Fas Ligand–Positive Membranous Vesicles Isolated from Sera of Patients with Oral Cancer Induce Apoptosis of Activated T Lymphocytes

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ABSTRACT

Objective: In patients with oral squamous cell carcinoma, a high proportion of T cells in the tumor undergo apoptosis, which correlates with Fas ligand (FasL) expression on tumor cells. The present study was done to identify mechanisms responsible for apoptosis of T cells seen in the peripheral circulation of these patients.

Methods: Sera of 27 patients, normal donor sera, and supernatants of cultured normal or tumor cells were fractionated by size exclusion chromatography and ultracentrifugation to isolate microvesicles. The presence of microvesicle-associated FasL was studied by Western blots, blocking with anti-Fas reagents, and immunoelectron microscopy. Biological activities of microvesicles were tested including the ability to induce apoptosis of Jurkat and T-cell blasts. Semiquantitative analysis of FasL in microvesicles was correlated with caspase-3 activity, DNA fragmentation, cytochrome c release, loss of mitochondrial membrane potential, and TCR-ζ chain expression in lymphocytes.

Results: FasL-positive (FasL+) microvesicles were detected in sera of 21 of 27 patients. Microvesicles contained 42 kDa FasL. These microvesicles induced caspase-3 cleavage, cytochrome c release, loss of mitochondrial membrane potential, and reduced TCR-ζ chain expression in target lymphocytes. Biological activity of the FasL+ microvesicles was partially blocked by ZB4 anti-Fas monoclonal antibody. Microvesicle-associated FasL levels correlated with the patients' tumor burden and nodal involvement.

Conclusion: Sera of patients with active oral squamous cell carcinoma contain FasL+ microvesicles, which induce the receptor and mitochondrial apoptotic pathways in Jurkat and activated T cells.

INTRODUCTION

Malignant diseases are known to be associated with immune suppression, which facilitates escape of tumors from the host immune system. Although the mechanisms responsible for dysfunction of immune cells in patients with cancer are not well defined, several different pathways used by tumors to escape immune surveillance have been described (1–4). We previously reported that T cells found at tumor sites and in the peripheral circulation of patients with cancer showed high rates of spontaneous apoptosis and signaling abnormalities, including TCR-ζ down-regulation (5–7). Interestingly, apoptosis of circulating T cells seems to be a generalized phenomenon in patients with cancer, as it is observed in melanoma, ovarian and breast carcinomas, and head and neck cancer (8–10). This apoptosis preferentially targets CD8+ effector T cells, and circulating tumor-specific tetramer+ T cells are highly susceptible to apoptosis in patients with cancer (11).

A convincing explanation for apoptosis of T cells at the tumor site was previously provided by the demonstration of surface and intracytoplasmic Fas ligand (FasL) expression in tumor cells. The ability of these tumor cells to induce apoptosis in Fas+ T lymphocytes both *in vivo* and *in vitro* was previously shown (5, 12). However, the mechanism(s) inducing apoptosis of circulating peripheral blood T cells remains undefined. A possibility has been considered that tumor-derived soluble FasL could contribute to demise of Fas+ T cells in the circulation of patients with cancer (5, 13), although other mechanisms have been suggested as well (14–16).

The involvement of Fas (CD95), a type I transmembrane glycoprotein, and FasL, a type II transmembrane protein, both belonging to the tumor necrosis factor superfamily of receptor and ligands, in a demise of CD8+ T cells is well documented (reviewed in ref. 1). The Fas/FasL pathway seems to play a dual role *in vivo* as it mediates proinflammatory effects as well as immune cell apoptosis (17). Apoptosis of T cells infiltrating tumor tissue or T cells coincubated with tumor cells has been correlated with expression of cell membrane–bound FasL on the surface of tumor cells (12). FasL exists in soluble or cell membrane–bound forms (18). The soluble form of FasL (26 kDa) is thought to be released from tumor cells after enzymatic cleavage of membrane-bound FasL (37-42 kDa) by matrix metalloproteinases (18). Its ability to cross-link the receptor and induce apoptosis of Fas (CD95+) cells is reduced relative to membrane-bound FasL (19). The soluble form of FasL was previously reported by us to be present in sera of patients with oral squamous cell carcinomas (OSCC) but showed no correlation with spontaneous apoptosis of circulating T cells (5).

To provide a rational explanation for apoptosis of CD95+ T cells in the circulation of patients with cancer, we and others have considered a possibility that membranous vesicles, long known to be present in sera of patients with cancer (20, 21), could be involved in a demise of T cells. To this end, we have isolated these microvesicles from sera of patients with OSCC by exclusion chromatography and ultracentrifugation. In this article, we describe biological activities of the microvesicles, including their ability to induce apoptosis of Jurkat cells and activated normal T cells.

MATERIALS AND METHODS

Patients and Controls. Twenty-seven patients with OSCC and 20 healthy normal controls were included in this study. All patients underwent surgery for treatment of the malignant disease at the University of Mainz Hospital (Mainz, Germany). The patients all signed the informed consent form approved by the University of Mainz Hospital Institutional Review Committee. All normal controls and additional patients with OSCC signed a consent form approved by the IRB at the University of Pittsburgh (IRB 980633). Sera of patients and normal controls were obtained from venous blood and stored at −80°C until use. All patients donated blood for this study before any therapy. The pathologic and clinical characteristics of the patients with OSCC included in this study are listed in Table 1. Twenty-one patients had advanced disease (stages III or IV) and six had early disease (stages I or II). Fifteen patients had tumors classified as T4, 15 patients had nodal metastases (N1, N2, or N3), and 3 patients had distant metastases (M1). Sera of

additional OSCC patients with active disease seen in Pittsburgh were used to obtain microvesicles for confirmatory experiments.

Cell Lines. The OSCC cell line, PCI-13, was established in our laboratory and maintained as previously described (22). It was transfected with the FasL gene obtained from Dr. S. Nagata (Osaka Biosciences Institute, Osaka, Japan), using a retroviral vector as previously reported (12, 23). Transfected PCI-13 supernatants, which contained both sFasL and the 4-kDa membranous form of FasL (12, 23), were used as positive controls in our experiments.

Jurkat cells were obtained from American Tissue Culture Collection (Manassas, VA) and were cultured in RPMI 1640 supplemented with 10% (v/v) bovine fetal serum, L-glutamine, and antibiotics. Cultures in the log phase of growth were used for all experiments. The Jurkat cell line resistant to Fas-mediated apoptosis was obtained from Dr. H. Rabinowich (Department of Pathology, University of Pittsburgh, PA) (24). Human fibroblasts were cultured from skin explants as previously described (25).

