Antitumor Activity of SS(dsFv)PE38 and SS1(dsFv)PE38, Recombinant Antimesothelin Immunotoxins against Human Gynecologic Cancers Grown in Organotypic Culture *in Vitro*[1]

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ABSTRACT

Purpose: **Mesothelin, a cell surface glycoprotein overexpressed in ovarian cancer, mesotheliomas, and some squamous cell carcinomas, is an attractive candidate for targeted therapy because it is not shed in significant amounts into the bloodstream and is not present in significant amounts on normal human tissues except for mesothelial cells. The objective of this study was to determine the antitumor activity of SS1(dsFv)PE38, a recombinant antimesothelin immunotoxin, against human gynecologic tumors grown in short-term culture *in vitro*.**

Experimental Design: **Tumor cells obtained from primary cultures of five ovarian and one cervical tumor were mixed with an equal proportion of NIH-3T3 fibroblasts and plated inside collagen gels in tissue culture plates. After 4–7 days of growth, these organotypic cultures were treated with media alone, SS1(dsFv)PE38, and a control immunotoxin RFB4(dsFv)PE38, which targets the CD22 antigen not present on gynecologic tumors, every other day × 3. The organotypic culture gels were then formalin fixed, paraffin embedded, and evaluated for immunotoxin sensitivity using light microscopic examination of H&E-stained slides and also evaluated for apoptosis using the terminal deoxynucleotidyl transferase-mediated nick end labeling assay.**

Results: **Tumors expressing mesothelin showed a significant dose-dependent sensitivity to SS1(dsFv)PE38 even at concentrations as low as 1 ng/ml, whereas no antitumor activity was seen at 100 ng/ml in tumors that did not express mesothelin. This activity was specifically attributable to mesothelin targeting because RFB4 (dsFv)-PE38 had no activity against mesothelin-expressing tumors.**

Conclusions: **These results demonstrate that ovarian and cervical tumor cells obtained from patients can be grown in short-term culture using an organotypic culture model. Our results also show low concentrations of an immunotoxin targeting mesothelin is cytotoxic to mesothelin-expressing human tumors by inducing apoptosis.**

INTRODUCTION

Chemotherapy remains the mainstay of therapy for patients with advanced ovarian and cervical cancer. Despite initial responses, the majority of patients continue to have progressive disease and ultimately die from their cancer (1, 2). Because development of resistance to chemotherapeutic drugs plays a major role in tumor progression (3, 4), development of agents that act by a mechanism different from chemotherapy is needed. One such approach involves targeting cell surface receptors overexpressed on cancer cells using monoclonal antibodies (5). Despite initial disappointing results, monoclonal antibodies are now proving to be valuable anticancer agents in the clinic (6, 7). Monoclonal antibodies have also been used to develop immunotoxins by linking them with bacterial or plant toxins (8). These immunotoxins are extremely potent molecules and several such compounds have recently been shown to be effective in treating patients with cancer (9, 10).

Mesothelin is a M_r 40,000 glycosylphosphatidylinositol-linked cell surface glycoprotein that is overexpressed in certain tumors, especially ovarian cancer, malignant mesotheliomas, and some squamous cell cancers (11–14). The biological function of mesothelin is not known, and studies of the mouse mesothelin gene have shown that it is not required for normal mouse development or reproduction (15). Mesothelin is an attractive candidate for targeted therapy because it is not present on most normal tissues, except the mesothelial lining of body cavities and basal cells of the trachea (12). Because the murine monoclonal antibody against mesothelin, Mab K1, by itself is not cytotoxic to mesothelin-expressing cells, we developed immunotoxins targeting mesothelin. SS(dsFv)PE38 and SS1(dsFv)PE38 are disulfide-stabilized single-chain Fv antimesothelin immunotoxins that have significant *in vitro* and *in vivo* activity against a transfected and an established cell line ex-

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Table 1 Clinical characteristics of patients whose tumors were grown in organotypic culture

