LETTERS TO THE EDITOR

Gastric cancer cell lines lack Fas ligand (FasL) expression but kill T cells via a FasL independent pathway

EDITOR,—Bennett et al (Gut 1999;44:156-162) reported that in each of 30 paraffin wax specimens of human gastric adenocarcinomas, FasL mRNA and protein co-localised to neoplastic epithelial cells. TUNEL staining revealed the high number of tumour infiltrating lymphocytes (TIL) displayed apoptotic features. From these results and from their findings of FasL expression in human colon and oesophageal cancer,1 the authors propose that FasL might be a mediator of immune privilege in gastrointestinal cancers.

We studied intrinsic FasL expression in gastric cancer cell lines derived from primary (RF-1, SNU-1) or from metastatic sites (Kato-III, N-87, RF-48). We did not detect FasL mRNA or protein in any of the six cell lines analysed by RT-PCR and by flow cytometry (table 1).2 We then performed the JAM assay to rule out the presence of a functional FasL expression below the detection limit of our assays.2 Although we found that gastric cancer cells were able to induce DNA fragmentation in the Fas sensitive T-acute lymphocytic leukaemia cell line CEM-C7H2 (fig 1A), blocking FasL on the effector cell site did not reduce the extent of cytotoxicity. This result was confirmed by replacing the target cell line by a subclone stably expressing the viral protein crmA, which inhibits activation of caspases 1 and 8 and thereby mediates resistance to Fas triggering (fig 1B).3

Owing to the discrepancy between our results (all靶子 were FasL negative) and those of Bennett et al (all 30 primary neoplasias were FasL positive), we wondered whether tissue derived factors such as tumour necrosis factor (TNF) α and interferon (IFN) γ might upregulate FasL in vivo, thus explaining the differences observed. In our setting, neither of the cytokines was able to modify FasL expression on gastric cancer cell lines (table 1). In addition, killing of T cell lines was not mediated via secretion of TNF-α as blocking the cytokine using a monoclonal antibody did not influence the result of the JAM assay (fig 1A). How can the

Table 1 Expression of FasL and Fas in gastric cancer cell lines and their sensitivity toward Fas triggering by the CH11 monoclonal antibody

<table>
<thead>
<tr>
<th>Cell line</th>
<th>FasL mRNA</th>
<th>Constitutive NOK-1/1H1</th>
<th>+TNF-α (100 ng/ml)</th>
<th>+IFN-γ (100 ng/ml)</th>
<th>Fas expression</th>
<th>Control (%)</th>
<th>CH11 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RF-1</td>
<td>Negative</td>
<td>1.1/1.0</td>
<td>1.0</td>
<td>0.8</td>
<td>8.3</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>RF-48</td>
<td>Negative</td>
<td>1.3/1.0</td>
<td>0.9</td>
<td>0.9</td>
<td>6.1</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>Kato-III</td>
<td>Negative</td>
<td>0.9/1.2</td>
<td>Not done</td>
<td>Not done</td>
<td>1.4</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>SNU-1</td>
<td>Negative</td>
<td>1.1/1.0</td>
<td>0.9</td>
<td>0.9</td>
<td>4.9</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>SNU-16</td>
<td>Negative</td>
<td>1.0/1.2</td>
<td>Not done</td>
<td>Not done</td>
<td>1.1</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>N-87</td>
<td>Not done</td>
<td>1.0/1.2</td>
<td>Not done</td>
<td>Not done</td>
<td>2.2</td>
<td>Not done</td>
<td></td>
</tr>
</tbody>
</table>

1 RT-PCR analysis was done as follows: total RNA from about 10⁶ cells was extracted by the acid guanidinium thiocyanate-pheno/ chloroform protocol described by Chomczynski and Sacchi;1 1 μg RNA together with 250 ng of oligo (dT)₁₅ primer was diluted in a.d. to a final volume of 14 μl, denatured by heating up to 70°C for five minutes and immediately chilled on ice. To each reaction, 6 μl RT mixture containing 4 μl 5× buffer, 2 pmol each of dATP, dCTP, dGTP and dTTP, and 200 units Moloney-murine leukaemia virus reverse transcriptase, was added (all reagents from Promega, Wisconsin, USA). For cDNA synthesis all samples were incubated at 37°C for 60 minutes. The reaction was stopped by heating the sample to 80°C for two minutes; 100 ng cDNA obtained was amplified by 50 cycles with 1° C; annealing, 60 seconds each at 63°C with a secondary fluorescein isothiocyanate (FITC) labelled rabbit anti-mouse antibody (Dako, Vienna, Austria; dilution 1 in 10). Cells were washed and immediately analysed by flow cytometry for their specific fluorescence signals. Specific fluorescence intensities (MFI) were calculated as the ratio of mean fluorescence intensity achieved with the specific antibody/isotype matched negative control antibody. A ratio ≥ 1.5 was considered positive. The mean value of MFI for three independent experiments is given.

