Epithelial Ovarian Cancer Cells Secrete Functional Fas Ligand[1]

Vikki M. Abrahams, Shawn L. Straszewski, Marijke Kamsteeg, Bozena Hanczaruk, Peter E. Schwartz, Thomas J. Rutherford, and Gil Mor[2]

Department of Obstetrics and Gynecology, Yale University School of Medicine, New Haven Connecticut 06520

ABSTRACT

The Fas/Fas ligand (FasL) system has been suggested to play an important role in the establishment of an immune privilege status of the tumor by inducing Fas-mediated apoptosis in tumor-specific lymphocytes. However, the role of cell surface-expressed FasL in tumor cell protection has recently become controversial. In this study, we have demonstrated that ascites-derived epithelial ovarian cancer cells lack membranal FasL but constitutively secrete whole, intracellular FasL (37 kDa) via the release of microvesicles. In contrast, normal ovarian surface epithelial cells express, but do not secrete, FasL. We have also identified a heavily glycosylated form of secreted FasL (48 kDa), associated with microvesicles isolated directly from the ascites fluid of patients with ovarian cancer. Following the disruption of the microvesicle membrane, both the 37-kDa and 48-kDa forms of secreted FasL were able to trigger Fas-mediated apoptosis in Jurkat T cells. These results suggest that the release of secreted FasL, and not the membrane form, may provide a mechanism by which tumors might counterattack Fas-bearing immune cells, thus facilitating their escape from immune surveillance and promoting tumor cell survival.

INTRODUCTION

A successful tumor cell is one that can escape immune surveillance. Many tumors develop mechanisms to avoid immune recognition, whereas some actively fight immune responses by triggering the death of tumor-specific lymphocytes (1). The latter has been thought of as the tumor establishing itself as a site of immune privilege through the mechanism of "counterattack" (2). The Fas/FasL[3] apoptotic pathway is thought to play an important role in promoting immune privilege in both normal and malignant tissues (3). Therefore, it has been proposed that FasL-expressing tumor cells may induce Fas-mediated apoptosis in tumor-specific immune cells (2, 4).

FasL is a type II transmembrane protein, expressed by NK cells and activated T cells and within immune-privileged sites, such as the eye and brain (5, 6). FasL is a member of the TNF family, which also includes TNF-α and CD40 ligand (7). Membranal FasL is expressed on the surface of cells as a ~37-kDa protein and is proteolytically cleaved by MMP to generate its soluble, 26-kDa form (sFasL) (8). Both forms can self-associate, and after trimerization, FasL binds to and activates its receptor, Fas (CD95). However, trimerization and bioactivity of membranal FasL are more efficient than that of its soluble form (9, 10). Fas is a type I membranal protein that is a member of the TNF receptor family and is expressed by a wide variety of cell types. Upon cross-linking by either FasL or an agonistic anti-Fas mAb, Fas intracellularly recruits Fas-associated death domain and procaspase-8, forming the death-inducing signaling complex, after which the caspase cascade can be activated, resulting in apoptosis and cell death (11).

Ovarian cancer is one of the leading causes of death in women in the Western world and is the fifth most common cancer affecting females (12). Several studies have shown the expression of FasL in ovarian cancer cells (13–15), and its expression has been correlated with severity of disease (16, 17), suggesting a role for FasL in the progression of ovarian cancer. Although *in vitro* studies have shown that FasL expressed on the surface of tumor cells can induce Fas-mediated T-cell apoptosis (13, 18–20), such observations have been recently challenged. *In vivo* models have demonstrated that tumors expressing cell surface membranal FasL are rejected in association with a neutrophilic inflammatory response, whereas this is not the case with cleaved, sFasL (10, 21–23), raising the question of whether FasL mediates immune privilege or inflammation (24, 25).

We have found that epithelial ovarian cancer cells, isolated from malignant ovarian ascites, express large amounts of intracellular FasL that is secreted via microvesicles. In addition, we have isolated FasL-containing microvesicles directly from malignant ovarian ascites obtained from ovarian cancer patients. This form of secreted FasL is different from the soluble FasL, which is a proteolytic fragment produced as a result of the action of MMP. In this study, we demonstrate that secreted FasL induces Fas-mediated apoptosis in Jurkat T cells. We propose that the secreted form of FasL represents a mechanism by which epithelial ovarian cancer cells may, in a paracrine manner, induce apoptosis in Fas-bearing immune cells distant from the primary tumor site, establishing a state of immune privilege and facilitating tumor cell escape from immune surveillance.

MATERIALS AND METHODS

Patients and Samples. Ascites were collected from stage III/IV ovarian cancer patients undergoing surgical debulking. All patients signed consent forms, and the use of patient samples was approved under Yale University's Human Investigations Committee.

Reagents and Antibodies. Camptothecin and Triton X-100 were purchased from Sigma (St. Louis, MO). The pan-caspase inhibitor (Z-VAD-FMK) and the agonistic mouse IgM anti-FasL (clone E0S9.1) were obtained from PharMingen (San Diego, CA). Immunohistochemical staining for FasL was performed using the rabbit polyclonal N-20 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and the mouse mAb G247.4 (PharMingen), and for Western blot using the mouse mAb, clone 33 (Transduction Laboratories, Lexington, KY). Western blot analysis of FasL was confirmed using clone G247.4. Colocalization studies for FasL with Lamp-1 (CD107a) were performed using the anti-FasL polyclonal (N-20) antibody and a mouse anti-Lamp-1 mAb (PharMingen; clone H4A3). FITC-conjugated horse antimouse and Texas Red-conjugated goat antirabbit secondary antibodies were used (Vector Laboratories, Burlingame, CA). The mouse anti-caspase-8 mAb was purchased from Oncogene Research Products (San Diego, CA), the mouse anti-caspase-9 mAb was purchased from R&D Systems, Inc. (Minneapolis, MN); the rabbit anti-caspase-3 polyclonal antibody was obtained from Santa Cruz Biotechnology, Inc. The mouse mAb for β-actin was purchased from Sigma. Specific signals were detected using either a peroxidase-conjugated horse antimouse or a peroxidase-conjugated goat antirabbit secondary antibody (Vector Laboratories).

Isolation of Epithelial Ovarian Cancer Cells from Ascites. Ovarian cancer cells were isolated from freshly collected malignant ovarian ascites as

described previously (26, 27). Briefly, the ascitic fluid was centrifuged at 1500 rpm at room temperature for 15 min. The supernatant was collected for microvesicle isolation. The cellular pellet was resuspended in HBSS (Invitrogen, Carlsbad, CA), and the suspension was centrifuged at 1500 rpm for 10 min at room temperature. The pellet was then resuspended in HBSS and layered onto LSM lymphocyte separation medium (ICN Biomedicals, Inc., Aurora, IL) and centrifuged at 2000 rpm for 25 min at room temperature. The mononuclear cell-containing layer was collected and incubated with an anti-CD45 mAb conjugated to magnetic beads (Dynal, Oslo, Norway) at 4°C with rotation for 30 min. After this incubation, the cells were placed into a magnet, and the unbound epithelial cells were collected, washed, and placed into culture media. Purity of the epithelial ovarian cancer cells was 100% as determined by immunostaining for cytokeratin (Sigma; Ref. 28).

