T-Cell Apoptosis and Suppression of T-Cell Receptor/CD3-ζ by Fas Ligand-Containing Membrane Vesicles Shed from Ovarian Tumors

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Abstract

Purpose: The accumulation of shed plasma membrane vesicles in the peripheral circulation is unique to cancer. Because these membrane fragments (MFs) express biologically active components, such as Fas ligand (FasL), the objective of this study was to define the link between the presence of shed membrane vesicles, apoptosis, and suppression of T-cell receptor/CD3-ζ expression in T lymphocytes of patients with ovarian cancer.

Experimental Design: MF shedding was measured chromatographically in sera from women with ovarian cancer (n = 11) and, as controls, non-cancer-bearing females (n = 9) and women with benign ovarian disease (n = 4). FasL associated with these shed fragments was assayed by Western immunoblots, whereas HLA class I expression was defined by slot-blotting. The effect of shed MFs on CD3-ζ expression was evaluated using a T-cell bioassay, and apoptosis of circulating T cells was measured by a cell-death ELISA and electrophoretic analysis of caspase-3.

Results: MFs were undetectable in control sera, and their levels were significantly elevated in sera from women with ovarian cancer. These tumor-derived MFs expressed 41-kDa FasL and HLA class I antigens. In co-incubation experiments, dose-dependent suppression of T-cell receptor/CD3-ζ expression by MFs was observed. Decreases in ζ expression correlated with the level of FasL in MFs but not with the level of HLA. The suppression of CD3-ζ by MFs appeared to be linked to the induction of apoptosis and caspase-3 within T cells.

Conclusion: Our results suggest that FasL associated with tumor-derived MFs is responsible for apoptosis of T lymphocytes and a concomitant loss of ζ-chain expression in patients with ovarian carcinoma.

Introduction

Patients with advanced malignancies, including ovarian carcinoma, exhibit progressively impaired immune responses, suggesting that their tumor cells have developed mechanisms to subvert the immune system and suppress antitumor immune reactivity (1). The impairments are often subtle, as reflected by variably decreased, but not absent, functions of lymphocytes. Recent studies have demonstrated that alterations in expression and function of the TcRζ-ζ-associated signal transducing ζ chain are responsible for deficient immune responsiveness of T cells in patients with cancer, including ovarian cancer (2, 3). Although a transient decrease in expression of CD3-ζ normally accompanies antigenic stimulation, the persistent loss of ζ in tumor-infiltrating lymphocytes, for example, has been correlated with reduced proliferative responses and reduced cytokine production by these cells (3, 4). Importantly, decreased expression or absence of ζ chain in the T lymphocytes of cancer patients is biologically significant because it correlates with poorer prognosis and shorter 5-year survival (5).

Investigations into the molecular mechanisms responsible for reduced ζ expression in T cells of patients with cancer have identified several alternatives (reviewed in Ref. 6). One of the possibilities is that the loss of CD3-ζ and CD3-ζ expression results from apoptosis of T lymphocytes mediated by interactions with FasL-expressing tumor cells (7). In vitro studies of the mechanism of ζ-chain loss and in situ observations in human tumors demonstrated that the level of T-cell ζ expression was diminished by direct contact between FasL-positive tumor cells and Fas-negative lymphocytes (7, 8). The susceptibility of T lymphocytes to Fas-mediated apoptosis appeared to be dependent on cellular activation in that neither apoptosis nor loss of ζ chain expression was observed in resting T lymphocytes (9). This finding appears to be in agreement with reports by Shores et al. (10), who investigated activation-induced cell death.

The Fas/FasL system is an important mediator of apoptosis in the immune system, whereby interactions with cells expressing cell-surface FasL induce apoptosis within Fas+ lymphocytes. FasL is a 40-kDa type II transmembrane protein, of the tumor necrosis factor family (11). Co-expression of Fas and FasL by activated T cells leads to activation-induced cell death (12). The expression of FasL in normal tissues is limited to
activated T lymphocytes (13), natural killer cells (14), and to specific immunoprivileged tissues, such as the eye (15), testis (16), and placenta (17, 18). Recent evidence indicates that FasL is expressed by many human tumors, including melanoma, colon, lung, and oral carcinomas as well as ovarian carcinomas (19). This tumor-associated FasL has been postulated to trigger apoptosis of activated Fas+ lymphocytes, providing tumors with a survival advantage (20).