Patient	Age	Tumor site	Pathological diagnosis
O2	54	Ovary	Serous papillary carcinoma
O3	35	Ovary	Hemorrhagic follicular cyst
O4	64	Ovary	Endometrioid carcinoma
O10	32	Cervix	Squamous cell carcinoma
O12	38	Ovary	Micropapillary serous carcinoma
O15	61	Ovary	Endometrioid carcinoma

pressing mesothelin (16–18).[3] Because SS1(dsFv)PE38, which was obtained by mutations in the complementarity determining region 3 of SS V_L, exhibits higher affinity and cytotoxicity than SS(dsFv)PE38, this is the antimesothelin immunotoxin that we decided to study. As a control in these studies, we used RFB4 (dsFv)PE38 (BL22), which is similar in construction to the antimesothelin immunotoxins, except that it contains the Fv region of the antibody targeting the CD22 antigen that is overexpressed in many B-cell neoplasms (10).

Evaluation of the antitumor activity of new drugs most commonly involves the use of established tumor cell lines. However, use of such established cell lines may not be helpful in evaluating agents' targeting cell surface receptors if the expression level is not similar to that seen on tumor specimens. In case of mesothelin, many established ovarian cancer cell lines express mesothelin to varying extent, but only in the case of A-1847 does the antigen expression approximate that seen on human tumors (12). Therefore, to conduct preclinical studies of immunotoxins targeting mesothelin, we transfected a human epidermoid carcinoma cell line (A431) with the plasmid encoding mesothelin. This transfected cell, A431-K5, has stable and uniform expression of mesothelin (19), similar to that seen on immunohistochemical examination of tumor specimens obtained from patients, and is very sensitive to several antimesothelin immunotoxins (16, 20).

Because immunotoxins active against transfected or established cell lines may lack clinical applicability for treating patients, we decided to evaluate the antitumor activity of immunotoxins targeting mesothelin against fresh tumor cells obtained from patients. To study this, we decided to use a three-dimensional *in vitro* culture system because the physical contact of cells with each other and the structural environment regulate gene expression as well as influence the sensitivity of cells to drug treatment (21, 22). Organotypic cultures, an example of three-dimensional *in vitro* culture system, in which cells are grown in fibroblast containing collagen gels have been used to grow a variety of different cells, including skin keratinocytes, human ovarian surface epithelial cells, as well as human tumor cell lines (23–25). These cultures have also been used as models for preclinical evaluation of novel therapies such as topical gene therapy *in vitro* (26).

[3] P. S. Chowdhury and I. Pastan, unpublished data.
[4] The abbreviations used are: MEM, modified Eagle medium; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.

MATERIALS AND METHODS

Patients. Tumors used to establish primary cultures and grown in organotypic culture (O2, O3, O4, O10, O12, and O15) were obtained from patients with gynecologic cancers undergoing surgery at the University of Oklahoma Health Sciences Center (Oklahoma City, OK). The Institutional Review Board at the University of Oklahoma Health Sciences Center approved this study. A piece of the tumor specimen obtained during surgery to be used for establishing primary cultures was transported from the operating room to the laboratory in a sterile container. The clinical characteristics of the patients whose tumors were evaluated are shown in Table 1.

Primary Culture of Tumors. Fresh tumor samples obtained from patients were processed under sterile conditions in a tissue culture hood. The tumors were cleaned of any fat, blood, or necrotic tissue and rinsed 2–3 times in PBS. The tumor was cut into small fragments, vortexed vigorously, and plated onto 100-mm tissue culture dishes containing MEM supplemented with 10% fetal bovine serum, nonessential amino acids, sodium pyruvate, and antibiotics/antimycotics (complete MEM). The tissue culture plates were incubated in a 37°C CO_2 incubator. Monolayer cultures that developed from single floating cells and outgrowths of the small tumor pieces were passaged. The time required by tumor cells to become confluent was variable with most of the plates becoming confluent in 10–12 days. Tumor cells from passages 1 to 3 were used for making organotypic cultures.

