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Research Article

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Lymphocyte Apoptosis Induced by Fas Ligand–expressing Ovarian Carcinoma Cells

Implications for Altered Expression of T Cell Receptor in Tumor-associated Lymphocytes

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Abstract

We have recently reported that tumor-associated lymphocytes obtained from ascitic fluids of women with ovarian carcinoma (OvCA) demonstrate a marked decrease in expression of cytoplasmic CD3-ζ and surface CD3-ϵ chains, which is associated with altered function of T cell receptor (TcR). We now demonstrate that OvCAs in situ and in culture express functional Fas ligand (FasL), capable of triggering an intrinsic cell death program in Fas-expressing T cells. The possibility of a relationship between cell death and altered expression of TcR was examined. The data indicate that alterations in expression of CD3-ζ and CD3-ϵ chains in T cells coincubated with OvCA are related to tumor-induced apoptosis, as the addition of pan-caspase inhibitors, DEVD-cho or YVAD-cho, prevents both the in vitro induction of T cell death by OvCA cells and the changes in the level of expression of CD3-ζ and CD3-ϵ chains. In the presence of Fas–Fc fusion protein, but not Fc-control protein, the loss in expression of CD3-ζ and CD3-ϵ chains induced in T cells by FasL$^+$ OvCA cells was prevented. These results suggest that the loss in expression of CD3-ζ and CD3-ϵ chains in T lymphocytes interacting with OvCA cells is associated with apoptosis mediated by FasL-expressing tumor cells. (*J. Clin. Invest.* 1998. 101:2579–2588.) Key words: caspases • CD3-ζ • programmed cell death • protease inhibitors • signal transduction

Introduction

Patients with advanced malignancies have progressively impaired immune responses, indicating that tumor cells have developed mechanisms to subvert the immune system. Various mechanisms have been shown to be involved in the tumor escape from the immune response, including soluble tumor–derived inhibitory factors (1), aberrations in antigen presentation by the downregulation of the major histocompatibility complex expression on tumor cells (2), or aberrant signal transduction in lymphocytes (3). Recent studies from ours, as well as other laboratories, have suggested that alterations in expression and function of signal transducing molecules associated with TcR are responsible for deficient immune responsiveness of T cells in several types of malignancies (4–8). We have shown that tumor-associated lymphocytes (TAL)[1] obtained from ascitic fluids of women with advanced ovarian carcinoma (OvCA) demonstrate a marked decrease in expression of cytoplasmic CD3-ζ and surface CD3-ϵ chains, which are essential for T cell receptor (TcR) signaling (6, 9). The reduced expression of ζ and ϵ chains in TAL-T cells was associated with altered TcR function, as demonstrated by decreased Ca^{2+} flux and reduced kinase activity associated with cross-linked and immunoprecipitated TcR (10).

The mechanisms responsible for the decreased expression of certain signaling proteins in T lymphocytes of patients with cancer are unknown. It was reported that splenic macrophages from tumor-bearing mice or normal spleen macrophages activated with zymosan A and LPS were able to induce size-related changes in CD3-ζ, -γ, -δ, and -ϵ, by contact-dependent interactions (11). Another study has demonstrated that hydrogen peroxide secreted by tumor-derived macrophages downmodulated expression of the ζ chain (12). However, the molecular mechanisms involved in macrophage-mediated loss in expression of CD3-ζ and CD3-ϵ chains have not yet been elucidated.

The identification of Fas ligand (FasL) expression on cells in immuneprivileged sites, such as testis (13) and the anterior chamber of the eye (14), has suggested that FasL may be important in tolerance induction and immunosuppression. Indeed, it has been reported that when inflammatory cells in the anterior chamber of the eye undergo Fas-mediated apoptosis after infection with herpes simplex virus (HSV-1), a systemic tolerance to this antigen develops (14). Recent studies from several laboratories have demonstrated expression of FasL on human tumors, including colon (15), hepatocellular carcinoma (16), melanoma (17), and lung carcinoma (18), which may be involved in induction of apoptosis in Fas-sensitive T cells.

In the present study, we show that OvCAs in situ and in culture also express functional FasL, which is capable of triggering an intrinsic cell death program in activated T cells. Apoptosis of T lymphocytes induced by OvCA cells is Fas-mediated and involves activation of caspase-1– and 3–like proteases. Our in vitro experiments indicate that a direct link might exist between Fas-mediated apoptosis of activated T lymphocytes and the loss in expression of CD3-ζ and CD3-ϵ. Thus, the data suggest that two of the mechanisms responsible for immunosuppression in tumor-bearing hosts, namely, altered expression of

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1. *Abbreviations used in this paper:* FasL, Fas ligand; ICE, interleukin-1β-converting enzyme; OvCA, ovarian carcinoma; TAL, tumor-associated lymphocytes; TdT, terminal deoxynucleotide transferase; TUNEL, TdT-mediated dUTP nick-end labeling.

signal-transducing molecules and lymphocyte apoptosis, are manifestations of the same tumor-induced process.

Methods

Patients. OvCA tissue, ascitic fluids, and peripheral blood were collected from 11 patients with ovarian carcinoma admitted to Magee Women's Hospital, University of Pittsburgh Medical Center (Pittsburgh, PA) or Louisiana State University Hospital (Shreveport, LA). The patients ranged in age from 42 to 81 yr, and the ovary was the primary site of malignancy for all patients. All the patients had advanced adenocarcinoma (stages III and IV) with prominent ascites. Nine of the patients were untreated, and two patients had received chemotherapy in the past but were end-stage at the time of specimen collection and were not receiving any treatment. The study was approved by the Institutional Review Boards at the University of Pittsburgh and Louisiana State University Medical Centers. Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque gradient centrifugation from heparinized blood obtained from patients or normal donors. To select for PBL, monocytes were removed on nylon wool columns (6).

Isolation of ovarian TAL, TAL-T, or tumor cells. Ascitic fluids (850–1300 ml) were obtained from 11 patients with ovarian carcinoma. Ascitic fluid cells were washed twice in RPMI 1640, placed on Ficoll-Hypaque discontinuous density gradients, and centrifuged to harvest TAL and tumor cells as described by us earlier (6). The TAL preparations were used for further purification of T cells, and fractions enriched in tumor cells were used for purification of tumor cells. To select for T lymphocytes, TAL were incubated in the presence of anti-CD14, anti-CD16, and anti-CD19 mAbs (10 μg/ml, DAKO Corp., Carpinteria, CA) for 30 min at 0°C. To negatively purified OvCA cells, the tumor-enriched fraction of the discontinuous Ficoll-Hypaque gradient was incubated in the presence of anti-CD3, anti-CD5, anti-CD14, anti-CD16, anti-CD19, and anti-CD56 mAbs for 30 min at 0°C. The cells were then washed twice and incubated with magnetic beads coated with goat anti–mouse immunoglobulins (GAMIg, 1 cell:30 beads, PerSeptive Diagnostics, Cambridge, MA) for 30 min at 0°C. After each of two successive incubations with magnetic beads, a magnet was used to separate beads with attached cells. Two-color flow cytometry was used to determine the purity of the negatively selected T or OvCA cell populations. The negatively selected tumor cells were 99% $CD3^-CD14^-CD16^-CD19^-CD56^-$.

