table 2).

Intetumumab Inhibited Integrin-Mediated USPC Cell Adherence to a Vitronectin Substratum

The α V-integrins are known receptors for ECM proteins, such as vitronectin, tenascin, and fibronectin.⁶ We therefore investigated the ability of intetumumab to inhibit the adhesion of USPC cell lines (ie, USPC-ARK-2, USPC-ARK-3, USPC-ARK-4, and USPC-ARK-6) to vitronectin-coated plates in vitro. As representatively shown in Figure 2 for 1 USPC primary cell line (ie, USPC-ARK-3), we found that different concentrations of intetumumab were

able to specifically inhibit the α V-integrin–vitronectin interaction in a dose-dependent fashion in all cell lines tested. The range of inhibition with intetumumab was 30% to 65% when compared with control USPC cells where no mAb was added ($P < 0.003$ for each condition). No significant inhibition was reported in the presence of rituximab (1% inhibition, $P = 0.717$), independent of the concentration used.

Intetumumab Inhibited Serum-Induced Migration of USPC Cells

The ability of intetumumab to block the binding of α V-integrins to ECM protein vitronectin suggested potential intetumumab effects on cell motility because this event is known to be mediated by integrin-ECM interactions.⁶ We therefore investigated the ability of intetumumab to inhibit the migration of USPC cell lines (ie, USPC-ARK-2, USPC-ARK-4, and USPC-ARK-6) through an 8.0- μ m pore polycarbonate membrane. We used FBS 10% as chemoattractant to stimulate the migration of tumor cells kept in starvation medium (serum free RPMI 1640) for 24 hours before performing the experiments. We found that intetumumab concentrations from 1.25 μ g/mL up to 20 μ g/mL were able to significantly inhibit the migration of 17% to 27% of USPC-ARK-2 cells through the membrane when compared with control cells with no mAb added ($P < 0.03$; Fig. 3). Lower doses of intetumumab (ie, 0.15 μ g/mL, 0.3 μ g/mL, or 0.625 μ g/mL; inhibition range, 3%–12%; P values between 0.724 and 0.142) or rituximab (up to the highest dose, 20 μ g/mL, inhibition of 4%; $P = 0.724$) did not significantly affect migration of tumor cells. Similar results were obtained using the USPC-ARK-4 and USPC-ARK-6 cell lines (data not shown).

DISCUSSION

Uterine serous papillary carcinoma histology is considered a prognostically unfavorable factor in endometrial cancer because of the high rates of chemotherapy resistance, recurrence, and mortality secondary to the diagnosis with this rare but highly aggressive variant of endometrial tumor.^{4,5} Importantly, because USPCs, similar to ovarian cancer, have the tendency to early spread throughout the peritoneal cavity, the discovery of novel, effective treatment options to inhibit USPC migration and metastases would be highly desirable. The α V-containing integrins are known to play a major role in tumor growth, adhesion, movement, angiogenesis, and metastasis formation in several types of tumors.⁶ Accordingly, in the current study, we have carefully evaluated the surface expression of α V-integrins in multiple primary USPC cell lines and analyzed for the first time the potential therapeutic effects of intetumumab, a fully human monoclonal antibody against α V-integrins, to inhibit USPC cell adhesion and migration in vitro.

We found significant α V-chain surface expression in all 6 primary USPC cell lines available to this study. Consistent with the results obtained for α V-chain with intetumumab, when single heterodimeric integrin expression was evaluated by flow cytometry with specific antibodies, integrins α V β 3, α V β 5, and α V β 6 also were found upregulated in primary USPC cell lines at different levels. Although limited information is currently available in the literature regarding expression of α V-integrins in endometrial cancer, our results in USPC are consistent with those of recent reports demonstrating significant expression of the integrins α V β 5 and α V β 6 in endometrial endometrioid tumors.^{11,12} Of interest, in the study by Hecht et al,¹² although α V β 6 integrin was only weakly or not expressed in normal epithelium, it was found upregulated in most endometrial endometrioid carcinomas, especially high-grade tumors and those characterized by the presence of metastatic disease. These observations, combined with our results, are consistent with the possibility that high expression of α V-integrins in biologically aggressive tumors, such as poorly differentiated endometrioid tumors¹² and USPC, may at least partially account for their enhanced invasive behavior and their rapid pattern of metastatic spread.