Antibodies. Anti-FasL polyclonal antibodies, Ab-3 (Oncogene/EMD Biosciences, San Diego, CA) and C-20 (Santa Cruz Biotechnology, Santa Cruz, CA), as well as anti-FasL monoclonal antibodies (mAb), G247-4, and NOK-1 (PharMingen, San Diego, CA) and Ab-33 (Transduction Technology, Lexington, KY) were purchased for FasL detection. The antibody for caspase-3 detection was from PharMingen. Anti-Fas (CH-11) agonistic mAb, IgM isotype control for CH-11, anti-Fas blocking mAb, clone ZB4, and isotype IgG1 control for ZB4 were all purchased from Upstate Biotechnology (Lake Placid, NY). Antibodies used for flow

Table 1 Clinicopathologic characteristics of the patients with OSCC included in this study*

Patient no.	Age (y)	Gender	Tumor-Node-Metastasis	Stage	Tumor localization
1	79	F	T4N2M0	IV	Floor of the mouth
2	49	F	T3N0M0	III	Base of the tongue
3	83	F	T4N1M0	IV	Buccal mucosa
4	60	M	T4N2M0	IV	Base of the tongue
5	66	M	T4N0M0	IV	Maxilla
6	56	M	T1N0M0	I	Mandible
7	54	M	T4N2M1	IV	Floor of the mouth
8	50	M	T1N3M0	IV	Tonsil
9	41	F	T4N0M0	IV	Maxilla
10	62	M	T4N0M0	IV	Retromolar trigone
11	45	M	T2N2M0	IV	Floor of the mouth
12	62	M	T4N2M0	IV	Retromolar trigone
13	51	M	T2N0M0	II	Mandible
14	43	M	T4N2M0	IV	Floor of the mouth
15	50	F	T2N0M0	II	Base of the tongue
16	66	F	T3N0M0	III	Retromolar trigone
17	33	M	T2N0M0	II	Buccal mucosa
18	69	M	T4N2M0	IV	Retromolar trigone
19	61	M	T4N2M0	IV	Tongue
20	60	F	T4N0M0	IV	Mandible
21	82	M	T4N2M1	IV	Retromolar trigone
22	40	F	T4N2M1	IV	Palate
23	74	M	T1N0M0	I	Mandible
24	61	F	T1N0M0	I	Buccal mucosa
25	61	M	T1N1M0	III	Floor of the mouth
26	49	M	T4N1M0	IV	Retromolar trigone
27	70	M	T2N1M0	III	Floor of the mouth

Abbreviations: OSCC, oral squamous cell carcinoma.
*The TNM staging of tumors was performed according to the AJCC grading at the time of surgery.

cytometry included anti-CD3, -CD4, -CD8, and -CD247 (TCR-ζ chain) all purchased from Beckman Coulter (Miami, FL). mAbs to cytochrome *c* were purchased from Santa Cruz Biotechnology.

Preparation of Microvesicles. Microvesicles were prepared from sera of OSCC patients and normal controls as well as from culture supernatants of the parental or FasL-transduced PCI-13 cell lines and of normal human fibroblasts (12, 23, 25). Microvesicles were isolated as previously described (26). Briefly, sera or tumor culture supernatants were fractionated by a two-step procedure, including size exclusion chromatography and ultracentrifugation. First, 0.5-mL aliquots of sera were applied to a Sepharose 2B (Amersham Biosciences, Piscataway, NJ) column (1.0 × 35 cm) equilibrated with PBS. Fractions (1 mL) were collected, and the protein content was monitored by measuring absorbance at 280 nm. The void volume peak material, containing proteins of >50 million kDa, was then centrifuged at 105,000 × g for 1 hour at 4°C. The pellet was resuspended in 1 mL of sterile PBS, and this material, shown to contain microvesicles, is referred to as the "microvesicle fraction" throughout the article. Protein concentration in each microvesicle fraction was estimated by a Bradford protein assay (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin used as a standard.

Western Blot Assays. Western blots were used to identify FasL in the microvesicle fractions isolated from the patients' sera. Each microvesicle fraction equivalent to 25 μg of protein was subjected to 12% SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes. The membranes were blocked in 5% fat-free milk in TBST (0.05% Tween 20 in Tris-buffered saline) for 1 hour at room temperature and then incubated overnight at 4°C with anti-FasL antibodies, either Ab-3 or G247-4, or anti-caspase 3 antibody at the final concentration of 1 μg/mL. After washing (3 × 15 minutes) with TBST at room temperature, membranes were incubated with horseradish peroxidase–conjugated secondary antibody at 1:150,000 dilution (Pierce Chemical Co., Rockford, IL). The signal was detected with a SuperSignal detection system (Pierce Chemical Co.). The band intensity of FasL was semiquantitated using Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA). We have previously shown that four of the anti-FasL antibodies we purchased (Ab3, C-20, G247-4, and Ab-33) specifically recognize FasL in immunoblots (12). Because Ab-3 and G247-4 were previously shown to detect the 42-kDa form of FasL in addition to the soluble 27-kDa form (12), we selected these antibodies to study FasL in microvesicles.

Coincubation of Activated Normal T Cells or Jurkat Cells with Sera or Microvesicles Containing FasL. Normal human peripheral blood mononuclear cells were isolated on Ficoll-Hypaque gradients and washed in medium. Mononuclear cells were then incubated in culture flasks (T75) for 2 hours at 37°C to remove plastic-adherent cells. The nonadherent cells were collected, and used as a source of T cells. T cells were activated in the flasks precoated with OKT3 antibody (UPCI Pharmacy Pittsburgh, PA) at the concentration of 1 μg/mL in AIMV medium (Invitrogen, Grand Island, NY) at 37°C in 5% CO₂ in air. After 24 hours, [³H]thymidine was added to cells for overnight incubation to be used in JAM assays or cells were incubated in AIMV medium and harvested after 24 to 48 hours, counted in trypan blue, and used in other assays. CH-11 antibody–sensitive or –resistant Jurkat T cells were in the log phase of growth (100% viability by a trypan blue exclusion test) on the day of experiment. To test patients' sera for biological

activity, activated T lymphocytes or Jurkat cells were plated at 2 × 10⁶ cells/mL per well in 12-well plates in medium and incubated with a 0.5-mL aliquot of serum overnight (T cells) or for 4 days (Jurkat). Activated T cells were more sensitive to serum-induced apoptosis; hence incubation time was 24 hours. Microvesicles (0.20-0.25 mg protein) were coincubated with all cell targets for 24 hours. Jurkat cells or T cells were harvested, washed, and then used to measure mitochondrial membrane potential ($\Delta\Psi_m$) and cytochrome *c* release as well as expression of TCR-ζ. In preliminary experiments, dose-dependent activity of microvesicles was measured by using various dilutions of microvesicles.

DNA Fragmentation (JAM) Assay. Jurkat cells or activated normal T cells were coincubated with microvesicles or patients' sera to estimate apoptosis of lymphocytes using the JAM assay. Fas-sensitive and Fas-resistant Jurkat cells or normal activated T cells were labeled overnight with [³H]thymidine, plated at 2.5 × 10⁴ cells per well in a 96-well plate, and coincubated with 6.25 μg of a microvesicle fraction or serum at 25% final concentration per well in triplicate wells. As a positive control, anti-Fas mAb at 200 ng/mL was used and IgG isotype served as a negative control. Cells were harvested into fiberglass filters and radioactivity corresponding to undamaged DNA was counted. Percent apoptosis was quantitated using the following formula:

$$\% \text{ Specific apoptosis} = \frac{(\text{CPM}_{\text{spontaneous}} - \text{CPM}_{\text{experimental}})}{(\text{CPM}_{\text{spontaneous}} - \text{CPM}_{\text{maximum}})} \times 100$$

CPM$_{\text{spontaneous}}$ indicates spontaneous apoptosis of Jurkat T cells and CPM$_{\text{maximal}}$ indicates maximal apoptosis after treatment of Jurkat cells with 5% (v/v) Triton X-100 in buffer.