Isolation of Normal Surface OSE Cells. Normal OSE cells were obtained as described previously (29). In short, fragments of normal surface epithelium were detached from the surface of human ovary biopsy tissues, which were obtained from normal cycling females who had undergone surgery at the Yale-New Haven Hospital for benign conditions. Epithelial origin was confirmed by immunostaining for cytokeratin as described previously (28).

Culture of Primary and Continuous Cell Lines. All cells were maintained at 37°C/5% CO_2. Epithelial ovarian cancer cells, after isolation from ascitic fluid and normal surface ovarian epithelial cells, were cultured in 50% 199 media and 50% MCDB 105 media (Sigma), supplemented with 10% fetal bovine serum (Hyclone, South Logan, UT), 10 mM HEPES, 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, 100 nm penicillin/streptomycin (Life Technologies), and 4 ng/ml epidermal growth factor (Sigma). Jurkat cells, a human T-cell leukemia line (American Type Cell Culture, Manassas, VA), were maintained in RPMI 1640 (Life Technologies) supplemented with 10% fetal bovine serum, 10 mM HEPES, 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, and 100 nm penicillin/streptomycin.

Immunohistochemistry. The cellular localization of FasL expression in tissue samples from ovarian tumors was performed as described previously (4). In short, samples were fixed with 4% paraformaldehyde and then paraffin embedded. Sections (5 μm) of ovarian tumors or paraformaldehyde-fixed epithelial ovarian cancer cells, previously adhered to glass slides, were blocked with 10% goat or horse serum in PBS for 1 h at room temperature. After three washes with PBS, samples were incubated at room temperature for 2 h with either the rabbit polyclonal N-20 antibody (1:200 dilution) or the mouse G247.4 (2 μg/ml) anti-FasL antibody. Rabbit IgG or mouse IgG1 served as isotype controls. After three washes with PBS, specific staining was detected by incubating with either a peroxidase-conjugated goat antirabbit or horse antimouse antibody (1:1000 dilution) for 1 h, followed by a 5-min incubation with 3,3′-diaminobenzidine substrate (Vector Laboratories). Cells and tissue sections were then counterstained with hematoxylin (Sigma) before dehydration with ethanol and Histosolve (Shandon, Inc., Pittsburgh, PA). Slides were then mounted with Permount (Fisher Scientific, Pittsburgh, PA).

Colocalization of FasL with Lamp-1. Epithelial ovarian cancer cells, previously adhered to glass slides, were fixed with 4% paraformaldehyde and then blocked with 10% goat and 10% horse serum in PBS for 1 h at room temperature. After three washes with PBS, samples were incubated overnight at 4°C with the rabbit polyclonal anti-FasL antibody (N-20; 1:200 dilution) and the mouse anti-Lamp-1 mAb (H4A3; 10 μg/ml). Rabbit serum and mouse IgG1 served as isotype controls. After three washes with PBS, cells were incubated with a FITC-conjugated horse antimouse antibody and a Texas Red-conjugated goat antirabbit antibody (both at 1:1000) for 1 h at room temperature in the dark. After three washes with PBS, slides were mounted and visualized by fluorescent confocal microscopy (Olympus) using Magnafire software (Microsoft).

Isolation of Microvesicles. Microvesicles were isolated from both ascites from patients and epithelial ovarian cancer cell culture supernatants. The ascitic fluid or cell culture supernatants were centrifuged twice at 1800 rpm for 20 min at 4°C to remove any cellular debris. The cell-free fluid or supernatant was then ultracentrifuged at 25,000 rpm at 4°C for 3 h. The supernatant was discarded, and the microvesicle-containing pellet was resuspended in sterile PBS. After treatment with or without 2% Triton X-100 at 4°C for 30 min, the microvesicle suspension was centrifuged at 14,000 rpm and 4°C for 1 h. The supernatant was collected, and the pellet was resuspended in sterile PBS; both were stored at −40°C until further use.

Removal of Sialic Acid from Ascites-derived FasL. Sialic acid was removed from the ascites-derived secreted FasL as described previously (30). Briefly, 30 μM protein was incubated with 5 units of neuraminidase (Sigma) in 50 mM sodium acetate, 150 mM sodium chloride, and 4 mM calcium chloride (pH 5.5) at 37°C overnight. After incubation, proteins were either diluted with gel loading buffer for Western blot analysis or adjusted to pH 7.2 for functional analysis.

Western Blot Analysis. Microvesicles were prepared as described above and then analyzed by Western blot. For analysis of intracellular proteins, cells were lysed using 1% NP40 and 0.1% SDS in the presence of protease inhibitors. Protein concentrations were calculated by bicinchoninic assay (Pierce Biotechnology, Rockford, IL). Proteins were then diluted with gel loading buffer to 20 μg and boiled for 5 min. Proteins were resolved under reducing conditions on either 10 or 12% SDS-PAGE gels and then transferred onto polyvinylidene difluoride paper (NEN Life Sciences, Boston, MA). Membranes were blocked at room temperature for 1 h with 5% FFPM in PBS/0.05% Tween 20 (PBS-T). After three washes for 10 min, each with PBS-T, membranes were incubated overnight at 4°C with primary antibody in PBS-T/1% FFPM. After this incubation, membranes were washed three times as before and then incubated at room temperature for 1 h with the appropriate secondary antibody conjugated to peroxidase (Vector Laboratories) in PBS-T/1% FFPM. After three washes for 10 min each with PBS-T and three washes for 10 min each with distilled water, the peroxidase-conjugated antibody was detected by enhanced chemiluminescence (NEN Life Sciences). All experiments were repeated at least three times, and the intensity of the signals was analyzed using a digital imaging analysis system and 1D Image Analysis Software (Scientific Imaging Kodak Company). Where appropriate, β-actin was used as internal control, in addition to Ponceau Red, to validate the amount of protein loaded onto the gels.

Quantification of FasL. Secreted FasL was quantified by ELISA according to the manufacturer's instructions (Cell Sciences, Norwood, MA). This ELISA uses antibodies specific for the extracellular domain of human FasL and can, therefore, be used for detecting both soluble and whole FasL.

Assessment of Secreted Fas Ligand Bioactivity. The bioactivity of the secreted FasL was determined using the CellTiter 96 viability assay (Promega Corp., Madison, WI), as described previously (18, 31). Jurkat cells were plated in wells of a 96-well plate at 5×10^5 cells/well in the reduced serum medium, Opti-MEM (Life Technologies), and cultured overnight at 37°C. Jurkat cells were then incubated with either the secreted FasL, medium as a negative control, or an agonistic anti-Fas mAb (500 ng/ml) as a positive control. Cells were incubated at 37°C for 24 h, after which the CellTiter substrate, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfonyl)-2H-tetrazolium, was added. After a 1–4-h incubation at 37°C, absorbances were read at 490 nm. Cell viability was calculated as described previously (32) and presented as a percentage of the control. All samples were assayed in triplicate.