Histochemical and Immunohistochemical Evaluation. A piece of the tumor specimen obtained for establishing primary cultures was placed in buffered formalin and paraffin embedded. Tissue sections (5-μm thick) were cut from these formalin-fixed, paraffin-embedded blocks and stained with H&E using a standard staining procedure. These slides were then evaluated by a pathologist to confirm the histological diagnosis. Mesothelin expression in tumor specimens was evaluated using immunohistochemistry. Briefly, tissue sections (5-μm thick) were cut from formalin-fixed, paraffin-embedded blocks onto Superfrost/Plus slides (Fisher Scientific, Fair Lawn, NJ); deparaffinized in xylene; rinsed repeatedly in 100, 95, and 70% ethanol; and rehydrated in distilled water. The antigen was recovered by incubating the slides in 3M urea for 40 min in a vegetable steamer. Slides were then rinsed in PBS and incubated with blocking serum for 20 min. After shaking off excess blocking serum, the slides were incubated with antimesothelin monoclonal antibody K1 (10 μg/ml) overnight at 4°C (12). Slides were then washed with PBS, and *in situ* localization of antigens was accomplished by using a biotin-avidin antigen detection method (Ready to Use Vectastain kit; Vector Laboratories, Burlingame, CA). After extensive washing in PBS, sections were incubated in a diaminobenzidine solution and counterstained with a hematoxylin solution. Slides were then dehydrated by ethanol washing and cleared in xylene, and coverslips were placed on the slides. The slides were evaluated for the intensity as well as extent of mesothelin staining. Mesothelin positivity was identified by brownish staining of the surface of the tumor cells and graded as negative if $<30\%$ of cells expressed mesothelin and positive if $\geq 30\%$ of tumor cells stained for mesothelin.

Recombinant Immunotoxins. SS(dsFv)PE38, SS1-(dsFv)PE38, and BL22 were produced in the Laboratory of Molecular Biology at the National Cancer Institute, NIH. SS1(dsFv)PE38 was also supplied by NeoPharm, Inc. (Lake Forest, IL). The immunotoxins in 0.9% saline containing 0.2% human serum albumin were stored at −70°C and thawed at room temperature immediately before use in the experiments.

Organotypic Cultures. Tumor cells grown in primary culture were trypsinized, and cells were counted with a particle counter. Volumes containing $0.9–2 \times 10^5$ tumor cells and $0.9–2 \times 10^5$ NIH-3T3 fibroblasts were mixed and centrifuged. The pellet was resuspended in a 4°C solution containing complete MEM and rat tail collagen I (Collaborative Biomedical Products, Bedford, MA), a solution that is liquid at 4°C. This solution was poured into Falcon cell culture inserts with transparent membranes containing 0.4-μm pores (Becton Dickinson, Franklin Lakes, NJ) that were placed in 6-well tissue culture plates. This collagen/cell mix was allowed to solidify at 37°C for 1 h, and then 3 ml of complete MEM were added to each well so as to completely immerse the inserts in the media. In one culture (O2), tumor cells (0.9×10^5) were added on top of the collagen gel on the fourth day of culture. The media surrounding the collagen gels in the 6-well tissue culture plates were changed every other day and given the following treatments after 4–7 days of growth: media alone; 100 ng/ml BL22 (an immunotoxin that targets CD22 antigen expressed by hematological malignancies) as a control for the nonspecific toxicity of PE38; or different concentrations of SS1(dsFv)PE38, *i.e.*, 1, 10, 100, and 1000 ng/ml. The samples were treated with the media alone or with the immunotoxins every other day × 3. After completing the treatment, the collagen gels were removed from the insert, fixed in formalin, and subsequently embedded in paraffin. Two 5-μm thick sections were cut from each paraffin block in a random fashion to be used for subsequent morphological and apoptosis evaluation.

Except for the O4 organotypic culture in which only tumor cells were added to the collagen gel, all other cultures were made by mixing the tumor cells with the NIH-3T3 fibroblasts in the collagen gel. The organotypic culture of O2 was treated with SS(dsFv)PE38, whereas all other cultures were treated with SS1(dsFv)PE38. As a control for the nonspecific action of immunotoxins, all organotypic cultures except O2 were also treated with BL22.

