Diffuse and intestinal type gastric carcinomas differ in their expression of apoptosis related proteins

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Gastric cancer can be divided into adenocarcinomas of the diffuse and the intestinal type according to the Lauren classification.1 Intestinal type gastric carcinomas are associated with Helicobacter pylori associated chronic gastritis, atrophy, and intestinal metaplasia, which are thought to be precursors of the dysplastic changes that evolve into this type of carcinoma.2 Gastric carcinomas of the diffuse type are also associated with H pylori infection, but not with atrophy and intestinal metaplasia; they are usually less well differentiated, characterised by sheets of cells without gland formation, with the occasional presence of signet ring cells and mucin, and are associated with a poor prognosis compared with the intestinal type of tumour.3

The aim of our study was to investigate whether there are differences in the expression of apoptosis related proteins in intestinal type and diffuse type gastric carcinoma

Dysregulation of apoptosis is a hallmark of malignant transformation of tissues and our hypothesis is that this dysregulation is more pronounced in diffuse type gastric cancers. Important apoptosis related proteins that determine sensitivity to apoptosis include Fas, Bax, Bcl-2, and Bcl-xl. Therefore, the aim of our study was to investigate whether there are differences in the expression of these apoptosis related proteins in intestinal type and diffuse type gastric carcinoma. Moreover, we investigated the extent of apoptosis (active caspase 3) and proliferation (Ki-67) in diffuse type and intestinal type gastric carcinomas. Finally, because activation of the inflammation related transcription factor nuclear factor κB (NF-κB) contributes to resistance to apoptosis and facilitates proliferation, we also investigated the expression of the NF-κB regulated proteins inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) in the two types of gastric carcinoma.

Background: Gastric carcinomas can be divided into intestinal and diffuse types, with the last type having a worse prognosis.

Aims: To investigate whether specific patterns in the expression of apoptosis related proteins correlate with carcinoma type and/or prognosis

Methods: The expression of Fas, Bcl-2, Bax, Bcl-xl, cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) was studied immunohistochemically and the extent of apoptosis and proliferation was investigated in 11 cases of intestinal type and in eight cases of diffuse type carcinoma.

Results: Fas was expressed in all intestinal type and in one diffuse type carcinoma. Bcl-xl was expressed in 10 of 11 intestinal type and in one of eight diffuse type carcinomas. Bcl-2 was expressed in lamina propria immune cells. iNOS was expressed in six of 11 intestinal type and in four of eight diffuse type carcinomas, and COX-2 was expressed in eight of 11 intestinal type and in six of eight diffuse type carcinomas.

Conclusion: Fas and Bcl-xl expression can differentiate between intestinal type and diffuse type gastric carcinomas. No differences in apoptosis and proliferation between intestinal type and diffuse type gastric carcinomas were observed.

MATERIALS AND METHODS

Biopsies from tumours and resected tumours from patients diagnosed with either diffuse or intestinal type gastric carcinoma in the period between 1998 and 2000 were re-graded by one pathologist according to the classification of Lauren.

Immunohistochemical analysis

Sections, 4 μm thick, were cut from paraffin wax embedded tissues and de-waxed.

Staining for Fas, iNOS, COX-2, Bcl-xl, Bcl-2, Bax, active caspase 3, and Ki-67

For antigen retrieval, sections were heated in a microwave (700 W) or pressure cooker under the conditions described in table 1. After treatment, sections were allowed to cool to room temperature for 15 minutes. Endogenous peroxidase activity was quenched by incubation in 0.3% H2O2 in phosphate buffer saline.

Table 1 lists the monoclonal antibodies used for Fas, iNOS, COX-2, Ki-67, and Bcl-xl immunohistochemistry. Biotinylated goat antimouse immunoglobulin (15 μg/ml; Ventana Medical Systems, Tucson, Arizona, USA) and hors eradish peroxidase conjugated avidin (Ventana Medical Systems) were used as secondary and tertiary reagents, respectively. All antibody incubations were performed for one hour at room temperature. Incubations were performed in a Ventana ES automated staining system, according to the manufacturer’s instructions. Peroxidase activity was detected using diaminobenzidine as substrate. Slides were counterstained with haematoxylin and eosin and mounted in mounting medium. Human tonsil was used as a positive control for Fas staining, and Reed-Sternberg cells were used as a positive control for Bcl-xl staining.

