

The Fas Counterattack In Vivo: Apoptotic Depletion of Tumor-Infiltrating Lymphocytes Associated with Fas Ligand Expression by Human Esophageal Carcinoma¹

Michael W. Bennett,^{2*} Joe O'Connell,^{2*} Gerald C. O'Sullivan,[†] Ciaran Brady,*
Desmond Roche,* J. Kevin Collins,* and Fergus Shanahan^{3*}

Various cancer cell lines express Fas ligand (FasL) and can kill lymphoid cells by Fas-mediated apoptosis in vitro. FasL expression has been demonstrated in several human malignancies in vivo. We sought to determine whether human esophageal carcinomas express FasL, and whether FasL expression is associated with increased apoptosis of tumor-infiltrating lymphocytes (TIL) in vivo, thereby contributing to the immune privilege of the tumor. Using in situ hybridization and immunohistochemistry, respectively, FasL mRNA and protein were colocalized to neoplastic esophageal epithelial cells in all esophageal carcinomas (squamous, $n = 6$; adenocarcinoma, $n = 2$). The extent of FasL expression was variable, with both FasL-positive and FasL-negative neoplastic regions occurring within tumors. TIL were detected by immunohistochemical staining for the leukocyte common Ag, CD45. FasL expression was associated with a mean fourfold depletion of TIL when compared with FasL-negative areas within the same tumors (range 1.6- to 12-fold, $n = 6$, $p < 0.05$). Cell death of TIL was detected by dual staining of CD45 (immunohistochemistry) and DNA strand breaks (TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling). There was a mean twofold increase in detectable cell death among TIL in FasL-positive areas compared with FasL-negative areas (range 1.6- to 2.4-fold, $n = 6$, $p < 0.05$). In conclusion, we demonstrate a statistically significant, quantitative reduction of TIL concomitant with significantly increased TIL apoptosis within FasL-expressing areas of esophageal tumors. Our findings suggest Fas-mediated apoptotic depletion of TIL in response to FasL expression by esophageal cancers, and provide the first direct, quantitative evidence to support the Fas counterattack as a mechanism of immune privilege in vivo in human cancer. *The Journal of Immunology*, 1998, 160: 5669–5675.

Esophageal carcinoma has a 5-yr survival rate of less than 4%, and the incidence of this disease is increasing in the developed world (1). While the introduction of multimodal therapy has produced some gain in survival, the prognosis for esophageal cancer still ranks with those of the most aggressive cancers (2, 3). The discovery of effective immune-based modalities of therapy will depend on better understanding of how esophageal cancer evades the host's immune system. While esophageal tumors have been shown to cause local immune suppression (4), and to express soluble immunosuppressive factors (5), none of the mechanisms of immune evasion of esophageal cancer has been elucidated at the molecular level.

Antigenically distinct cancers must overcome the host's antitumor immunity to develop. Although a number of classical mechanisms have been proposed to account for tumor evasion of the immune system (these include defective Ag presentation, interference with tumor/T cell interaction, and production of immunosup-

pressive factors), new evidence points to expression of Fas ligand (FasL)⁴ as a possible mediator of tumor immune privilege (6). There are three basic findings that support this theory: first, FasL, by inducing FasR-mediated apoptosis of activated leukocytes, serves a number of negative regulatory functions in immunity, including 1) tolerance acquisition (7), 2) immune response termination (8, 9), 3) maintaining immune privilege in certain organs (10, 11), and 4) immunologic protection of allografts in animal transplantation experiments (12–15). Second, various cancer cell lines express FasL and kill lymphoid cells by Fas-mediated apoptosis in vitro (16–20), and third, various human tumors have been shown to express FasL in vivo (17–21). There is as yet a paucity of direct evidence to confirm that FasL-expressing human cancers mount an effective Fas counterattack against Fas-sensitive antitumor immunocytes in vivo.

The aims of this study were to determine 1) whether human esophageal carcinomas express FasL, and 2) whether FasL expression is associated with increased apoptosis of tumor-infiltrating lymphocytes (TIL), thereby contributing to the immune privilege of the tumor. Using human esophageal carcinoma sections, we investigated FasL expression at the mRNA and protein levels. We quantified and compared both the number and cell death rate of CD45-positive TIL in FasL-positive vs FasL-negative tumor regions.

*Department of Medicine, Cork University Hospital, and [†]Department of Surgery, Mercy Hospital, National University of Ireland, Cork, Ireland

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² M.W.B. and J.O. contributed equally and share first authorship.