Measurement of the Mitochondrial Membrane Potential. Jurkat cells or activated normal T cells coincubated with microvesicles for 24 hours or control lymphocytes alone were incubated for 15 minutes at 37°C in the medium containing 200 nmol/L tetramethylrhodamine methyl ester (Molecular Probes, Eugene, OR). An aliquot of T cells was treated with 5 mmol/L mitochondrial uncoupler, carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (Sigma Chemical Co., St. Louis, MO) during the staining period as a positive control for mitochondrial depolarization. The red tetramethylrhodamine methyl ester fluorescence was analyzed using a flow cytometer (EPICS XL, Coulter) immediately after staining.

Measurement of Cytochrome *c* Release. Jurkat cells or activated normal T cells were fixed with 1% (w/v) paraformaldehyde in PBS at room temperature for 10 minutes and then permeabilized with ice-cold cell lysis and mitochondria intact buffer (CLAMI) containing 250 mmol/L sucrose, 80 mmol/L KC1, and 50 mg/mL digitonin in PBS for 5 minutes at 4°C. The cells were stained with FITC-conjugated anti-human cytochrome *c* mAb for 20 minutes at 4°C. FITC-conjugated mouse IgG1 was used as an isotype-matched control. After washing in the cell lysis and mitochondria intact buffer, the cells were analyzed by flow cytometry. Permeabilization of the cell membrane was confirmed by staining of cells with 0.1% (v/v) trypan blue in PBS.

Measurement of TCR-ζ Expression. Jurkat cells or activated normal T cells incubated for 24 hours with the microvesicle fractions or analogous serum fractions obtained from normal controls were first fixed with 1% (w/v)

paraformaldehyde at room temperature for 10 minutes. The cells were then permeabilized with 0.1% (v/v) saponin in PBS for 5 minutes at 4°C. After permeabilization, the cells were stained for 30 minutes at 4°C with phycoerythrin-conjugated anti-human TCR-ζ mAb. Phycoerythrin-conjugated mouse IgG1 mAb was used as an isotype-matched background control. After washing in 0.1% saponin solution, the cells were resuspended in the paraformaldehyde solution and analyzed by flow cytometry.

Blocking with mAbs. Anti-Fas neutralizing mAb, ZB4 (1 or 10 μg/mL), or IgG1 isotype control was added to Jurkat cells or activated normal T cells before JAM assays. The cells were then incubated with microvesicle-containing fractions as described above, and the percent of inhibition was calculated. Pan-caspase inhibitor Z-VAD-FMK (BD Biosciences, PharMingen) was used in selected blocking experiments at the concentration of 20 μmol/L.

Transmission Electron Microscopy. Transmission electron microscopy of microvesicles was done in the Center for Biologic Imaging at the University of Pittsburgh Medical School. For transmission electron microscopy, the microvesicle pellets were fixed in 2.5% (w/v) glutaraldehyde in PBS, dehydrated, and embedded in Epon. Ultrathin sections (65 nm) were cut and stained with uranyl acetate and Reynold's lead citrate. The sections were examined in a Jeol 1210 transmission electron microscope.

Immunoelectron Transmission Microscopy. Copper grids (200 mesh) were formvar coated using 0.125% (v/v) formvar in chloroform. Microvesicles (1-10 μL) were loaded onto grids by centrifugation in an Airfuge ultracentrifuge (Beckman, Palo Alto, CA) using the EM-90 rotor. Following centrifugation at $100,000 \times g$ for 5 minutes, the grid was removed and excess sample solution was wicked away with filter paper. Microvesicles were fixed with 2% (w/v) paraformaldehyde in PBS for 5 minutes. Microvesicles were washed thrice with PBS, then thrice with PBS containing 0.5% bovine serum albumin and 0.15% glycine (PBG buffer) followed by a 30 minutes incubation with 5% normal goat serum in PBG. Microvesicles were labeled with anti-FasL mAb (clone NOK-1 pretested for use in immunostaining) for 1 hour at room temperature. Microvesicles were washed four times with PBG buffer and then grids were labeled with 5-nm colloidal gold (Amersham, goat anti-mouse at 1:25) at room temperature for 1 hours. Sections were washed thrice in PBG, thrice in PBS, then washed six times in double-distilled water. The grid was then placed on a drop of 0.45-μm-filtered 2% phosphotungstic acid (pH 6.0) in milli-Q H_2O for 30 to 60 seconds. Excess stain was wicked away and samples were viewed on a JEOL JEM 1210 transmission electron microscope at 80 kV. Under these conditions, no labeling was observed when secondary antibodies alone or irrelevant isotype control antibodies were used.

Statistical Analysis. Differences between normal controls and patients with OSCC were analyzed using Mann-Whitney U test for unpaired comparisons. Correlations were calculated by linear regression analysis. Associations between levels of FasL in microvesicle fractions and the disease stage or nodal involvement were analyzed using a χ^2 and Cochran-Armitage tests. A P value lower than 0.05 was considered to be statistically significant.

RESULTS

Presence of Microvesicles in Sera of Patients with OSCC. Before microvesicle isolation, sera of the patients with OSCC were tested in JAM assays for evidence of biological activity. Representative results (Fig. 1A) show that these sera induced apoptosis of Jurkat cells, which was partially inhibited in the presence of Z-VAD-FMK. These sera also induced apoptosis of T-cell blasts, which was inhibited (20-40%) by Z-VAD-FMK (data not shown) and by anti-Fas (ZB4) antibody (Fig. 1B). Following fractionation of the sera (0.5 mL) on a Sepharose 2B column and ultracentrifugation to obtain microvesicles, the protein content of each recovered fraction was determined. Microvesicle fractions isolated from sera of patients had significantly higher mean protein content ($P < 0.001$) than the analogous fractions of normal control sera. These microvesicle fractions induced apoptosis in Jurkat cells and T-cell blasts, which was partially but significantly inhibited by Z-VAD-FMK (Fig. 2A and B). The microvesicle fractions were also examined for the presence of microvesicles by electron microscopy. As shown in Fig. 3, serum fractions obtained from patients but not normal controls contained membranous structures of ~60 to 100 nm in size. Supernatants of PCI-13 transduced with the FasL gene (PCI-13/FasL) but not supernatants of normal human fibroblasts (not shown) also contained microvesicles.

Western Blots for Detection of the Membrane Form of FasL. Previous studies suggested that tumor cell–associated FasL exists in two forms: a soluble form, which on Western blots gives a 27kDa band and a membrane form, which is 42 kDa in molecular weight (19). We have previously determined that anti-FasL antibodies, Ab-3 and G247-4, can detect the membrane form of FasL (12) and, therefore, we selected these antibodies for immunoblotting of microvesicle fractions. To confirm that semiquantitative densitometric analysis of FasL immunoblots is valid, we used either rFasL (12) or microvesicles obtained from supernatants of PCI-13/FasL, which were immunoblotted at different protein concentrations, followed by densitometry of the FasL bands. A linear correlation between the signal intensity (in pixels) of each band and the concentration of FasL was established (12). Subsequently, microvesicle fractions were tested in Western blots and, as shown in Fig. 4A, also showed a strong 42-kDa band, corresponding to the band identified in the microvesicles obtained from supernatants of PCI-13/FasL cells (12). Of 27 microvesicle fractions obtained from OSCC patients, 21 contained FasL detectable by immunoblots (Fig. 4B). Semiquantitative densitometry of FasL bands showed that the microvesicles contained various levels of FasL. In contrast, FasL was detectable in only 1 of 8 equivalent protein fractions isolated from normal control sera and tested in the same assay.