2 Conditions of Fas expression were determined using two different monoclonal antibodies, NOK-1 (Pharmingen, San Diego, California, USA) and HI1 (Alexis, Läufelfingen, Switzerland). For detection of FasL expression, 0.5 × 10⁶ cells were fixed with parafformaldehyde, permeabilised with a buffer containing 0.05% saponin and 1% borax serum albumin and stained with 1 μg of the respective specific monoclonal antibody or a relevant isotype matched negative control antibody for 30 minutes at 4°C. In the case of staining with NOK-1, cells were incubated for 20 minutes at 4°C with a secondary fluorescein isothiocyanate (FITC) labelled rabbit anti-mouse antibody (Dako, Vienna, Austria; dilution 1 in 10). Cells were washed and immediately analysed by flow cytometry for their specific fluorescence signals. Mean specific fluorescence intensities (MFI) were calculated as the ratio of mean fluorescence intensity achieved with the specific antibody/isotype matched control antibody. A ratio ≥ 1.5 was considered positive. The mean value of MFI for three independent experiments is given.

3 Time kinetics (1-3 days’ stimulation) were performed and values are given for day 3. Tumour necrosis factor (TNF) α and interferon (IFN) γ were purchased from R&D Systems (Minneapolis, Minnesota, USA). Flow cytometric analysis was performed using the NOK-1 monoclonal antibody.

4 Since the assay was done as follows: total RNA from about 10⁶ cells was extracted by the acid guanidinium thiocyanate-pheno/ chloroform protocol described by Chomczynski and Sacchi;1 1 μg RNA together with 250 ng of oligo (dT)₁₅ primer was diluted in a.d. to a final volume of 14 μl, denatured by heating up to 70°C for five minutes and immediately chilled on ice. To each reaction, 6 μl RT mixture containing 4 μl 5× buffer, 2 pmol each of dATP, dCTP, dGTP and dTTP, and 200 units Moloney-murine leukaemia virus reverse transcriptase, was added (all reagents from Promega, Wisconsin, USA). For cDNA synthesis all samples were incubated at 37°C for 60 minutes. The reaction was stopped by heating the sample to 80°C for two minutes; 100 ng cDNA obtained was amplified by 50 cycles with 1° C; annealing, 60 seconds each at 63°C with a secondary fluorescein isothiocyanate (FITC) labelled rabbit anti-mouse antibody (Dako, Vienna, Austria; dilution 1 in 10). Cells were washed and immediately analysed by flow cytometry for their specific fluorescence signals. Specific fluorescence intensities (MFI) were calculated as the ratio of mean fluorescence intensity achieved with the specific antibody/isotype matched control antibody. A ratio ≥ 1.5 was considered positive. The mean value of MFI for three independent experiments is given.

5 Cells were incubated with the CH11 monoclonal antibody (250 ng/ml) for 24 hours and the proportion of apoptotic cells was determined using the propidium iodide assay. Even after 72 hours’ incubation, there was only a very small increase in the percentages of apoptotic cells (e.g. in the SNU-1 cell line the increase was from 3% (control) to 5% (CH11)).
differences between in situ and in vitro results be explained?

Bennet et al mentioned that CD45+ TIL express FasL mRNA, but they did not analyse Fas expression and sensitivity, features that together characterize activation induced cell death. Although an immunohistochemical examination of slides the authors excluded the possibility of lymphocytes being killed by infiltrating neutrophils potentially attracted by the expression of FasL on the tumour cells, it is possible that lymphocytes succumbed to apoptosis owing to bystander or suicide. This mechanism could well be under the (cytokine) control of the tumour as has been discussed for other diseases. Alternatively, lymphocytes could indeed be killed by the tumour cells but by a mechanism independent of the Fas system, a hypothesis supported by our data (fig 1).

Bennet et al did not use the standard Lauren classification system. It has been shown that gastric carcinoma cells of the intestinal and diffuse type (according to Lauren) differ in morphology, growth pattern and risk factors, and also in their expression of molecules involved in apoptosis such as Fas or p53. There is evidence that at least in some tumour models Fas and FasL expression are under transcriptional control of p53. Loss-of-function mutations or deletions of p53 have been reported to be involved in gastric carcinogenesis and the frequency of these events differs between intestinal and diffuse gastric cancers. Also, a correlation between p53 mutation, Fas expression and gastric carcinoma cell differentiation has been demonstrated. Further studies of the impact of differentiation and p53 functional status on FasL expression are therefore mandatory in gastric carcinoma cells.