³ Address correspondence and reprint requests to Dr. Fergus Shanahan, Department of Medicine, Clinical Sciences Building, University Hospital, Cork, Ireland. E-mail address: Fshanahan@irucevax.ucc.ie

⁴ Abbreviations used in this paper: FasL, Fas ligand; FasR, Fas receptor; TIL, tumor-infiltrating lymphocyte; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

Materials and Methods

Tissues

Human esophageal carcinomas (squamous, $n = 6$; adenocarcinoma, $n = 2$) were collected following surgical resection of esophageal carcinomas ($n = 7$) or investigative endoscopic biopsy ($n = 1$) at Mercy Hospital (Cork, Ireland), following a protocol approved by University Teaching Hospital's Ethics Committee. None of the patients had received chemo-, radio-, or immunotherapy before tissue collection.

Immunohistochemical detection of FasL and FasR

Paraffin-embedded sections of esophageal tumors were deparaffinized in xylene and rehydrated before analysis. Slides were washed twice for 5 min in a wash buffer containing 50 mM Tris-Cl, pH 7.6, 50 mM NaCl, and 0.001% saponin, and endogenous peroxidase was quenched with 3% hydrogen peroxide in methanol for 5 min. Slides were washed as before, except that the wash buffer for this and all subsequent steps included 1% normal goat serum. Slides were then blocked for 1 h in wash buffer containing 5% normal goat serum. Slides were washed and incubated overnight at 4°C with a rabbit polyclonal anti-human FasL-specific IgG (Santa Cruz Biotechnology, Santa Cruz, CA) at $0.2 \mu\text{g ml}^{-1}$ in wash buffer. Ab binding was localized using a biotinylated secondary Ab, avidin-conjugated horseradish peroxidase, and diaminobenzidine substrate, contained within the Vectastain ABC detection kit (Vector Laboratories, Burlingame, CA). The immunizing peptide (FasL, N-terminal amino acids 2–19; Santa Cruz Biotechnology) was included at $2 \mu\text{g ml}^{-1}$ during primary Ab incubation in control staining. FasR was immunohistochemically detected using the same protocol, except that a rabbit polyclonal anti-human FasR-specific IgG (Santa Cruz Biotechnology) was used, along with its corresponding immunizing peptide (FasR, amino acids 316–335; Santa Cruz Biotechnology) in control staining. Slides were counterstained with hematoxylin.

Generation of a FasL-specific RNA probe (riboprobe)

A digoxigenin-labeled RNA hybridization probe (344 bp) was generated corresponding to codons 96–210 of the human FasL cDNA sequence. The riboprobe was synthesized by *in vitro* transcription using digoxigenin-11-UTP and T7 RNA polymerase (Boehringer Mannheim, Mannheim, Germany). Template for the *in vitro* transcription reaction was generated by PCR amplification of a fragment (codons 96–210) of FasL cDNA using a proofreading thermostable polymerase (UITma DNA polymerase; Perkin-Elmer, Norwalk, CT) and an antisense primer to which a T7 promoter sequence was added.

RNA was isolated from FasL-expressing cells by homogenization in guanidine thiocyanate (Sigma, St. Louis, MO), followed by phenol extraction and ethanol precipitation. cDNA was synthesized using the AMV reverse transcriptase (Promega, Madison, WI) and random hexanucleotide primers (Boehringer Mannheim).

PCR was performed on the cDNA using the following sense and antisense primers, respectively: FasL, GGATTGGCCTGGGGATGTTTCA and [pT7]-TTGTGGCTCAGGGGCAGGTTGTTG.

PCR primers were designed using the DNASTAR Lasergene Primers-elect program (DNASTar, Madison, WI). Primer pairs were chosen to span introns in the FasL genomic sequence, thus ensuring mRNA-specific amplification. Primers were selected that showed no significant homology to any other genes in the EMBL DNA sequence database.

Thermal cycling (40 cycles) was as follows: denaturation at 96°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 3 min. Primers were used at a final concentration of 0.1 μM each, dNTPs at 50 μM , and MgCl_2 at 1.5 mM. One unit of UITma DNA polymerase was used per 50- μl reaction. PCR product specificity was confirmed by restriction mapping.

Using this PCR-amplified FasL cDNA fragment as template, a riboprobe was synthesized by *in vitro* transcription using digoxigenin-11-UTP and T7 RNA polymerase (Boehringer Mannheim), according to the manufacturer's instructions. The nucleotide sequence of the FasL probe showed no significant homology to any other sequence in the EMBL DNA sequence database. An unlabeled riboprobe was also synthesized for use in competitive control hybridizations.

