Expression of Fas ligand by human gastric adenocarcinomas: a potential mechanism of immune escape in stomach cancer

M W Bennett, J O’Connell, G C O’Sullivan, D Roche, C Brady, J Kelly, J K Collins, F Shanahan

Abstract
Background—Despite being immunogenic, gastric cancers overcome antitumour immune responses by mechanisms that have yet to be fully elucidated. Fas ligand (FasL) is a molecule that induces Fas receptor mediated apoptosis of activated immunocytes, thereby mediating normal immune downregulatory roles including immune response termination, tolerance acquisition, and immune privilege. Colon cancer cell lines have previously been shown to express FasL and kill lymphoid cells by Fas mediated apoptosis in vitro. Many diverse tumours have since been found to express FasL suggesting that a “Fas counterattack” against antitumour immune effector cells may contribute to tumour immune escape.

Aim—To ascertain if human gastric tumours express FasL in vivo, as a potential mediator of immune escape in stomach cancer.

Specimens—Thirty paraffin wax embedded human gastric adenocarcinomas.

Methods—FasL protein was detected in gastric tumours using immunohistochemistry; FasL mRNA was detected in the tumours using in situ hybridisation. Cell death was detected in situ in tumour infiltrating lymphocytes using terminal deoxynucleotidyl transferase mediated dUTP nick end labelling (TUNEL).

Results—Prevalent expression of FasL was detected in all 30 resected gastric adenocarcinomas examined. In the tumours, FasL protein and mRNA were co-localised to neoplastic gastric epithelial cells, confirming expression by the tumour cells. FasL expression was independent of tumour stage, suggesting that it may be expressed throughout gastric cancer progression. TUNEL staining disclosed a high level of cell death among lymphocytes infiltrating FasL positive areas of tumour.

Conclusions—Human gastric adenocarcinomas express the immune downregulatory molecule, FasL. The results suggest that FasL is a prevalent mediator of immune privilege in stomach cancer.

Keywords: Fas ligand; gastric cancer; immune escape; apoptosis; tumour; mRNA

Despite expression of tumour rejection antigens such as MAGE 1–3 and the presence of tumour specific cytotoxic T cells, the immune system fails to contain gastric carcinoma. Evidence suggests that a poor local immune response contributes to the potential for lymph nodal metastatic spread of gastric tumours. The mechanisms by which gastric cancers overcome antitumour immunological responses are poorly understood.

Fas ligand (FasL) triggers apoptotic cell death of sensitive lymphoid cells which express its cell surface receptor (Apo-1/CD95). FasL mediated apoptosis contributes to the regulation of the immune system through its roles in tolerance acquisition, T cell activation induced cell death, and immune response termination. FasL expressed in indigenous cells of the eye and the testis mediates immune privilege by inducing apoptosis in infiltrating proinflammatory immunocytes. FasL has been shown to confer immunological privilege in tissue transplantation experiments. In rodents, successful allograft survival was obtained of FasL expressing tissues and of non-immune privileged cells (pancreatic islets) co-transplanted with FasL expressing cells. Allograft transplantation of murine tumour cells stably transfected with the FasL gene showed that FasL can cause local suppression of both humoral and cellular allograft specific immune responses.

The possibility that FasL is expressed by non-lymphoid tumours as a mediator of immune evasion was initially raised by our finding that colon cancer cell lines express...
Table 1 Tumour, node, metastases (TNM) classification of gastric adenocarcinomas

<table>
<thead>
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<th>Histological type</th>
<th>Number</th>
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Table 2 Grade of differentiation of gastric adenocarcinomas

<p>| | | | | |</p>
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<tr>
<td>Poor*</td>
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<tr>
<td>Moderate</td>
<td>11</td>
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*All signet-ring cell carcinomas (n = 5) examined were poorly differentiated.

Materials and methods

Materials

Thirty human gastric adenocarcinomas of disparate pathological stages (table 1) were collected after surgical resections performed at the Mercy Hospital, Cork, following a protocol approved by the University Teaching Hospitals ethics committee. None of the patients had received chemo-, radio-, or immuno-therapy before resection. Tumours were moderately (n = 11), poorly (n = 15), or moderately to poorly (n = 4) differentiated (table 2). Of the poorly differentiated tumours, five were signet ring cell carcinomas.

