APOPTOSIS IN (PRE-) MALIGNANT LESIONS IN THE GASTRO-INTESTINAL TRACT

JANNEKE VAN DER WOUDE

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CHAPTER 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Inflammatory conditions are characterized by activation of the transcription factor nuclear factor kappa B (NF-kB), resulting in the expression of NF-kB-regulated, inflammation-related genes, such as inducible nitric oxide synthase (iNOS) and cyclo-oxygenase-2 (COX-2). Expression of these genes contributes to the survival of cells. Indeed, exposure to pro-inflammatory cytokines in the absence of NF-kB activation leads to apoptosis[1,2]. Chronic inflammatory conditions are accompanied by constitutive activation of NF-kB and hence, to the continuous expression of pro-survival genes, as has been observed in chronic gastritis[3]. Although beneficial for the survival of cells during exposure to inflammatory stress, the continuous activation of NF-kB may also pose a risk: cells with a pro-survival phenotype may give rise to continuously proliferating cells and may thus be tumorigenic. Progression to a malignant phenotype of these cells will most likely involve additional changes in the expression of non-NF-kB regulated genes e.g. a shift in the balance of pro- and anti-apoptotic genes towards a more anti-apoptotic phenotype.

1.1. Actions of iNOS and COX-2

iNOS is one of three NO synthases responsible for the production of nitric oxide from L-arginine. Whereas endothelial NOS (eNOS, NOS-III) and neuronal NOS (nNOS, NOS-I) are calcium-dependent NO synthases and responsible for picomolar nitric oxide concentrations, iNOS is a calcium-independent NO synthase and responsible for NO production in the nanomolar range[4]. NO, produced by nNOS and eNOS, is necessary for physiological functions in the human body[5,6]. In contrast to the eNOS and nNOS that are active only when intracellular calcium concentrations are elevated, iNOS activity is calcium-independent. The expression of iNOS is induced by various inflammatory cytokines, in particular tumor necrosis factor alpha (TNFα), interferon-gamma and bacterial cell wall products like lipopolysaccharide (LPS)[7,8,9]. Anti-apoptotic actions of NO include the inhibition of caspases, the proteases involved in apoptosis and elevation of cyclic nucleotides[10]. In addition, NO is able to cause DNA-damage and simultaneously inhibit DNA repair mechanisms[11]. This results in the preservation and propagation of DNA damage in proliferating cells. The COX-isoenzyme COX-2 is normally expressed at very low levels but is rapidly induced at sites of inflammation[12,13]. COX-2 predominates in inflammatory conditions

and is also induced in cancer cells[14,15]. Products of COX-2 promote cell survival: the COX-2 specific inhibitor celecoxib causes regression of polyps in patients with familial adenomatous polyposis[16]. In cell lines expressing COX-2, inhibition of COX-2 sensitizes these cells to apoptotic stimuli. Therefore COX-2 expression may be a target for the chemoprotective effect of NSAIDs in pre-malignant conditions.

1.2 Apoptosis and apoptosis-related proteins

Apoptosis is important for the removal of unnecessary, aged or damaged cells. Abnormal resistance to apoptosis entails malformations, autoimmune disease or cancer due to the persistence of unwanted cells. Apoptosis is regulated through different pathways[17]. In general, apoptosis is initiated after activation of death-receptors at the plasma membrane. These receptors include the TNF receptor, activated by TNF, Fas activated by FasLigand and TRAIL- or DR-receptors, activated by TRAIL[17]. In contrast to Fas and TRAIL receptor, activation of the TNF receptor also activates a survival pathway, regulated by NF-kB. Stimulation of death receptors will lead to the activation of initiator caspases, such as caspase-8 and caspase-10. Initiator caspases activate down-stream effector caspases such as caspase-3 that cleave essential cellular proteins leading to cell death. Activation of caspase-3 is amplified by pro-apoptotic signals released from damaged mitochondria[18]. These pro-apoptotic proteins from mitochondria include cytochrome-c, Diablo/Smac and HtrA2/Omi[18]. The release of these proteins into the cytoplasm causes activation of capsase-3. Therefore, the integrity of mitochondrial membranes is essential to avoid apoptosis. This is regulated to a large extent by members of the Bcl-2 family[19]. This family comprises both anti-apoptotic members (e.g. Bcl-2, Bcl-xl, Bfl1/A1) and pro-apoptotic members (e.g. Bid, Bak, Bad, Bax). Pro-apoptotic members of the Bcl-2 family contribute to the formation of pores in the outer mitochondrial membranes, facilitating leakage of pro-apoptotic proteins into the cytoplasm. Anti-apoptotic members of the Bcl-2 family antagonize the pro-apoptotic members, thus inhibiting the formation of pores. The balance between pro- and anti-apoptotic Bcl-2 family members within a cell determines its relative resistance or sensitivity to apoptosis[19]. A shift in this balance towards a more anti-apoptotic phenotype may result in transformation of a normal cell into a continuously proliferating malignant tumor cells. Some members of the Bcl-2 family, including anti-apoptotic A1/Bfl-1 and Bcl-xl are under the control of the transcription factor NF-kB. Overexpression of Bcl-2 family

members in several cancer cell types has been reported, e.g. hepatocellular carcinoma and leukemias [20,21].