Localization of FasL mRNA expression by *in situ* hybridization

In situ hybridization was performed on paraffin-embedded human esophageal tumor sections (4 μm thick), mounted on aminopropylethoxysilane (APES)-treated slides. Prehybridization treatments involved washing twice 5 min each in 1) PBS, 2) PBS and 0.1 M glycine, 3) PBS and 0.3% Triton X-100, and 4) PBS again. Sections were digested for 30 min at 37°C with proteinase K (10 $\mu\text{g/ml}$ in 100 mM Tris-HCl, 50 mM EDTA, pH 8), fixed

for 5 min at 4°C in 4% paraformaldehyde and PBS, and then acetylated twice for 5 min in fresh 0.25% acetic anhydride and 0.1 M triethanolamine (pH 8). Sections were incubated at 37°C for 10 min in a prehybridization buffer consisting of 50% deionized formamide in 4 \times SSC. Hybridization was performed at 42°C overnight in hybridization buffer (50% formamide, 10% dextran sulfate, 1 \times Denhardt's reagent, 4 \times SSC, 10 mM DTT, 500 $\mu\text{g/ml}$ yeast tRNA, and 1 mg/ml heat-denatured herring sperm DNA) containing 1 ng/ μl digoxigenin-labeled riboprobe. After hybridization, tissues were washed with increasing stringency to 0.1 \times SSC at 37°C. Hybridized probe was detected immunologically using alkaline phosphatase-conjugated sheep anti-digoxigenin Ab (Boehringer Mannheim) and visualized with nitroblue tetrazololium/5-bromo-4-chloro-3-indolyl phosphates (NBT/BCIP; purple/black precipitating product). Control slides involved competitive inhibition of hybridization by adding a 10-fold excess of unlabeled riboprobe to the digoxigenin-labeled riboprobe before hybridization. This resulted in a marked reduction of signal intensity, thus confirming the specificity of hybridization.

CD45 (leukocyte common Ag) staining

To identify tumor-infiltrating immunocytes, CD45 (leukocyte common Ag) staining was performed on paraffin-embedded esophageal tumor sections. Following deparaffinization and rehydration, sections were pretreated by microwave irradiation in 0.01 M citrate buffer for 5 min at 370W. The sections were cooled rapidly by immersing in 0.1 M PBS. The slides were then incubated with a mouse anti-human CD45 monoclonal IgG (Dako, Carpinteria, CA) at a dilution of 1/70 for 1 h. All incubations were conducted at room temperature. Next the slides were washed for 5 min in 0.1 M Tris-buffered saline (TBS), which was used for all washes. Following incubation with a secondary rabbit anti-mouse IgG (Dako) at a dilution of 1/25 for 30 min, washing was repeated. Sections were then incubated for 30 min with alkaline phosphatase-conjugated anti-alkaline phosphatase (APAAP) complex (Dako), at a dilution of 1/50. Following washing, the secondary Ab and APAAP complex incubations were repeated (10 min each), to enhance staining. Sections were then incubated for 10 min with an alkaline phosphatase substrate solution (fast blue; Sigma). CD45-positive immunocytes appeared blue when viewed under light microscopy, and were almost exclusively of lymphoid morphology (i.e., TIL).

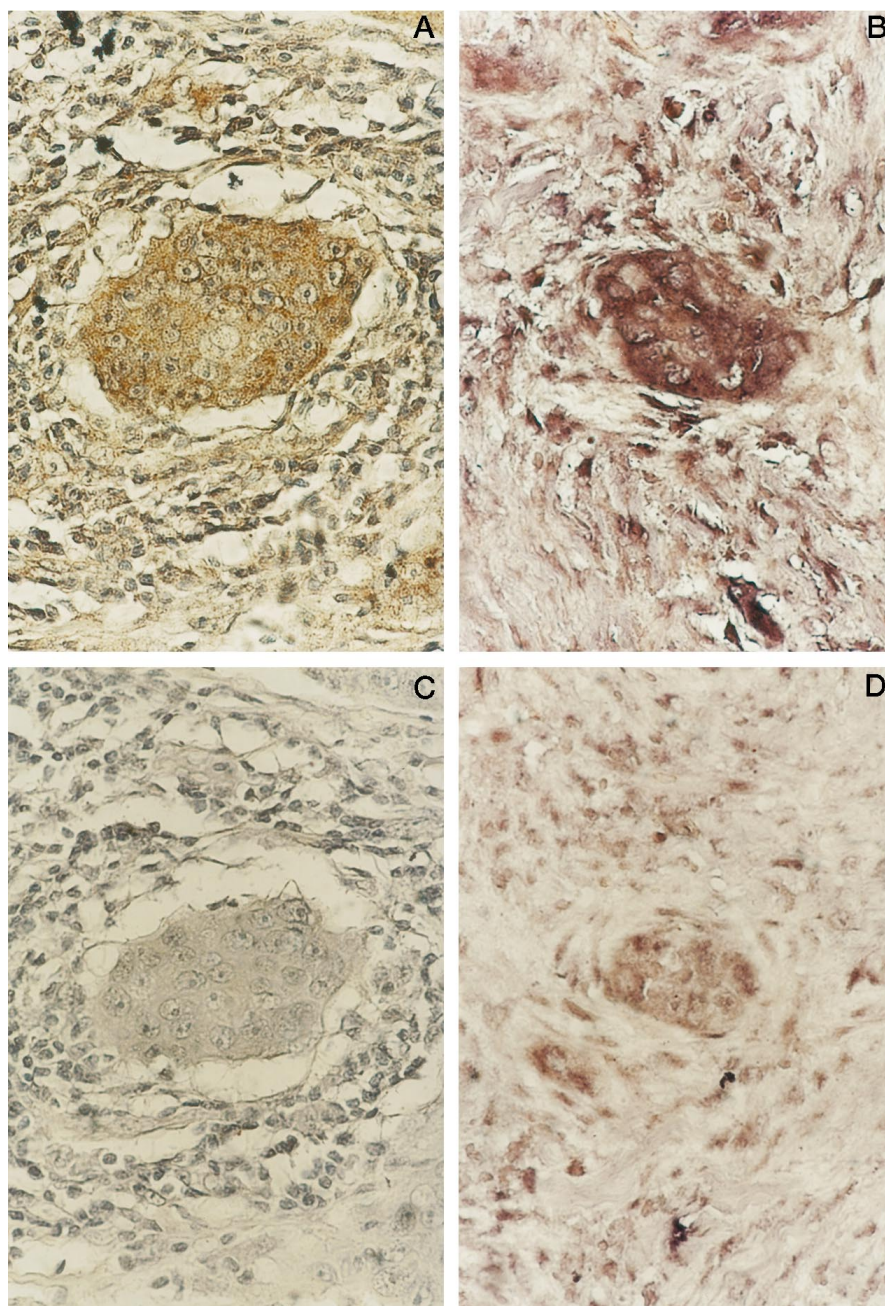
Immunocyte cell death detection by CD45/TUNEL dual staining