Immunoelectron Microscopy for FasL in Microvesicles. To confirm the association of FasL with microvesicles, immunoelectron microscopy was done using microvesicle fractions obtained from the patients' sera and from PCI-13/FasL supernatants. As shown in Fig. 3B (inset), immunoelectron microscopy revealed dense dots approximately 15 nm in diameter associated with microvesicles. The dots represent FasL-antibody complexes located on the surface of microvesicles. No labeling outside the membranous structures was observed.
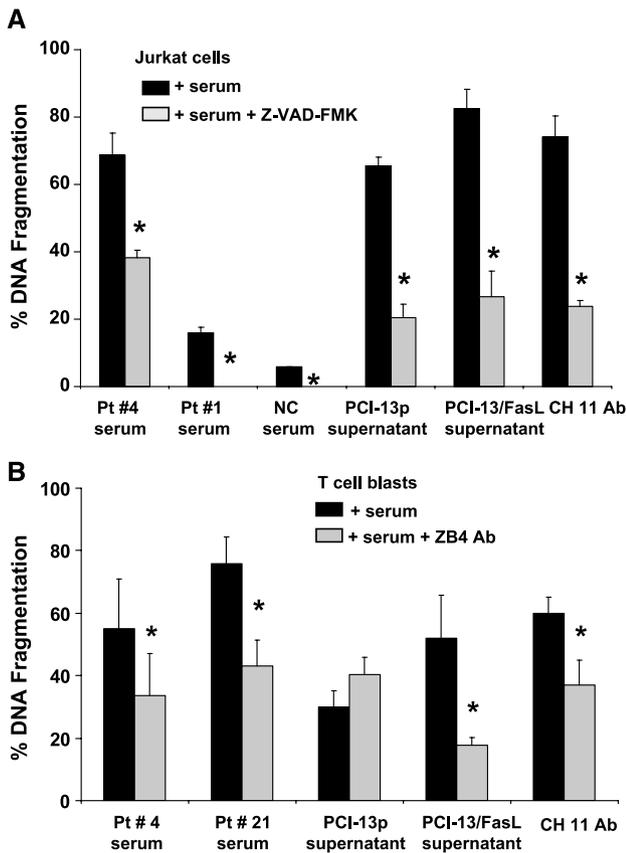
Fig. 1 Sera of the patients with OSCC induce DNA fragmentation in Jurkat cells (*A*) or activated normal T lymphocytes (*B*). Jurkat cells were incubated with sera or supernatants for 4 days and T-cell blasts were incubated for 24 hours. Culture supernatants of PCI-13 and PCI-13/FasL were adjusted with medium to the final concentration of 25%. CH-11 antibody was used as a positive control. Jurkat cells (*A*) were incubated with the pan-caspase inhibitor Z-VAD-FMK (20 μmol/L) and T-cell blasts (*B*) were incubated with anti-Fas (ZB4) antibody before the addition of sera. *, $P < 0.01$ (± inhibitors). Representative results with sera of patients 1, 4, and 21.

Biological Activity of Microvesicle-Associated FasL. To show that 42-kDa FasL associated with microvesicle fractions was biologically active, two types of assays were done, initially using Jurkat cells and then T-cell blasts. DNA fragmentation in target cells (Jurkat) incubated with microvesicle fractions for 24 hours was first tested (Fig. 4*B*). Biological activity was detected in microvesicles obtained from all but one patient (patient 16) and in none of the control fractions (data not shown). Microvesicles obtained from 6 of 21 patients were negative for FasL expression by immunoblots but were able to induce DNA degradation in Jurkat cells sensitive to Fas-mediated apoptosis (Fig. 4*B*) as well as Jurkat cells resistant to Fas-induced apoptosis (data not shown). To confirm these results, changes in the MMP of Jurkat cells coincubated with microvesicle fractions were determined, using flow cytometry. Figure 5*A* shows that the microvesicle fraction obtained from serum of patient 26 induced a substantial shift in the MMP of Jurkat cells, whereas the equivalent fraction obtained from normal control did not. Furthermore, this MMP loss was partially inhibited by anti-Fas antibody, ZB4, or Z-VAD-FMK

(Fig. 5*B*). All other microvesicle fractions obtained from sera of patients with OSCC were also tested for the ability to alter the MMP potential of Jurkat cells, and the microvesicle fractions positive for FasL in immunoblots were found to have the highest biological activity relative to FasL-negative microvesicle fractions ($P = 0.017$). Overall, 21 of 27 microvesicle fractions were FasL positive (FasL+) and induced DNA fragmentation or decreased the MMP potential in Jurkat cells.

In additional experiments, the loss of MMP in Jurkat cells coincubated with microvesicles was correlated to apoptosis of these cells and showed a strong positive relationship ($P = 0.00005$). Nevertheless, 6 of 27 microvesicle fractions obtained from patients' sera were FasL- and apoptogenic, an indication that microvesicle-associated molecules other than FasL may be responsible for biological activity seen with FasL-microvesicle fractions. This conclusion was supported by the lack of a significant correlation between expression of FasL on microvesicles and DNA fragmentation in Jurkat cells for this cohort of 27 patients. Similar experiments done with activated normal T cells confirmed that microvesicle fractions positive for FasL induced DNA degradation as well as an MMP shift in these cells and that T-cell blasts were more sensitive to microvesicle-induced apoptosis than Jurkat cells (data not shown).
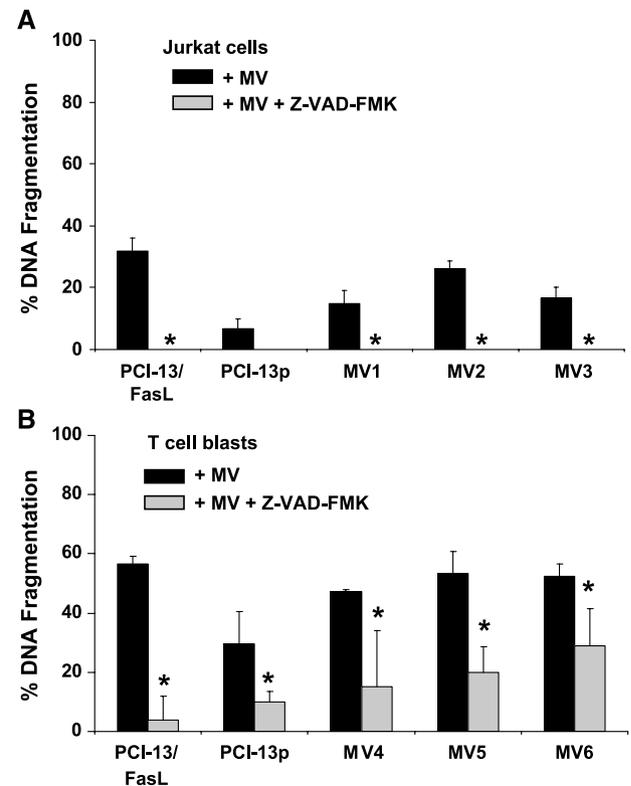