IMMUNOHISTOCHEMICAL DETECTION OF FasL PROTEIN

Paraffin wax embedded surgically resected tumour sections were deparaffinised in xylene and rehydrated before analysis. The slides were washed twice for five minutes in a wash buffer containing 50 mM Tris/HCl, pH 7.6, 50 mM NaCl, and 0.001% saponin. Endogenous peroxidase was quenched with 3% hydrogen peroxide in methanol for five minutes. The slides were then washed as before except that the wash buffer for this and all subsequent steps included 1% normal goat serum. They were then blocked for one hour in wash buffer containing 5% normal goat serum. This was followed by a further wash and incubation overnight at 4°C with affinity purified rabbit polyclonal anti-human FasL specific IgG (Santa Cruz Biotechnology, Santa Cruz, California, USA) at 0.1 µg/ml in wash buffer. Antibody binding was localised using a biotinylated secondary antibody, avidin conjugated horseradish peroxidase, and diaminobenzidine substrate, contained within the Vectastain ABC detection kit (Vector Laboratories, Burlingame, California, USA). The immunising peptide to which the antibody was raised (FasL: N-terminal amino acids 260–279; Santa Cruz Biotechnology) was included at 1 µg/ml during primary antibody incubation as a direct internal competitive control for antibody specificity. In a separate control for specificity of the Santa Cruz FasL specific polyclonal antibody, we previously found that specific antisense oligonucleotide mediated inhibition of FasL expression14 converted SW620 cells from positive to negative by immunohistochemistry with this antibody. The FasL specificity of the Santa Cruz antibody has also been previously verified in immunohistochemistry by others, staining FasL mRNA negative cell lines,19 and staining in concordance with other FasL specific monoclonal20 and polyclonal21 antibodies. FasL detection in the gastric tumours was confirmed by immunohistochemistry with a FasL specific monoclonal antibody (Pharmigen, San Diego, California, USA; clone G247–4) as described above. The monoclonal antibody was used at a concentration of 5 µg/ml, and an isotype matched control antibody was also used. Slides were counterstained with haematoxylin.

GENERATION OF A FasL SPECIFIC RNA PROBE (RIBOPROBE)

A digoxigenin labelled RNA hybridisation probe (344 bp) was generated corresponding to codons 96–210 of the human FasL cDNA sequence. The riboprobe was synthesised by in vitro transcription using digoxigenin-11-UTP and T7 RNA polymerase (Boehringer-Mannheim GmbH, Mannheim, Germany). Template for the in vitro transcription reaction was generated by polymerase chain reaction (PCR) amplification of a fragment (codons 96–210) of FasL cDNA using a proofreading thermostable polymerase (U Telecom DNA polymerase; Perkin-Elmer, Norwalk, Connecticut, USA) and an antisense primer to which a T7 promoter sequence was added. RNA was isolated from FasL expressing cells by homogenisation in guanidine thiocyanate (Sigma Chemical Co, St Louis, Missouri, USA) followed by phenol extraction and etha-
nol precipitation. cDNA was synthesised using the avian myeloblastosis virus reverse transcriptase (Promega, Madison, Wisconsin, USA) and random hexanucleotide primers (Boehringer-Mannheim).

PCR was performed on the cDNA using the following sense and antisense primers respectively: FasL: dGGATTGGGCCTGGGGAT-GTTTCA and d[pT7]-TTGTGGCTACGGGGAGTTGTTGG. PCR primers were designed using the DNASTAR Lasergene Primerselect program (DNASTAR Inc, Madison, Wisconsin, USA). Primer pairs were chosen to span introns in the FasL genomic sequence, thus ensuring mRNA specific amplification. Primers that showed no significant homology to any other genes in the EMBL DNA sequence database were selected.

Primer select program (DNASTAR Inc, Madison, Wisconsin, USA) and random hexanucleotide primers were used as template to synthesise a riboprobe 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 unlabelled riboprobe was also synthesised for use in competitive control hybridisations.