FACS Analysis. After treatment, Jurkat cells (1×10^6) were washed twice with cold PBS and centrifuged at 1500 rpm for 5 min at 4°C. The cell pellet was then resuspended in 1 ml of cold PBS and incubated on ice for 20 min with propidium iodide (Sigma) at 1 μg/ml and Hoechst 33342 dye (Molecular Probes) at 5 μg/ml. Unstained cells served as a negative control. Samples were then analyzed using a FACS Vantage (Becton Dickinson) with 488 nm/UV dual excitation. Propidium iodide staining was detected in the FL-2 channel, and Hoechst staining was detected in the SSc-W channel. Data were analyzed using CellQuest software (Becton Dickinson).

Statistical Analysis. Data are expressed as mean ± SD. Statistical significance ($P < 0.05$) was determined using the Student t test.

RESULTS

Epithelial Ovarian Cancer Cells Express Intracellular FasL. Ovarian cancer cells are known to express FasL; however, its cellular distribution has not been elucidated. We first evaluated FasL expression and its intracellular localization in paraffin sections of ovarian tumor samples by immunohistochemistry. As shown in Fig. 1a, sections of ovarian tumors showed positive immunoreactivity for FasL with expression predominantly localized in the cytoplasm. To examine this intracellular expression of FasL in greater detail, an *in vitro* system was used in which epithelial ovarian cancer cells were isolated
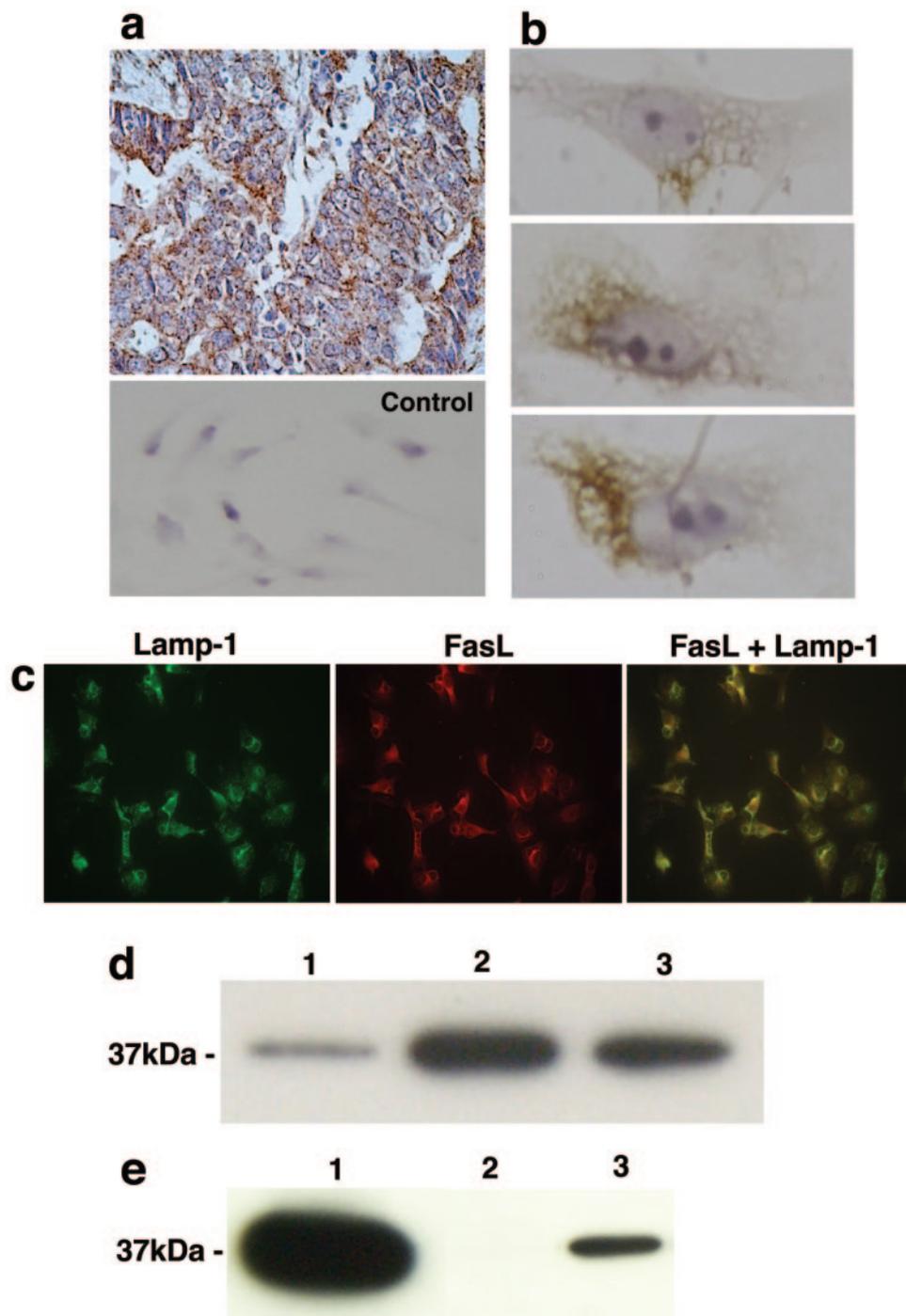
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Fig. 1. Intracellular FasL is secreted from epithelial ovarian cancer cells via microvesicles. Tissue sections of paraffin-embedded ovarian tumor (×10; *a*) and ascites-derived epithelial ovarian cancer cells (×60; *b*) were stained for FasL using a rabbit polyclonal antibody (N-20) and a peroxidase-conjugated goat antirabbit antibody. Note the strong positive immunoreactivity localized to the cytoplasm both in the tumor and isolated cells. Control cells treated with a nonspecific primary antibody showed no staining (×20). *c,* epithelial ovarian cancer cells were double stained for FasL (rabbit polyclonal antibody N-20) and a Texas red-conjugated goat antirabbit, and for Lamp-1 (mAb H4A3) and a FITC-conjugated horse antimouse (×40). Note that the intracellular FasL is colocalized with Lamp-1 (*yellow*); however, not all Lamp-1 is colocalized with FasL, as seen by *green* punctuate staining. *d,* Western blot analysis of microvesicles isolated from culture supernatants of three epithelial ovarian cancer cell lines shows the presence of FasL (37 kDa). *Lanes 1–3,* ovarian cancer cell lines R127, R182, and R203. Results were reproduced in microvesicles isolated from all other cell lines tested (R220, R177, R178, R247, and R250). *e,* microvesicles isolated from the culture supernatants of, and cell lysates prepared from, normal OSE cells were analyzed for FasL expression. Western blot shows 37-kDa FasL present in Jurkat cell lysates (*Lane 1*) and normal OSE lysates (*Lane 3*) but not in the microvesicles isolated from the culture supernatant of the normal OSE cells (*Lane 2*).

from ascitic fluid of ovarian cancer patients. As shown in Fig. 1*b,* FasL was absent from the cell surface of these epithelial ovarian cancer cells; however, positive immunoreactivity for FasL was found intracellularly, comparable with that observed in the primary tumor. Furthermore, this intracellular FasL was distributed in the cytoplasm in a polarized manner, indicative of a protein that is secreted (Fig. 1*b*).