Fig. 2 Microvesicles obtained from sera of OSCC patients induce apoptosis of Jurkat cells (*A*) or activated T lymphocytes (*B*). Target cells were incubated with microvesicles for 24 hours. Microvesicles obtained from supernatants of PCI-13/FasL and of parental PCI-13 were also tested. DNA fragmentation in Jurkat cells and T-cell blasts was partially but significantly inhibited in the presence of Z-VAD-FMK; *, $P < 0.01$. Experiments were done with microvesicles isolated from sera of three representative patients with OSCC.
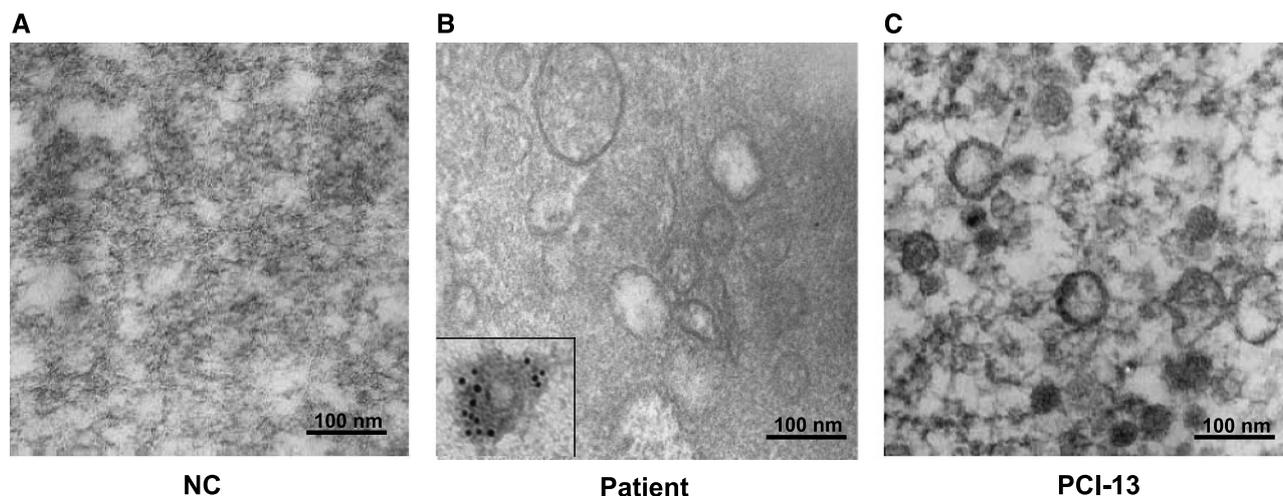
Fig. 3 Electron micrographs of microvesicles fractionated from serum of patient 12 with OSCC (*B*) or supernatants of the PCI-13/FasL cells (*C*). The membranous microvesicles are 60 to 100 nm in diameter. Inset in B, shows immunoelectron microscopy of FasL associated with the membranous structures (~ *15 nm dots*). The FasL was localized to microvesicles with anti-FasL antibody, NOK-1, as described in Materials And Methods. No microvesicles were seen in concomitantly fractionated sera of normal donors (*A*).

Expression of Fas on Jurkat Cells and T-cell blasts. The observed sensitivity of Jurkat cells and activated normal T lymphocytes to sera of patients with OSCC (Fig. 1) and to microvesicle fractions containing 42-kDa FasL (Fig. 4) implies that Fas (CD95) is expressed and active in these cells. As reported previously, Jurkat cells and activated T cells in the log phase of growth are strongly positive for Fas by flow cytometry and negative for FasL expression (10).

Blocking of FasL Activity Associated with Microvesicles. To confirm that microvesicle-associated FasL is responsible for apoptosis of Jurkat or activated normal T cells, blocking of microvesicle-induced DNA fragmentation in these cells by anti-Fas (ZB4) antibodies was performed. (Fig. 6*A*). Blocking of microvesicle-induced apoptosis as well as CH-11 antibody–induced apoptosis was significant ($P < 0.01$ versus DNA degradation without ZB4 antibody) but not complete. This again suggested that in addition to FasL, other microvesicle-associated molecules probably contributed to apoptosis of target cells.

To confirm that the receptor-mediated apoptosis occurred in Jurkat cells coincubated with microvesicles, we also measured caspase-3 cleavage by Western blots. Fig. 6*B* shows that the representative FasL+ microvesicle fractions (e.g., microvesicles of patients 19 and 24) induced caspase-3 activation and cleavage in Jurkat cells. These target cells pretreated with anti-Fas mAb (ZB4) or the pan caspase inhibitor, Z-VAD-FMK, did not show caspase-3 cleavage (Fig. 6*B*).

Down-Regulation of TCR-ζ by Microvesicle Factions. Pretreatment of T cells with FasL has been reported to induce a loss of TCR-ζ protein (27). Similar losses in TCR-ζ were previously observed by us in T cells incubated with analogous microvesicles obtained from sera of patients with ovarian carcinoma (26). When Jurkat cells or T-cell blasts were coincubated with the microvesicle fractions isolated from sera of OSCC patients, ζ chain expression was shown to be down-regulated (Fig. 7*A*). This down-regulation of TCR-ζ was completely inhibited in Jurkat cells preincubated with ZB4

antibody or Z-VAD-FMK (Fig. 7*A*). Jurkat cells incubated with individual microvesicles isolated from sera of all 27 patients expressed a mean of 36,000 ± 7,000 molecules of equivalent soluble fluorochrome (MESF) units of ζ protein, whereas untreated or control fraction–treated Jurkat cells expressed
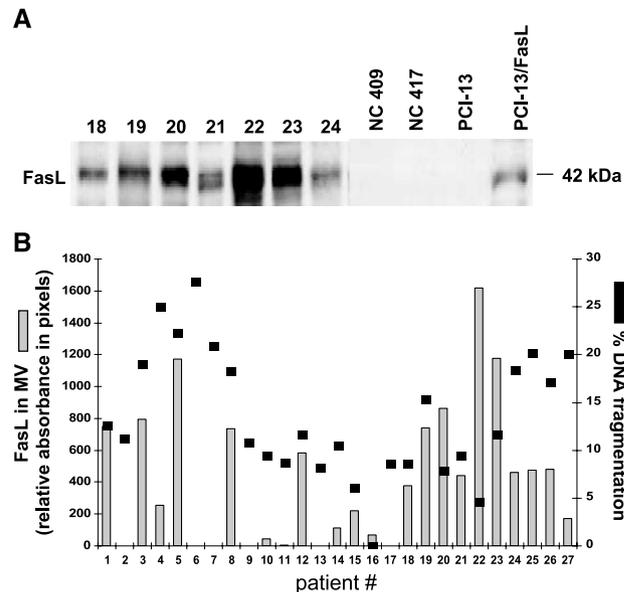

