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

Michael W. Bennett, Joe O'Connell, Gerald C. O'Sullivan, Ciaran Brady, Desmond Roche, J. Kevin Collins and Fergus Shanahan

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Eosophageal 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, interferon-mediated inhibition of immune response terminations, 3) maintaining immune privilege in certain organs including 1) tolerance acquisition (7), 2) immune response terminations (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.

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.

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Michael W. Bennett,2* Joe O’Connell,2* Gerald C. O’Sullivan,† Ciaran Brady,∗ Desmond Roche,* J. Kevin Collins,* and Fergus Shanahan3*
Materials and Methods

Tissues

Human esophageal carcinoma tissues (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 0.09% sodium azide overnight. Proteinase K (10 µg/ml in 100 mM Tris-HCl, 50 mM EDTA, pH 8) was used, and 1 mg/ml heat-denatured herring sperm DNA containing 1 ng/ml digoxigenin-labeled riboprobe. After hybridization, tissues were washed with increasing stringency to 0.1× SSC at 37°C. Hybridized probe was detected immunologically using alkaline phosphatase-conjugated sheep anti-digoxigenin Ab (Boehringer Mannheim) and visualized with nitroblue tetrazolium/5-bromo-4-chloro-3-indoly phosphate (NBT/BCIP; purple/black precipitating product). Competition 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, Carpenteria, CA) at a dilution of 1/170 for 1 h. All incubations were conducted at room temperature. Next the slides were washed for 5 min in 0.1 M phosphate-buffered saline (PBS), 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 protease K (20 µg/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 peroxidase 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 coveredslipped 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

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 aminopropylhexylamine (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 µg/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× SSC. Hybridization was performed at 42°C overnight in hybridization buffer (50% formamide, 10% dextran sulfate, 1× Denhardt’s reagent, 4× SSC, 10 mM DTT, 500 µg/ml sheared tRNA, and 1 mg/ml heat-denatured herring sperm DNA) containing 1 ng/ml digoxigenin-labeled riboprobe. After hybridization, tissues were washed with increasing stringency to 0.1× SSC at 37°C. Hybridized probe was detected immunologically using alkaline phosphatase-conjugated sheep anti-digoxigenin Ab (Boehringer Mannheim) and visualized with nitroblue tetrazolium/5-bromo-4-chloro-3-indoly phosphate (NBT/BCIP; purple/black precipitating product). Competition 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.
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 homogenously 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 metases (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.
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), including the possible role of FasL in the periphery (7), in downregulating 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).

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 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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References


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

<table>
<thead>
<tr>
<th>Tumor Specimen</th>
<th>FasL-positive Tumor Region (% TIL)*</th>
<th>FasL-negative Tumor Region (% TIL)*</th>
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<td>Mean</td>
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<td>19.27</td>
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* The % TIL was calculated as the % CD45-positive cells per 2000 total nuclei counted within FasL-positive/negative tumor regions.

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

<table>
<thead>
<tr>
<th>Tumor Specimen</th>
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<th>FasL-negative Tumor Region (% apoptotic TIL)*</th>
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* 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.