Reverse-transcription polymerase chain reaction (RT-PCR) for Fas and FasL. Cellular RNA was extracted from 3×10^6 to 6×10^6 OvCA cells purified by negative selection from ascitic fluids. RNA extraction was performed by acid-guanidium phenol-chloroform method (TRI REAGENT, Molecular Research Center, Cincinnati, OH), and RT-PCR was performed using RNA PCR kit (Perkin Elmer, Norwalk, CT). Cellular RNA (100 ng) was reverse transcribed into cDNA in a reaction mixture containing 5 mM $MgCl_2$, 1 mM each of deoxynucleotide triphosphate (dNTP), 1 U RNase inhibitor, and 2.5 U reverse transcriptase. After incubation at 42°C for 15 min, the reaction was terminated by heating at 95°C for 5 min. PCR was performed on the cDNA using the following sense/antisense primers, respectively: Fas: CAGAACTTGGAAGGCCTGCATC and TCTGTTCTGCTGTGTCTTGGAC; FasL: GGATTGGGCCTGGGGATGTTTCA and TTGTGGCTCAGGGGCAGGTTGTTG (15); β-actin: GGGTCAGAAGGATTCCTATG and GGTCTCAAACATGATCTGGG. The Fas primers span exons 3–6 and, thus, enable amplification of the five splice variants of Fas mRNA identified in normal activated lymphocytes and tumor cell lines that code for soluble forms of Fas (19–21). The PCR reaction buffer (25 μl) consisting of 2 mM $MgCl_2$, 0.5 μM of each primer, 1 μCi [α-^{32}P]dCTP, 5 μl of each RT solution, and 1 U Ampli Taq DNA polymerase was added to each amplification tube. The amplification was performed by denaturing at 94°C for 15 s, annealing at 64°C for 30 s, and DNA chain extension at 72°C for 3 min. This process was repeated for a total of 35 cycles. Aliquots of amplified product (20 μl) were separated on 5% polyacrylamide gels. After electrophoresis, the gels were dried and exposed to BioMax film (Kodak, Rochester, NY) for 1 h.

Flow cytometry analysis. Expression of surface proteins was assessed as previously described (6). Assessment of cytoplasmic CD3-ζ, CD3-ε, or FasL expression by flow cytometry was performed on lymphocytes fixed in 1% paraformaldehyde in PBS for 20 min on ice, and then incubated in permeabilization buffer (1% FCS, 0.1% sodium azide, 0.1% saponin in PBS, pH 7.5) for 30 min on ice (9). The degree of permeabilization was assessed by trypan blue uptake. The cells were then incubated with mouse anti-ζ mAb (TIA-2, Coulter Corp., Miami, FL), anti-ε (clone 452, DAKO), anti-FasL (NOK-2), or IgG isotype control at the same concentration, for 30 min on ice. The cells were washed three times with permeabilization buffer, and then incubated with FITC-GAMIg for 20 min on ice. After three washes in staining buffer, the cells were incubated in the presence of mouse IgG (DAKO, 50 μg/ml) for 20 min on ice, to block binding capability of GAMIg. After the blocking treatment, the cells were stained with CD3-PE or similarly conjugated isotype control. The cells were then fixed with 2% paraformaldehyde and analyzed by flow cytometry.

DNA fragmentation assays. Fas-resistant Jurkat cells were obtained by multiple cycles of treatment of Fas-sensitive Jurkat cells with agonistic anti-Fas Ab (CH-11, 0.2 μg/ml), followed by selection for Fas-positive cells by FACS® sorting. Fas-sensitive and Fas-resistant Jurkat T lymphocytes were cocultured with OvCA cells purified from ascitic fluids and assessed for cell death by the JAM assay (22). DNA labeling of Jurkat target cells was performed by incubation of the cells in the presence of 5 μCi/ml [^{3}H]TdR for 18–24 h at 37°C. OvCA cells were cocultured with [^{3}H]TdR-labeled target cells for 16 h at 37°C at range of 40:1 to 2.5:1 E/T ratio. At the end of the coincubation period, the cells were harvested (Mach IIM, TOMTEC) onto glass fiber filters. The radioactivity of unfragmented DNA, retained on the glass fiber filters was measured by liquid scintillation counting. Specific cell death was calculated, according to the following formula: percentage apoptosis $= 100 \times (S - E)/S$, where S is retained DNA in the absence of effector cells (spontaneous), and E is experimentally retained DNA in the presence of effector cells.

To identify fragmented DNA in Jurkat cells, ConA lymphoblasts, or PBL from OvCA patients, a TdT-mediated dUTP nick-end labeling (TUNEL) assay was performed and assessed by either histochemistry or flow cytometry (Boehringer Mannheim, Indianapolis, IN) (23). The cells (10^6/sample) were washed in PBS and fixed in 2% paraformaldehyde for 30 min at room temperature. After fixation, the cells were washed twice in PBS containing 0.01% BSA, and resuspended in TUNEL reaction mixture containing fluorescein dUTP and terminal deoxynucleotidyl transferase (TdT). Control cells were resuspended in TUNEL reaction mixture containing fluorescein dUTP without TdT. Fluorescein incorporated in DNA strand breaks was detected by flow cytometry. For histochemistry, incorporated fluorescein was detected by sheep antifluorescein Ab, conjugated with horseradish peroxidase. After substrate reaction, stained cells were analyzed under light microscope. To analyze tissue sections for in situ apoptotic DNA fragmentation, frozen tissue sections were fixed by 4% paraformaldehyde, washed with PBS, pretreated with microwave (370 W, 5 × 5 min), blocked with 0.3% hydrogen peroxide, and incubated with the TUNEL mixture (24). TUNEL positive cells were evaluated, and counted with Nikon Labophot fluorescence microscope.

Coculture of T lymphocytes with OvCA cells, rFasL, or anti-Fas Ab. OvCA cells (1.5×10^6/ml) purified from ascitic fluids of OvCA patients were incubated with autologous or allogeneic PBL-T cells, ConA lymphoblasts, or Jurkat cells (0.5×10^6/ml) in either 96- or 24-multiwell tissue culture dishes for 16–24 h. Control lymphocytes were plated with medium alone or in the presence of rFasL (10–100 ng/ml; Alexis, San Diego, CA), anti-Fas Ab (CH-11, IgM 200 ng/ml, Upstate Biotechnology Inc., Lake Placid, NY), or etoposide (VP-16, 20 mM, Sigma Chemical Co., St. Louis, MO). At the end of the incubation period, the cells were aspirated and assayed for viability using trypan

blue, apoptosis by TUNEL, and cytoplasmic and surface expression of CD3-ζ and CD3-ϵ, respectively. Tetra-peptide aldehyde inhibitors of caspases, including Ac-YVAD-cho or Ac-DEVD-cho (500 ng/ml each; Bachem Bioscience, King of Prussia, PA) were added at the start of the coculture. In preliminary experiments, these inhibitors, used at similar range of concentrations, were determined not to have toxic effects on either T or OvCA cells when cultured alone. In experiments using Fas–Fc fusion protein or control Fc protein (10 μg/ml, Immunex, Seattle, WA), OvCA cells were preincubated with these proteins for 1 h at 37°C before the seeding of T cells.