Fig. 4 FasL (42 kDa) is detected in microvesicle fractions obtained from sera of seven representative patients (patients 18-24) with OSCC (*A*). The FasL bands show variable densities in different microvesicle fractions tested in Western blots. microvesicle fractions obtained from all patients were tested in one experiment. Normal controls (*NC*) represent samples of normal controls. Microvesicle fractions obtained from supernatants of PCI-13 and PCI-13/FasL served as negative and positive controls for FasL, respectively. *B,* to measure biological activity of microvesicles, Jurkat cells were incubated with microvesicles for 24 hours as described in MATERIALS AND METHODS, and DNA fragmentation was evaluated in JAM assays. Expression of FasL in microvesicles was semiquantitatively determined from densitometric scans of Western blots as previously described (12).
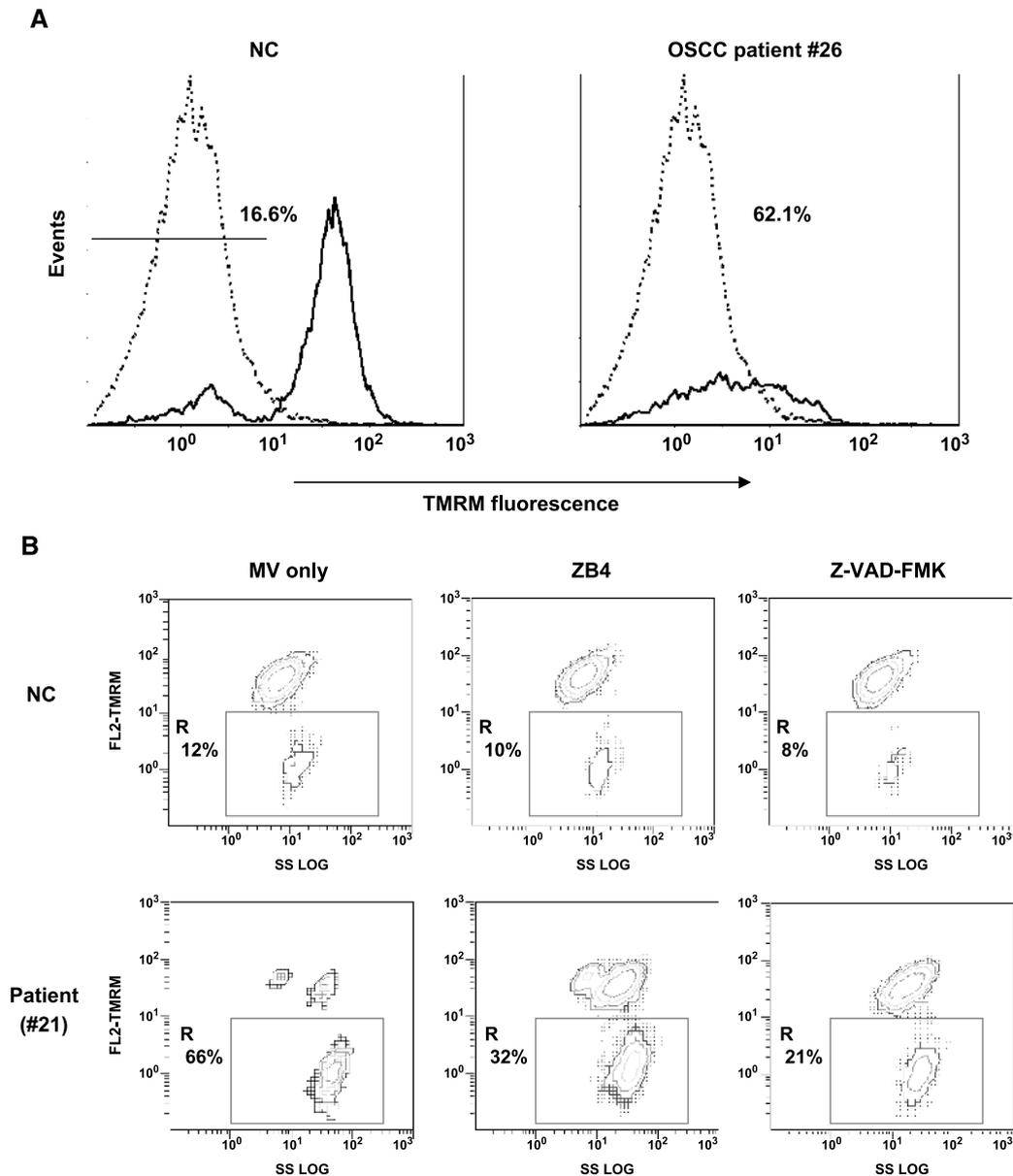
Fig. 5 A loss of the MMP potential in Jurkat cells coincubated with microvesicle fractions isolated from the patients' sera. Following 24 hours coincubation with microvesicles, flow cytometry was used to measure the MMP shift in target cells as described in MATERIALS AND METHODS. *A*, percent loss of the MMP is shown in a representative patient (patient 26) and a normal control (*NC*). *B*, inhibition of the MMP loss in Jurkat cells in the presence of anti-Fas (ZB4) antibody or Z-VAD-FMK. Microvesicles isolated from serum of patient 21 were used to induce the MMP loss.

$48,000 \pm 4,800$ (mean $\pm$ SD) MESF units of ζ protein. This difference was statistically significant at $P < 0.02$. Furthermore, when FasL expression in the various microvesicle fractions was correlated to ζ expression in Jurkat cells coincubated with these microvesicle fractions, a significant ($P = 0.04$) negative relationship was observed (Fig. 7B), because microvesicle fractions with a higher FasL content induced the greatest reduction in ζ expression.

Intracellular Mechanisms Responsible for Biological Activity of Microvesicle Fractions. We next determined that activation of the mitochondrial pathway upon coincubation of Jurkat cells with microvesicles resulted in cytochrome *c* release from mitochondria as measured by flow cytometry (Fig. 8).

Cytochrome *c* release was not present in controls (Jurkat cells incubated with an analogous microvesicle fraction of normal serum). The changes in cytochrome *c* release were inhibited in the presence of ZB4 antibodies, suggesting they were mediated by Fas/FasL interactions. Furthermore, cytochrome *c* release correlated with the percent loss of MMP ($P = 0.0001$, data not shown) in Jurkat cells incubated with microvesicles. These data are consistent with the conclusion that the microvesicle fractions induced both Fas-mediated and mitochondrial changes associated with apoptosis in Jurkat cells.

FasL Content of Microvesicles and Disease. Based on the level of FasL expression in immunoblots, the microvesicle fractions obtained from sera of patients with OSCC could be
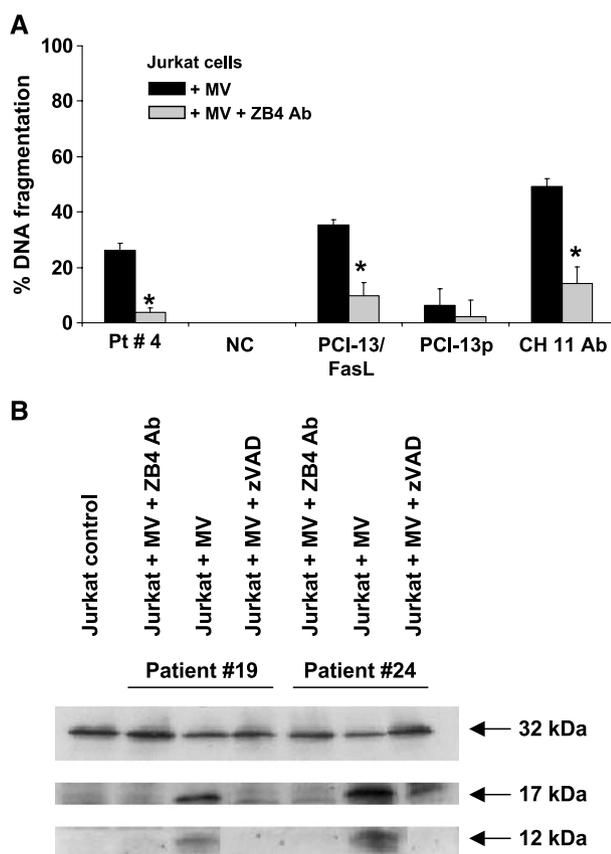
Fig. 6 Blocking of apoptosis (DNA fragmentation or caspase-3 cleavage) induced by microvesicles coincubated with Jurkat cells in the presence of anti-Fas ZB4 antibody or Z-VAD-FMK. *A,* microvesicles were obtained from serum of OSCC patient 4, normal controls (*NC*) or from supernatants of PCI-13 and PCI-13/FasL and coincubated with target cells. CH 11 mAb was also used to induce receptor-mediated apoptosis in Jurkat cells. In all cases, blocking of DNA fragmentation was significant but not complete; *, $P < 0.01$. *B,* caspase-3 prodomain (32 kDa) cleavage in Jurkat cells coincubated with microvesicles isolated from sera of patients 19 and 24. Note the absence of caspase-3 cleavage products (17 and 12 kDa) in immunoblots of Jurkat cells pretreated with anti-Fas ZB4 antibody or Z-VAD-FMK. *A* and *B* are from the same Western blot exposed to X-ray film for different periods and then photographed.