Abbreviations: COX-2, cyclooxygenase 2; FasL, Fas ligand; iNOS, inducible nitric oxide synthase; NF-κB, factor nuclear factor κB; NO, nitric oxide
The monoclonal antibodies used as primary antibodies for Bcl-2 and Bax staining were also listed in table 1. Horseradish peroxidase conjugated rabbit antimouse immunoglobulin (1/50 dilution; Dako, Glostrup, Denmark) and horseradish peroxidase conjugated goat antirabbit immunoglobulin (1/50 dilution; Dako) were used as the secondary and tertiary antibody, respectively. For staining of active caspase 3, a polyclonal antibody was used as the primary antibody (table 1). Horseradish peroxidase conjugated goat antirabbit immunoglobulin (1/50 dilution; Dako) and horseradish peroxidase conjugated rabbit antigot immunoglobulin (1/50 dilution; Dako) were used as the secondary and tertiary antibody, respectively. All antibody incubations were performed for one hour at room temperature. Staining was developed using diaminobenzidine as chromogen. Slides were counterstained with haematoxylin and eosin and mounted in mounting medium.

Scoring of immunoreactivity

The immunohistochemical sections stained for iNOS, COX-2, Fas, Bcl-xl, Bcl-2, and Bax were scored separately by three different observers. When differences in interpretation occurred the sample was scored again and a consensus was reached. Scoring was performed based on the percentage of positive staining tumour cells. No staining was scored as 0; 0–10% was scored as 1; 11–50% was scored as 2; and 51–100% was scored as 3.

Statistical analysis

To compare differences between the intensity in expression of each separate protein in the intestinal type and diffuse type carcinomas, a Somers’ d test was performed. The analysis was performed using Sigmaplot Scientific Software (SPSS Inc, Chicago, Illinois, USA).

A p value < 0.05 was considered significant.

RESULTS

Material

Eleven tissue samples containing intestinal type and eight tissue samples containing diffuse type carcinoma were used for staining. The mean age of the patients with intestinal type carcinoma was 73 years (range, 58–92), and for diffuse type carcinoma, 66 years (range, 56–92). Tissue was obtained both from biopsies (seven diffuse type and eight intestinal type) and resected tumours (one diffuse type and three intestinal type).

Immunohistochemical staining of tumour cells of the diffuse and intestinal type carcinomas

See table 2 for an overview of the results.

Staining was membranous/cytoplasmic for Fas; nuclear for Ki-67; and cytoplasmic for iNOS, COX-2, Bcl-xl, Bcl-2, Bax, and active caspase 3.

Table 1: Immunohistochemistry methods

<table>
<thead>
<tr>
<th>Protein</th>
<th>Section</th>
<th>Antigen retrieval</th>
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<tr>
<td>Fas</td>
<td>Paraffin wax</td>
<td>MW 3×10 min at 98°C in 1mM EDTA; pH 8.0</td>
<td>Mouse monoclonal at 1/400 dilution; Upstate Biotechnology, Lake Placid, USA</td>
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<td>Bcl-xl</td>
<td>Paraffin wax</td>
<td>MW 2×15 min at 98°C in 0.1M Tris HCl; pH 9.0</td>
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<td>Bax</td>
<td>Paraffin wax</td>
<td>MW 1×8 min boiling in 10mM citrate; pH 6.0</td>
<td>Mouse monoclonal at 1/400 dilution; Santa Cruz Biotechnology P19, Santa Cruz, California, USA</td>
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<td>COX-2</td>
<td>Paraffin wax</td>
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<td>Mouse monoclonal at 1/50 dilution; Transduction Laboratories, Lexington, Kentucky, USA</td>
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<td>Mouse monoclonal at 1/50 dilution; Transduction Laboratories, Lexington, Kentucky, USA</td>
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<tr>
<td>Active caspase 3</td>
<td>Paraffin wax</td>
<td>MW; 1×8 min boiling in 10mM citrate buffer; pH 6.0</td>
<td>Rabbit polyclonal at 1/100 dilution; Cell Signalling Technology, Beverly Massachusetts, USA</td>
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<td>Ki-67</td>
<td>Paraffin wax</td>
<td>3×15 min at 115°C in 0.2% SDS in maleate buffer; pH 6.0</td>
<td>Mouse monoclonal MIB-1 at 1/400 dilution; Immunotech, Marseille, France</td>
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For the Fas staining human tonsil was used as the positive control, for Bcl-xl and Bax staining Reed-Sternberg cells were used as the positive control. COX-2, cyclooxygenase 2; iNOS, inducible nitric oxide synthase; MW, microwave at 700 W; SDS, sodium dodecyl sulfate.