Immunoblot analysis. Proteins were separated by SDS-PAGE using 10% polyacrylamide gels and transferred to PVDF membranes. After blotting with specific Ab, the protein bands were detected by enhanced chemiluminescence (Pierce, Rockford, IL).

Results

Expression of FasL on OvCA cells. Recent studies have suggested that FasL expressed on nonhematopoietic tumors is actively engaged in downregulation of the immune response (15–18). To investigate the role of FasL in immunosuppression of TAL in women with OvCA, we examined its expression in OvCA cells at the gene and protein levels. Expression of mRNA for FasL in OvCA cells, purified by negative selection from ascitic fluids obtained from eight patients with OvCA, was evaluated by RT-PCR. These OvCA cells were found to express mRNA for FasL at a level comparable to that of ConA–T lymphoblasts or Jurkat T cell (Fig. 1). The same cDNA preparations were also used to examine mRNA expression of Fas in these tumor cells. The Fas primers we have used span exons 3–6 and, thus, enable amplification of additional splice variants reported to code for soluble forms of Fas (15). Expression of five soluble forms of Fas (sFas) from splice variants of Fas mRNA lacking the transmembrane exon has been shown previously (20, 21). In all specimens of OvCA cells examined for Fas mRNA expression, two splice variants were detected, whereas only mRNA for the full-length Fas was expressed in ConA–T lymphoblasts or Jurkat T cells studied under identical experimental conditions. These results suggest that OvCA cells, freshly isolated from malignant ascites, contain the genetic information required for expression of both Fas and FasL.

Surface expression of Fas and FasL on OvCA cells or TAL was examined by flow cytometry of cells stained with mouse mAbs specific for Fas (M31; Immunex) or FasL (NOK-2, a gift from S. Nagata, University of Osaka, Osaka, Japan). OvCA cells, purified from ascitic fluids of seven out of nine patients, expressed surface FasL and no or only low levels of surface Fas (Fig. 2). In contrast, several TAL preparations expressed Fas but no FasL.

Because all specimens of OvCA cells tested expressed FasL mRNA, whereas surface expression of FasL protein was detected in only seven out of nine tumor specimens, the intracellular expression of this protein was examined by Western blot analysis of whole cell lysates. As demonstrated in Fig. 3, FasL was present in cell lysates obtained from all specimens of tumor cells purified from ovarian ascites. Similar results were also obtained by flow cytometry of permeabilized OvCA cells stained with anti-FasL Ab (NOK-2). Of note, intracytoplasmic expression of FasL was also detected in the two OvCA specimens that did not demonstrate surface expression by flow cytometry.

Consequences of FasL expression on OvCA cells. The observed expression of FasL on OvCA cells and of Fas on TAL suggests that lymphocytes present at the tumor site might be able to readily activate their death program. As expected, numerous apoptotic lymphocytes were detected by TUNEL assay performed in conjunction with CD3 staining in cryosections of OvCA tissues (Fig. 4). To directly test the ability of

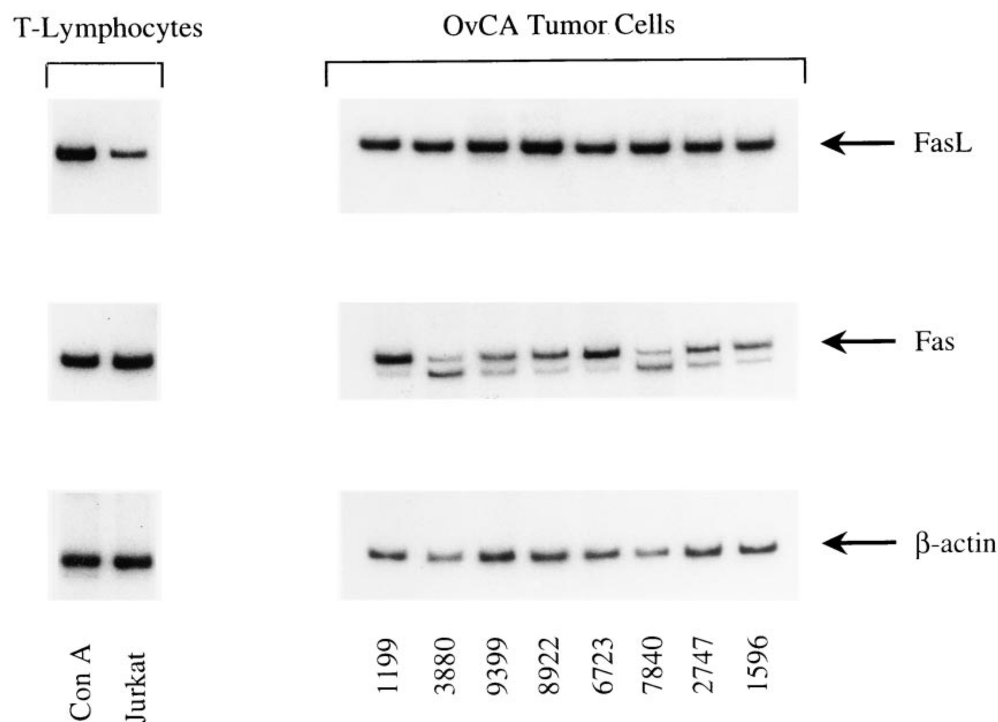


Figure 1. FasL and Fas mRNA expression in OvCA cells purified from ascitic fluids. ConA–activated lymphocytes and Jurkat T cells were used as controls. Expression was analyzed by RT-PCR of equal amounts of RNA. mRNA-specific amplification product bands for FasL (344 bp), Fas (682 bp), and β-actin (237 bp) are shown. The bottom band (619 bp) of Fas, obtained in all OvCA cells tested, represents a mRNA splice variant that encodes a soluble form of Fas.