1.3 Aim of this thesis

The aim of this thesis is to investigate the expression of the NF-kB-regulated anti-apoptotic genes iNOS and COX-2 in chronic inflammatory, pre-malignant and malignant disorders of the gastrointestinal tract. In addition, the expression of the apoptosis-related genes Bcl-2, Bcl-xl and Bax was investigated in these disorders. Finally, the extent of apoptosis, using activated caspase-3 as a specific parameter of apoptosis, was determined.

1.4 Outline of the thesis

In **Chapter 2** an overview of the existing literature on chronic inflammation, apoptosis and (pre-) malignant lesions in the gastro-intestinal tract is given. In **Chapter 3** the expression of the NF-kB-regulated proteins iNOS and COX-2 as well as the expression of the apoptosis-related proteins Fas, Bcl-2, Bax and Bcl-xl in Barrett's esophagus and Barrett's esophagus associated adenocarcinoma is reported. In the subsequent two chapters alterations in apoptosis and apoptosis-related proteins in the stomach are described. In **Chapter 4** the expression of COX-1 and COX-2 and iNOS in gastritis of various etiology and in intestinal metaplasia as a precursor for the sequence to intestinal type gastric cancer is reported. In addition, the possibilities of using these proteins as targets for chemoprotective strategies are evaluated. In **Chapter 5** apoptosis and the expression of apoptosis-related proteins in intestinal type and diffuse type gastric carcinoma are compared. In **Chapter 6** the issue of apoptosis in celiac disease using two novel and more specific assays to detect apoptosis is re-addressed: immunohistochemical staining for active caspase-3 using an antibody recognizing only processed and active caspase-3 and immunohistochemical staining for caspase-cleaved cytokeratin-18 (Cytodeath). In addition, the expression of the apoptosis-related genes Fas, Bax, Bcl-xl, Bcl-2 and iNOS in active and inactive celiac disease is determined. In longstanding colitis it is often difficult to discriminate between a true adenoma and dysplasia associated neoplastic lesions. In this disorder endoscopic surveillance is needed, which is very demanding for the patient. In **Chapter 7** we describe the expression of apoptosis-

related proteins in the malignant transformation of longstanding colitis. Finally, the results of the thesis are summarized and discussed in **Chapter 8.**

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CHAPTER 2

CHRONIC INFLAMMATION, APOPTOSIS AND (PRE-)MALIGNANT LESIONS IN THE GASTRO-INTESTINAL TRACT

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1. NF-κB, iNOS AND COX-2 EXPRESSION IN BARRETT'S ESOPHAGUS

Barrett's esophagus (BE) is a typical pre-malignant condition of the esophagus. Normal epithelium is replaced by columnar epithelium and eventually this can evolve to adenocarcinoma[1-3].

iNOS was reported to be induced in BE and BE associated carcinoma[4-6]. COX-2 expression in BE is induced in both pre-cancerous and cancerous lesions. This could have implications for chemopreventive therapy. Although data are limited, selective inhibition of COX-2 in esophageal adenocarcinoma cells suppresses growth and induces apoptosis[7-9]. It was demonstrated that selective and non-selective COX-2 inhibitors can inhibit inflammation, COX-2 activity, and development of adenocarcinoma induced by reflux[10]. On the other hand, in a retrospective trial among patients with BE, no difference in cancer risk in BE was found in the presence or absence of COX-inhibitors of the NSAID-family[11]. Finally, one report observed no COX-2 expression in dysplastic lesions in BE[12].

In summary: iNOS is induced in BE. Due to the scarcity of investigations, it is not clear what the exact consequences of iNOS in BE epithelium are with respect to survival and apoptosis. The use of iNOS inhibitors as chemopreventive intervention in BE has not been reported yet. The data on COX-2 expression and chemoprevention in BE are conflicting. No studies report on NF-kB activation in BE.

2. APOPTOSIS AND APOPTOSIS-RELATED PROTEINS IN BARRETT'S ESOPHAGUS

Apoptosis measured by counting apoptotic cells in different stages of BE was found to be increased in BE compared to normal fundus epithelium, whereas apoptosis determined by the TUNEL assay in BE was almost absent[13,14]. In BE, with or without dysplasia or carcinoma, decreased Fas expression has been reported[15]. This suggests a protective mechanism against apoptosis in BE and BE-associated adenocarcinoma. In addition to gastric acid in reflux esophagitis there is also reflux of bile in BE. Bile acids have been shown to activate Fas, inducing apoptosis in liver cells. The decreased expression of Fas may be an adaptation of epithelium against exposure to pro-apoptotic bile acids resulting in decreased sensitivity to apoptosis. Furthermore, bile acids have been shown to promote survival of cholangiocyte cell lines by activating the Epidermal Growth Factor (EGF) receptor[16]. If confirmed, this is