Cell death was detected *in situ* in resected esophageal tumors by enzymatic labeling of DNA strand breaks using TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) (Boehringer Mannheim), according to the manufacturer's instructions. CD45/TUNEL dual staining was performed to allow apoptotic tumor-infiltrating immunocytes to be identified and enumerated. CD45 staining was performed first on esophageal tumor sections, as described. Following treatment with proteinase K (20 $\mu\text{g/ml}$ in 10 mM Tris-Cl, pH 7.6) for 30 min, sections were washed in PBS and endogenous peroxidase activity was blocked in 3% hydrogen peroxide in methanol for 30 min. Next, the slides were washed. This and all subsequent washes were in PBS. The sections were treated with permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 15 min. After washing, the labeling reaction was performed using a solution containing terminal deoxynucleotidyl transferase, its buffer, and fluorescein-dUTP. During this step, slides were coverslipped and incubated at 37°C for 60 min in a humidity chamber. Terminal deoxynucleotidyl transferase was omitted from negative control slides, which were included in each run. To localize cells containing labeled DNA strand breaks, sections were washed and incubated with a sheep anti-fluorescein Ab Fab fragment conjugated with horseradish peroxidase at 37°C in a humidity chamber for 30 min. Following washing, TUNEL-positive color development (brown) was obtained by incubating the sections with a diaminobenzidine substrate solution for 15 min. When viewed under light microscopy, CD45 single-positive cells stained blue, while CD45/TUNEL dual-positive cells appeared with brown nuclear staining and blue cytoplasmic/cell surface staining.

Cell counting and labeling indices

To quantify CD45-positive TIL infiltration of FasL-positive vs FasL-negative tumor regions, stained tumor sections were analyzed under light microscopy, as follows. FasL-positive and FasL-negative areas were located on a FasL-stained esophageal tumor section by one investigator. Tumor areas staining with high intensity similar to or greater than that of nearby FasL-positive lymphoid cells were regarded as strongly positive. Tumor areas staining with weak intensity close to the background staining observed in the corresponding peptide control slide were regarded as negligible/negative. A reference slide was used to assure consistent selection of FasL strongly positive or negligible/negative tumor areas. Areas of tumor

FIGURE 1. Human esophageal carcinomas express Fas ligand. Immunoperoxidase staining using a FasL-specific rabbit polyclonal IgG was performed on paraffin-embedded esophageal carcinoma sections. Slides were counterstained with hematoxylin. *A*, FasL-positive immunohistochemical staining (brown) is shown in a representative squamous esophageal tumor island. *C*, As a control for specificity of Ab detection, the FasL-immunizing peptide was included during primary Ab incubation. Competitive inhibition of staining by the immunizing peptide confirms FasL specificity. Tumor sections sequential to those shown in *A* and *C* were used to detect FasL mRNA by in situ hybridization, using a digoxigenin-labeled FasL-specific riboprobe. *B*, Positive purple hybridization signal was obtained from the same tumor island that stained immunohistochemically positive for FasL. *D*, In a control hybridization, a 10-fold excess of unlabeled probe caused direct, competitive displacement of the labeled probe, confirming the specificity of hybridization. These results are representative of six squamous and two adenocarcinomas of the esophagus.