divided into "high-FasL" and "low-FasL" categories. The mean relative absorbance of FasL bands in all microvesicle fractions was calculated, and those with absorbance exceeding the mean were assigned to the high-FasL category, whereas those with absorbance lower than the mean, to the low-FasL category (Table 2). We initially determined that the loss of TCR-ζ chain was significantly greater in Jurkat cells incubated with the high-FasL microvesicles compared with the low-FasL microvesicles ($P < 0.007$). Thus, consistently high FasL microvesicles showed higher biological activity than "low-FasL" microvesicles. We then considered possible clinical implications of FasL+ microvesicles and their biological activity in patients with OSCC.

When the content of FasL in microvesicles and the disease status of the patients were compared, a significant association ($P = 0.0094$) emerged only for the T stage and the presence of high-FasL microvesicles (Table 3). Among patients with high-FasL microvesicles in their sera, 11 of 14 were T4 and 10 of 14 had

nodal disease as compared with 5 of 13 and 5 of 13, respectively, in the low-FasL microvesicle group (Table 3). The data suggest that sera of patients with stage IV disease and positive nodes often contain microvesicles with a high level of FasL. These patients have more advanced disease and poor prognosis. Thus, it is possible that the FasL content of microvesicles could have prognostic significance in patients with OSCC.

DISCUSSION

Microvesicles ranging in size between 60 and 100 nm and containing a membrane form (37-42 kDa) of FasL have been reported to be present in sera of patients with ovarian carcinoma (26, 28) and melanoma (29, 30). The microvesicles mediate biological activities, and Jurkat cells incubated in the presence of these fractions have been shown to undergo apoptosis and down-regulate expression of TCR-associated ζ chain (26–30). The
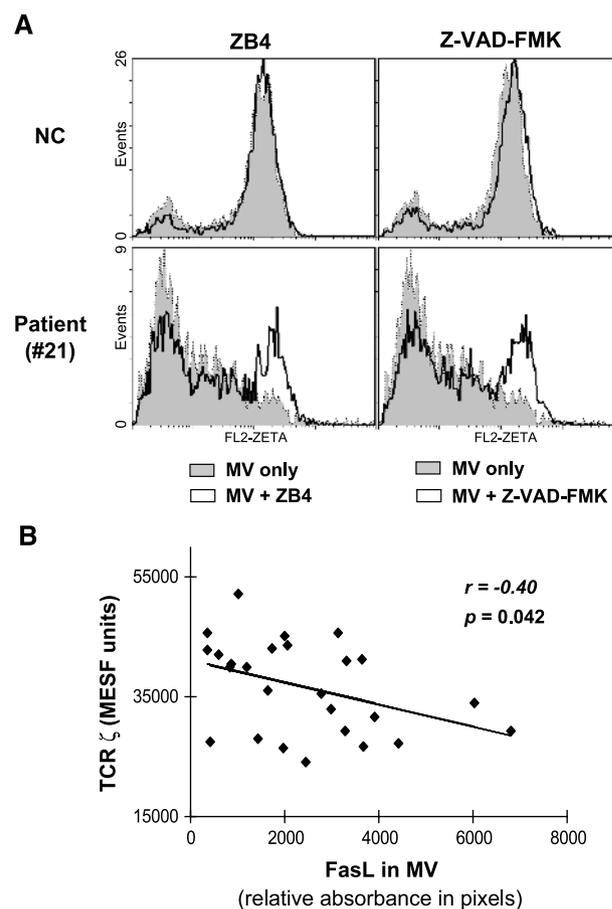

Fig. 7 Effects of microvesicles on expression of the ζ chain in Jurkat cells. *A,* down-regulation of ζ chain expression was observed in Jurkat cells coincubated for 24 hours with the microvesicle fraction obtained from serum of patient 21 relative to the equivalent microvesicle fraction obtained from normal control (*NC*) serum. Incubation of Jurkat cells in the presence of anti-Fas (ZB4) antibody or Z-VAD-FMK prevented the ζ chain down-regulation. *B,* a linear but reverse correlation between the FasL content of microvesicles obtained from all patients with OSCC and expression of ζ chain in Jurkat cells coincubated with these microvesicle fractions for 24 hours. The FasL content of microvesicles was determined by semiquantitative densitometry as described in MATERIALS AND METHODS.

presence of microvesicles in sera of patients with cancer, but only rarely in sera of normal donors, suggests that these structures are associated with disease. The origin of microvesicles is uncertain, although it has been hypothesized that microvesicles are tumor-derived in patients with cancer and active disease (26). Tumors have the ability to release or shed intact, vesicular portions of the plasma membrane, originally referred to as membrane fragments (20). The rate of shedding is significantly increased in most neoplastic as compared with normal cells (20), and the micro-vesicles accumulate in patients' sera. Previously, it has been shown that these shed microvesicles express biochemical markers present in the plasma membrane of the tumor (20, 21, 26). In primary tumor cultures established from the patients with microvesicles in their sera, similar microvesicular structures were showed in culture media (20). Based on this evidence, it is reasonable to assume that microvesicles originate from the tumor. A recent discovery in microvesicles of FasL in the membrane form provides an explanation for systemic effects exerted by tumors on T lymphocytes and resulting in apoptosis of circulating CD95+ T cells as reported by us earlier (5, 8).