LOCALISATION OF FasL mRNA EXPRESSION BY IN SITU HYBRIDISATION

In situ hybridisation was performed on paraffin wax embedded human gastric tumour sections (4 µm thick), mounted on aminopropyltriethoxysilane treated slides. Prehybridisation treatments involved two washes of five minutes each in (a) phosphate buffered saline (PBS), (b) PBS/0.1 M glycine, (c) PBS/0.3% Triton X-100, and (d) PBS again. Sections were digested for 30 minutes at 37°C with proteinase K (10 µg/ml in 100 mM Tris/HC1/50 mM EDTA, pH 8.0), fixed for five minutes at 4°C in 4% paraformaldehyde/PBS and then acetylated twice for five minutes in fresh 0.25% acetic anhydride/0.1 M triethanolamine, pH 8.0. Sections were incubated at 37°C for 10 minutes in a prehybridisation buffer consisting of 50% deionised formamide in 4 × SSC (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate). Hybridisation was performed at 42°C overnight in hybridisation buffer (50% formamide, 10% dextran sulphate, 1 × Denhardt’s reagent, 4 × SSC, 10 mM dithiothreitol, 500 µg/ml yeast tRNA, and 1 mg/ml heat denatured herring sperm DNA) containing 1 ng/µl digoxigenin labelled riboprobe. After hybridisation, tissues were washed with increasing stringency to 0.1 × SSC at 37°C. Hybridised probe was detected immunologically using alkaline phosphatase conjugated sheep anti-digoxigenin antibody (Boehringer-Mannheim) and visualised with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP, purple/black precipitating product). Control slides involved direct internal competitive inhibition of hybridisation. This was performed exactly as described above except that a tenfold excess of unlabelled FasL specific riboprobe was added to the digoxigenin labelled FasL specific riboprobe before hybridisation. This resulted in direct competitive displacement of positive hybridisation leading to a marked reduction in signal intensity, confirming the FasL specificity of hybridisation.

LEUCOCYTE COMMON ANTIGEN (CD45) STAINING

To identify TILs, CD45 staining was performed on consecutive paraffin wax embedded sections. After deparaffinisation and rehydration, sections were pretreated by microwave irradiation in 0.01 M citrate buffer for five minutes at 370 W. The sections were rapidly cooled by immersion in 0.1 M PBS. The slides were then incubated with a mouse anti-human CD45 monoclonal IgG (clone 2B11+PD7/26; Dako Corp, Carpinteria, California, USA) at a dilution of 1:70 for one hour. All incubations were carried out at room temperature. Next the slides were washed for five minutes each in 0.1 M Tris buffered saline which was used for all washes. After incubation with a secondary rabbit anti-mouse IgG (Dako Corp) at a dilution of 1:25 for 30 minutes, washing was repeated. Alkaline phosphatase conjugated anti-alkaline phosphatase (APAAP) complex (D0651; Dako Corp), at a dilution of 1:50, was incubated on the slides for 30 minutes and they were washed again. To enhance staining, the secondary antibody incubation and APAAP complex incubation were repeated once as above, but the duration of the incubation steps was reduced to 10 minutes each. Alkaline phosphatase substrate solution was then incubated on the slides for 10 minutes. The phosphatase substrate used was Fast Red (Sigma Chemical Co, St Louis, MO, USA). Sections were counterstained with haematoxylin and viewed by light microscopy.

CELL DEATH DETECTION IN SITU BY TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE (TdT) MEDIATED DUTP NICK END LABELLING (TUNEL)

Cell death was detected in situ in resected tissues by enzymic labelling of DNA strand breaks using a TUNEL assay (Boehringer-Mannheim) according to the manufacturer’s instructions. Paraffin wax embedded surgically resected tumour sections were deparaffinised in xylene and rehydrated before analysis. After treatment with proteinase K (20 µg/ml in 10 mM Tris/HCl, pH 7.6) for 30 minutes, sections were washed in PBS and endogenous peroxidase activity was blocked in 0.3% hydrogen peroxide in methanol for 30 minutes. Next, the slides were washed. This and all subsequent washes were in PBS. The sections were treated with permeabilisation solution (0.1% Triton X-100 in 0.1% sodium citrate) for 15 minutes. After a wash, the labelling reaction was performed using a solution containing TdT, its
buffer and fluorescein conjugated dUTP. During this step, slides were coverslipped and incubated at 37°C for 60 minutes in a humidity chamber. TdT was omitted from negative control slides. To localise cells containing labelled DNA strand breaks, sections were washed and incubated with a sheep anti-fluorescein antibody Fab fragment conjugated with hors eradish peroxidase at 37°C in a humidity chamber for 30 minutes. After a wash, colour was developed by incubating sections with a diaminobenzidine substrate solution for 15 minutes. After a final wash, sections were viewed by light microscopy. Only those cells with positive TUNEL staining and of apoptotic morphology were considered apoptotic.