Importantly, because α V-chain expression was found in 100% of the primary USPC cell lines tested, targeting these receptors with intetumumab, a fully human mAb directed against α V-integrins, may represent a novel, potentially effective therapy to prevent USPC metastatic spread. Consistent with this hypothesis, we were able to demonstrate a significant inhibition of USPC cell adhesion to ECM proteins, such as vitronectin, using low doses of intetumumab. In this regard, vitronectin was the substrate chosen for our in vitro model because it is the specific ligand of α V-subunit, whereas fibrinogen and fibronectin also can be recognized by other α -integrins, that is, α 3, α 4, α 5, α 8, and α IIb.²¹ Importantly, our results are consistent with the data of other groups who have demonstrated intetumumab's ability to inhibit adhesion in other human cancers, such as melanoma¹³ and breast cancer.¹⁶

Next, to mimic the passage of tumor cells through the endothelium to reach the bloodstream and exit from it to spread throughout the body (ie, a key process in metastasis formation), we have tested the in vitro ability of intetumumab to inhibit the migration of USPC cells through an 8.0- μ m pore polycarbonate membrane. We found intetumumab to significantly inhibit the migration of USPC cells through the membrane. These results clearly demonstrate that intetumumab may have the ability to inhibit the migration of USPC cells in vitro and potentially in vivo. Our data are therefore in agreement with previous reports demonstrating the ability of this fully human anti- α V-integrin antibody to inhibit migration and invasion and spontaneous metastasis in multiple human tumor models.^{13,16}

Although not directly tested in the current work because of the lack of a suitable USPC immunocompetent animal model, multiple studies have previously demonstrated the in vivo activity of intetumumab to inhibit the growth of several human tumor models in animals when administered both intraperitoneally^{13,14} or intravenously.^{13,16} In this regard, rat and monkey models are the most suitable for in vivo studies because intetumumab has no reactivity against mouse α V-integrin, has limited cross-reactivity with rat α V-integrin ($K_d = 220$ nM), and can recognize cynomolgus macaque integrins with similar binding affinity as human cells ($K_d = 5.1$ and 5.2 nM, respectively).¹³ Of interest, intetumumab in combination with radiation therapy has been recently tested in rat models by Ning and colleagues,¹⁴ showing that it is active in tumor growth delay, better when administered just before the radiotherapy session and when used in maintenance therapy. In that study, to further assess the safety and tolerability of the antibody, they also checked the effect of intetumumab on intestinal crypt stem cells, which are sensitive to radiation, and showed that intetumumab did not affect the stem cells when administered in combination with radiation therapy.¹⁴

Importantly, intetumumab is currently under investigation in several clinical trials. Before proceeding with human clinical trials, the safety of long-term administration of intetumumab was tested on cynomolgus macaque, resulting in no signs of toxicity and no histopathological changes in any tissue.¹⁵ Furthermore, the effects of intetumumab on physiological angiogenesis have been tested in additional wound healing studies conducted on cynomolgus macaques.¹⁵ Two phase I studies have shown that intetumumab is generally active, safe, and well tolerated, both alone¹⁷ or when used in combination with docetaxel in patients with castrate-resistant metastatic prostate cancer.¹⁸ Major common adverse events were fever, headache, chills (responding to acetaminophen or ibuprofen),¹⁷ neutropenia, nausea, and neutropenic fever.¹⁸

In summary, our study with intetumumab in primary USPC cell lines demonstrates for the first time that α V-integrins are overexpressed on the cell surface of primary uterine serous tumors and that intetumumab is able to significantly inhibit USPC cell adhesion and migration pathways in vitro. On the basis of these findings, intetumumab may represent a novel treatment option to combine with other active regimens for the therapy of patients harboring this rare but highly aggressive variant of endometrial cancer.