an example of an adaptation to inflammatory stress, resulting in an anti-apoptotic phenotype and predisposing to cancer. Reports on the expression of Bax in BE demonstrated a positive association between progression to adenocarcinoma and Bax expression[17]. Increased Bax expression alone in these cells may not be pro-apoptotic perse. Only in response to an apoptotic trigger does Bax translocate from cytoplasm into mitochondrial membranes forming pores. In contrast, anti-apoptotic Bcl-2-family members are constitutively located in intracellular membranes including mitochondria, and therefore increased expression of these Bcl-2 members directly contribute to a more apoptosis-resistant phenotype. Reports on Bcl-2 expression in the neoplastic transformation to adenocarcinoma are scarce. Some studies showed an increased expression of Bcl-2 in neoplastic transformation, but others failed to demonstrate Bcl-2 expression at all in the epithelium of Barrett's esophagus[18-20]. In contrast, expression of the anti-apoptotic Bcl-2 family member Bcl-xl increased in the sequence towards adenocarcinoma[21] and this increase may compensate for the observed increase in Bax expression.

In summary: There are hardly any reports on apoptosis in BE. Distinct changes in the expression of Bcl-2 family members occur, but the consequences for the resistance against apoptosis are not clear.

3. NF-κB, iNOS AND COX-2 EXPRESSION IN PRE-MALIGNANT AND MALIGNANT CONDITIONS IN THE STOMACH

According to the Lauren classification[22], gastric adenocarcinomas can be divided into those of the diffuse and those of the intestinal type. Atrophic gastritis and intestinal metaplasia can eventually result in the development of pre-malignant and malignant lesions in intestinal type cancer[23]. It is well accepted that Helicobacter pylori-associated gastritis is causally linked to both types of gastric cancer[24-26].

Compared to normal gastric antral mucosa, NF-kB in Helicobacter pylori (Hp) gastritis is activated and translocated to the nuclei of epithelial cells and its expression correlates with the activity of gastritis[3,27-30]. NF-kB is not only activated in epithelial cells but also in endothelial cells, macrophages and B lymphocytes in the lamina propria. Several studies have demonstrated the activation of NF-kB by Helicobacter pylori in human gastric cancer cell lines and in vivo activation of NF-kB was demonstrated in intestinal type gastric carcinoma[31,32]. In the latter report a correlation

was found between NF-kB activity and clinicopathological features of the carcinoma. iNOS is induced in the gastric epithelium of patients with Helicobacter pylori-induced gastritis[33,34] and also in epithelium of intestinal metaplasia[35,36]. Reports on the expression of iNOS in pre-malignant and malignant lesions showed an increased expression of iNOS[37-42]. Furthermore, in these studies, expression of iNOS correlates with tumor invasiveness, metastatic potential and a worse prognosis. A relationship between NF-kB activity and iNOS expression in Helicobacter pylori associated gastritis of humans has been demonstrated[43,44]. In these studies inhibition of NF-kB prevented iNOS expression and NO production. The authors suggested that iNOS inhibition was restricted to epithelial cells and did not occur in inflammatory cells of the lamina propria. Most studies report induction of COX-2 expression in Helicobacter pylori gastritis[45,46,57]. The localisation of COX-2 expression remains controversial: some studies showed COX-2 expression in both epithelial cells and lamina propria immune cells whereas other studies showed only expression in lamina propria immune cells. In addition, COX-2 expression has been demonstrated in epithelium of gastic atrophy and intestinal metaplasia and in both diffuse and intestinal type gastric adenocarcinoma[47,48], no difference in COX-2 expression between diffuse and intestinal type gastric carcinoma was observed and not all tumor cells were positive for COX-2[49]. Inhibition of NF-kB resulted in inhibition of COX-2 expression and inhibition of proliferation of gastric cancer cells[50].

In summary: NF-kB, iNOS and COX-2 are induced in Hp-gastritis, intestinal metaplasia, dysplasia and adenocarcinoma of the stomach. The localisation and degree of expression varies between studies. NF-kB activation is involved in the expression of iNOS, COX-2 and cell proliferation. Some data suggest that inhibition of NF-kB activation or NF-kB-regulated genes may sensitize gastric cancer cells to apoptosis or inhibit their proliferation.