Insensitivity towards Fas is usually an early step in tumour development, allowing tumour cells to resist the attack of the immune system and to avoid suicide when FasL expression is acquired. Furthermore, a sequence of Fas resistance and FasL expression has been demonstrated for hepatocellular carcinoma. Secondary loss of the Fas gene or of its expression during continuous culture of gastric adenocarcinoma cells is unlikely for the following reasons: (I) All cell lines were resistant to Fas and thus loss of Fas expression does not seem to be a prerequisite for their survival, and (ii) to our knowledge, no data are available from other cell (line) systems that tumour cell lines lose FasL expression during long-term culture.

In conclusion, we think that Bennett et al’s data suggest that CD45+ lymphocytes die in the immediate proximity of neoplastic cells. Although their data are compatible with Fas induced TIL cell death, our functional data from cell lines suggest that other tumour mediated mechanisms of killing immunocompetent cells might also exist in gastric cancer. Further work clarifying the sequence of Fas/FasL expression and function during the transformation and metastatic processes is needed.

*MIdo*
Vector manometry and LOS dynamics

EDITOR,—We read with interest the recent Vector manometry and LOS dynamics regarding the effect of hiatus hernia on gastro-oesophageal junction pressures. This study drew some interesting conclusions. On the appearance of a normal sphincter (LOS) vector volume from 10 pull-throughs at end tidal expiration in a single normal volunteer. Coefficient of variance=43%.

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Vector volume (mm Hg/mm²).

Figure 1 Variation of lower oesophageal sphincter (LOS) vector from 10 pull-throughs at end tidal expiration in a single normal volunteer. Coefficient of variance=43%.

We believe that three factors contribute to the poor reproducibility of vector manometry. Firstly, the point at which respiration is suspended is critical in defining vector volume. It is likely that the point at which respiration is suspended varies from patient to patient and from pull-through to pull-through—that is, not all patients suspend respiration at the end tidal point. Secondly, it is unlikely that the diaphragm is completely relaxed during a 15 second expiratory breath hold. It is speculated that crural activity would therefore be expected. Finally, there can be significant minute to minute variation in lower oesophageal sphincter tone. The poor reproducibility of vector manometry has been described previously by Benelman et al using rapid pull-through vector manometry (8 channel catheter, 0.7 ml/s pull-back speed). They showed that LOS pressure varied from 20 to 80 mm Hg in 20 pull-throughs performed in one hour in the same patient.

Kahrlas et al did not mention the number of pull-throughs for each patient or the reproducibility of vector profiling. It is therefore difficult to draw accurate conclusions on the size and position of high pressure zones, particularly when the study population is limited to seven patients.

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The study by Neurath et al using an oesophageal pressure baseline. This is in contrast with previous studies which have uniformly used a gastric baseline in vector analysis and profiling. If a gastric baseline had been applied to this study, the distal ‘crural’ high pressure zone (3 mm Hg) would have been less evident. These authors have thus presented a fundamental change in the methodology of vector profiling.

Our own experiences with vector manometry of the LOS have shown that this technique has poor reproducibility. We have performed rapid pull-through vector manometry (8 channel catheter, 0.5 ml/min perfusion, 0.5 cm/s pull-back speed) 10 times each on 17 volunteers. Using a gastric baseline we found a median coefficient of variance of 42% for LOS vector volume and 19% for LOS pressure with widely differing three dimensional vector profiles in individual patients (unpublished observation; fig 1).

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Mycophenolate mofetil for Crohn’s disease

EDITOR,—On the basis of a study reported recently by Neurath et al (Gut 1999;44:625–628), commentators in Gut and the Lancet suggested that mycophenolate mofetil (MMF) should be used in patients with Crohn’s disease who have either not responded to or are intolerant of azathioprine or 6-mercaptopurine. This advice is premature: firstly, because the study was flawed and, secondly, because it examined only management of acute inflammation, not the place of MMF in maintaining remission and in steroid sparing (a fact acknowledged in both commentaries).

The study by Neurath et al compared the effect of MMF 15 mg/kg daily with azathioprine 2.5 mg/kg daily, both with high dose steroids, in the treatment of active chronic Crohn’s disease (six months’ follow up). The main conclusions were that activity, as measured by the Crohn’s disease activity index (CDAI), dropped further at one month in patients given MMF plus steroids than in those given azathioprine plus steroids, and that this was as a result of a faster effect in more severe disease. The major drawbacks of the study were as follows. As pointed out by the authors, neither patients nor investigators were blinded. Four (11%) of 35 patients in the MMF group were lost to follow up compared with none in the azathioprine group: thus results may have looked different if analysed on an intention to treat basis. The MMF group had higher starting CDAIs: if the levels of CDAI reached at one month were compared between groups, rather than the fall of CDAI, the groups may not have been significantly different. The division of patients into those with moderate and severe activity was retrospective: thus conclusions based on this division should be regarded as hypothesis generating only, especially as differences between the groups do not reach formal statistical significance if adjustments for multiple comparisons are made. Finally, steroid usage in the two groups is not recorded: one can imagine the possible early poor response would lead to more steroids being given and so to a better overall result.