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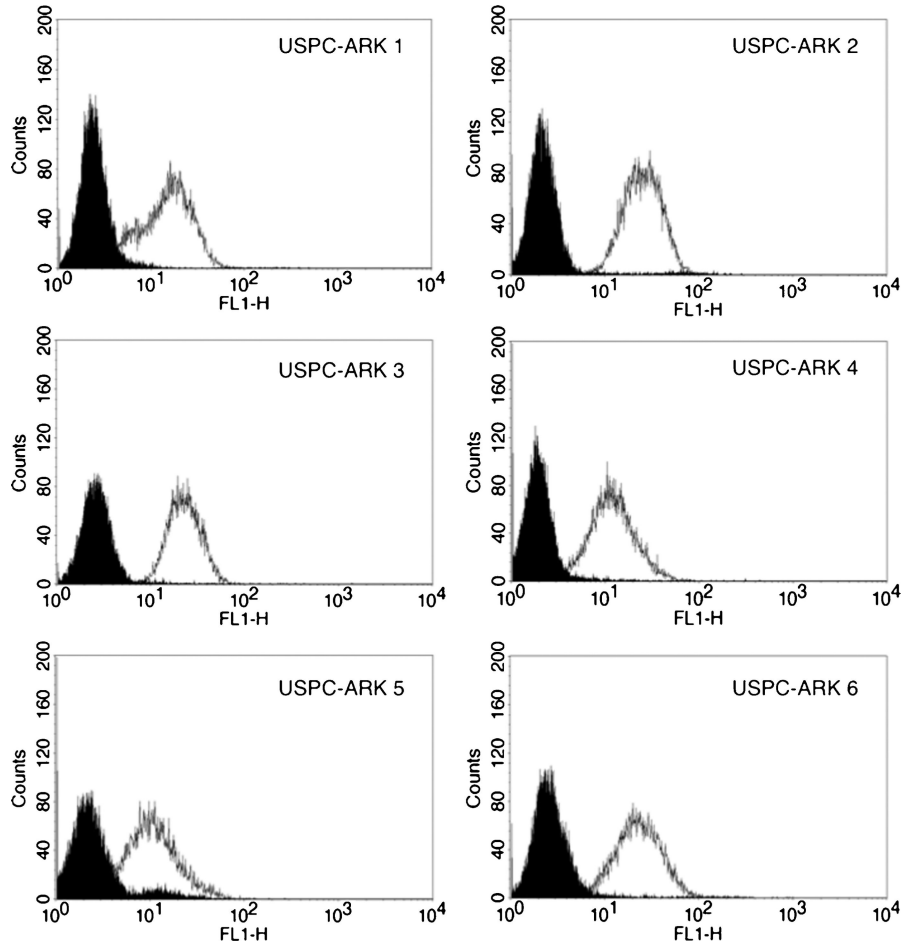


FIGURE 1. α V-integrin expression on cell surface by flow cytometry analysis. Representative expression of α V-integrins on the cell surface of 6 primary USPC cell lines when evaluated by flow cytometry. 2.5 μ g/mL of intetumumab, as well as isotype control, were used to target α V-integrins. Rituximab anti-CD20 control antibody (solid black) and intetumumab (white peak).

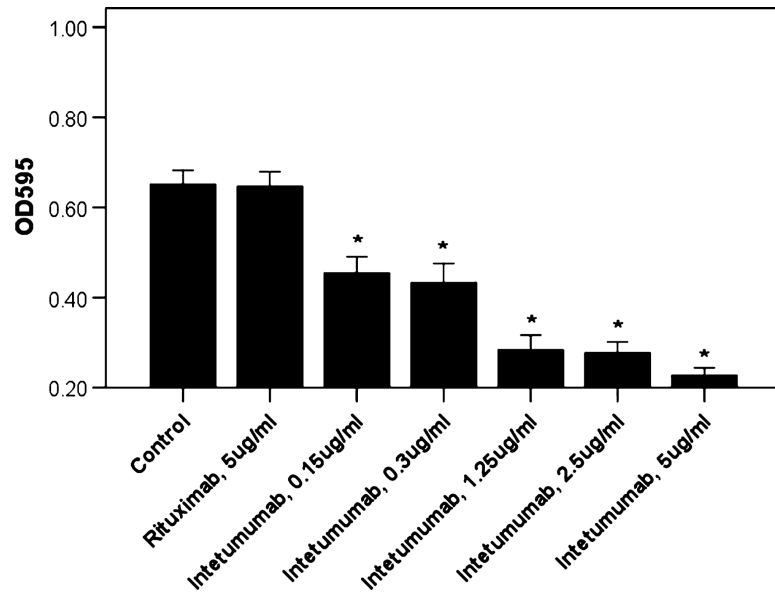


FIGURE 2.