4. APOPTOSIS AND APOPTOSIS-RELATED PROTEINS IN PRE-MALIGNANT AND MALIGNANT CONDITIONS IN THE STOMACH

Gastric intestinal metaplasia is associated with increased apoptosis compared to normal gastric mucosa[51]. Increased apoptosis, determined using the TUNEL assay, was demonstrated in intestinal type gastric carcinomas but other studies failed to confirm this finding. Since the TUNEL assay is prone to artefacts, other ways of

determining apoptosis should clarify this apparent discrepancy[52-54]. In one study activated caspase-3 was not detected in gastric cancer cells nor in the gastric mucosa surrounding the gastric cancer whereas in normal gastric mucosa activated caspase 3 expression was detected. This suggests that inhibition of apoptosis, as indicated by the lack of caspase-3 activation, is involved in the transformation to gastric carcinoma[55]. In normal gastric mucosa Fas expression is hardly detectable in epithelial cells. Fas expression increases in gastric atrophy and intestinal metaplasia and is detectable in all cases with dysplasia[56]. Vollmers et al reported Fas expression in the diffuse type carcinoma but not in the intestinal type carcinoma[57], whereas we observed exactly the opposite result[58]. Another group reported high expression of Fas in gastric cancer cells and reduced Fas expression with the advancement of the carcinoma[59]. The increased Fas expression on malignant cells compared to normal gastric epithelium is difficult to explain. It remains to be determined whether the increased Fas expression really results in increased sensitivity to apoptosis. Possibly, increased Bcl-2 expression may counteract the increased Fas expression in terms of sensitivity to apoptosis: in normal gastric mucosa Bcl-2 expression is confined to only a few regenerating epithelial cells of the mucous neck region. Bcl-2 expression is increased in chronic gastritis, intestinal metaplasia and dysplasia[60-64]. Kyokane et al demonstrated Bcl-2 expression in early gastric cancer of the elevated type. This elevated type probably resembles adenomatous polyps in the colon[65]. Others demonstrated Bcl-2 expression in tumor cells of both intestinal type carcinoma as well as diffuse type gastric carcinoma, but mostly in a small percentage of the tumor cells[66,67]. However, the expression of Bcl-2 seems to be higher in intestinal type gastric cancer compared to diffuse type cancer[68,69,75,76]. Bax expression is reported in both intestinal and diffuse type carcinomas but seems to be decreased in comparison to the surrounding non-tumorous tissue, favouring an anti-apoptotic phenotype in gastric cancers[70,83].

In summary: Apoptosis as determined by the expression of activated caspase-3 is reduced in gastric cancer compared to normal gastric mucosa. Reports on apoptosis using the TUNEL assay are conflicting. Expression of Fas and Bcl-2 proteins are increased in intestinal metaplasia, dysplasia and adenocarcinoma compared to the normal gastric mucosa, whereas Bax expression is reduced in gastric cancer cells.

5. NF-kB, iNOS AND COX-2 EXPRESSION IN INFLAMMATORY BOWEL DISEASES

NF-kB activity varies in inflammatory bowel diseases (IBD). NF-kB activation has been observed in macrophages in the lamina proptria and in epithelial cells[71,72]. IL-10, sulphasalazine and immunosuppressive drugs have been reported to inhibit NF-kB activity in the mucosa of patients with Crohn's disease and ulcerative colitis[73-76]. iNOS is clearly expressed in epithelial cells of the inflamed gut[77-79]. The expression of COX-2 in surface epithelial cells and in lamina propria immune cells in areas of inflammation in Crohn's colitis and ulcerative colitis is strongly induced[80,81]. COX-2 overexpression has been described in sporadic colonic neoplasia and in colitis-associated neoplasia but its exact role in neoplastic transformation is not yet clear. One group reported COX-2 overexpression in ulcerative colitis associated neoplasia and in this study the increase in COX-2 expression could not be explained by inflammatory activity alone[82]. However, in this report the expression of COX-2 in adenocarcinoma in longstanding colitis was not as uniform as in the dysplastic regions.

In summary: Only a limited amount of data concerning NF-kB activation and COX-2 and iNOS expression in IBD-related carcinogenesis has been published. Although these proteins are induced in IBD, their role in oncogenesis is not known.

6. APOPTOSIS AND APOPTOSIS-RELATED PROTEINS IN INFLAMMATORY BOWEL DISEASES

In normal intestinal epithelium apoptosis is observed in the crypt and at the luminal surface[83]. Bcl-2 is expressed in the bases of crypts, whereas epithelial cells on the luminal surface express less Bcl-2[84,85]. Bax, Bcl-xl and Bak expression are confined to areas of colonic epithelial cells of the luminal surface[86,106]. There is a higher expression of Bak in the left colon compared to the right colon[87]. Fas is strongly expressed in all epithelial cells of the normal colon throughout the crypt[88]. In ulcerative colitis, apoptotic colonocytes are increased in number throughout the crypt[89]. In the same report Fas expression in the intestinal epithelium of ulcerative colitis patients was comparable to that of normal epithelial cells. Another report confirmed this[90]. In both reports Fas ligand was highly expressed compared to normal colonic epithelium. The reports on expression of apoptosis-related proteins in the epithelium of patients with ulcerative colitis and Crohn's colitis are limited. In

active colitis, no change in Bcl-2 expression compared to normal colonic epithelium was observed[96]. Bcl-2 overexpression was observed in ulcerative colitis-associated neoplasia[91]. Compared to adenomas in areas involved in ulcerative colitis, Bcl-2 expression in ulcerative colitis-associated dysplastic lesions is less frequent[92]. The expression of Bcl-2 in ulcerative-colitis-associated colorectal cancer is significantly lower compared to that in sporadic colorectal cancer[93]. Another report failed to demonstrate a significant difference in Bcl-2 expression between ulcerative colitis associated neoplasia and sporadic adenocarcinomas, although this study revealed less apoptosis in the ulcerative colitis associated neoplasia compared to sporadic adenocarcinomas[94]. Bax expression is reduced in ulcerative colitis compared to normal colonic mucosa[95]. Other reports on expression of apoptosis-related proteins in IBD are mainly focussed on lamina propria T cells.