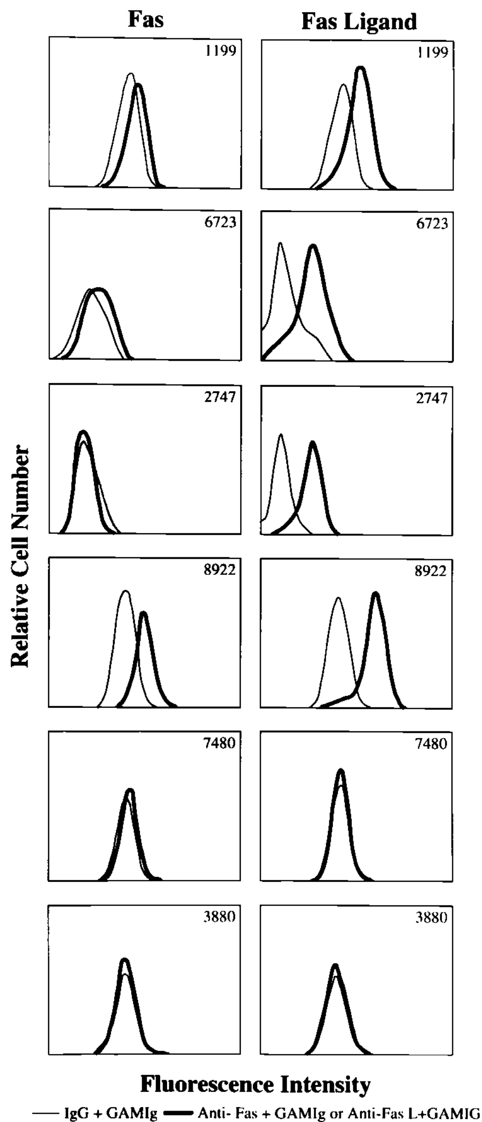


Figure 2. Surface expression of Fas and FasL on OvCA cells. Tumor cells purified by negative selection from ascitic fluids of patients with OvCA were stained with mouse anti–human Fas (M31) or mouse anti-FasL mAbs (NOK-2), followed by staining with a secondary FITC-conjugated goat anti–mouse IgG. Fas and FasL expression was determined by flow cytometry.

Table I. Expression of Surface CD3-ε and Cytoplasmic CD3-ε in PBL-T Cells Coincubated with Autologous OvCA Cells

	Mean flourescence intensity*	
	Surface CD3-ε	Cytoplasmic CD3-ε
Pt. PBL + OvCA tumor	88	565
Pt. PBL + medium	332	1685
Pt. PBL + fibroblasts	315	2145
Normal PBL + medium	292	1720
Normal PBL (fresh)	262	2226

*At the end of a 24-h coincubation period, the cells were fixed, permeabilized, and stained with anti-ε mAbs. Mean fluorescence intensity was assessed by flow cytometry.

tumor cells to induce apoptosis in T lymphocytes, purified OvCA cells were coincubated with [^{3}H]TdR-labeled Fas-sensitive Jurkat T cells. Specific DNA fragmentation in Jurkat cells was quantified by assessing the loss in labeled DNA in the JAM assay. In these experiments, Jurkat cells apoptosis appeared to be Fas-dependent as it was induced by surface FasL$^+$, but not by surface FasL$^-$ OvCA cells (Fig. 5 *A*). To confirm the involvement of the Fas-induced apoptotic pathway in specific killing of Jurkat lymphocytes, OvCA cells were preincubated with Fas–Fc fusion protein or control Fc protein before the coincubation with [^{3}H]TdR-labeled target cells. This Fas–Fc fusion protein binds FasL with high affinity and blocks its activity. Treatment with Fas–Fc fusion protein significantly inhibited Jurkat cell apoptosis induced by FasL$^+$ OvCA cells (Fig. 5 *B*).

To further confirm the involvement of Fas–FasL pathway in OvCA-induced apoptosis in Jurkat cells, a Fas-resistant Jurkat cell line was used as [^{3}H]TdR-labeled target cells in the JAM assay. Despite a high level of expression of Fas, Fas-resistant cells were not sensitive to Fas-ligation by agonistic anti-Fas Ab (Fig. 6). Similarly, tumor-induced cell death of Fas-resistant Jurkat cells was significantly reduced, as compared to that of Fas-sensitive cells (tumors 1199 and 2747). However, an additional pathway of death might be induced in Jurkat cells by tumor 6727, because apoptosis was only partially blocked by Fas–Fc (Fig. 5 *B*) or in Fas-resistant Jurkat cells (Fig. 6).

Association of the loss in expression of ζ and ε chains with

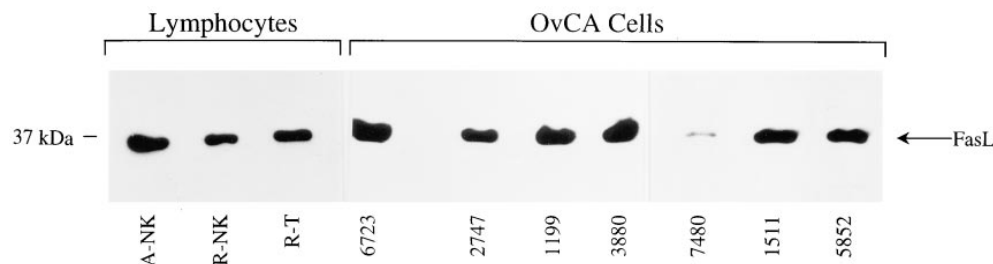


Figure 3. Western blot analysis of expression of FasL in OvCA tumor cells purified from ascitic fluids. Lymphocytes used as controls were purified by negative selection from peripheral blood of normal donors and included: IL-2–activated NK cells (*A-NK*), resting NK cells (*R-NK*), or resting T cells (*R-T*). Lysates of 0.5×10^6 cells/lane were electrophoresed in 10% SDS gels. The separated proteins were electrophoretically transferred to Immobilon-P membrane and blotted with mouse anti-FasL mAb (0.1 mg/ml; Transduction Laboratories, Lexington, KY). Proteins were detected by enhanced chemiluminescence.