One general characteristic of tumors is their ability to release or shed intact, vesicular portions of the plasma membrane (termed MFs), which was described by us several years ago (21). The precise mechanisms of shedding remain unclear; however, shedding is an energy requiring phenomenon, modulated by extracellular signals. The rate of shedding is significantly increased in most neoplastic cells and occurs continuously (22). Increased shedding of MFs and their accumulation appear to be important in the malignant transformation process. Although extracellular shedding of plasma membrane-derived vesicles might occur in several types of cells, including non-neoplastic cells under specific physiological conditions, the accumulation of these shed MFs is rarely observed. In contrast, MFs shed by tumor cells accumulate in patients’ sera. Furthermore, tumor-derived MFs appear to represent selected domains (micromaps) of the plasma membrane in each parent cell (22).

In this report, we describe the presence of a circulating factor in sera of patients with ovarian cancer that induces apoptosis and suppresses expression of the ζ chain in T lymphocytes. At the same time, we demonstrate that sera contain abundant MFs enriched in FasL. The presence and levels of FasL+ MFs are correlated with T-cell apoptosis and suppression of ζ expression in T cells.

Materials and Methods

Patient-Derived Materials. Blood specimens were obtained from women diagnosed with stage IIIc papillary serous adenocarcinoma of the ovary (n = 11) and from women with benign ovarian adenoma (n = 4) at the Gynecologic Oncology Clinic of the Department of Obstetrics and Gynecology of the University of Louisville School of Medicine. Blood samples were also obtained from age-matched normal female volunteers (n = 9) at the Gynecology Clinic of the Department of Obstetrics and Gynecology of the University of Louisville School of Medicine. The University Human Studies Committee of the University of Louisville approved this study, and informed consent was obtained from each patient. Blood samples were allowed to clot and then were centrifuged at 400 × g for 10 min to sediment the cells and clot. The serum was removed, aliquoted, and stored at −70°C until analysis. For all of the samples studied, the mean (±SD) age of the non-tumor-bearing female volunteers was 57 ± 4.1 years, compared with 61.5 ± 4.5 years for women with ovarian cancer.

Isolation of Circulating MFs and Association with FasL and MHC Class I Antigens. MFs were isolated from sera by a two-step procedure developed in our laboratory (23). Initially, 500 µL of serum was applied to a Bio-Gel A50m column (1.5 × 45 cm) equilibrated with PBS. Fractions (1 ml) were collected, and the elution was monitored by the absorbance at 280 nm. The void peak, containing material >50 × 106 Da was then centrifuged at 100,000 × g for 1 h at 4°C. The pelleted MFs were resuspended in PBS, and the quantity of protein was determined by the Bradford microassay method (Bio-Rad Laboratories, Hercules, CA) with BSA as a standard. The distributions of proteins were examined by SDS-PAGE on a 12.5% acrylamide gel (24), followed by silver staining (Bio-Rad Laboratories).

To define the level and form of FasL expression associated with shed MFs, we applied 25 µg of protein from each fragment isolate to each lane to a 12.5% SDS-PAGE gel. The proteins were separated electrophoretically by the method of Laemmli (24) and analyzed by Western immunoblot as described previously (25), with probing overnight at 4°C with rabbit anti-FasL (1 µg/ml; Calbiochem, San Diego, CA) as the primary antibody and peroxidase-conjugated antirabbit immunoglobulin as the secondary antibody. The bound immune complexes were visualized by enhanced chemiluminescence (ECL; Amersham Life Sciences, Arlington Heights, IL) and quantitated by densitometry (Un-Scan-it Software; Silk Scientific Corp., Orem, UT).