Histological and Apoptosis Evaluation of Organotypic Cultures. Five-μm thick sections cut from the formalin-fixed, paraffin-embedded organotypic gels were stained with H&E and also examined for apoptosis using the TUNEL assay (*i.e.*, terminal deoxynucleotidyl transferase-mediated dUTP transferase nick-end labeling; Ref. 27). Apoptosis was evaluated using the Klenow-FragEL DNA Fragmentation Kit (Oncogene Research Products, Cambridge, MA) according to the manufacturer's instructions.

Pathology Review. A pathologist experienced in immunohistochemistry (S. A. L.), evaluated the original biopsies of the tumors for mesothelin expression. The same pathologist also reviewed all stained slides from the organotypic gels for tumor morphology and apoptosis in a blinded manner. The following characteristic features in H&E-stained sections were used to identify apoptotic cells: nuclei that were shrunken and markedly hyperchromatic; multiple small pieces of nuclear material in the cell; and cell cytoplasm that contained some fragment of a nucleus and cell cytoplasm that resided as a small dense segment without nuclear material. Apoptosis was also evaluated in the TUNEL-stained slides, which were screened at ×10 magnification, and then the individual cells were examined at ×40 magnification. Cells that had granular brown pigment in the nucleoplasm were scored as positive. Cells were scored as negative if no staining was present or if the brown pigment was smooth and nongranular. For each H&E slide, the pathologist counted the total number of cells per section as well as the percent of these cells, which showed apoptosis using the TUNEL assay.

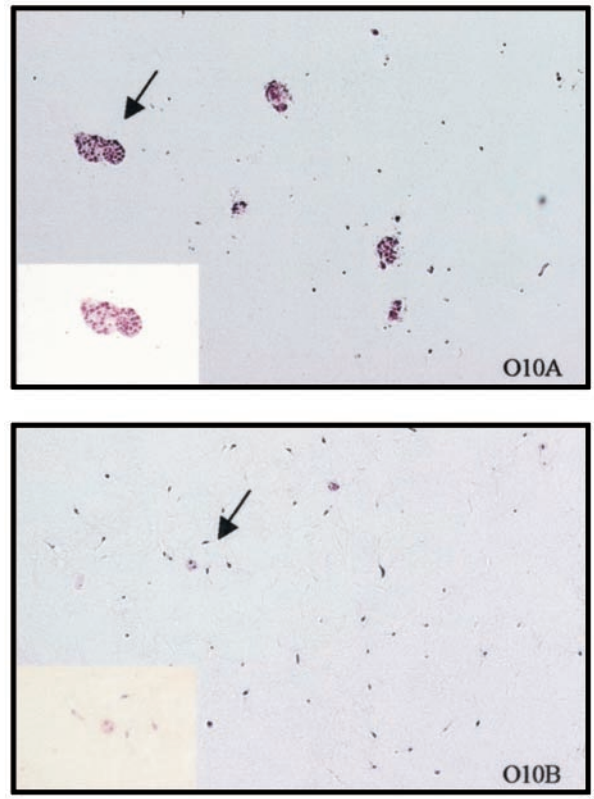

Fig. 1 Organotypic culture of O10 (cervical squamous cell carcinoma). *A*, culture of O10 tumor cells with NIH-3T3 cells present in the collagen gel. ×5 (inset higher magnification ×20). *B*, culture of O10 tumor cells without NIH-3T3 fibroblasts present in the collagen gel. ×5 (inset higher magnification ×20).

RESULTS

Patient Characteristics. Six tumor specimens that showed sufficient tumor cell growth in organotypic culture were evaluated for immunotoxin sensitivity. Five were ovarian tumors, and one was a squamous cell carcinoma of the cervix (O10). Of the five ovarian tumors, one, O3, was a benign hemorrhagic follicular cyst, whereas the other four (O2, O4, O12, and O15) were malignant tumors of the ovary. The clinical characteristics of these patients are shown in Table 1.