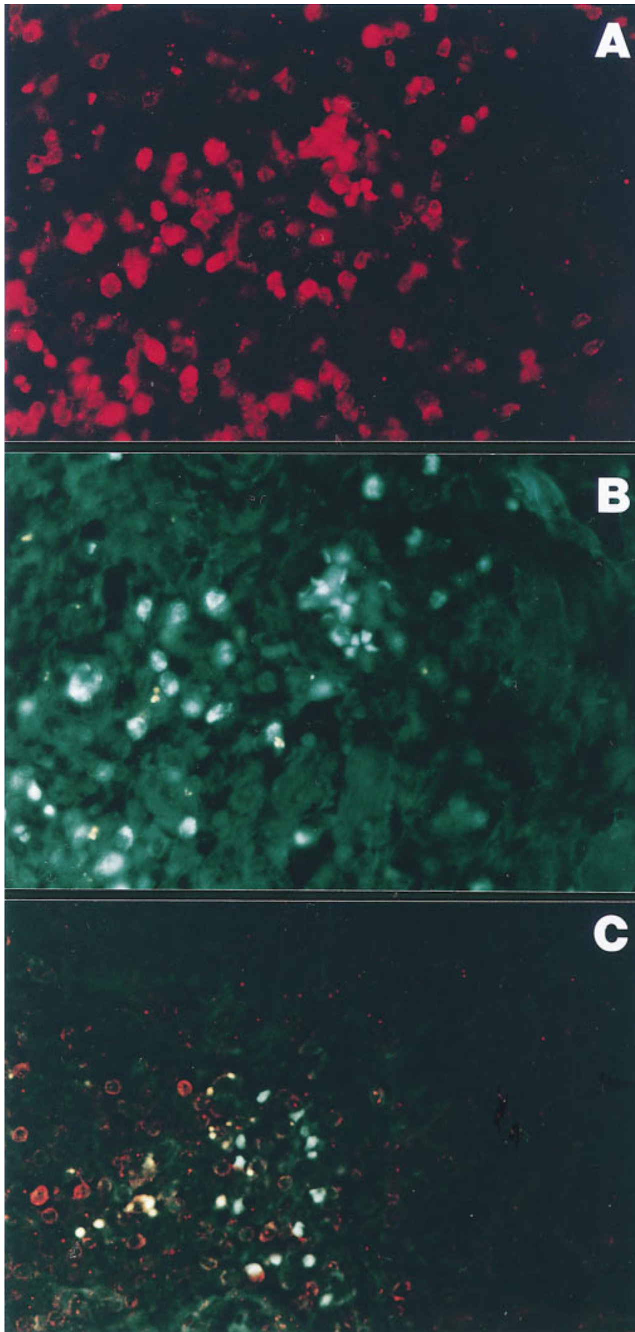


Figure 4. Immunohistology and TUNEL signal of TAL in paraffin sections of an OvCA. TAL were stained with anti-CD3 (*A*, *red*), with TUNEL (*B*, *green*), and double staining of CD3 and apoptotic cells performed in conjunction (*C*, *red* and *green-blue*). Original magnification ×400.

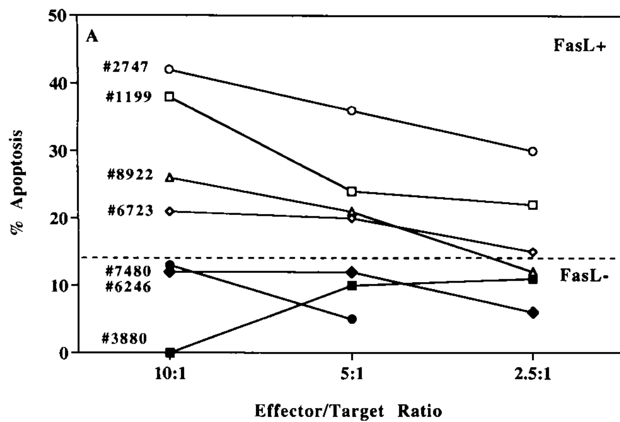
tumor-induced apoptosis in vitro. To examine a possible association between OvCA-induced apoptosis in T lymphocytes and alterations in expression of ζ and ϵ chains, purified tumor cells were coincubated with autologous PBL-T, shown in preliminary experiments to have a normal level of ζ chain expression. Reduced expression of ζ chain (Fig. 7) or ϵ chain (Table I), as assessed by flow cytometry of permeabilized CD3$^+$ cells stained with ζ- or ϵ-specific mAb, was observed after 24 h of coculture with autologous OvCA cells. However, no changes in the levels of expression of ζ or ϵ chains were observed in control T cells, incubated in the presence of normal human fibroblasts or medium alone (Fig. 7, Table I). Simultaneously, the presence of apoptotic lymphocytes was assessed in the same cocultures by performing a TUNEL assay on cytospin preparations at the end of a 24-h coincubation. T cells with fluorescein-labeled single-strand DNA breaks were enumerated. The elevated proportion of TUNEL$^+$ T cells detected in the cocultures of tumor cells and autologous T lymphocytes (Fig. 7, *parentheses*) demonstrated that the observed reduction in ζ chain or ϵ chain expression coincided with the appearance of apoptotic lymphocytes in these cocultures.

To further explore the possibility of a correlation between tumor-induced apoptosis of T lymphocytes and a loss in ζ chain expression, similar coincubation experiments were performed in the presence of the tetra-peptide aldehydes caspase inhibitors, Ac-YVAD-cho or Ac-DEVD-cho (25). Both apoptosis of T cells, as assessed by TUNEL flow cytometry assays (Fig. 8), and a loss in expression of ζ and ϵ chains, as assessed by flow cytometry of permeabilized cells (Fig. 9), were blocked in the presence of each of these inhibitors. Tumor-induced apoptosis of T lymphocytes and a loss in ζ chain expression were observed in PBL or ConA–activated lymphocytes obtained from OvCA patients. However, neither apoptosis nor loss in ζ chain expression was observed in resting T lymphocytes from normal donors (Figs. 8 and 9). These findings suggest that at least some patients with OvCA may have circulating T cells that can be sensitized to initiate an intrinsic mechanism of apoptotic cell death in the presence of tumor cells.

Involvement of Fas-mediated apoptosis in the loss of expression of CD3-ζ and CD3-ϵ chains. To examine the role of the Fas–FasL pathway in the loss of ζ or ϵ chain expression, Fas-sensitive Jurkat T cells were treated with agonistic anti-Fas Ab or rFasL, and tested for changes in levels of expression of CD3-ζ and CD3-ϵ (Fig. 10). Whereas cross-linking of Fas by either anti-Fas or rFasL resulted in the loss of CD3-ζ expression, the expression of CD3-ϵ was markedly reduced, but not lost.

To investigate the involvement of FasL expressed on OvCA in the loss of ζ or ϵ chain expression, coincubation experiments of FasL$^+$ OvCA cells with ConA–activated lymphocytes were performed in the presence of Fas–Fc fusion protein or control Fc protein. Pretreatment of OvCA cells with Fas–Fc fusion protein, but not with control Fc-containing protein, strongly inhibited the loss in ζ chain expression induced by FasL$^+$ OvCA cells during a 24-h coincubation (Fig. 11, *A* and *B*). Similarly, expression of CD3-ϵ, both in the cytoplasm and on the T cell surface, was significantly decreased after coculture of lymphocytes with OvCA cells, but was not significantly altered in the presence of Fas–Fc chimeric protein (data not shown).

To determine whether an apoptotic signal different from Fas will also converge on ζ and ϵ chains and induce their degradation, T cells were treated with etoposide VP-16. As demonstrated in Fig. 12, incubation of Jurkat cells with VP-16 (20 mM, 16 h), which induced apoptosis of 40% of these cells, as measured in TUNEL assay, also led to a loss in expression of cytoplasmic CD3-ζ and CD3-ϵ. These observations suggest that not only Fas–FasL interactions, but also other apoptotic signals, can induce alterations in TcR protein expression.



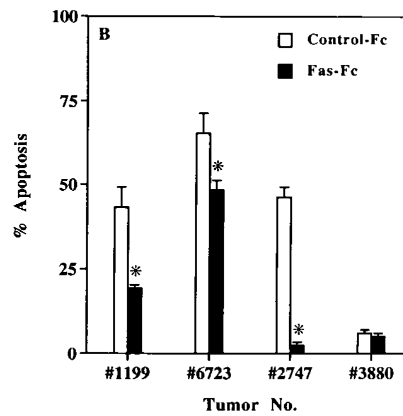


Figure 5. Apoptosis of Jurkat T cells by FasL-expressing OvCA tumor cells. In *A*, [^{3}H]TdR-labeled Jurkat cells were cocultured with FasL$^+$ or FasL$^-$ OvCA tumor effector cells at the indicated E/T ratios. In *B*, OvCA tumor cells purified from ascitic fluids were pretreated with Fas–Fc fusion protein or control-Fc protein (10 μg/ml) for 1 h at 37°C before coincubation with [^{3}H]TdR-labeled Jurkat cells. Target cell death was determined at 8 h by measuring DNA fragmentation of ^{3}H-labeled target cell DNA (JAM assay). Each percentage of apoptosis value represents the mean of sextuplicate coculture cell-killing assays. Error bars, SEM; *, significant difference ($P < 0.05$ Mann-Whitney U) between Fas–Fc and control Fc–treated OvCA cells.