Results

Expression of FasL Protein by Gastrointestinal Adenocarcinomas

With the use of an affinity purified FasL specific rabbit polyclonal IgG (Santa Cruz Biotechnology) raised against a synthetic FasL peptide (N-terminal amino acids 260–279), FasL expression by tumour cells was immunohistochemically detected in all (30/30) surgically resected gastric adenocarcinomas (fig 1A). FasL specificity was confirmed using the FasL peptide immunogen (FasL amino acids 260–279) as an internal competitive control. Inclusion of the soluble peptide immunogen during immunohistochemistry resulted in direct competitive displacement of positive staining in consecutive control sections (fig 1B). The FasL specificity of the Santa Cruz polyclonal antibody used (C-20) had been previously verified by us14 and others.15 FasL detection in the gastric tumours was confirmed using immunohistochemistry with a FasL specific monoclonal antibody (Pharmingen; FasL clone G247–4), which resulted in a pattern of staining identical with that detected with the Santa Cruz polyclonal antibody (not shown). An isotype-matched monoclonal antibody did not stain in control sections.

Positive staining of neoplastic tissue varied in intensity and extent, both within individual tumours and between tumours. Intensity of staining varied from weakly positive neoplastic areas to intensely staining tumour regions, where staining was stronger than that observed in local FasL positive TILs. FasL staining intensity was locally uniform within nests of tumour cells. FasL positive and negative staining neoplastic areas were frequently found to occur within the same tumour. However, all tumours examined were predominantly FasL positive (>70% of tumour area).

Apoptosis of TILs

CD45 immunohistochemistry showed immunocyte infiltration in all 30 carcinomas (fig 2B). Tonsil sections included as positive controls stained strongly, and omission of the primary antibody abolished staining. Most of the CD45 positive cells were of lymphoid morphology. Recently, FasL expression by human oesophageal carcinoma was found to be associated with increased cell death of TILs.20 Apoptosis was detected by TUNEL among TILs adjacent to FasL positive areas of gastric carcinomas. These TUNEL positive TILs exhibited morphological features of apoptosis, including nuclear condensation and fragmentation (fig 2C). This was a consistent finding in all the tumours examined (n = 8).

Discussion

In this report, we show that stomach cancers express FasL, an inducer of immunocyte apoptosis. Expression of FasL, potentially enables stomach tumours to counterattack and kill Fas sensitive antitumour immune effector cells (fig 3). We have previously shown that FasL expressed by colon adenocarcinoma cells in vitro is biologically active: SW620 cells induced Fas mediated apoptosis of co-cultured Jurkat T cells. As an established mediator of immune privilege and immunological tolerance in the eye and testis, our finding that gastric tumours express FasL suggests that FasL contributes to the immune evasion of stomach cancer.

Expression of FasL in vivo in human gastric cancers was prevalent; all 30 resected gastric adenocarcinomas were found to express FasL mRNA and protein. Co-localisation of FasL mRNA and protein confirmed that FasL was expressed by gastric epithelial tumour cells. Since activated lymphocytes are known to shed FasL,21 confirmation of tumour cell expression of FasL mRNA precludes the possibility that the detected FasL protein was derived from TILs. FasL staining was variable, in both intensity and extent, within tumours. Extensive expression (>70% of the tumour area) occurred in all tumours irrespective of tumour stage or degree of differentiation (tables 1 and 2 respectively), suggesting that FasL may be...
expressed throughout gastric tumour progression. Whereas we found that FasL was undetectable by in situ hybridisation in control normal gastric epithelial sections (n = 7; not shown), FasL protein could be detected using both FasL specific monoclonal and polyclonal antibodies (7/7). In contrast with the extensive expression detected in the gastric carcinomas, in the normal epithelium FasL expression was consistently restricted to only those epithelial