Table 2 Antitumor activity of SS1(dsFv)PE38 against human gynecologic tumors grown in organotypic culture

Tumor	Pathological diagnosis	Mesothelin expression[a]	Immunotoxin concentrations (ng/ml)[b]	Total no. of cells/section[c]	Percentage of apoptotic cells (TUNEL)[d]
O2	Papillary serous ovarian cancer	Positive	0 (media)	509	19
			10	666	55
			100	410	78
			1000	206	92
O3	Hemorrhagic ovarian follicular cyst	Negative	0 (media)	325	20
			10	330	22
			100	305	18
			1000	300	18
			BL22-100	315	14
O4	Ovarian endometrioid carcinoma	Positive	0 (media)	42	Too few cells to quantitate apoptosis
			10	16	
			100	8	
			1000	2	
			BL22-100	38	
O10	Cervical squamous cell carcinoma	Positive	0 (media)	1210	4
			1	1248	54
			10	936	58
			100	897	72
			1000	288	74
			BL22-100	1072	38
O12	Papillary serous ovarian carcinoma	Positive	0 (media)	525	5
			1	498	12
			10	478	10
			100	200	26
			1000	178	32
			BL22-100	504	3
O15	Ovarian endometrioid carcinoma	Negative	0 (media)	925	0
			1	966	14
			10	890	16
			100	875	8
			1000	815	8
			BL22-100	974	12

[a] Mesothelin expression was quantitated by immunohistochemistry using MabK1. Negative = <30% of cells express mesothelin; positive = ≥30% of cells express mesothelin.

[b] All the organotypic cultures except O2 were treated with different concentrations of SS1 (dsFv)PE38. O2 was treated with SS(dsFv)PE38. Except O2, the organotypic cultures were also treated with 100 ng/ml BL22 as a control immunotoxin because it targets CD22 antigen not present on gynecologic tumors.

[c] Five-μm sections of paraffin-enbedded organotypic cultures were stained for H&E and evaluated by a pathologist for the number of tumor cells per section.

[d] The percentage of apopotic cells in the different treatment groups was evaluated by using a TUNEL assay.

Primary Cultures. Fifteen tumor specimens obtained from patients undergoing surgery as initial treatment for their malignancy were obtained and processed for primary cultures. Ten of the 15 tumors grew as primary cultures, and nine of these primary cultures were grown in organotypic culture. Of these nine tumors grown in organotypic culture, six had sufficient tumor cell growth to examine for immunotoxin sensitivity.

Mesothelin Expression in the Tumors. All tumors were evaluated for mesothelin expression by immunohistochemistry. The O2, O4, O10, and O12 tumors were positive for mesothelin expression, whereas the O3 (benign ovarian cyst) and O15 (ovarian endometrioid carcinoma) tumors were negative for mesothelin expression.

Organotypic Cultures. All five of the organotypic cultures grown with NIH-3T3 fibroblasts showed growth of tumor cells inside the collagen gel. Only in one of four organotypic cultures (*i.e.*, O4) made without adding NIH-3T3 fibroblasts to the collagen gel were we able to grow tumor cells. However, the tumor cells in the O4 organotypic culture were mostly single cells and also greatly decreased in number compared with culture of tumor cells mixed with NIH-3T3 fibroblasts in the collagen gel. The results presented are for the five organotypic cultures where both tumor cells and NIH-3T3 fibroblasts were cultured together (O2, O3, O10, O12, and O15), as well as the O4 organotypic culture where tumor cells were grown without NIH-3T3 fibroblasts. Except for the O3 and O4 organotypic cultures in which the cells were seen as single tumor cells, the majority of the tumor cells in the other cultures were present in colonies.