To identify the presence HLA class I antigens associated with shed MFs, we applied 25 µg of protein from each fragment isolated to nitrocellulose membrane by use of a vacuum-slot blot apparatus (Bio-Rad Laboratories). The membranes were blocked with 5% nonfat dried milk and probed overnight at 4°C with mouse anti-HLA-class I (W6/32, 1 µg/ml; eBioscience, San Diego, CA) as the primary antibody and peroxidase-conjugated antimouse immunoglobulin as the secondary antibody. The bound immune complexes were visualized by enhanced chemiluminescence (ECL) and quantitated by densitometry (Un-Scan-it Software).

Expression of TcR/CD3-ζ Protein and mRNA. Jurkat E-61 cells (American Type Culture Collection, Rockville, MD), a human T-cell lymphoma with a functional TcR/CD3 complex capable of synthesizing interleukin-2, were used as an in vitro assay for lymphocyte modulation by sera-derived MFs. Jurkat cells were grown in RPMI 1640 supplemented with 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 200 mM L-glutamate, 100 µg/ml streptomycin, and 100 IU/ml penicillin in a humidified 5% CO2 chamber at 37°C. Cell viability was evaluated by trypan blue exclusion. All cultures used for this study were >95% viable. Viable Jurkat cells (106 cells/ml) were incubated in a medium supplemented with 400 µg/ml isolated MFs for 4 days and were compared with unexposed Jurkat cells or Jurkat cells exposed to the analogous chromatographic fractions from control sera. After 4 days, the cells were centrifuged, and the cell pellet was washed and used for either protein or mRNA analysis.

To assess CD3-ζ protein, the cell pellet was lysed with 50 mM HEPES (pH 7.2), 150 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate, 2.5% Triton X-100, 200 µg/ml trypsin/chymotrypsin inhibitor, 200 µg/ml chymostatin, and 2 mM phenylmethylsulfonyl fluoride. The cell lysate was assayed for protein by the Bio-Rad protein assay. The modulation of CD3-ζ was analyzed by Western immunoblot using a 15% SDS-PAGE gel, as described above, with mouse monoclonal anti-CD3-ζ antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as the primary antibody. As an additional loading control, blots were also probed with rabbit polyclonal anti-β-actin (Santa Cruz Biotechnology).

To define the effect on the mRNA encoding CD3-ζ, we extracted total cellular RNA from the treated Jurkat cell pellet and performed reverse transcription-PCR as described previ-
in the same subset of samples. After sera-derived MFs (400 μg/ml) were incubated with viable Jurkat cells (10⁶ cells/ml) for 24 h, cell lysate samples were applied to a 12.5% SDS-PAGE gel. The proteins were separated electrophoretically and analyzed by Western immunoblotting using mouse anti-caspase-3 monoclonal antibodies (Santa Cruz Biotechnology).

**Statistical Analysis.** TcR/CD3-ζ expression densitometry was standardized to a control lane included on each gel and compared by the Kruskal–Wallis test. In the remainder of experimental data, all relative absorbance determinations were performed at least twice, and the mean ± SE for each sample was calculated. Comparisons between noncontrol and cancer patient populations were performed by the Kruskal–Wallis test. Tests with P < 0.05 were considered statistically significant. Correlations between expression of FasL and suppression of CD3-ζ and between loss of CD3-ζ and induction of apoptosis were calculated by linear regression analysis. Statistical analysis was performed using Stata version 6.0 (College Station, TX).