In summary: Reports on apoptosis in IBD and associated neoplasia are limited. Conflicting data exist on the expression of Bcl-2 in colitis-associated neoplasia compared to sporadic carcinoma. Little is known about the expression in epithelium of other apoptosis-related proteins in the sequence from colitis to carcinoma.

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CHAPTER 3

EXPRESSION OF APOPTOSIS-RELATED PROTEINS IN BARRETT'S METAPLASIA-DYSPLASIA-CARCINOMA SEQUENCE: A SWITCH TO A MORE RESISTANT PHENOTYPE

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SUMMARY

Barrett's esophagus (colomnar-lined esophagus, CLE) is a pre-malignant disorder in which the stratified squamous epithelium is replaced by metaplastic epithelium. To gain more insight into the process of carcinogenesis in CLE we studied several factors involved in the apoptotic pathway in biopsies with gastric metaplasia (GM), intestinal metaplasia (IM), dysplasia, and/or adenocarcinoma. Immunohistochemistry was performed for Fas, Bcl-2 , Bax , Bcl-xl, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). Fas staining was positive in epithelium of all biopsies from patients with CLE but negative in normal gastric mucosa. Fas staining was positive in all tumor cells of the 8 cases containing adenocarcinoma. Bcl-2 was positive in lamina propria immune cells of all specimens. Bax staining was positive in the epithelium of all biopsies, including tumor cells. Bcl-xl was positive in dysplasia and tumor cells, but negative in 8 of 17 biopsies containing IM. iNOS was positive in 20 of 21 biopsies with IM and in 4 of 8 dysplasia biopsies. COX-2 was positive in 7 of 8 adenocarcinomas. We conclude that the apoptotic balance in the transformation from IM to adenocarcinoma switches to an anti-apoptotic phenotype because of increased Bcl-xl expression and decreased Bax expression. Fas can be used as a marker for the differentiation of gastric mucosa and metaplasia in the esophagus. iNOS is highly positive in CLE-associated intestinal metaplasia. COX-2 negative in non-malignant CLE. Therefore pharmacological inhibition of COX-2 activity is unlikely to be effective in the prevention of CLE-associated adenocarcinoma. There was no clear correlation between iNOS expression and activation of pro- and anti-apoptotic genes.

1. INTRODUCTION

In Barrett's esophagus, or colomnar-lined esophagus (CLE), the normal stratified squamous epithelium lining the esophagus has been replaced by metaplastic columnar epithelium containing goblet cells[1]. This replacement is a risk factor for neoplastic transformation, and there is evidence for the sequential development of adenocarcinoma via intestinal metaplasia and low grade and high grade dysplasia[2,3] Therefore, periodic surveillance endoscopy with multiple biopsies is recommended for CLE patients. Other modalities to evaluate the esophagus for Barrett's metaplasia

and impending malignant degeneration have been investigated, but histologic examination remains the gold standard[4-7]. To simplify surveillance new preventive treatment options are needed[8-12].

Disturbances in apoptosis are supposed to play an important role in the sequential development of dysplasia and cancer. To gain better insight in these disturbances we studied the expression of 4 apoptosis-related proteins Fas, Bcl-2, Bax and Bcl-xl. We further studied the expression of 2 other closely apoptosis-related proteins: inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). Nitric oxide, produced by iNOS, has been demonstrated to inhibit apoptosis by inhibiting caspase activity. However, chronic exposure to high levels of nitric oxide can also promote apoptosis[13].

High COX-2 expression has been demonstrated in human colorectal adenomas and in gastric adenocarcinomas[14,15]. Inhibition of COX-2 activity promotes apoptosis and could be a promising modality for chemoprevention of these tumors, as has been shown in patients with familial adenomatous polyposis[16,17].

2. MATERIALS AND METHODS

2.1 Patient selection and tissue collection

We studied tissue samples taken from patients who participated in an endoscopy surveillance program between January 1998 and December 2000 at the University Hospital Groningen. All patients used omeprazole at the time of endoscopy. Samples were stained with haematoxylin and eosin and periodic acid-Schiff. Standard histological examination was performed on these stained samples with attention given to the type of metaplasia, presence and degree of inflammation and dysplasia and presence of adenocarcinoma. The most distal samples from the esophagus from each patient were used in this study. Samples from patients with esophagitis graded according to Savary and Miller[18] during endoscopy and from those with histologically active inflammation, graded according to Paull et al[19], were excluded from this study. After exclusion of these samples and samples without metaplasia, dysplasia or adenocarcinoma, the samples were histologically graded by a single pathologist. Samples were categorized as GM or specialized columnar metaplasia[19]. Dysplasia was scored as absent, indefinite-low grade and high grade[20]. Different histological gradations could coexist in one sample.