Representative adhesion assay on USPC-ARK 3. Increasing concentrations of intetumumab inhibit the adhesion of USPC-ARK-3 cells to the plate. Extent of cell adhesion in the presence of various concentrations of antibody is shown as absorbance of the solution after lysis of stained adherent cells. Data shown are mean (\pm SEM) of at least 3 different experiments. * $P < 0.003$ versus the condition in the absence of any antibody.

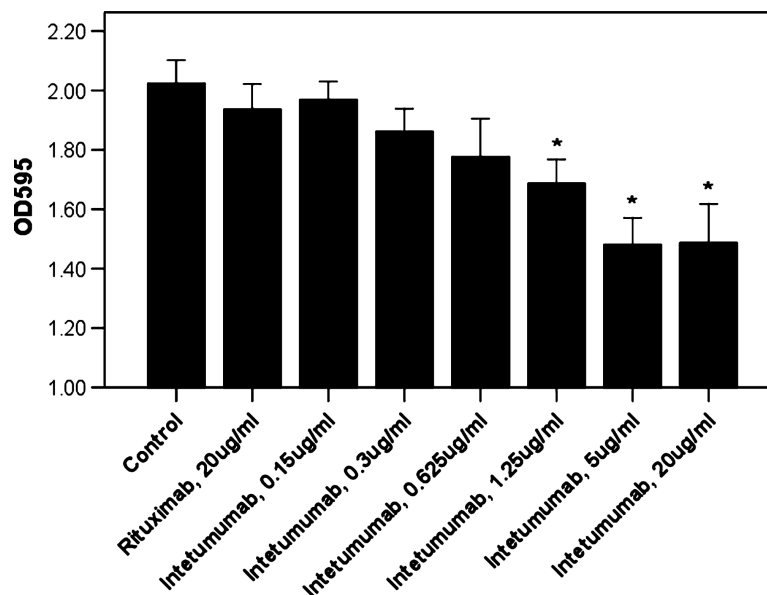


FIGURE 3.

Representative migration assay on USPC-ARK 2. Increasing concentrations of intetumumab inhibit the migration of USPC-ARK-2 cells through an 8.0- μ m pore polycarbonate membrane, mediated by a chemoattractant stimulus. Extent of cell migration in the presence of various concentrations of antibody is shown as absorbance of the solution after lysis of stained migrated cells. Data shown are mean (\pm SEM) of at least 3 different experiments. * P 0.03 versus the condition in the absence of any antibody.

TABLE 1

Patient characteristics, from which the 6 USPC cell lines were established

Patient	Age (yr)	Race	FIGO Stage	USPC Histopathology
USPC-ARK-1	62	AA	IVA	Pure
USPC-ARK-2	63	AA	IVB	Pure
USPC-ARK-3	59	AA	IVB	Mixed
USPC-ARK-4	73	W	IVB	Pure
USPC-ARK-5	73	AA	IIIC	Pure
USPC-ARK-6	62	W	IB	Mixed

AA, African American; W, white; FIGO, International Federation of Gynecology and Obstetrics.

TABLE 2

Cell surface expression analysis of integrins in a representative flow cytometry experiment

Patient	Intetumumab	αV	$\beta 1$	$\alpha V\beta 3$	$\alpha V\beta 5$	$\alpha V\beta 6$
USPC-ARK-1	100	97.2	15.6	36.5	28.1	9.1 % Positive
	32.2	29.9	11.3	14.2	24.1	6.5 MFI
USPC-ARK-2	100	100	18.2	33.3	62.3	27.6 % Positive
	34.9	29.7	7.9	8.0	9.2	7.1 MFI
USPC-ARK-3	100	100	8.4	12.0	48.2	35.7 % Positive
	28.9	28.8	7.4	16.2	11.2	6.2 MFI
USPC-ARK-4	100	100	28.1	50.9	39.2	14.5 % Positive
	21.9	31.6	7.3	6.5	10.5	6.8 MFI
USPC-ARK-5	100	94.0	2.4	35.3	5.7	2.7 % Positive
	13.1	24.7	22.1	14.8	17.3	8.7 MFI
USPC-ARK-6	100	100	2.3	56.7	8.6	8.1 % Positive
	39.5	48.4	13.5	14.2	32.5	11.5 MFI

MFI, Mean Fluorescence Intensity; USPC, Uterine Serous Papillary Carcinoma.