ulceration were excluded. A consecutive, CD45-stained slide from the same tumor was superimposed on the FasL-stained slide. Using histologic landmarks, the corresponding FasL-positive and FasL-negative areas were located on this slide. The FasL-stained section was removed, and a second investigator, blinded as to the local status of FasL expression, counted the number of CD45-positive TIL per 2000 total nuclei in the area located by the first investigator. A similar approach was used to enumerate CD45/TUNEL dual-positive TIL within FasL-positive vs FasL-negative areas of esophageal tumors. For each type of staining, all slides were stained in a single experiment.

Labeling indices for TIL infiltration were expressed as the percentage of CD45-positive cells per 2000 total nuclei counted. Labeling indices for TIL apoptosis were expressed as the percentage of CD45/TUNEL dual-positive cells per 500 total CD45-positive cells counted.

Results

Esophageal carcinomas express FasL

Using a FasL-specific rabbit polyclonal IgG, FasL-positive immunohistochemical staining was detected in all esophageal tumors

(squamous, $n = 6$; adenocarcinoma, $n = 2$) (Fig. 1). FasL-binding specificity was confirmed since staining was inhibited by inclusion of the immunizing peptide as a competitive inhibitor before primary Ab incubation. The staining within the tumors varied from strongly positive to negligible/negative. Although FasL-positive and FasL-negative areas usually occurred within the same tumor, in all specimens at least 70% (range 70–100%, $n = 8$) of the tumor tissue stained strongly positive. FasL expression was confirmed by detection of FasL mRNA in consecutive tumor sections using in situ hybridization. FasL mRNA detection corresponded closely with immunohistochemical staining for FasL protein (Fig. 1). Hybridization was specific, as a 10-fold excess of unlabeled riboprobe competitively inhibited hybridization of labeled riboprobe in control sections.

Using immunohistochemistry, all of the esophageal tumors were found to express FasR essentially homogeneously throughout the tumor mass. Specificity of detection was confirmed since staining

was inhibited by inclusion of the FasR-immunizing peptide as a competitive inhibitor before primary Ab incubation. Coexpression of FasL and FasR, which occurred over large areas of the tumors, did not result in an enhanced rate of tumor cell apoptosis, as assessed by TUNEL (not shown).

Reduced TIL infiltration of FasL-expressing esophageal tumor islands

All eight esophageal tumors showed infiltrates of cells staining immunohistochemically positive for CD45 (leukocyte common Ag), which were almost exclusively of lymphoid morphology, permitting identification of TIL (Fig. 2). Tonsil sections included as positive controls stained strongly, and omission of the primary Ab abolished staining (data not shown). FasL expression in the eye (10) and testis (11) has been shown to preclude infiltration of these organs by proinflammatory, Fas-sensitive leukocytes, thereby maintaining a state of immune privilege at these locations. To evaluate whether FasL expression by esophageal tumor cells limited immune effector cell infiltration of the tumor, TIL numbers infiltrating FasL-positive vs FasL-negative tumor areas were enumerated and compared (Table I).

Of the eight esophageal tumors examined, one squamous carcinoma expressed FasL over 100% of the tumor area, so comparison between FasL-positive vs FasL-negative areas was not possible. One biopsied adenocarcinoma did not contain sufficient tumor tissue to allow significant comparison between FasL-positive vs FasL-negative areas. In all six esophageal tumor sections that were amenable to analysis, a significant reduction of tumor infiltration by TIL was observed in FasL-expressing tumor regions (Fig. 2). FasL expression by the tumors was associated with a statistically significant, mean fourfold reduction in TIL (range 1.6- to 12-fold, $n = 6$, $p < 0.05$; Wilcoxon Signed Ranks) when compared with FasL-negative areas within the same tumors. Thus, the expression of FasL by esophageal cancer cells appears to have a dramatic inhibitory effect on TIL infiltration of the tumor.