FasL Is Secreted via Microvesicles. Recent studies have suggested that FasL may be released from immune cells in association with either secretory lysosomes, which fuse with the plasma membrane (33), or with microvesicles (34). Therefore, we tested whether the FasL present in the cytoplasm of ovarian cancer cells was also secreted:

Firstly, we determined whether the intracellular FasL expressed by

epithelial ovarian cancer cells had the potential to be secreted by staining the cells for FasL and the specialized secretory lysosome maker, Lamp-1. Fluorescent confocal microscopy revealed that, although staining for Lamp-1 could be seen both alone as punctate staining (green), as well as in colocalization with FasL (yellow), the majority of FasL staining (red) was colocalized with that for Lamp-1 (yellow; Fig. 1*c*). This observation suggested that the intracellular form of FasL expressed by ovarian cancer cells was associated with a specialized secretory pathway.

Secondly, we isolated microvesicles from the culture supernatants collected from epithelial ovarian cancer cells and analyzed for FasL expression by Western blot. Microvesicles isolated from the culture supernatant of ovarian cancer cells were found to be associated with
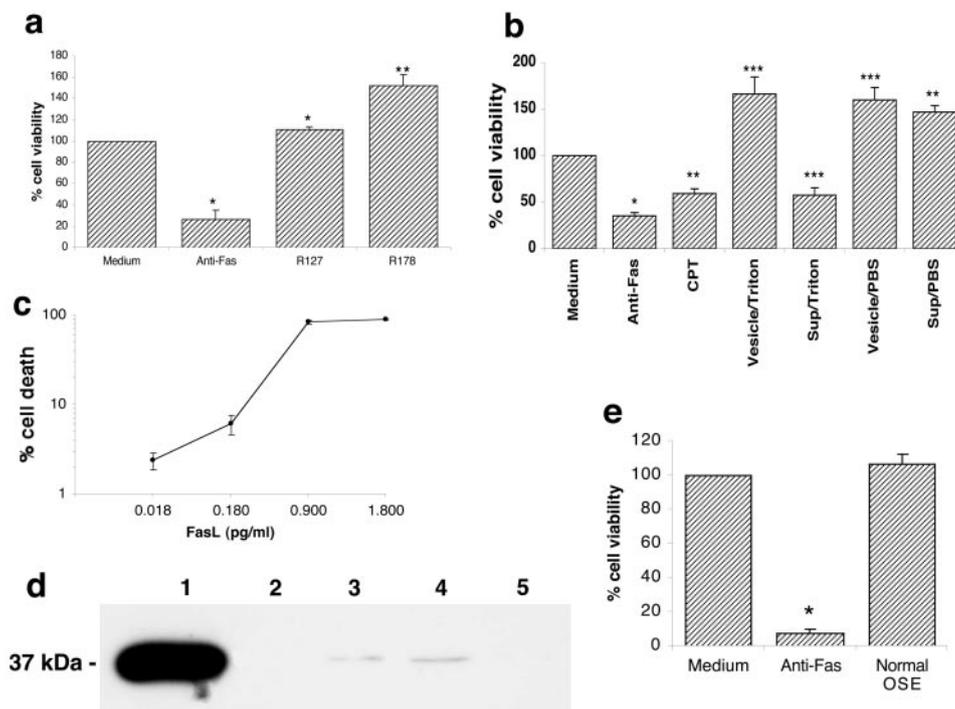
Fig. 2. Secreted FasL is bioactive. *a,* Jurkat cells (5×10^5) were incubated for 24 h with microvesicles isolated from the culture supernatants of ovarian cancer cells at 1:10 dilution. Cell viability was then determined using the CellTiter 96 assay. As a positive control, Jurkat cells were incubated with an agonistic anti-Fas mAb (500 ng/ml). *Bar chart* shows percentage cell viability relative to the untreated control (medium). Treatment with anti-Fas mAb significantly reduced Jurkat cell viability (∗, $P < 0.0001$), whereas treatment with whole microvesicles (R127 and R178) significantly increased Jurkat cell viability (∗, $P < 0.0001$; ∗∗, $P < 0.001$). This figure is representative of three independent experiments. *b,* after isolation from the culture supernatant of epithelial ovarian cancer cell lines, microvesicles were treated either with PBS (intact microvesicles) or 2% Triton X-100 to disrupt microvesicle integrity and release its contents. Supernatant fractions and vesicle fractions were then separated by centrifugation and analyzed for FasL bioactivity. Jurkat cells (5×10^5) were incubated with either the supernatant fractions from Triton X-100-treated vesicles (*sup/triton*), vesicle fraction from Triton X-100-treated vesicles (*vesicle/triton*), supernatant fraction from PBS-treated vesicles (*sup/PBS*), or vesicle fraction from PBS-treated vesicles (*vesicle/PBS*) at a 1:1000 dilution for 24 h. Cell viability was then determined using the CellTiter 96 assay. As positive controls, Jurkat cells were incubated with either an agonistic anti-Fas mAb (500 ng/ml) or camptothecin (*CPT*; 4 μM). *Bar chart* shows percentage cell viability, relative to the untreated control (medium). Treatment with anti-Fas mAb, camptothecin or the supernatant fraction from the Triton X-100-treated microvesicles significantly reduced cell viability (∗, $P < 0.0001$; ∗∗, $P < 0.0005$; ∗∗∗, $P < 0.005$). All other fractions significantly increased cell viability. This figure is representative of three independent experiments using preparations from four different cells lines. *c,* secreted FasL induces cell death in a dose-dependent manner. Jurkat cells were incubated for 24 h with the supernatant fraction from Triton X-100-treated microvesicles in serial dilutions (1:500, 1:1000, 1:5000, and 1:50000). The *graph* shows percentage cell death relative to untreated Jurkat cells. The dilution factors correspond to the following FasL protein concentrations, as determined by ELISA: 1.8, 0.9, 0.18, and 0.018 pg/ml. This figure is representative of three independent experiments. *d,* Western blot analysis for the presence of FasL (37 kDa) in Jurkat cell lysate (*Lane 1*), Triton X-100-treated vesicle fraction (*Lane 2*), Triton X-100-treated supernatant fraction (*Lane 3*), PBS-treated vesicle fraction (*Lane 4*), and PBS-treated supernatant fraction (*Lane 5*). The figure shows the results obtained from the cell line R220. Similar results were obtained with microvesicles isolated from four different epithelial ovarian cancer cell culture supernatants. *e,* Jurkat cells (5×10^5) were incubated for 24 h with either the supernatant fraction from Triton X-100-treated microvesicles isolated from the culture supernatant of normal OSE cells (1:1000), medium alone, or the agonistic anti-Fas mAb (500 ng/ml). Jurkat cell viability was then determined using the CellTiter 96 assay. The *bar chart* shows percentage cell viability relative to medium control. The anti-Fas mAb significantly reduced Jurkat cell viability (∗, $P < 0.00001$), whereas the supernatant fraction from the Triton X-100-treated OSE microvesicles had no effect. This figure is representative of three independent experiments.