I agree with the authors and commentators that alternatives to azathioprine/6-mercaptopurine are needed. I also agree that the therapeutic effect of MMF in chronic active Crohn’s disease should be assessed in properly performed trials, and perhaps more importantly that its effect in maintaining remission and in steroid sparing should be assessed. However, until then, MMF should be considered to have no clear indications for use in Crohn’s disease.

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Reply

EDITOR,—Mycophenolate mofetil (MMF) is an immunosuppressive drug that is often used in organ transplantation. It is an oral prodrug of mycophenolic acid that inhibits inosine monophosphate dehydrogenase and potently suppresses lymphocyte proliferation. Furthermore various clinical trials have shown its efficacy in suppressing autoimmune and chronic inflammatory disorders, such as rheumatoid arthritis, pemphigus vulgaris, and psoriasis. There are several case reports and also our controlled study indicating that MMF can be successfully used in patients with Crohn’s disease. In our study treatment of patients with moderately active Crohn’s disease with MMF/corticosteroid led to a significant reduction in clinical activity score compared with treatment with azathioprine/corticosteroids. These data suggested that treatment of chronic active Crohn’s disease with MMF/corticosteroids would be effective in inducing remission. As corticosteroids were given to patients in addition to
MMF, the data available do not show unequivocally that MMF alone is effective in the maintenance of remission in Crohn's disease. This question is currently under study in a double blind, randomised controlled trial in Europe and the USA, in which the effects of MMF on maintenance of remission will be analysed.

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The first edition of this handbook was a valuable resource to both junior hospital staff and family doctors for its practical coverage of basic gastroenterology. In the seven years since it was first published, there have been many advances in gastroenterology and these have been included in the new edition, which is a rapid reference book which the authors hope will be of interest to doctors and health professionals in clinics, accident and emergency departments and family doctors when indicated; it will continue to be a valuable resource for surgeons in training as well as those in practice.

M R B KEIGHLEY

The clear track record of success of Emer gency Abdominal Surgery is proved by the publication of its third edition. The authors, who are all from Aberdeen, classify themselves as general surgeons and the book is dedicated to the general surgeons of the future. As we enter the millennium, general surgery is still vital to the management of patients with serious injuries, vascular emergencies, gynaecological disorders, and medical aspects of the acute abdomen. They are also to be praised for acknowledging in their preface that, nowadays, vascular surgery should be performed by specialists as should colorectal emergency surgery. Nevertheless, the emphasis in this book is on the clarity of decision making, by gynaecologists where appropriate, and by specialists when indicated. It is to continue to be a valuable resource for surgeons in training as well as those in practice.

R A HARRY

Inflammation, and Sepsis will be held in The 5th World Congress on Trauma, Shock, 2950 West Cypress Creek Road, Fort Lauderdale, USA, on 17–19 February 2000. Further information from: Professor Günther Schimpfl, Department of Paediatric Surgery, Auenbruggerplatz 34, A-8036 Graz, Austria. Tel: +43 316 385 3762; fax: +43 316 385 3775; email: kinderchirurgie@kfunigraz.ac.at

European Courses from the European Postgraduate Gastro-Surgical School

The Board of Directors of the European Postgraduate Gastro-Surgical School announce the following events for 2000:

• 2nd European Update conference will be held at the Academic Medical Centre, Amsterdam, The Netherlands, on 14 and 15 December 2000. Registration fee: NLG 450.

• Functional Disorders of the Colon and Rectum will be held at the Academic Medical Centre, Amsterdam, The Netherlands, on 19 and 20 October 2000. Registration fee: NLG 450.

• Diagnostic and Therapeutic Endoscopic Intervention in Paediatric Gastroenterology will be held at the Academic Medical Centre, Amsterdam, The Netherlands, on 16 and 17 November 2000. Registration fee: NLG 450.

• The 3rd Amsterdam International Update conference on Hepatology will be held at the Academic Medical Centre, Amsterdam, The Netherlands, on 8 and 9 June 2000. Registration fee: NLG 350.

• 3rd World Conference on Digestology will be held in Beijing, China, on 8–11 September 2000. Further information from: World Second Conference on Digestology, PO Box 2345, Beijing 100023, China. Tel: +86 10 6589 1901; fax: +86 10 6589 1893; email: wedj@public.bta.net.cn

Second World Conference on Digestology

The Second World Conference on Digestology will be held in Beijing, China, on 8–11 September 2000. Further information from: World Second Conference on Digestology, PO Box 2345, Beijing 100023, China. Tel: +86 10 6589 1901; fax: +86 10 6589 1893; email: wedj@public.bta.net.cn