Increased cell death of TIL associated with FasL expression by esophageal cancers

FasL expressed in the eye (10) and testis (11) causes apoptosis of infiltrating Fas-sensitive immune effector cells, precluding proinflammatory immune responses and thus maintaining immune privilege in these organs. We wished to investigate whether the diminished TIL infiltration observed in FasL-expressing esophageal tumor tissue was a result of apoptotic depletion. CD45/TUNEL dual staining was used to identify apoptotic TIL in the esophageal cancer sections (Fig. 2). CD45-positive TIL were first stained immunohistochemically (blue), followed by detection of DNA strand breaks on the same tumor section using TUNEL (brown). Dual-stained, apoptotic TIL therefore appeared as cells with brown TUNEL-positive, condensed, or fragmented nuclei surrounded by blue cytoplasmic/cell surface CD45 staining. TIL in immediate contact with tumor cells were examined, and the number that was CD45/TUNEL dual positive was expressed as a percentage per 500 total CD45-positive TIL counted in the same area. Levels of apoptotic TIL were quantified in FasL-positive vs FasL-negative areas of the same tumor (Table II).

A statistically significant, mean twofold increase in TUNEL-positive cell death of CD45-positive TIL was observed in FasL-expressing areas of the esophageal tumors relative to FasL-negative areas (range 1.6- to 2.4-fold, $n = 6$, $p < 0.05$; Wilcoxon signed ranks). As a comparative control, the cell death levels of extratumoral leukocytes in the surrounding stroma were also quantified (data not shown). In the stroma, the levels of TUNEL-positive immunocytes (mean 17.1%, $n = 4$) were similar or slightly

less than those in FasL-negative areas of tumor (mean 23.6%, $n = 6$). Therefore, infiltration of stromal lymphocytes into FasL-negative tumor regions resulted in only a marginal increase in cell death. The twofold increase in TIL apoptosis associated with FasL-expressing tumor tissue suggests that Fas-mediated apoptosis of TIL triggered by tumor-expressed FasL may contribute to the depletion of CD45-positive TIL observed in these tumor regions.

Discussion

The results show that tumors from all eight esophageal carcinoma patients studied expressed FasL, a potent mediator of immunologic tolerance and privilege, as well as immune response termination. Cell lines derived from tumors of diverse origin, including colon carcinoma (16), melanoma (17), hepatocellular carcinoma (18), lung cancer (19), and astrocytoma (20), have been shown previously to express biologically active FasL, suggesting that tumor-expressed FasL is invariably functional. FasL-expressing cell lines of all origins were shown to induce Fas-mediated apoptosis of cocultured Fas-sensitive lymphoid target cells *in vitro*, supporting a Fas counterattack hypothesis for tumor immune escape (16, 6). This proposes that tumors, by expressing FasL, may induce apoptosis of Fas-sensitive antitumor immune effector cells, thus rendering the tumor immunologically privileged. In all cases, tumors and cell lines themselves exhibit resistance to FasL-mediated apoptosis due to various acquired defects in Fas signal transduction (6).

Although FasL expression has been reported in a number of human malignancies *in vivo*, including melanoma (17), hepatocellular carcinoma (18), lung cancer (19), astrocytoma (20), primary tumors (O'Connell et al., manuscript submitted), and liver metastases (21) of colon adenocarcinomas, there is a paucity of direct evidence to confirm that tumor-expressed FasL mediates apoptosis of TIL *in vivo* in human cancer.

In the present study, the effect of esophageal tumor expression of FasL on effective TIL infiltration was analyzed directly. Both the number of TIL and the level of apoptosis among TIL were quantified in FasL-expressing vs FasL-negative regions of tumors. While more than 70% of neoplastic tissue expressed FasL in all of the esophageal tumors, there were sufficient FasL-negative areas to permit significant comparison between FasL-positive and FasL-negative regions in close proximity within the same tumor. This paired analysis within individual tumors minimized the possible contribution of differences other than FasL expression to our results. Factors such as tumor immunogenicity, patient immune competence, tumor stage, and other factors were therefore relatively controlled. In addition, a recent study demonstrated that in esophageal carcinoma, the total number and activation of tumor-infiltrating T cells are independent of such factors as tumor stage and expression of molecules usually stimulatory to immune responses, including MHC or ICAM-1 (22).

A striking reduction (a mean fourfold) in CD45-positive TIL was observed in FasL-positive areas of tumor relative to FasL-negative regions. Of those CD45-positive TIL that resided within FasL-positive tumor islands, the frequency of cell death detected *in situ* by TUNEL was a mean twofold greater than that of CD45-positive cells within FasL-negative tumor zones. The cell death rate of TIL detected in FasL-negative tumor areas was only marginally greater than that of extratumoral, stromal leukocytes. These findings suggest that FasL expressed by esophageal tumor cells *in vivo* causes enhanced apoptosis of leukocytes that infiltrate the tumor, resulting in significant depletion of TIL. The clinical consequence of TIL depletion is suggested by a recent study of colorectal carcinoma, in which lower levels of TIL were associated