2.2 Immunohistochemical analysis

All stainings were performed on deparaffinized 4 micrometer thick sections. These sections were cut from formalin-fixed and paraffin embedded tissues.

An overview of the methods is given in table 1.

Table 1: Immunohistochemistry methods

Protein	Section	Antigen retrieval	Primary antibody
Fas	Paraffin	2x15min at 98°C in 1mM EDTA; pH6.0	Mouse monoclonal at 1:400 Upstate Biotechnology, Lake Placid; cat. nr. 05-201
Bcl-xl	Paraffin	2x15min at 98°C in 0,1M Tris HCl; pH9.0	Mouse monoclonal at 1:100 Zymed Laboratories, South San Francisco; cat. nr. 33-6300
Bax	Paraffin	MW (700W) 8 min in 10mM citrate; pH6.0	Mouse monoclonal at 1:400 Santa Cruz Biotecnology P19, Santa Cruz ; cat. nr. SC-526
Bcl-2	Paraffin	MW (700W) 8 min in 10mM citrate; pH6.0	Mouse monoclonal at 1:50 Dako Glostrup, Denmark; cat. nr. M 0887
COX-2	Paraffin	2x15min at 98°C in 1mM EDTA; pH6.0	Mouse monoclonal at 1:50 Transduction Laboratories;cat. nr. C-22420
iNOS	Paraffin	2x15min at 98°C in 1mM EDTA; pH6.0	Mouse monoclonal at 1:50 Transduction Laboratories: cat. nr. N-39120

2.3 Quantitation of immunoreactivity

The immunohistochemical sections stained with Fas, Bcl-2, Bax, Bcl-xl, iNOS and COX-2 were scored by 3 different observers for the percentages of epithelial cells stained. In case of differences in interpretation, the sample was scored again, and a consensus was reached. Absence of staining was scored as 0; 0 to 10% staining was scored as 1; 11 to 50% staining as 2; and 51 to 100% was scored as 3. Tissue samples stained with Fas, Bcl-xl and Bax were also scored for intensity of staining in each individual epithelial cell on a scale of 0 to 3, with 0 being negative; 1, weak; 2, moderate; 3, strong.

Staining of tumor cells was scored in the same way.

2.4 Statistical analysis

The relationship between grade and intensity in expression of each separate protein on the one hand and the histological parameters on the other was evaluated using Somers'd test was performed. The analysis was performed using Sigmaplot Scientific Software (SPSS Inc., Chicago, IL, USA). A p value < 0.05 was considered significant.

3. RESULTS

3.1 Patients

Samples from 28 patients (20 male, 8 female) were included. The age of these patients was 31 to 86 years (mean 58). Six samples contained gastric metaplasia (GM), 21 contained intestinal metaplasia (IM), 8 contained indefinite for and low grade dysplasia (D) and 6 contained high grade dysplasia and carcinoma (CA). The adenocarcinoma group was expanded with archival resection material from 4 patients with CLE-associated adenocarcinoma.

3.2 Immunohistochemistry (table 2 and 3)

3.2.1 Fas staining in epithelium and tumor cells

Three of 21 tissue samples containing IM, 2 of 8 samples containing dysplasia and 1 of 10 samples containing CA were not stained, because of a lack of material. Fas staining was present in the epithelium of all tissue samples, including GM (fig. 1a). Tumor cells were all positive (fig. 1b). Fas staining of normal gastric mucosa in patients with GM and in controls was negative (fig. 1c). The correlation between staining grade and sequence from IM to CA was significant: r = 0.527 (± 0.126), p= 0.01. The correlation between staining intensity and sequence from IM to CA was also significant: r = 0.329 (± 0.126), p < 0.001.

Table 2 . Staining *intensity* of Fas, Bax and Bcl-xl expression

Intensity	Fas*				Bax**				Bcl-xl***			
	GM	IM	D	CA	GM	IM	D	CA	GM****	IM	D	CA
0	0	1	0	0	0	0	0	1		8	0	1
1	0	4	2	0	1	4	4	6		8	1	0
2	3	9	2	3	1	7	2	2		1	4	3
3	3	4	2	6	3	9	2	0		0	3	6

* a significant difference from intestinal metaplasia to cancer, ** a significant difference from intestinal metaplasia to cancer
*** a significant negative difference from intestinal metaplasia to cancer, **** not scored