Figure 1  Expression of Fas ligand (FasL) in human gastric adenocarcinomas. Immunoperoxidase staining using a FasL specific rabbit polyclonal IgG was performed on paraffin wax embedded gastric carcinoma sections. Slides were counterstained with haematoxylin. (A) FasL positive immunohistochemical staining (brown) is shown in a representative gastric adenocarcinoma. (B) As a control for specificity of antibody detection, the FasL immunising peptide was included during primary antibody incubation. Competitive displacement of staining by the immunising peptide confirms FasL specificity. Tumour sections sequential to those shown in (A) and (B) were used to detect FasL mRNA by in situ hybridisation, using a digoxigenin labelled FasL specific riboprobe. (C) Positive purple hybridisation signal was obtained from the tumour area that stained immunohistochemically positive for FasL protein. (D) In a control hybridisation, a tenfold excess of unlabelled probe caused direct competitive displacement of the labelled probe, confirming the specificity of hybridisation. These results are representative of 30 adenocarcinomas of the stomach.
cells at the luminal surface (7/7). Hence a deregulation and upregulation of FasL expression occurs during the transformation process. A role for FasL in tumour immune escape is supported by the many downregulatory roles that this molecule normally plays in immunity. FasL is involved in mediating immune privilege in the eye, testis, and placenta, in contributing to immunological tolerance in the periphery, in downsizing immune responses, and in supporting experimental allograft survival in most cases. All of these roles involve FasL mediated apoptosis of lymphocytes. FasL has broad immunosuppressive effects: activated T, B, and NK cells, neutrophils, and monocytes have all been shown to be sensitive to FasL mediated apoptosis. Surprisingly, in a few experiments, allografts of murine pancreatic islets or tumour cells genetically manipulated to express FasL were damaged as the result of proinflammatory neutrophil infiltration, for reasons that are as yet unclear. Conversely, adenovirus mediated overexpression of FasL in mouse ankle joints ameliorated collagen induced arthritis, providing powerful evidence for an anti-inflammatory role for FasL in vivo.

Significant neutrophil infiltration was absent from all FasL expressing areas of the gastric tumours examined, suggesting that, in the context of spontaneous human gastrointestinal cancers, FasL expression mediates immune privilege rather than proinflammatory neutrophil recruitment.

Several cancers have been reported to express FasL: melanoma, hepatocellular carcinoma, lung cancer, astrocytoma, and liver metastases of colon adenocarcinomas. In all cases, tumour derived cell lines induced apoptosis in Fas sensitive, but not Fas resistant co-cultured lymphoid target cells in vitro. All of these roles involve FasL mediated apoptosis of lymphocytes. FasL has broad immunosuppressive effects: activated T, B, and NK cells, neutrophils, and monocytes have all been shown to be sensitive to FasL mediated apoptosis. Surprisingly, in a few experiments, allografts of murine pancreatic islets or tumour cells genetically manipulated to express FasL were damaged as the result of proinflammatory neutrophil infiltration, for reasons that are as yet unclear. Conversely, adenovirus mediated overexpression of FasL in mouse ankle joints ameliorated collagen induced arthritis, providing powerful evidence for an anti-inflammatory role for FasL in vivo. Significant neutrophil infiltration was absent from all FasL expressing areas of the gastric tumours examined, suggesting that, in the context of spontaneous human gastrointestinal cancers, FasL expression mediates immune privilege rather than proinflammatory neutrophil recruitment.

Evidence that FasL inhibits tumour rejection in vivo comes from studies using a murine FasL expressing melanoma cell line. When injected into syngeneic host mice, this cell line quickly developed tumours. In syngeneic hosts expressing the defective mutant Fas receptor (lpr, or lymphoproliferation), tumour formation was retarded. The greater efficiency of tumour restriction by these syngeneic-lpr mice may have been due to the insensitivity of their
lymphocytes to tumour expressed FasL. Although other immune escape mechanisms allowed the eventual establishment of tumours in FasL insensitive lpr mutant mice, these experiments showed that FasL contributed to the immune privilege of the tumour, expediting tumour formation in wildtype mice. A recent study involving allograft transplantation of murine tumour cells stably transfected with the FasL gene showed that FasL caused profound local suppression of both humoral and cellular allograft specific immune responses.13

Immunogenic tumour cells are probably subjected to a barrage of cell mediated cytotoxic antitumour immune assaults. The fact that most tumour cells are efficiently killed by LAK cells in vitro11 suggests that cancer cells probably exhibit some degree of susceptibility to cell mediated cytotoxic mechanisms. Expression of a molecule to defuse antitumour immune challenge clearly offers a protective advantage to tumour growth and development. As an established mediator of immunological tolerance and privilege, FasL is such a molecule. Our findings conclusively show, at both the mRNA and protein levels, that human gastric tumours express FasL. Stomach cancer may therefore be added to the growing list of malignancies that appear to be immunologically privileged through FasL expression. The high prevalence of FasL expression in the tumours suggests that this molecule may be critical to tumour immune privilege. In conclusion, the Fas counterattack appears to prevail as a potentially critical mechanism of immune privilege in human stomach cancer.

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