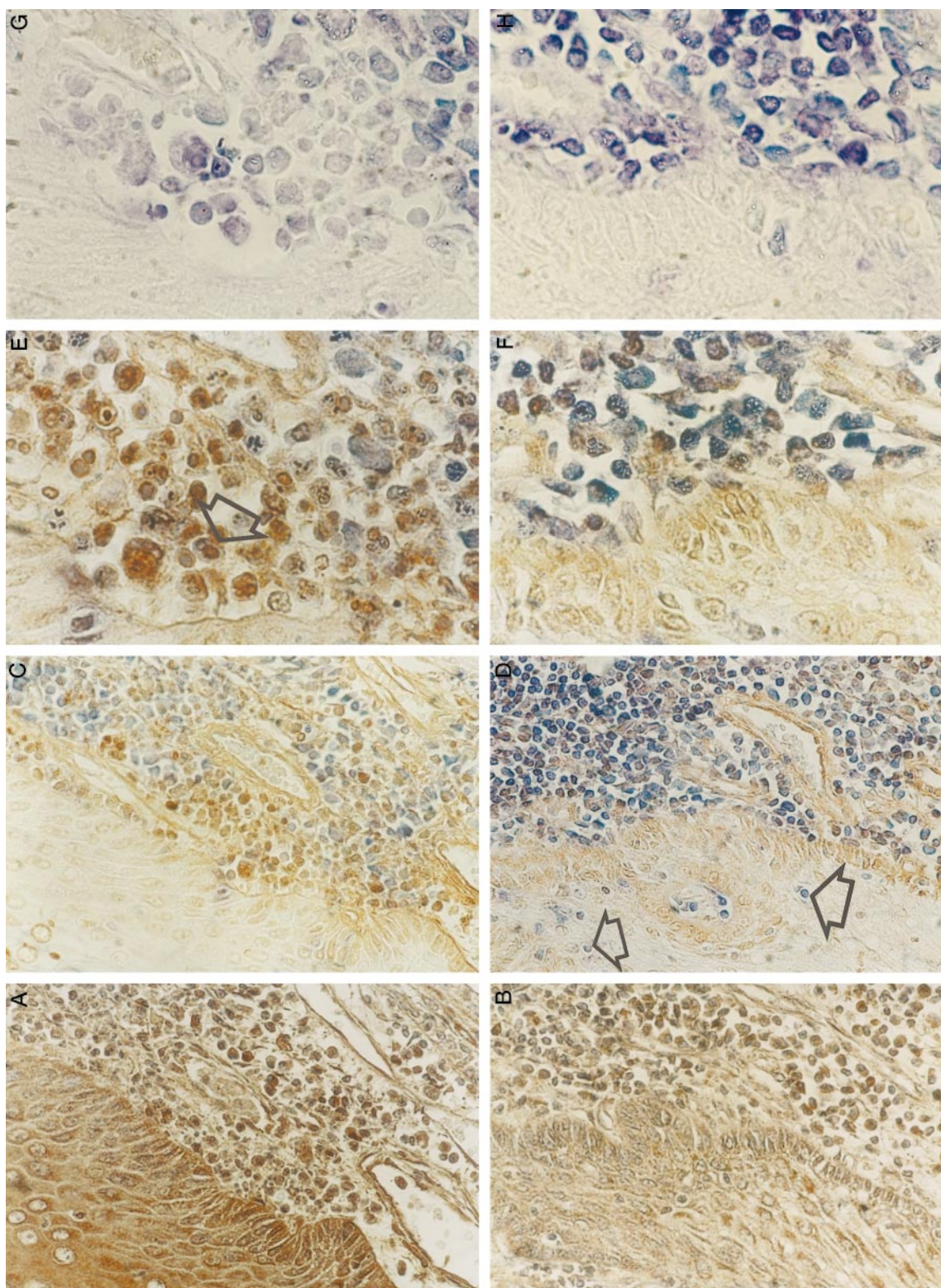


FIGURE 2. Apoptotic depletion of TIL in FasL-expressing regions of esophageal carcinoma. Immunoperoxidase staining (brown) using a FasL-specific Ab allowed identification of FasL-expressing (A) and FasL-negative (B) tumor regions in close proximity within individual esophageal carcinomas. FasL expression was confirmed by in situ hybridization (Fig. 1). On a consecutive tumor section, apoptotic TIL were identified by CD45-TUNEL dual staining. C, A high proportion of TIL in contact with the FasL-expressing tumor tissue shown in A is apoptotic, exhibiting brown TUNEL-positive nuclear staining with blue CD45-positive cytoplasmic/cell surface staining. E, At higher magnification, condensation and fragmentation of TUNEL-positive nuclei can be seen against a blue, CD45-staining cytoplasmic background (arrow), confirming apoptotic TIL. D, Apoptosis is less frequent among TIL in contact with the FasL-negative tumor region shown in B. TUNEL staining is largely absent from these blue, CD45-stained TIL, seen at higher magnification in F. There is a striking lack of infiltration of the FasL-expressing tumor region by viable TIL (C) compared with the FasL-negative tumor region (D), which shows infiltration by blue, CD45 single-positive, nonapoptotic TIL (arrows). G and H show another consecutive section from the same tumor stained for CD45 alone. CD45 single staining (blue) of cells observed in the same areas as E and F, respectively, confirms the identity of TIL. These results are representative of five squamous and one adenocarcinoma of the esophagus. Quantitative results for both TIL infiltration and apoptosis in FasL-positive vs FasL-negative tumor regions are shown in Tables I and II, respectively.