Table 3. Staining *grade* of iNOS, Fas, Bax and Bcl-xl expression

Grade	iNOS*				Fas**				Bax***				Bcl-xl****			
	GM	IM	D	CA	GM	IM	D	CA	GM	IM	D	CA	GM*****	IM	D	CA
0	6	1	4	6	0	1	0	0	0	0	0	1		8	0	1
1	0	3	4	0	0	5	2	0	0	3	0	0		7	1	0
2	0	14	0	0	2	9	1	0	2	3	0	0		2	3	2
3	0	3	0	0	4	3	3	9	3	14	8	8		0	4	7

* significant negative difference from intestinal metaplasia to cancer, ** significant difference from intestinal metaplasia to cancer
*** not significant difference from intestinal metaplasia to cancer, **** significant difference from intestinal metaplasia to cancer
***** not scored

3.2.2 Bcl-2 staining in epithelium and tumor cells

Bcl-2 staining was negative in epithelium of CLE and was also not present in tumor cells. Lamina propria immune cells showed positive staining (fig. 1d).

3.2.3 Bax staining in epithelium and tumor cells

Because of a lack of material 1 tissue sample of the GM, one of the IM group and one of the CA group was excluded from the Bax staining series. In all groups, epithelial cells stained positive (fig. 1e). In tumor cells, Bax staining was also clearly positive (fig. 1f).

The correlation between staining grade and sequence from IM to CA was not significant: $r = 0.302$ (± 0.197), $p = 0.141$. The correlation between staining intensity in each individual epithelial cell and sequence from IM to CA was significant.
$r = –0.443$ (± 0.101), $p = 0.001$.

3.2.4 Bcl-xl staining in epithelium and tumor cells

Because of a lack of material, 4 tissue samples of the IM group were excluded from the Bcl-xl staining series. Staining in dysplasia and tumor cells (fig. 1g) was mostly positive with a strong intensity. The correlation between staining grade and sequence from IM to CA was significant: $r = 0.600$ (± 0.085), $p < 0.001$. The correlation between staining intensity and sequence was also significant: r = 0.600 (± 0.088), $p < 0.001$.

3.2.5 iNOS staining in epithelium and tumor cells

iNOS staining was intensely positive in epithelium of IM (fig. 1h). Epithelial staining was positive in 4 out of 8 samples containing dysplasia (fig. 1i). GM and tumor cells

were negative for iNOS. The correlation between staining grade and sequence from IM to CA was significant: $r = –0.678$ (± 0.084), $p < 0.001$.

3.2.6 COX-2 staining in epithelium and tumor cells

COX-2 expression was not present in epithelium of CLE and associated dysplasia. Lamina propria immune cells and myofibroblasts showed positive staining (fig. 1j). In the adenocarcinoma group, tumor cells, but not normal epithelium, were positive for COX-2 in 9 of 10 samples. In these biopsies however, only a minority of tumor cells stained positive (fig. 1k).

4. DISCUSSION

In CLE, iNOS is highly expressed in IM and in 50% of samples containing dysplasia, but not in CLE associated adenocarcinoma. All our samples containing high-grade dysplasia were positive for iNOS, as reported by Wilson et al[21]. However, in contrast to these authors, we did not observe iNOS expression in CLE-associated adenocarcinomas. Nitric oxide, the product of iNOS, is able to inhibit apoptosis in low concentrations, due in part to inhibition of caspase activity[13]. In high concentrations, it can induce apoptosis. We could not detect apoptosis in CLE intestinal metaplasia using staining for caspase-cleaved cytokeratin 18 (cytodeath) (data not shown). Whether this means that iNOS inhibits apoptosis in CLE IM remains to be established, because apoptosis was also absent in iNOS negative CLE dysplasia.

The role of apoptosis in the sequence of IM to adenocarcinoma is not clear[22,23]. Our results suggest that Bcl-2 is not involved in the carcinogenesis of CLE, because only lamina propria immune cells, not the epithelium, showed positive staining. Bax, a pro-apoptotic member of the Bcl-2 family, was positive in all samples. Although no significant differences in staining grade was observed among the different groups, there was a significant negative correlation between intensity of Bax staining in each individual epithelial cell and the transformation of IM to adenocarcinoma. According to these observations, the epithelial cells transform into less Bax-positive cells and thus more apoptosis-resistant cells. These results contrast with previous reports[24] that found a positive association between progression to adenocarcinoma in CLE and Bax expression.

Members of the Bcl-2 family play an important role in the regulation of apoptosis. This family contains pro-apoptotic members (Bax, Bid, Bad, Bak) and anti-apoptotic members (Bcl-2, Bcl-xl). Bcl-2 proteins regulate the permeability of the mitochondrial membrane. Increased mitochondrial permeability allows leakage of cytochrome C from mitochondria into cytoplasm, triggering caspase activation and apoptosis. Proapoptotic Bcl-2 proteins increase mitochondrial membrane permeability, whereas antiapoptotic members antagonize the effects of proapoptotic Bcl-2 proteins[25]. The continuous expression of Bax could be triggered by overexpression of mutated p53 (proapoptotic) found in earlier reports[26,27].