Our discovery of FasL+ microvesicles in the sera of a small but immunologically and clinically well-characterized cohort of patients with OSCC is important for several reasons. First, it indicates that the presence of microvesicles in sera of patients with cancer is a generalized phenomenon not restricted by a tumor type. Although microvesicles with a variety of sizes have been recovered from sera or ascites of patients with ovarian carcinoma (26, 28), and recently also from sera of patients with melanoma (29), ours is the first report of FasL+ microvesicles in sera of patients with OSCC. Second, it offers a compelling rationale and a mechanistic explanation for apoptosis of CD95+ T lymphocytes observed in the peripheral

Table 2 The relative FasL content of microvesicles obtained from the patients' sera and the patients' clinicopathologic characteristics

	High FasL						Low FasL				
	Disease						Disease				
	Classification						Classification				
ID	T	N	M	Stage	FasL	ID	T	N	M	Stage	FasL
22	4	2	1	IV	1,618	4	4	2	0	IV	254
23	1	0	0	I	1,175	15	2	0	0	II	222
5	4	0	0	IV	1,174	27	2	1	0	III	169
20	4	0	0	IV	861	14	3	0	0	III	114
3	4	1	0	IV	795	16	3	0	0	III	69
1	4	2	0	IV	748	10	4	0	0	IV	44
19	4	2	0	IV	738	11	2	2	0	IV	4
8	1	3	0	IV	736	13	2	0	0	II	1
12	4	2	0	IV	582	6	1	0	0	I	0
26	4	1	0	IV	479	2	4	2	0	IV	0
25	1	1	0	III	477	7	4	2	1	IV	0
24	1	0	0	I	460	9	4	0	0	IV	0
21	4	2	1	IV	443	17	2	0	0	II	0
18	4	2	0	IV	376						

NOTE. Patients were divided into two groups based on relative FasL band intensities in the individual microvesicle fractions studied by Western immunoblots. Patients with microvesicles containing greater than mean FasL value are in the high-FasL group and those with microvesicles containing less than mean FasL value are in the low-FasL group.

Abbreviations: ID, patient identification number, which is identical in all the results shown in this article; FasL, results are relative band intensities (relative absorbance) in Western immunoblots.

circulation of the patients with OSCC (31). Our current results confirm that FasL+ microvesicles mediated apoptosis of activated normal T lymphocytes *ex vivo* and provide evidence for the involvement of the receptor and mitochondrial pathways in microvesicle-mediated death of these cells. Because a majority of circulating T cells express surface CD95 in patients with head and neck cancer (31), we surmise that microvesicles present in the patients' sera induce T cell apoptosis *in vivo*. Third, it offers an opportunity for exploring associations of FasL expression in microvesicles and their biological activities with the patients' disease. Thus, it is interesting to note that even in the small cohort of 27 OSCC patients, a high content of FasL in microvesicles was significantly correlated with the T stage, and that its association with nodal involvement was
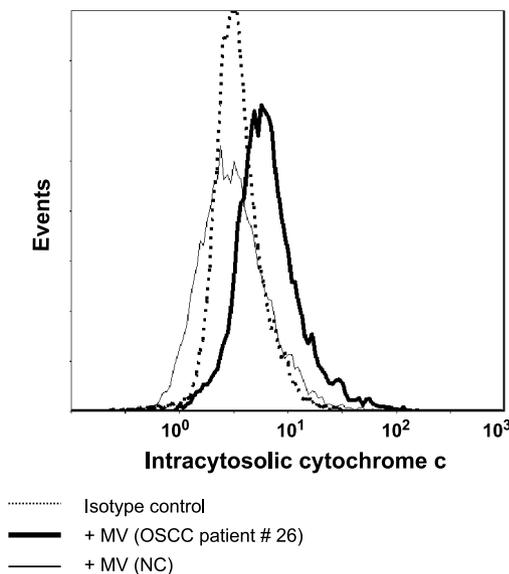

Fig. 8 The mitochondrial pathway is activated in Jurkat cells coincubated with microvesicle fractions obtained from sera of patients with OSCC. An increase in the intracytoplasmic content of cytochrome *c* is seen following coincubation of Jurkat cells with microvesicles obtained from patient 26 but not with the analogous microvesicle fraction obtained from a normal serum.

Table 3 Analysis of associations between the FasL content in microvesicles and the disease stage or nodal involvement in patients with OSCC

	FasL in microvesicles vs. T stage				FasL in microvesicles vs. N*				
	T1	T2	T3	T4	N0	N1	N2	N3	Total
High FasL	2	0	1	11	4	3	6	1	14
Low FasL	1	5	2	5	8	1	4	0	13
	P = 0.0094				*P* < 0.12				

NOTE. All 27 patients were divided into two groups based on the relative FasL content in microvesicle fractions as shown in Table 2. The high- and low-FasL groups were further subgrouped according to the T stage or nodal involvement, and the associations between them were analyzed as described in MATERIALS AND METHODS.

*The dotted line indicates that patients with no nodal disease were compared with all those with involved lymph nodes.

indicated although not statistically significant. Although preliminary, this finding suggests that microvesicle presence, their FasL content, and their biological activities might be of prognostic significance in OSCC, provided future studies confirm these associations.

Taking advantage of the available biologically active microvesicles, we analyzed molecular mechanisms responsible for their biological activity. Microvesicles induced apoptosis of Jurkat cells and T-cell blasts, as documented by DNA fragmentation, the MMP changes, caspase-3 cleavage, and cytochrome c release. The data are consistent with the conclusion that not only the receptor but also the mitochondrial pathway of apoptosis was induced in T cells incubated with the microvesicle. As shown previously with microvesicles isolated from sera of patients with ovarian carcinoma (26), down-regulation of ζ expression was a consistent finding in Jurkat cells incubated with the microvesicles of patients with OSCC. Furthermore, down-regulation of ζ expression showed significant inverse correlation with the FasL content of the microvesicles incubated with Jurkat cells. Together, these data clearly indicate that the molecular components of both apoptotic pathways are engaged in microvesicle-induced death of activated T cells.

Whereas microvesicles from all 27 patients mediated apoptosis of Jurkat cells, in 6 of 27 cases, FasL-microvesicle fractions showed apoptogenic activity. These observations indicate that microvesicle-associated molecules other than FasL contribute to apoptosis of activated T cells. Blocking with anti-Fas antibody or Z-VAD-FMK significantly decreased, but in most cases did not completely inhibit, proapoptotic activity of the microvesicle fractions known to contain FasL. It has been previously suggested that HLA class I molecules might mediate apoptosis of CD8+ T cells by mechanisms distinct from the Fas/FasL pathway (32). More recent experiments in one of our laboratories (DDT) indicated that microvesicles containing a ζ inhibitory protein referred to as ZIP (33) also suppressed JAK3 expression in T cells (34). Because a loss of JAK3 is linked to apoptosis induction via up-regulation of Bax levels, it is possible that microvesicle-associated apoptosis is induced by this mechanism. We recently reported that Bax expression is elevated in circulating T cells of patients with head and neck cancer relative to normal control T cells, and that the Bax up-regulation in the patients' T cells significantly correlates with the percent of Annexin V binding to these same T cells (31). These correlative data strongly imply that *in vivo*, microvesicles and their FasL content play a key role in regulating apoptosis of circulating CD95+ T cells. Because we have previously shown that this apoptosis preferentially targets tumor-specific effector cells (11),[4] the mechanism of T-cell demise via interactions with microvesicles may be critically important for antitumor defense.

Many questions remain unanswered with regard to microvesicles, including their possible origin from tumor cells, prevalence in body fluids of patients, and physiologic as well as therapeutic significance. Nevertheless, the fact that FasL+

microvesicles are able to induce death in activated T cells provides a strong rationale for further exploration of their role in tumor escape from the immune system.

ACKNOWLEDGEMENTS

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Fas Ligand−Positive Membranous Vesicles Isolated from Sera of Patients with Oral Cancer Induce Apoptosis of Activated T Lymphocytes

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