To determine whether NIH-3T3 fibroblasts were important for tumor growth in the collagen gel, primary cultures of O10

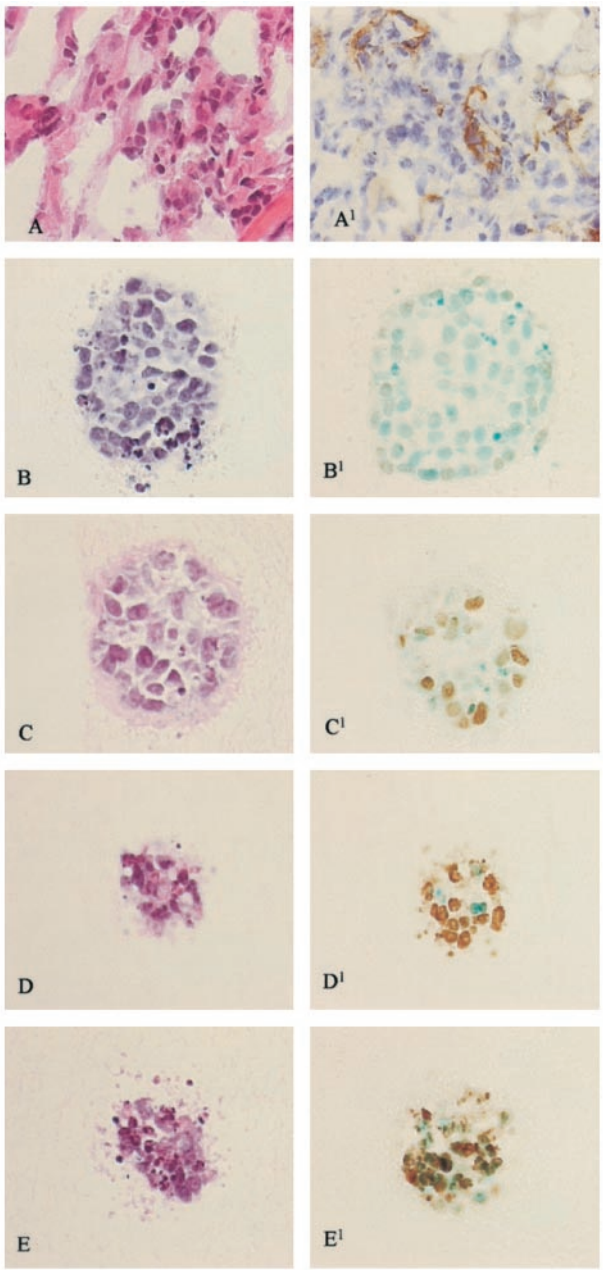

Fig. 2 Organotypic culture of O2 (serous papillary ovarian carcinoma) treated with SS(dsFv)PE38. *A*, H&E examination of O2 tumor. A^1, O2 mesothelin expression determined by immunohistochemistry. *B–E* represent H&E sections of O2 organotypic cultures treated with 0 (media), 10, 100, and 1000 ng/ml of SS(dsFv)PE38, respectively. B^1–E^1 represent evaluation of *B–E* organotypic cultures, respectively, for apoptosis using the TUNEL assay. (original magnification ×40).

and O12 were grown in organotypic culture, with and without NIH-3T3 fibroblasts. The cultures of tumor cells mixed with NIH-3T3 fibroblasts resulted in growth of large numbers of tumor cells, whereas organotypic cultures containing tumor cells without NIH-3T3 fibroblasts had very little if any growth of tumor cells. The results of the organotypic culture of O10 with