**Results**

**Presence of MFs in Sera of Ovarian Cancer Patients.** Sera obtained from normal volunteers did not contain detectable MFs. On the basis of high-molecular-weight exclusion chromatography, MFs were isolated from all serum samples obtained from women with ovarian cancer (Fig. 1A). As shown in Fig. 1B, the MFs consisted of proteins ranging in molecular weight from Mᵣ 10,000 to 205,000. These MFs were identified previously as being positive for placental-type alkaline phosphatase, confirming their tumor origin (data not shown). These MFs were demonstrated in the present study to express class I MHC antigens (Fig. 1C). When standardized to protein, the expression of class I antigens was 40,711 ± 12,769 pixels for MFs from ovarian cancer patients.
was not observed (\[H9256\] were present, a similar correlation with suppression of CD3-\[H9256\] of 0.906 was obtained. However, although MHC class I antigens fractions were analyzed for the presence of FasL and were found to be negative (data not shown). Although FasL expression was observed on shed MFs present in all patient sera, significant control sera failed to exhibit shed MFs, the analogous column form described previously as soluble FasL (27). Although the circulating shed MFs revealed the presence of FasL (Fig. 2), appearing as the intact 41-kDa form and not the 27-kDa cleaved form indicated.

**Detection of FasL.** Western immunoblot analysis of the circulating shed MFs revealed the presence of FasL. (Fig. 2), appearing as the intact 41-kDa form and not the 27-kDa cleaved form described previously as soluble FasL (27). Although the control sera failed to exhibit shed MFs, the analogous column fractions were analyzed for the presence of FasL and were found to be negative (data not shown). Although FasL expression was observed on shed MFs present in all patient sera, significant differences in the levels of FasL expression were observed, with a 3.2-fold range of expression.

**Suppression of TcR/CD3-\[H9256\].** To determine whether the MFs isolated from the sera of ovarian cancer patients could induce the loss of CD3-\[H9256\]-chain protein in T cells, Jurkat cells were incubated for 4 days in medium containing 400 \[H9256\]g/ml MFs or the analogous fractions from control sera, and the expression of \[H9256\] chain was determined by Western immunoblot. As shown in Fig. 3, \[H9256\] expression was variably decreased or absent in Jurkat cells incubated with the MFs. When the level of CD3-\[H9256\] suppression by 400 \[H9256\]g/ml MFs (defined as a percentage of the expression in controls) was compared with the expression of FasL associated with the MFs (defined as relative absorbance in pixels) obtained from patient sera, a correlation coefficient ($r^2$) of 0.906 was obtained. However, although MHC class I antigens were present, a similar correlation with suppression of CD3-\[H9256\] was not observed ($r^2 = 0.146$). We then showed that this suppression of CD3-\[H9256\] by MFs is dose dependent by incubating Jurkat cells with MF at 25, 100, and 400 \[H9256\]g/ml for 4 days. The suppression of CD3-\[H9256\] protein at each concentration paralleled the expression of FasL in MF (Fig. 4). In these experiments, the extent of MF-mediated down-modulation of CD3-\[H9256\] was measured after a 4-day incubation. To demonstrate the time-dependent down-regulation of \[H9256\] in Jurkat cells, we co-incubated MFs with these cells for 24, 72, and 96 h (Fig. 5). The change in CD3-\[H9256\] expression induced by exposure to MF over this time period was linear ($r = 0.92$).

The loss of CD3-\[H9256\]-chain expression observed in T cells on incubation with MFs could result from either increased degradation of the \[H9256\] protein or its reduced synthesis. To evaluate which of these mechanisms operated in the case of MFs, we performed semi-quantitative reverse transcription-PCR analysis of CD3-\[H9256\] chain mRNA in Jurkat cells incubated in the presence of MFs. The level of expression of this mRNA was analyzed after 25 cycles of PCR amplification, with \[H9256\]-actin levels used as a control. As shown in Fig. 6, T cells treated with MFs derived from patients' sera showed suppression of \[H9256\]-specific mRNA that correlated with the loss of \[H9256\]-chain protein.

**Induction of Apoptosis.** Because it has been hypothesized that induction of T-cell apoptosis by FasL is linked to the loss or decrease of TcR/CD3-\[H9256\]-expression, we also measured the capability of FasL-expressing MFs to induce apoptosis by quantitating the cytoplasmic histone-bound DNA in Jurkat cells, using a cell-death ELISA (Fig. 7). MFs isolated from the sera of ovarian cancer patients induced significant apoptosis ($P < 0.0001$) compared with untreated Jurkat cells, whereas addition of the analogous control fraction failed to induce apoptosis.