Discussion

Impaired lymphocyte function often seen in patients with OvCA appears to correlate with the tumor burden and may contribute to the progression of the disease. We have previously reported that dysfunction of the immune system in patients with OvCA is related to decreased expression and altered function of TcR/CD3 and FcγRIIIA complexes (6, 26). Decreased expression of CD3-ζ and CD16-ζ has been reported in lymphocytes obtained from patients with several types of malignancies (4, 5, 7, 8, 12). In this study, we demonstrate that a loss in expression of ζ and ϵ chains is directly induced by OvCA cells interacting with T lymphocytes in vitro. We also present evidence that links the altered expression of TcR components to apoptosis induced in T lymphocytes by FasL-expressing OvCA cells. The observed death of T lymphocytes interacting with OvCA cells involves the Fas–FasL pathway, as it is mediated mainly by FasL-expressing tumor cells and inhibited by blocking FasL with Fas–Fc. Additional evidence for the involvement of the Fas–FasL pathway is contributed by the use of Fas-resistant Jurkat cells, which demonstrate cross-resistance to apoptosis induced by either anti-Fas Ab (CH-11) or FasL-expressing OvCA cells. The altered expression of CD3-ζ and CD3-ϵ chains is apoptosis-related, as it can be blocked by either Fas–Fc or inhibitors of caspases.

Our results suggest that the loss in expression of CD3-ζ and

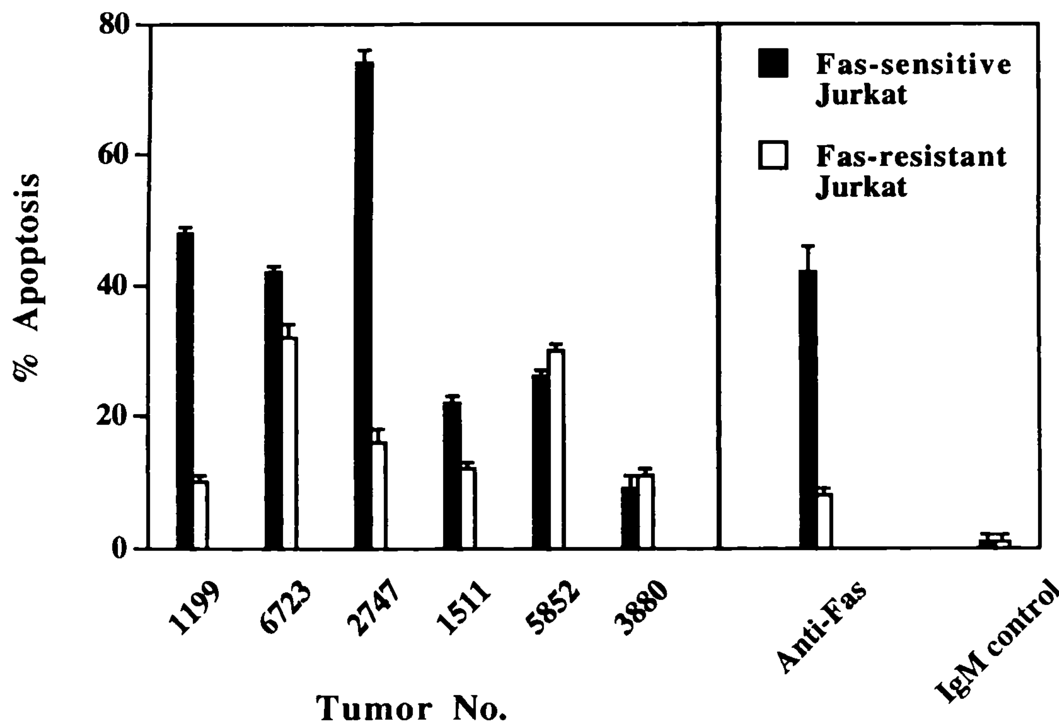


Figure 6. Apoptosis of Fas-sensitive or Fas-resistant Jurkat cells by OvCA cells purified by negative selection from ascitic fluids. Fas-sensitive and Fas-resistant Jurkat cells were incubated with OvCA cells (Tumor:Jurkat cell ratio 40:1), anti-Fas Ab (CH-11), or IgM isotype control for 18 h, and tested for apoptosis by the JAM assay. The bars represent SEM of eight replicates.

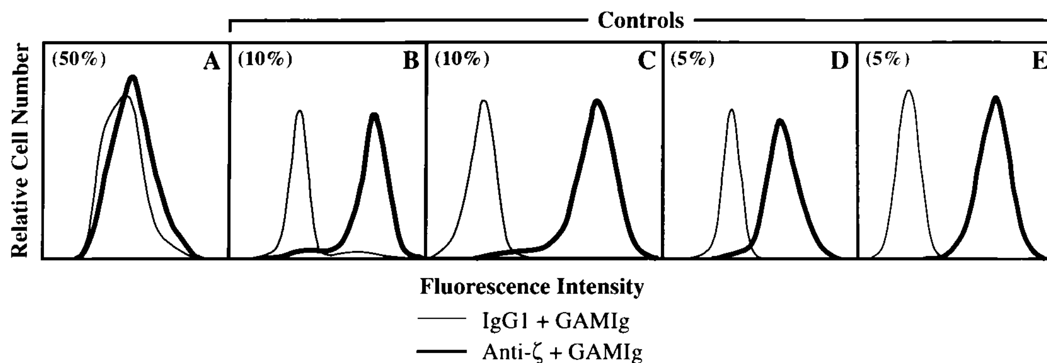


Figure 7. Expression of ζ chain and the presence of apoptotic lymphocytes in PBL-T coincubated with autologous OvCA cells purified by negative selection from ascites. PBL-T (3×10^6) obtained from an OvCA patient (No. 1199) were coincubated with autologous tumor cells (6×10^6) (*A*), fibroblasts (*B*), or medium (*C*) for 24 h. PBL-T from normal donors incubated in medium for 24 h (*D*) or freshly isolated (*E*) were used as controls. At the end of the coincubation period, the cells were examined for the presence of apoptotic lymphocytes and for cytoplasmic expression of the ζ chain. Apoptotic lymphocytes were detected by TUNEL assay performed on cytospins and the results are presented in parentheses. Expression of the ζ chain was assessed by flow cytometry after cell fixation, permeabilization, and staining with anti-ζ and anti-CD3 mAbs.