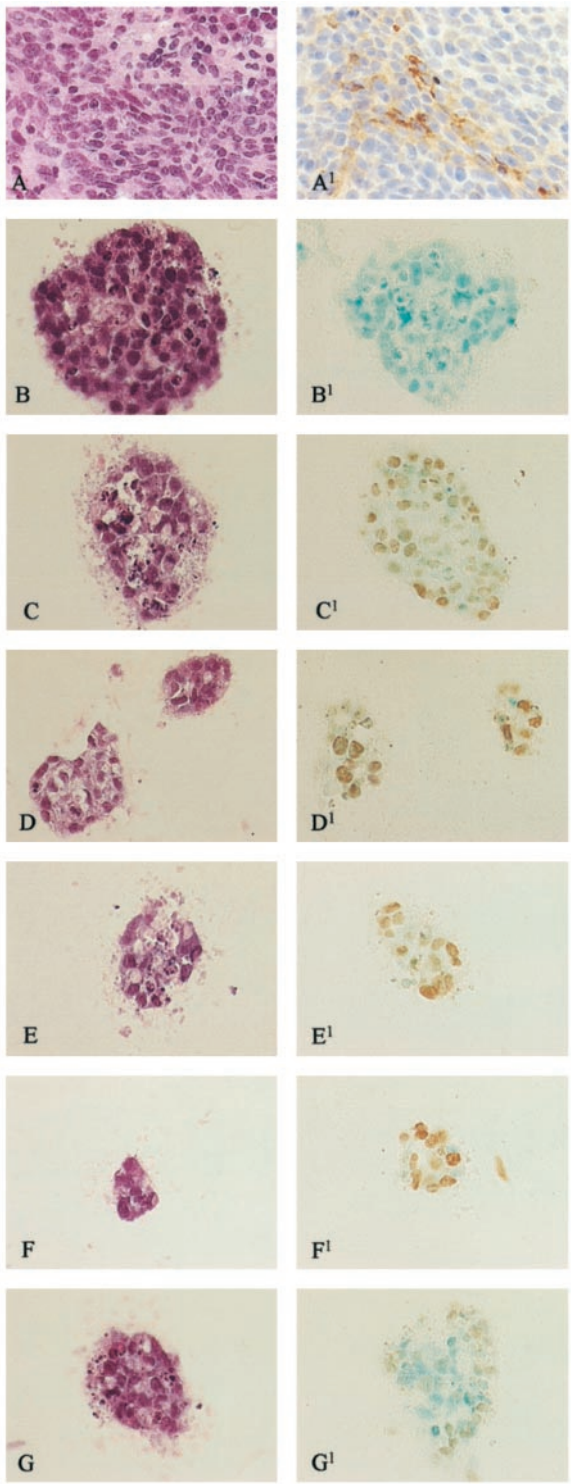

Fig. 3 Organotypic culture of O12 (micropapillary serous ovarian carcinoma) treated with SS1(dsFv)PE38. *A*, H&E examination of O12 tumor. A^1, O12 mesothelin expression determined by immunohistochemistry. *B–F* represent H&E sections of O12 organotypic cultures treated with 0 (media), 1, 10, 100, and 1000 ng/ml of SS1(dsFv)PE38, respectively. *G* represents H&E section of O12 organotypic culture treated with 100 ng/ml BL22. B^1–G^1 represent evaluation of *B–G* organotypic cultures, respectively, for apoptosis using the TUNEL assay. (original magnification ×40).

or without fibroblasts are shown in Fig. 1. We therefore used NIH-3T3 fibroblasts in all of our subsequent organotypic culture of tumor cells. Our results show that NIH-3T3 fibroblasts promote the survival and growth of cells obtained from primary cultures of ovarian or cervical tumors in collagen gels. The exact role of the fibroblasts in promoting growth of tumor cells in organotypic culture is unclear but is probably because of various growth factors secreted by these cells (23, 28).

Activity of Antimesothelin Immunotoxins against Tumors Grown in Organotypic Culture. To determine whether immunotoxins targeting mesothelin would be cytotoxic toward human cancers expressing mesothelin, tumor specimen obtained from patients were grown in organotypic culture and evaluated for immunotoxin sensitivity. H&E-stained sections of the paraffin-embedded organotypic culture gels treated with media alone or different concentrations of the immunotoxins were evaluated by the pathologist for morphology as well as number of tumor cells (whether in colonies or as single cells) per section. In organotypic cultures made from tumors that expressed mesothelin (O2, O4, O10, and O12), the number of tumor cells per section was decreased with increasing concentration of the immunotoxin, whereas no significant decrease was observed in cultures treated with the control immunotoxin BL22 (Table 2). In contrast, in tumors that did not express mesothelin (O3 and O15), there was no significant difference between the number of cells per section between the control and immunotoxin-treated cultures.