**MF Modulation of Apoptosis Regulators and Effectors.** The induction of Fas/FasL-dependent apoptosis is associated with activation of specific caspases, primarily caspase-3. The levels and activation of caspase-3 present in T cells after exposure to the circulating tumor-derived MFs was identified by Western immunoblot (Fig. 8). Within 24 h of Jurkat cell exposure to MFs, caspase-3 expression was significantly elevated ($P < 0.001$) compared with expression in untreated T cells. The increase in total caspase-3 resulted from both induction of the proenzyme (2.02-fold increase versus control) and its activation (1.89-fold increase).

**Discussion**

T lymphocytes isolated from peripheral blood mononuclear cells of women with ovarian cancer have previously been dem-
onstrated to have reduced or absent expression of CD3-\(\zeta\) chain (3). The \(\zeta\) chain is responsible for transducing activation signals from the antigen-binding TcR to the T-cell nucleus. The absence of \(\zeta\) chain alters the expression and functions of CD3/TcR complex (2). T-cells with suppressed \(\zeta\) chain have been demonstrated to exhibit diminished proliferation and production of cytokines (4, 5, 8). We have previously shown that the serum-mediated suppression of CD3-\(\zeta\) in T lymphocytes appears to be the consequence of a specific suppression or loss of \(\zeta\) (as well as a limited number of other proteins) and not nonspecific suppression of total protein synthesis within the T cell: the kinase \(lck\) is unaffected, whereas the \(\zeta\) chain is almost completely suppressed (28). T lymphocytes isolated from women who have cancer have been demonstrated to express diminished amounts of or no CD3-\(\zeta\) chain, and we demonstrated that factors present in sera of these women are able to down-modulate the expression of CD3-\(\zeta\) chain (19). The loss of \(\zeta\) chain alters the expression of the CD3/TcR complex and interferes with lymphocyte functions.

Our previous studies have demonstrated the unique association of MF shedding with cancer (22). The shed MFs found in sera of patients with cancer possess protein components expressed on the plasma membranes of the tumor (23). These MFs can also be demonstrated in the culture media of primary tumor cultures from these patients. In the present report, we

Fig. 4 A. Western immunoblots indicating the expression of CD3-\(\zeta\) chain by Jurkat cells after a 4-day incubation with membrane MFs (at 25, 100, and 400 \(\mu\)g/ml) obtained from ovarian cancer patients 2, 4, 6, and 10 and the analogous chromatographic fraction from normal female volunteers (Lane C). B. densitometric quantitation of CD3-\(\zeta\) expression by treated Jurkat cells.

Fig. 5 A. Western immunoblots indicating the expression of CD3-\(\zeta\) chain by Jurkat cells after incubation with MFs (at 100 \(\mu\)g/ml) obtained from ovarian cancer patients 1, 2, and 3 for 24, 48, and 72 h. B. densitometric quantitation of CD3-\(\zeta\) expression by treated Jurkat cells.

Fig. 6 Expression of mRNA for the CD3-\(\zeta\) chain and \(\beta\)-actin in Jurkat cells after a 4-day incubation with membrane fragments (at 400 \(\mu\)g/ml) isolated from patients 1 and 2. The control column corresponds to T cells incubated with the analogous fraction from control serum. Bars, SE.
demonstrated that FasL, MHC class I antigens, and placental-type alkaline phosphatase are expressed on shed MFs isolated from sera of patients with ovarian cancer. In contrast to previous reports, which detected soluble 27-kDa FasL in the sera of cancer patients (27), these shed MFs express the intact 41-kDa membrane-associated FasL (Fig. 2). Studies by Rabinowich et al. (7) demonstrated that co-incubation of T cells with FasL-expressing ovarian tumor cells led to suppression of CD3- 
expression. Our present findings show for the first time that CD3- 
expression can be mediated by circulating, shed tumor MFs (Fig. 3). In our hands, expression of FasL on MFs correlated with suppression of CD3- 
mediated by these MFs. Although studies by Maccalli et al. (29) suggested that circulating shed class I antigens could mediate suppression of CD3- 
or results failed to identify a correlation. In the study by Maccalli et al. (29), treatment of tumor culture supernatants with antibodies against MHC class I antigens led to a loss of a suppression, leading to their conclusion that HLA class I antigens mediated the suppression. However, as we have shown, because shed tumor-derived MFs express class I antigens, removal of class I antigens by immunosorption will remove both soluble individual class I antigens and class I antigens associated with MFs. Thus, their observed reduction in CD3- 
expression after adsorption of class I antigens may actually be the result of MF removal.