Table 2: Overview staining results for tumour cells

<table>
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<tr>
<th>Type</th>
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<th>iNOS</th>
<th>Bcl-2</th>
<th>Bax</th>
<th>Bcl-xl</th>
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</table>

0, 0% of tumour cells stained; 1, 0–10% of tumour cells stained; 2, 11–50% of tumour cells stained; 3, >50% of tumour cells stained.

COX-2, cyclooxygenase 2; iNOS, inducible nitric oxide synthase.
Staining for active caspase 3, indicating apoptosis, was seen in both intestinal and diffuse type carcinoma cells. However, not all tumours were positive for caspase 3 and not all tumour cells within one specimen were positive. In addition, no difference in staining pattern was seen between intestinal and diffuse type gastric carcinomas. As an example, active caspase 3 staining is shown for a diffuse type gastric carcinoma in fig 2. Ki-67 staining, which is a measure of proliferative cells, was seen in both types of carcinoma in all tumour specimens and also in crypts. For all staining procedures, no differences were seen between sections derived from biopsy material and sections derived from resected tumour material.

DISCUSSION

Our study revealed striking differences in the expression of two apoptosis related genes, Fas and Bcl-xl, between intestinal type and diffuse type gastric carcinomas. Fas expression was positive in all intestinal type carcinomas, but in only one diffuse type carcinoma. Fas is a member of the tumour necrosis factor receptor superfamily. Activation of this receptor by Fas ligand (FasL) activates caspase 8 and the apoptotic signal transduction pathway. Fas expressing cells are vulnerable to FasL induced cell death. FasL is predominantly expressed by lymphocytes, although other cells can also express FasL. Therefore, Fas mediated cell death can only occur when FasL positive cells are in close proximity to Fas positive target cells. Diffuse type carcinoma has a poor prognosis compared with intestinal type gastric carcinoma. This might be explained at least in part by the lack of Fas expression on diffuse type gastric carcinoma cells, resulting in less vulnerability to apoptosis induced by FasL expressing cells. This is in accordance with a previous report from Shinohara et al demonstrating significantly fewer apoptotic cells in poorly differentiated gastric tumours compared with well differentiated gastric tumours. Our results are in contrast to those of a previous study from Vollmers et al, who reported Fas expression in diffuse type carcinoma but not in intestinal type carcinoma. However, there were some differences in study design: the study of Vollmers et al used frozen sections and a different Fas specific antibody. On western blot, the Fas antibody used by Vollmers et al recognised three different bands, indicating non-specific binding.

Bcl-xl was expressed in 10 of 11 intestinal carcinomas and in only one of eight diffuse type carcinomas. Bcl-xl is a member of the Bcl-2 protein family. Members of this family play an important role in the regulation of apoptosis. This family contains both proapoptotic members (Bax, Bid, Bad, and Bak) and antiapoptotic members (Bcl-2 and Bcl-xl). Bcl-2 proteins regulate the permeability of the mitochondrial membrane. Increased mitochondrial permeability allows leakage of cytochrome c from mitochondria into the cytoplasm, triggering caspase activation and apoptosis. Proapoptotic Bcl-2 proteins increase mitochondrial membrane permeability, whereas antiapoptotic members antagonise the effects of proapoptotic Bcl-2 proteins.