CD3-ε chains is a common feature in T cell apoptosis, regardless of the nature of the apoptotic stimuli. Reduction in the levels of expression of these proteins was observed in T lymphocytes induced to activate an intrinsic programmed cell death by either FasL-expressing OvCA cells, rFasL, agonistic anti-Fas Ab (CH-11), or the etoposide, VP-16. These results suggest that the two different apoptotic signals, Fas-ligation or etoposide treatment of T cells, converge on common downstream apoptotic effector molecules. Moreover, recent studies have suggested that environmental stress mediated by exposure to γ-irradiation (27), ultraviolet light (28), and anticancer drugs such as etoposide or doxorubicin (29), induce upregulation in expression of Fas receptors and ligands, resulting in autocrine or paracrine cell death. However, the level of Fas expression is only one of the factors regulating the susceptibility to Fas-mediated apoptosis (30). Exposure to radiation, anticancer drugs, or other forms of stress may lead to apoptosis, not only by increasing surface expression of Fas, but also by affecting intracellular signaling molecules activated upon Fas ligation. Indeed, numerous drug-resistant cell lines were also found to be resistant to Fas-mediated apoptosis (31). These findings further support the hypothesis that apoptosis mediated by both chemotherapeutic agents and physiologic stimuli such as Fas ligation may share common downstream effector molecules.

The inhibition of both T cell apoptosis and the loss in ex-

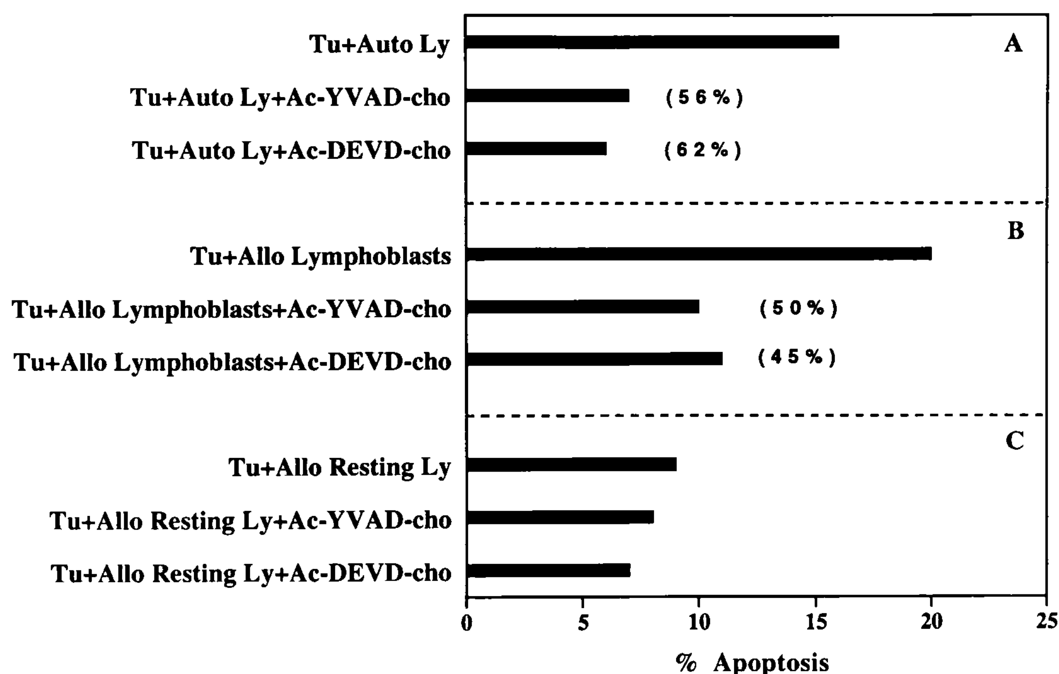


Figure 8. Inhibition of tumor-induced apoptosis of PBL by tetra-peptide aldehydes inhibitors of caspases. FasL-expressing OvCA cells were coincubated with autologous PBL (*A*), allogeneic ConA–activated lymphoblasts (*B*), or allogeneic resting PBL (*C*) for 16 h in the presence or absence of tetra-peptide inhibitors of caspases (Ac-YVAD-cho or Ac-DEVD-cho at 500 ng/ml). After coincubation, the cells were fixed, permeabilized, and incubated with TUNEL reaction mixture. Negative controls were incubated with label solution, in the absence of terminal transferase. Percent cell death was determined by flow cytometry. The results are corrected for the background apoptosis of lymphocytes in the absence of tumor cells. The percent of inhibition is shown in parentheses. *Tu*-Tumor; *Ly*-Lymphocytes.

pression of ζ and ε chains by pan-caspase inhibitors seen in our studies suggests that the two phenomena are related. However, the exact mechanisms involved in the proteolysis of ζ and ε chains remain unknown. One distinctive feature of caspases is the absolute requirement for an aspartic acid residue in the substrate P1 position. Recent studies divide the known caspases into three distinct groups of recognition cleavage sequences (32). As judged by its amino acid sequence, ζ chain does not contain any of the known sequences. However, it is currently uncertain whether all preferred peptide sequences of the 11 known caspases are included within the sequences described.

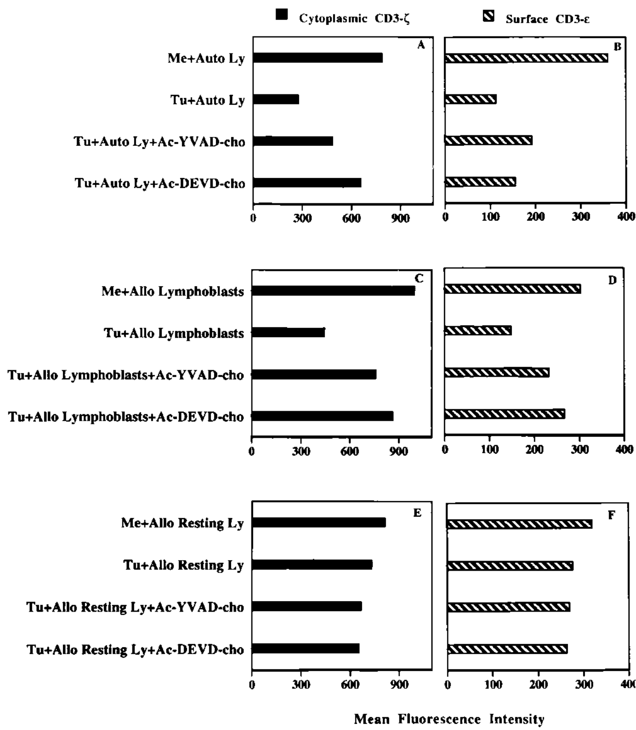


Figure 9. Effect of caspase inhibitors on tumor-induced reduction of expression of CD3-ζ and CD3-ε in T lymphocytes. FasL-expressing OvCA cells were coincubated with autologous PBL (*A* and *B*), allogeneic ConA–activated lymphoblasts (*C* and *D*), or allogeneic resting PBL (*E* and *F*) for 16 h in the presence or absence of tetra-peptide inhibitors of caspases (Ac-YVAD-cho or Ac-DEVD-cho at 500 ng/ml). After coincubation, the cells were fixed, permeabilized, and stained with mouse anti-ζ and anti-CD3 mAb (*A*, *C*, and *E*), or stained for surface expression of CD3 (*B*, *D*, and *F*). The levels of ζ and ε expression (*Mean Fluorescence Intensity*) were determined by flow cytometry. *Me*-Medium; *Tu*-Tumor; *Ly*-Lymphocytes.