whole, 37-kDa FasL (Fig. 1*d*). In contrast, although normal OSE cells expressed FasL, microvesicles isolated from the culture supernatant of these cells were negative for FasL (Fig. 1*e*). These results show that, unlike normal ovarian epithelial cells, malignant ovarian epithelial cells have the capacity to secrete FasL via microvesicles through a specialized secretory pathway.

Whole Microvesicles Have No Effect on Cell Viability. We then sought to determine whether this secreted FasL, released via microvesicles from epithelial ovarian cancer cells, was functional. Bioactivity of the microvesicle-associated FasL was assessed by means of the CellTiter viability assay, using the Fas-expressing Jurkat T cell line as a target. As shown in Fig. 2*a*, treatment of Jurkat cells with an agonistic anti-Fas mAb significantly reduced Jurkat cell viability to 27.0% ± 8.5 relative to the untreated control (∗, $P < 0.0001$). However, the intact FasL-expressing microvesicles, isolated from the culture supernatant of ovarian cancer cells, had no such effect (Fig. 2*a*).

Secreted FasL Is Only Bioactive When Released from Microvesicles. The above result suggested that either the secreted FasL was nonfunctional, or that, in association with the microvesicle, the secreted FasL was unable to elicit its activity. We first examined, by

FACS analysis, the localization of FasL in the purified microvesicles derived from epithelial ovarian cancer cells. No positive staining was detected on any of these preparations, indicating that the microvesicles lacked surface FasL (data not shown). Therefore, to free the putative encapsulated FasL, microvesicles were treated with the detergent, Triton X-100. After treatment with or without Triton X-100, the supernatant fractions were separated from the vesicle fractions by centrifugation, and both the vesicle and supernatant fractions were then tested for bioactivity using the CellTiter viability assay. As shown in Fig. 2*b,* the only fraction able to significantly reduce Jurkat cell viability was the supernatant from Triton X-100-treated vesicles. This fraction reduced Jurkat cell viability to 57.4% ± 8.0 (∗∗∗, $P < 0.005$) of the untreated control (Fig. 2*b*). In addition, this effect was dose dependent (Fig. 2*c*). All other fractions failed to induce cell death (Fig. 2*b*). To confirm that the secreted FasL was indeed the mediator of this decrease in cell viability, each of the four fractions was tested for the presence of FasL by Western blot. As shown in Fig. 2*d,* FasL was present, as expected, in the vesicle fraction of untreated microvesicles and was only detected in the supernatant fraction of the Triton X-100-treated vesicles. Furthermore, the Triton X-100-treated microvesicles isolated from the culture

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Fig. 3. Secreted FasL induces Fas-mediated apoptosis. *a,* Jurkat cells (1×10^6/ml) were incubated for 24 h with either no treatment, camptothecin as positive control for apoptosis (4 μM), or the secreted FasL at 9 pg/ml (1:100 dilution). After incubation, Jurkat cells were double stained with propidium iodide and Hoechst 33342 dye, and fluorescence intensities were analyzed by flow cytometry as described in "Materials and Methods." Treatment with secreted FasL increased the number of apoptotic cells to 91.53% as shown by double-positive-stained Jurkat cells. Data are representative of three independent experiments. *b,* Jurkat cells (1×10^6/ml) were incubated for 4 h with either no treatment, an agonistic anti-Fas mAb (500 ng/ml), or the secreted FasL (9 pg/ml). Note the active forms of caspase-8 (43/41 kDa), caspase-9 (36 kDa), and caspase-3 (17 kDa) in samples treated with secreted FasL (*R127*) and the anti-Fas mAb (α-*Fas*) but not in the untreated control (*NT*). β-Actin shows that equal amounts of protein loaded to all lanes. This is representative of three independent experiments. *c,* Jurkat cells (5×10^5) were incubated for 24 h with either no treatment, an agonistic anti-Fas mAb (500 ng/ml), or the secreted FasL in the presence or absence of a pan-caspase inhibitor (Z-VAD-FMK) at 20 μM. The presence of the pan-caspase inhibitor blocked the apoptotic effect of the secreted FasL (*R127*; *, $P < 0.05$) and the anti-Fas mAb (**, $P < 0.01$). The figure is representative of three independent experiments.
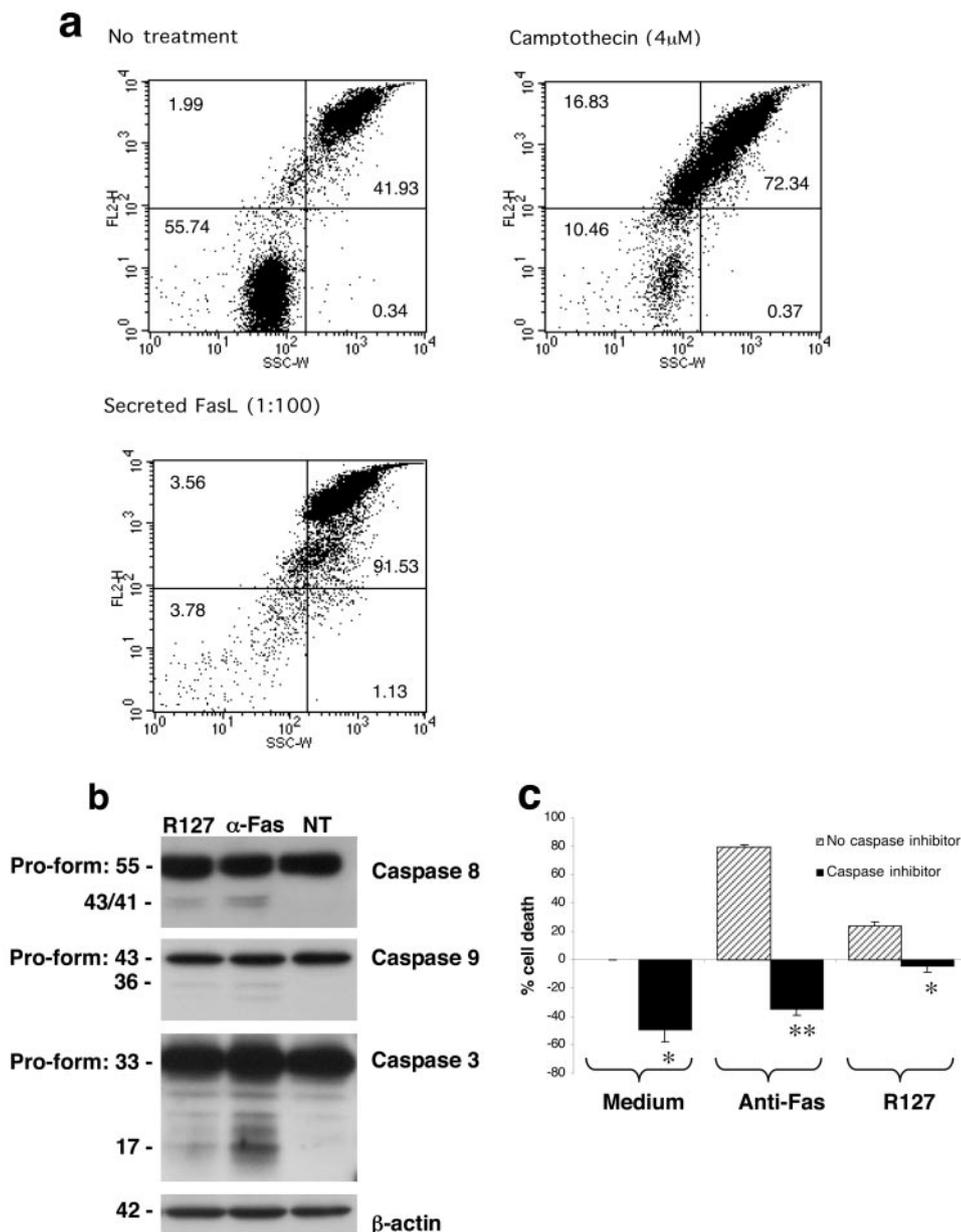
supernatant of normal OSE cells, which were negative for FasL (Fig. 1*d*), failed to induce Jurkat cell death (Fig. 2*e*). These results indicate that the receptor-binding domain of the secreted FasL is contained within the microvesicle and that binding of this FasL to its receptor, Fas, only occurs after membrane disruption of the vesicle. Therefore, the secreted FasL is only able to induce Fas-mediated apoptosis after release from the microvesicle.