“...The poor prognosis of diffuse type gastric carcinomas, which lack Fas, could result from reduced susceptibility to immune surveillance by Fas ligand expressing T cells and subsequently to increased metastatic potential”

To investigate whether the differences in Fas and Bcl-xl expression between diffuse type and intestinal type...
carcinomas is reflected in differences in apoptosis and/or proliferation, we analysed the expression of active caspase 3 as a marker for apoptosis and Ki-67 as a marker for proliferation in our tissue samples. No differences in these markers were seen between diffuse type and intestinal type gastric carcinoma. The apoptosis seen in the tumour cells in these samples may be indicative of enhanced proliferation, because these cells were also positive for Ki-67. This suggests that the differences in Bcl-xL and Fas expression between diffuse type and intestinal type gastric carcinoma are not related to large differences in the survival and proliferation of tumour cells. This does not exclude the possibility that the differences in the expression of Fas and/or Bcl-xL influence metastatic potential or susceptibility to immune surveillance. For example, the poor prognosis of diffuse type gastric carcinomas, which lack Fas, could result from reduced susceptibility to immune surveillance by FasL expressing T cells and subsequently to increased metastatic potential. Bax was found in both intestinal type carcinoma and diffuse type carcinoma and there was no difference in expression between the two groups. Bcl-2 was not expressed in tumour cells but only in lamina propria immune cells. Kyokane et al demonstrated Bcl-2 expression in early gastric cancer of the elevated type. Others demonstrated Bcl-2 expression in tumour cells of both intestinal type carcinoma and diffuse type gastric carcinoma, but mostly in a small proportion of the tumour cells. We did not investigate other Bcl-2 family members, apart from Bcl-2, Bax, and Bcl-xL, but it would be interesting to investigate whether other family members are differentially expressed in gastric carcinoma.

Activation of the transcription factor NF-κB is important in the resistance against apoptosis. NF-κB regulates the transcription of many inflammation associated genes, including antiapoptotic genes, such as iNOS and COX-2. We have previously reported on the protective role of NF-κB activation and iNOS in liver cells. We observed iNOS expression in both types of gastric carcinomas. Increased iNOS activity has been found in gastric cancer. iNOS synthesises nitric oxide (NO). NO may promote the generation and proliferation of tumour cells by various mechanisms. First, NO may inhibit apoptosis by inhibiting caspase activity as a result of nitrosylation of essential cysteine residues in the catalytic site of caspases. Second, NO produced by iNOS inhibits DNA repair enzymes, possibly resulting in the appearance of potentially tumorigenic cells, containing DNA mutations, as recently suggested by Jaiswal et al for the pathogenesis of cholangiocarcinoma. Therefore, the expression of iNOS in gastric carcinoma cells may endow these cells with a proliferation advantage. Similarly, COX-2 expression contributes to resistance against apoptosis. Various studies have demonstrated that the inhibition of COX-2—for example, in colon carcinoma cell lines—induces apoptosis of these tumour cells. However, there was no clear difference between COX-2 expression in the two carcinomas and this observation is in agreement with other reports.

In conclusion, intestinal type and diffuse type gastric carcinomas can be differentiated on the basis of Fas and Bcl-xL expression. These differences in Fas and Bcl-xL expression do not lead to clear differences in apoptosis or proliferation. However, the lack of Fas expression on diffuse type gastric carcinoma cells might protect these cells from immune surveillance.

**Take home messages**

- There were striking differences in the expression of two apoptosis related genes, Fas and Bcl-xL, between intestinal type and diffuse type gastric carcinomas.
- However, these differences did not lead to differences in apoptosis or proliferation as measured by caspase 3 and Ki-67, respectively.
- The lack of Fas expression on diffuse type gastric carcinoma cells might partly be responsible for the worse prognosis for this type, by protecting the cells from immune surveillance.

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