To further evaluate the antitumor activity of antimesothelin immunotoxins, untreated and treated samples were evaluated for apoptosis using the TUNEL assay. As can be seen in Table 2, mesothelin-positive tumors (O2, O10, and O12) exhibited increasing percentages of apoptotic cells with increasing concentrations of antimesothelin immunotoxin, in comparison to untreated cultures and cultures treated with BL22. In the O4 organotypic culture, which consisted of tumor cells cultured without the addition of NIH-3T3 fibroblasts, there were too few cells to evaluate for apoptosis. In the organotypic cultures made from mesothelin-negative samples (O3 and O15), there was little difference in the extent of apoptosis between the samples treated with media alone or with antimesothelin immunotoxins.

Fig. 2 illustrates the dose-dependent activity of SS(dsFv)PE38 against O2 organotypic culture, whereas Fig. 3 illustrates the activity of SS1(dsFv)PE38 against the O12 organotypic culture. Our results show that antimesothelin immunotoxins have significant dose-response antitumor activity, consisting of decreased tumor growth and increased apoptosis, at concentrations as low as 1–10 ng/ml against mesothelin-positive tumors grown in organotypic culture, whereas no antitumor activity was seen against tumors lacking mesothelin expression. Also the antitumor activity was because of specific targeting of mesothelin because BL22 did not kill mesothelin-positive or -negative cells.

DISCUSSION

Drugs demonstrating activity against established or transfected cell lines may lack clinical applicability because the phenotype of the cell lines may be different from the tumors present *in vivo*. This is especially true for agents targeting cell surface tumor antigens in which their expression is either lost or diminished in established cell lines. This appears to be the case for mesothelin, a cell surface glycoprotein present on the normal mesothelial cells, and overexpressed in certain cancers, including ovarian cancer (11). Although mesothelin is expressed by many established ovarian cancer cell lines, it is not present to the same degree as observed in tumor specimens obtained from patients (12).

To study the activity of antimesothelin immunotoxins against human tumors, we grew fresh tumor cells obtained from patients in short-term culture *in vitro* using an organotypic culture model. Our results demonstrate that cells obtained from primary cultures of ovarian and cervical tumors can be grown inside of a collagen gel and that tumor morphology can be easily evaluated on histological examination of sections obtained from paraffin blocks of the organotypic cultures. The NIH-3T3 fibroblasts appear to play a critical role for the growth of tumor cells using this model. Our results are in agreement with other published reports, which suggest that fibroblasts can modulate the growth of epithelial cells grown *in vitro*, using an organotypic model (23, 28).

Our results also demonstrate that antimesothelin immunotoxins can target and kill mesothelin-expressing tumor cells obtained from primary culture of human tumors and grown inside a collagen gel. Of the six tumors grown in organotypic culture, four tumors expressed mesothelin (three ovarian tumors and one cervical squamous cell carcinoma), whereas one benign and one malignant ovarian tumor did not express mesothelin. Mesothelin-expressing tumor cells grown in organotypic culture showed a dose-dependent effect when treated with SS(dsFv)PE38 or SS1(dsFv)PE38, even at concentrations as low as 1–10 ng/ml. This antitumor activity was demonstrated by decreased number of tumor cells present in organotypic sections with increasing concentration of the immunotoxins. A simultaneous increase in the percentage of TUNEL-positive cells demonstrated that the mechanism of cell death involved apoptosis. The specificity of this activity is demonstrated by the lack of significant antitumor activity in organotypic cultures treated with BL22, an immunotoxin that targets the cell surface antigen CD22 absent on gynecologic tumors. There was no significant activity of BL22 against mesothelin-positive tumors even at concentration as high as 100 ng/ml. Furthermore, in tumors that lacked mesothelin expression, there was no significant difference between untreated and immunotoxin treated samples in the number of cells present per slide or apoptosis.

In conclusion, our study demonstrates that both SS(dsFv)PE38 as well as SS1(dsFv)PE38 are highly effective against mesothelin-expressing tumors directly obtained from patients. These results are in keeping with the activity of these immunotoxins against a transfected cell line as well as the established ovarian cell line A-1847, which express mesothelin. Clinical trials of SS1(dsFv)PE38 have recently been initiated for treatment of patients with mesothelin expressing malignancies.

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