Secreted FasL Induces Apoptosis. To further confirm that the decrease in cell viability found with the secreted form of FasL was attributable to Fas-mediated apoptosis, Jurkat cells were incubated with FasL, isolated from the supernatant fraction of the Triton X-100-treated microvesicles. After incubation, the cells were double stained with propidium iodide and Hoechst 33342 dye and analyzed by flow cytometry. Treatment with the secreted FasL at a concentration of 9 pg/ml resulted in a 49.6% increase in the number of Jurkat apoptotic cells compared with the untreated control. As a positive control, Jurkat cells were treated with camptothecin, which increased the number of apoptotic cells by 30.4% (Fig. 3*a*).

Secreted FasL Activates the Fas Apoptotic Pathway. To demonstrate that the secreted form of FasL was able to induce apoptosis through the Fas pathway, we evaluated caspase activation by Western blot analysis. Untreated Jurkat cells expressed only the pro-forms of caspase-8 (55 kDa), caspase-9 (43 kDa), and caspase-3 (33 kDa; Fig. 3*b*). However, treatment with either the agonistic anti-Fas mAb, or the secreted FasL, resulted in caspase activation, evidenced by the presence of caspase-8, caspase-9, and caspase-3 cleavage products (43/41, 36, and 17 kDa, respectively; Fig. 3*b*). To further confirm these results, Jurkat cells were treated with secreted FasL or the anti-Fas mAb in the presence or absence of a pan-caspase inhibitor (Z-VAD-FMK). The presence of the pan-caspase inhibitor significantly reduced the amount of cell death induced by both the agonistic anti-Fas mAb and the secreted form of FasL (*, $P < 0.05$; **, $P < 0.001$; Fig. 3*c*).