Table I. Depletion of TIL in FasL-positive versus FasL-negative regions of esophageal carcinomas

Tumor Specimen	FasL-positive Tumor Region (% TIL) ^a	FasL-negative Tumor Region (% TIL) ^a
1	1.61	2.78
2	1.91	18.60
3	15.00	43.00
4	3.00	16.70
5	2.27	27.40
6	1.34	7.12
Mean	4.19	19.27

^a The % TIL was calculated as the % CD45-positive cells per 2000 total nuclei counted within FasL-positive/-negative tumor regions.

with poorer prognosis (23). It will be interesting to determine whether the dysfunctional, bystander TIL frequently observed in tumors (24) have a defective Fas pathway, rendering them resistant to FasL-mediated apoptosis.

These results are consistent with the substantial body of evidence that supports a role for FasL in mediating immune privilege in the eye (10), in contributing to immunologic tolerance in the periphery (7), in downsizing immune responses (8, 9), and in supporting allograft survival (12–15). All of these roles involve FasL-mediated apoptotic depletion of leukocytes. A recent study involving allograft transplantation of murine tumor cells stably transfected with the FasL gene showed that FasL caused local suppression of both humoral and cellular allograft-specific immune responses (15). The latter role is contradicted by the finding that in certain cases, murine allografts genetically manipulated to express FasL caused neutrophil infiltration and allograft destruction (25–27). It is unclear why FasL appeared to have such contradictory effects in these particular experimental settings (28). Recently, adenovirus-mediated overexpression of FasL in mouse ankle joints ameliorated collagen-induced arthritis, providing powerful evidence for an antiinflammatory role for FasL in vivo (29). No significant neutrophil infiltration was observed in any of the esophageal tumors examined, and our findings implicate FasL as a mediator of immune privilege in the context of spontaneous human cancers.

In all of the esophageal carcinomas, coexpression of FasL and FasR occurred throughout large areas of the tumor, without apparent enhancement of tumor cell apoptotic rate. This is similar to colon cancer cells (16) and lymphoid malignancies (30), which also coexpress FasL and FasR without apoptotic tumor cell suicide/fratricide. Hence, the esophageal tumor cells appear to be relatively resistant not only to FasL expressed by the tumor itself, but to FasL expressed as a cytotoxic mediator by antitumor immune effector cells, such as CTL or NK cells. Fas resistance may therefore contribute to immune evasion, augmenting the effectiveness of the FasL counterattack. Intracellular Fas signal-transduction defects reported in diverse cancers are many and varied (reviewed in Ref. 6), and may be heterogenous in any cancer type. High Bcl-2 expression has been reported in esophageal carcinomas (31), which may contribute to Fas resistance in a proportion of these tumors.

In conclusion, we have demonstrated a statistically significant, quantitative reduction of TIL concomitant with significantly increased TIL apoptosis within FasL-expressing areas of esophageal tumors in vivo. Our findings therefore suggest Fas-mediated apoptotic depletion of TIL in response to FasL expression by esophageal cancers. These results provide the first direct, quantitative evidence to support the Fas counterattack as a mechanism of immune privilege in vivo in human cancer. Fas resistance of the

Table II. Increased TIL apoptosis in FasL-positive versus FasL-negative regions of esophageal carcinomas

Tumor Specimen	FasL-positive Tumor Region (% apoptotic TIL) ^a	FasL-negative Tumor Region (% apoptotic TIL) ^a
1	45.35	28.70
2	47.40	24.80
3	41.90	25.50
4	49.10	26.40
5	54.90	22.80
6	25.20	23.30
Mean	43.98	25.25

^a The apoptotic TIL was calculated as the % CD45-TUNEL dual-positive cells per 500 total CD45-positive cells counted within FasL-positive/-negative tumor regions.

esophageal carcinomas may contribute to tumor immune evasion, protecting the tumor from FasL expressed by CTL and NK cells. Understanding the significance of the Fas counterattack as a mechanism of immune evasion in human cancer should facilitate more effective antitumor therapies. Fas sensitization of tumor cells or protection of TIL from FasL could represent promising targets for future therapeutic strategies.

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