We further demonstrated that these MFs induced dose- and time-dependent suppression of CD3- 
expression (Figs. 4 and 5, respectively). This suppression of TcR/CD3- 
expression at the mRNA level in T cells treated with the MFs derived from sera of cancer patients down-regulated mRNA for 
and correlated with the loss of CD3- 
chain protein (Fig. 6). This finding seems to contradict previous results suggesting that the loss of TcR/CD3- 
was attributable to increased degradation and not decreased synthesis (5). However, it should be noted that this previous conclusion was made based on results obtained with in vivo-derived lymphocytes that had been exposed to multiple factors within the host, including tumor-derived proteolytic enzymes and factors capable of activating degradative pathways.

The FasL-containing MFs were also analyzed for their ability to induce apoptosis because a link between CD3- 
expression and decreased CD3- 
expression has been proposed (30). In vitro exposure of T cells to MFs induced apoptosis (Fig. 7) as well as increased expression and activation of caspase-3 (Fig. 8). Analyses of the effect of T-cell exposure to shed MFs containing FasL demonstrated that apoptosis associated with CD3- 
loss was FasL dependent.

Some investigators have previously demonstrated specific suppression of the Th1 lymphocyte populations early in cancer development and progression (31, 32). Because circulating MFs expressing FasL would appear to be nonspecific and thus able to affect both Th1 and Th2 populations, this pathway might not be expected to account for the altered Th1/Th2 distributions observed in cancer. However, Th1 and Th2 subsets have been demonstrated to be differentially susceptible of FasL-mediated apoptosis, although their cell surface expression of CD95 (Fas) appears similar (33). The mechanism of the differential sensitivity of Th1 and Th2 cells to FasL-induced apoptosis appears to reside in the differentially regulated Fas-associated death domain/caspase-8 pathway. In the apoptosis-resistant Th2 cells, there is an incomplete processing of caspase-8 at the death-inducing signaling complex. Activation of phosphatidylinositol 3’-kinase has been demonstrated to block caspase-8 cleavage to its active form at the death-inducing signaling complex, preventing induction of apoptosis (34).

The loss of CD3- 
in peripheral-blood T cells has been demonstrated to correlate with poor prognosis in several tumor types (35). This study indicated that the presence of shed MFs in the peripheral circulation might be responsible for the loss of CD3- 
via the FasL-mediated apoptotic pathway. Similar multivesicular bodies, containing FasL and able to trigger Fas-dependent apoptosis of lymphoid cells, were recently reported to be produced by melanoma (36). Identification in the patients’ circulation of MF-containing FasL, which induced Fas-depend-
ent apoptosis of T lymphocytes, confirms their biological signif-
ificance. These MFs may also serve as a marker of the loss of
T-cell signaling molecules and subsequent absence of T-cell
activation. By defining the mechanism through which ovarian
cancers modulate ζ-chain levels, it might be ultimately possible
to control and perhaps reverse suppressive influences of the
tumor microenvironment. Thus, a better understanding the mo-
olecular mechanisms of tumor-mediated T-cell dysfunction is
likely to have an impact on future developments in the preven-
tion, diagnosis, and therapy of cancer.

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