Secreted FasL Is Present in the Ascites Fluid of Patients with Ovarian Cancer. To determine whether the secretion of FasL via microvesicles was a phenomenon of the epithelial ovarian cancer cells
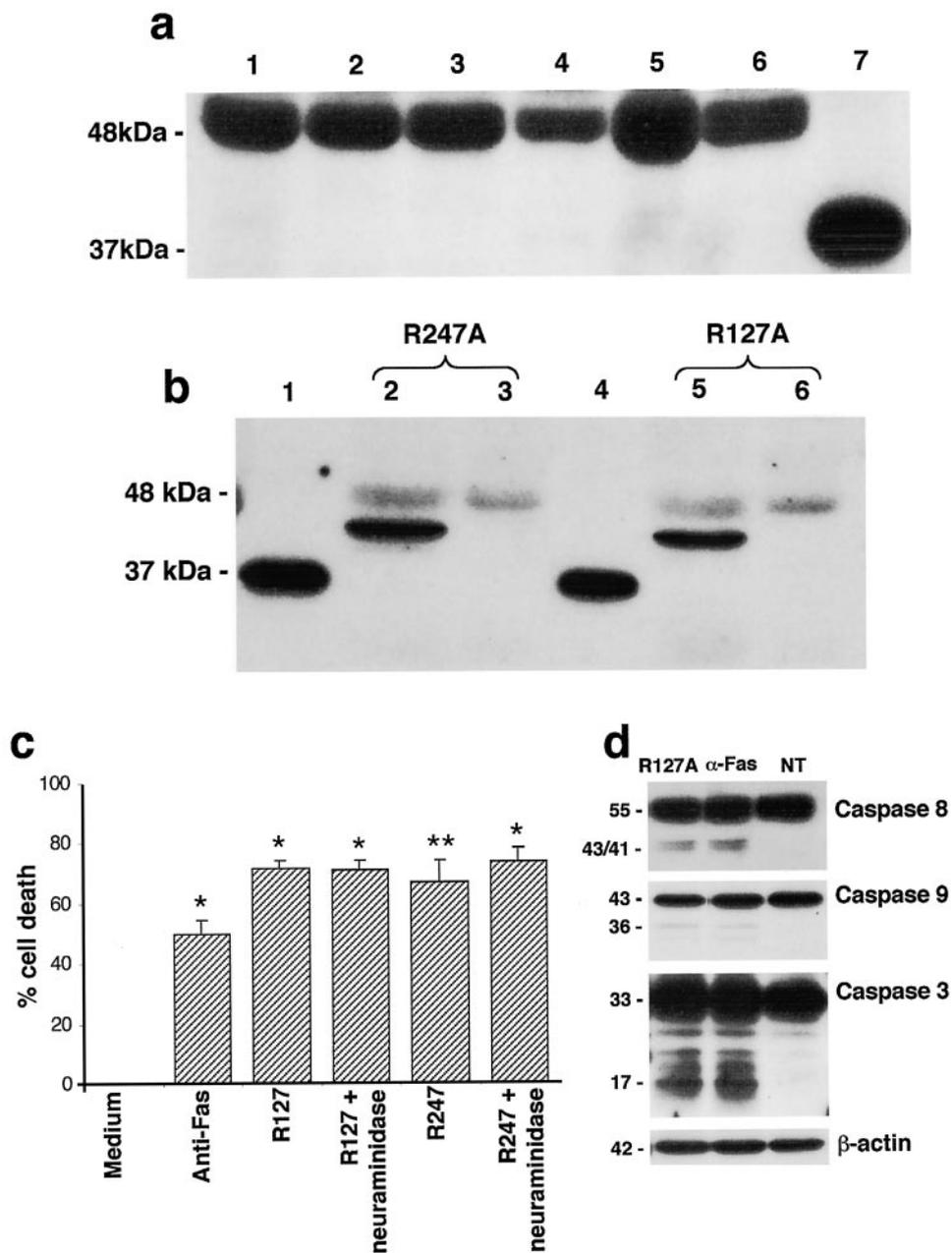
Fig. 4. Presence of a heavily glycosylated form of FasL associated with ascites-derived microvesicles. *a,* microvesicles isolated from the ascites fluid of patients with ovarian cancer were analyzed for FasL by Western blot. Immunoblot for FasL shows the presence of a ~48-kDa protein in microvesicles isolated from the ascites of six ovarian cancer patients. *Lanes 1–6,* R160, R161, R163, R165, R170, and R171; *Lane 7,* Jurkat cell lysate (37-kDa FasL). Similar results were obtained in microvesicles isolated from the ascites of six additional patients (R127, R247, R250, R256, R257, and R270). *b,* treatment of the ~48-kDa secreted FasL from ascites with neuraminidase decreased the molecular size as detected by Western blot. *Lanes 1* and *4,* FasL in Jurkat cell lysate (37 kDa); *Lanes 3* and *6,* ascites-derived secreted FasL without neuraminidase treatment (~48 kDa); *Lanes 2* and *5,* ascites-derived secreted FasL after neuraminidase treatment (~42 kDa). *c,* Jurkat cells were treated for 24 h with neuraminidase-treated and -untreated secreted FasL, and cell viability was determined using the CellTiter 96 assay. Treatment with either the anti-Fas mAb or the secreted FasL induced significant cell death (∗, $P < 0.00005$; ∗∗, $P < 0.0001$). However, there was no significant difference between the effect induced by the neuraminidase-treated and -untreated secreted FasL. This figure is representative of three independent experiments. *d,* ascites-derived secreted FasL (R127A) activates caspase-8 (43/41kDa), caspase-9 (36 kDa), and caspase-3 (17 kDa), similarly to the anti-Fas mAb (*α-Fas*) and unlike the no-treatment control (*NT*). β-Actin shows equal amounts of protein loaded to all lanes.

in culture, we assessed malignant ovarian ascites for the presence of microvesicles containing FasL. Using two different antibodies for FasL, we detected a positive band in the ascites-derived microvesicles, although the size of the protein did not correspond to the classical ~37 kDa. Instead, the size of the protein identified was approximately 48 kDa (Fig. 4*a*), raising the question of whether this protein was FasL.

Secreted FasL Associated with Ascites-derived Microvesicles Is Heavily Glycosylated. Under normal conditions, FasL displays various degrees of glycosylation, which is thought to influence expression levels but not receptor binding or bioactivity (7). Moreover, aberrant glycosylation is a common feature of malignancies (35). We hypothesized that the ~48-kDa band detected in the ascites-derived microvesicles was the result of a highly glycosylated form of FasL. Therefore, we subjected this preparation to deglycosylation followed by Western blot analysis. After treatment with neuraminidase, which removes sialic acid, this protein exhibited a size reduction from ~48 to ~42 kDa (Fig. 4*b*). These results suggested that the ~48-kDa

protein corresponded to FasL that has undergone additional glycosylation *in vivo*.

Glycosylated FasL Associated with Ascites-derived Microvesicles Is Bioactive. We sought to determine whether the hyperglycosylation of the ascites-derived FasL had an effect on its function. For this, Jurkat cells were treated with the glycosylated form of secreted FasL isolated from different patients, and cell viability was determined using the CellTiter assay. As shown in Fig. 4*c,* the ~48-kDa FasL, isolated from the ascites of two patients, had similar properties to the 37-kDa secreted FasL, isolated from the ovarian cancer cell culture supernatant, by significantly increasing Jurkat cell death (R127, $71.8 \pm 2.7\%$; R247, $67.3 \pm 7.2\%$; $P < 0.0001$). Furthermore, changes in glycosylation of the ~48-kDa secreted FasL had no impact upon its bioactivity, because both neuraminidase-treated and untreated FasL induced equal levels of Jurkat cell death (Fig. 4*c*). This decrease in Jurkat cell viability after treatment with the ~48-kDa secreted FasL correlated to caspase activation, as shown by the presence of the active forms of caspase-8, caspase-9, and

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caspase-3 (Fig. 4d). Furthermore, this effect of the ~48-kDa form of FasL was blocked by the presence of the pan-caspase inhibitor, Z-VAD-FMK (data not shown). These results indicate that *in vivo*, ovarian tumor cells release, into the abdominal cavity, a heavily glycosylated, fully active form of FasL via membrane microvesicles.

DISCUSSION

The Fas/FasL apoptotic pathway is thought to play an important role in the induction and maintenance of immune privilege, providing a mechanism by which tumor cells thrive. It has been postulated that tumor cells expressing FasL may protect themselves from immune surveillance by inducing Fas-mediated apoptosis in immune cells, potentially harmful to the tumor (2, 3). Recently, this theory has been challenged by *in vivo* data showing that cell surface, membranal FasL may actually promote tumor rejection rather than survival (10, 22, 23). In this study, we report the secretion of a whole, biologically active form of FasL from epithelial ovarian cancer cells via the release of microvesicles. Moreover, through the isolation of microvesicles from malignant ovarian ascites, we have, for the first time, demonstrated the presence of a hyperglycosylated form of FasL *in vivo*. Such release of a secreted form of FasL suggests a mechanism by which tumors might counterattack Fas-bearing immune cells while avoiding the inflammation observed with membranal FasL expression.