Anti-Fas (52% TUNEL+)

FasL (42% TUNEL+)

A

B

CD3-ζ

MFI CD3-ζ
Control: 614
FasL: 178
Anti-Fas: 138

C

D

CD3-ε

MFI CD3-ε
Control: 3508
FasL: 2040
Anti-Fas: 1353

Flourescence Intensity

-------- Isotype Control —— Untreated Cells ━━ Anti-Fas or FasL treated cells

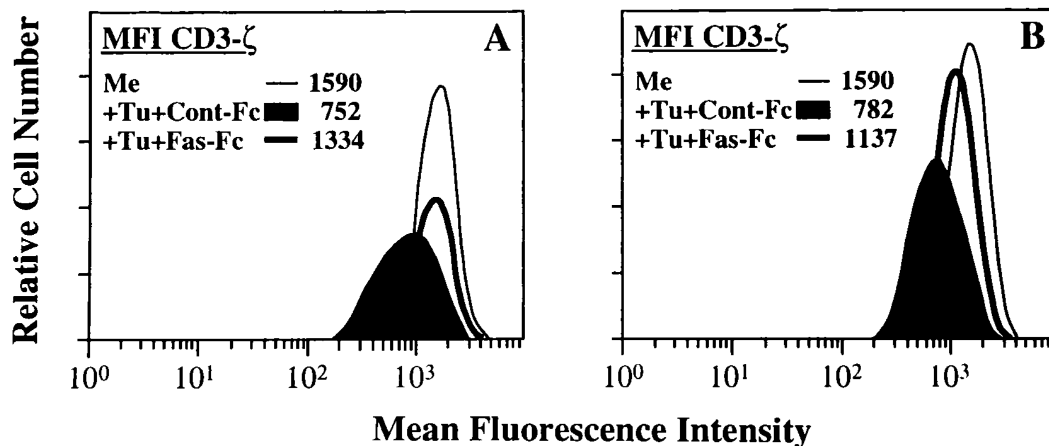


Figure 11. Effect of Fas–Fc fusion protein on OvCA-induced loss in expression of CD3-ζ in T lymphoblasts coincubated with OvCA cells. Tumor cells purified from OvCA ascitic fluids (Pt. 1199 in *A*; Pt. 2747 in *B*) were pretreated with Fas–Fc fusion protein or control Fc protein (10 μg/ml) for 1 h at 37°C before 16 h of coincubation with ConA–activated T cells. At the end of the coincubation period, the cells were fixed, permeabilized, and stained with mouse anti-ζ and anti-CD3 mAbs. *Me*-Medium; *Tu*-Tumor.

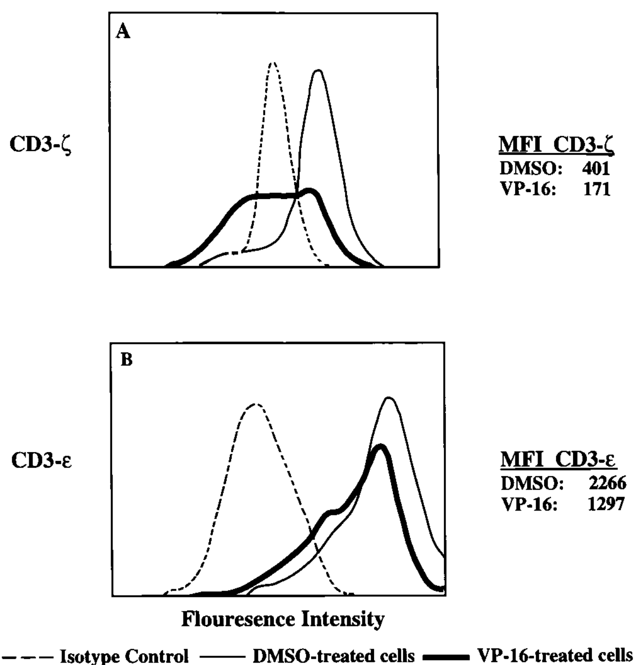


Figure 12. Reduced expression of CD3-ζ and CD3-ϵ in etoposide-treated Jurkat cells. Incubation of Jurkat cells with VP-16 (20 mM, 16 h) resulted in 40% apoptosis, as assessed by flow cytometry TUNEL. The cytoplasmic expression of CD3-ζ and CD3-ϵ was determined by flow cytometry of permeabilized cells, stained with specific antibodies.

Fas is preferentially expressed by CD45RO$^+$ memory, but not by CD45RO$^-$ naive T cells (33). Despite Fas expression on their cell surface, CD45RO$^+$ peripheral T cells are resistant to agonistic anti-Fas mAb. Therefore, it has been concluded that Fas expressed on mature peripheral T cells is nonfunctional. Because activated peripheral T cells are sensitive to agonistic anti-Fas mAb, it appears that susceptibility to Fas-mediated apoptosis depends on cellular activation. However, in our studies, not only ConA lymphoblasts, but also T cells obtained from patients, but not from peripheral blood of normal donors, were Fas-sensitive when coincubated with Fas-expressing autologous tumor cells. These results suggest that an increased proportion of the patients' circulating T cells is susceptible to Fas-mediated cell death. These results also indicate that membrane-bound FasL can mediate apoptosis of both fresh and in vitro–activated peripheral T cells. These findings are in agreement with studies from S. Nagata's group, demonstrating differential killing capability of membrane FasL and agonistic anti-Fas mAb (34).

In summary, our results suggest that the reduced expression of CD3-ζ and CD3-ϵ chains in T lymphocytes interacting with OvCA cells is related to an apoptotic process induced in T lymphocytes by the tumor cells. OvCA cells have the ability to counterattack activated lymphocytes with the lymphocytes' own cytotoxic tool: FasL. Surface expression of FasL by OvCA cells can conceivably confer immuneprivileged status to the tumor. To overcome tumor-induced apoptosis, it will be necessary to determine the mechanisms used by Fas-resistant lymphocytes to escape this mechanism of immunosuppression.

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Figure 10. Altered expression of CD3-ζ and CD3-ϵ in Fas–cross-linked Jurkat cells. Jurkat cells were incubated with agonistic anti-Fas Ab (CH-11, 200 ng/ml, Upstate Biotechnology Inc.) (*A* and *C*) or rFasL (50 ng/ml, Alexis, San Diego, CA) (*B* and *D*) for 16 h. The percentage of apoptotic lymphocytes was determined by flow cytometry TUNEL, and cytoplasmic expression of ζ and ϵ chains by flow cytometry of permeabilized cells.

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