Defining the precise role of FasL in both transplantation and tumor biology has proved to be contentious. A number of studies have demonstrated that the tumor cell surface expression of FasL can induce Fas-mediated apoptosis in T cells (13, 19). However, Allison *et al.* (36) found that FasL-expressing islet β cells transplanted into allogeneic animals, rather than being protected were rejected, and that this expression of FasL caused a neutrophilic infiltrate. These observations were subsequently supported by other studies using tumor cells and islet β cells engineered to express FasL (21, 22, 37). Some investigators have also claimed that FasL was undetectable on the surface of cancer cells, such as melanoma cells, although their Western blots demonstrated the presence of the protein (38). They concluded that the hypothesis that FasL may mediate immune privilege and immune escape was wrong (39). When we examined FasL expression in ovarian cancer tissue samples by Western blot analysis, we found high levels of expression. Immunohistochemistry revealed to us that FasL was not localized to the surface membrane, similar to that as described for melanoma cells (38). Careful examination of the intracellular localization of the FasL indicated that the protein was mainly cytoplasmic (see Fig. 1a).

In concurrence with the expression of FasL observed in primary ovarian tumors, ascites-derived epithelial ovarian cancer cells were also found to lack membranal FasL. Instead, these cells expressed large amounts of intracellular FasL in a polarized manner. In T and NK cells, intracellular FasL is reportedly targeted to specialized secretory lysosomes as a result of proline-rich domains within the cytoplasmic domain (33, 40). Although Bossi *et al.* (33) suggests that these immune cells release FasL from the fusion of secretory lysosomes with the plasma membrane, others have reported the association of released FasL with membranal microvesicles (34, 41). Similar to these reports, we have demonstrated that the intracellular form of FasL expressed by epithelial ovarian cancer cells is colocalized with specialized secretory lysosomes. Mincheva-Nilsson's group (42) has demonstrated intracellular FasL localized to microvesicles, further contained within the cytolytic granules of NK cells. We, therefore, propose that the release of FasL-containing microvesicles occurs after the fusion of secretory lysosomes with the plasma membrane of the ovarian cancer cells. In further support of this hypothesis, other proteins, such as interleukin 1β, which lack classical secretory sequences, are also released after cell activation, in association with membrane vesicles (43).

Constitutive membrane microvesicle shedding is a common feature among proliferating and cancerous cells (44). Here, we have demonstrated that ascites-derived epithelial ovarian cancer cells secrete whole FasL through the release of microvesicles. A recent study has reported the secretion of FasL from melanoma cells via microvesicles (45). However, in contrast to the report by Andreola *et al.* (45), as well as other studies (34, 41), the microvesicles described here lacked surface expression of FasL. Instead, the microvesicle-associated FasL isolated from ovarian cancer cells was functional when the integrity of the vesicle membrane was disrupted, analogous to that reported for microvesicle-associated interleukin 1β (43). How vesicle disruption occurs *in vivo* is presently unclear, although it is possible that changes in the microenvironment, such as pH, may play a role. Interestingly, in some of our experiments we found that the intact microvesicles promoted Jurkat cell proliferation rather than cell death. This may be a result of vesicles expressing either growth factors or stimulatory molecules on their surface. However, once liberated from the microvesicles, the secreted FasL was able to induce Jurkat cell death. Flow cytometric studies confirmed this cell death to be a result of the induction of apoptosis. Jurkat cells, under normal conditions express some spontaneous apoptosis. Despite this, treatment with the secreted FasL markedly increased Jurkat cell apoptosis through activation of the caspase cascade. These results suggested that the extracellular, Fas-binding domain of the secreted FasL was contained within the microvesicle, and once released, the secreted FasL could elicit its bioactivity. The lack of FasL on the vesicle surface suggested that within epithelial ovarian cancer cells these FasL-containing microvesicles may be further encapsulated by specialized secretory lysosomes, which can fuse with the plasma membrane of the cell (42, 46). Another significant difference between our study and that of Andreola *et al.* (45) is that we compared the malignant cell to its normal counterpart. Both epithelial ovarian cancer cells and normal OSE cells cultured *in vitro* express FasL (37 kDa). However, unlike the cancerous cells, normal OSE cells do not secrete FasL. This highlights the possible role for secreted FasL in tumor cell immune protection and survival.

To determine whether our observations of FasL secretion by epithelial ovarian cancer cells were not an *in vitro* phenomenon, microvesicles were isolated directly from ascitic fluid obtained from ovarian cancer patients. We found that these ascites-derived microvesicles also contain FasL, confirming the secretion of FasL *in vivo*. In support of our observations, Ginestra *et al.* (47) had previously demonstrated the presence of MMP-2 and MMP-9 localized in microvesicles isolated from ovarian cancer fluids. Moreover, Taylor *et al.* (17) have recently identified circulating tumor-derived membrane fragments containing FasL (41 and 26 kDa), MMP-2, and MMP-9 in the serum of patients with ovarian cancer. Interestingly, we found FasL isolated from malignant ovarian ascites to be abnormally glycosylated, an observation commonly linked with proteins in malignancies (35). Human FasL contains three potential sites for *N*-linked glycosylation within the extracellular domain. These sites are necessary for, and influence, expression levels but have no impact upon receptor binding or biological activity (7). In support of this, the heavily glycosylated form of secreted FasL (~48 kDa) that was isolated from ascites behaved similarly to the 37-kDa secreted FasL obtained from the ovarian cancer cell cultures. Furthermore, removal of the terminal sialic acid had no effect upon apoptotic efficacy. The variation in levels of glycosylation between the secreted FasL *in vitro* and *in vivo* may be a consequence of the microenvironment of the ovarian cancer cells. The role of the additional glycosylation in the ascites-derived secreted FasL may be to facilitate protein solubility

(48), self-association (49), or to protect the FasL molecule from degradation, particularly if secretion occurs via specialized lysosomes (40) or in microvesicles also expressing MMPs (17, 47).

We have identified a fully bioactive secreted form of FasL, associated with a secretory pathway and constitutively released via microvesicles from epithelial ovarian cancer cells, into the ascitic fluid of ovarian cancer patients. FasL secretion may provide an active process whereby tumor cells can establish immune protection, enhancing their survival through the elimination of tumor-specific immune cells. Such secretion of FasL avoids the cell-cell contact normally associated with Fas-mediated apoptosis and the antitumor inflammatory response that appears to occur when membranal FasL is expressed on the tumor cell surface (22). In agreement with studies using noncleavable mutants of FasL (50, 51), we propose that the membranal form of FasL in cancer cells is fully capable of inducing a neutrophilic response. On the other hand, the secreted form, after release via microvesicles, may initiate cell death in apoptosis-sensitive immune cells. Because epithelial ovarian cancer cells are resistant to Fas-mediated apoptosis (52–54), untimely liberation of the encapsulated FasL will fail to trigger tumor cell suicide or fratricide. Therefore, microvesicle-mediated secretion of FasL may provide a mechanism by which tumor cells establish a state of immune privilege for themselves by inducing apoptosis in Fas-bearing immune cells away from the primary tumor site, thus facilitating their escape from immune surveillance.

ACKNOWLEDGMENTS

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Epithelial Ovarian Cancer Cells Secrete Functional Fas Ligand

Vikki M. Abrahams, Shawn L. Straszewski, Marijke Kamsteeg, et al.

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