Bcl-xl, an antiapoptotic Bcl-2 family member, was highly positive in dysplasia and tumor cells but not in IM. The increase in Bcl-xl grade and intensity of staining in the transformation from IM to CLE-associated adenocarcinoma was significant. The reciprocal changes in the expression of Bax and Bcl-xl in the sequence from IM to adenocarcinoma indicate that these cells become increasingly more resistant to apoptotic cell death, giving these cells a survival and proliferation advantage.

Fas is a member of the tumor necrosis factor receptor superfamily. Activation of this receptor by its ligand activates caspase 8 and the apoptotic signal transduction pathway. Fas expressing cells are vulnerable to Fas ligand induced cell death. Fas ligand is predominantly expressed by lymphocytes, but can also be expressed by other cells. Therefore, Fas-mediated cell death can occur only when Fas ligand-positive cells are in close proximity to Fas-positive target cells[28]. Fas was not only present in CLE IM, but also in GM of the esophagus. Decreased Fas expression has been reported in CLE. However, in this study Fas staining of goblet cells was investigated[29]. Previously, Fas expression was not found in normal gastric mucosa[30] and we confirmed these results. Therefore, Fas expression can be used to differentiate between normal gastric mucosa and GM in the esophagus. The expression of Fas ligand has been reported during malignant transformation of Barrett's metaplasia[31]. However, the simultaneous expression of Fas and Fas ligand does not necessarily lead to apoptotic cell death. Various antiapoptotic mechanisms may exist in Fas/Fas ligand co-expressing cells that protect these cells against apoptosis[32].

Most CLE-associated adenocarcinomas were COX-2 positive but only in a minority of tumor cells. In IM and dysplasia, COX-2 staining was negative and only lamina propria immune cells showed COX-2 expression. This contrasts with previous

reports[33,34], although other reports support our findings[35]. Pharmacological inhibition of COX-2 activity has been proven effective in reducing colonic polyp formation in humans. COX-2 staining in this study was negative in the pre-cancerous state in CLE. Our results do not support a role for COX-2 inhibition in the prevention or treatment of Barrett's dysplasia and cancer and a recent report from Tsibouris et al found no differences in cancer occurence in CLE in the presence or absence of nonsteroidal anti-inflammatory drugs[36].

All patients in our study were using proton-pump inhibitors (PPI). Peters et al reported that high dose PPI treatment resulted in partial endoscopic regression of CLE,[37] and effective PPI treatment decreased proliferation in an earlier study[38]. However other factors, such as duodenogastroesophageal reflux, may contribute to the development of CLE. Therefore, the effects of PPI treatment on proliferation in Barrett's esophagus remains unclear. Likewise, nothing is known about the effect of PPI treatment on apoptosis in Barrett's esophagus. Considering the regression of metaplasia and decreased proliferation in Barrett's esophagus of patients using PPIs, a proapoptotic effect of PPI could be hypothesized, but data are lacking.

In conclusion, the apoptotic balance in the transformation from IM to adenocarcinoma switches to an antiapoptotic phenotype due to increased Bcl-xl expression and decreased Bax expression. Most Barrett's esophagus-associated adenocarcinomas are COX-2 positive but only in a minority of tumor cells. COX-2 is not positive in non-malignant Barrett's esophagus. Therefore, pharmacological inhibition of COX-2 activity is unlikely to be effective in the prevention of Barrett's esophagus-associated adenocarcinomas. iNOS is highly positive in intestinal metaplasia and Fas expression can be used as a marker for differentation between normal gastric mucosa and gastric metaplasia in the esophagus.

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CHAPTER 4

APOPTOSIS AND EXPRESSION OF iNOS, COX-1 AND COX-2 IN GASTRITIS AND INTESTINAL METAPLASIA: INDUCIBLE NITRIC OXIDE SYNTHASE IS HIGHLY SPECIFIC FOR INTESTINAL METAPLASIA

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SUMMARY

The NF-kB-regulated genes inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX-2) are induced in inflammation and are frequently expressed in gastric and intestinal cancer cells and may be involved in the protection against apoptosis.

Aim: To determine COX, iNOS and activated caspase-3 expression in the sequence normal gastric mucosa (controls), Helicobacter pylori associated gastritis and intestinal metaplasia.

Methods: iNOS, COX-1, COX-2 and activated caspase-3 were detected by immunohistochemistry. Grade and activity of gastritis were determined using the updated Sydney classification.

Results: iNOS expression was weakly positive in gastritis, but was strongly increased in epithelium of intestinal metaplasia. COX-1 and COX-2 were expressed in all tissue samples in lamina propria immune cells but not in epithelium. COX-2 expression was strongly increased around areas of intestinal metaplasia. Activated caspase-3 was absent in control biopsies but was present in lamina propria immune cells of gastritis and intestinal metaplasia but not in epithelial cells.

Conclusion: iNOS expression was highly and selectively induced in metaplastic epithelium, suggesting an important role for NO in the sequence to gastric carcinoma of the intestinal type. Increased expression of COX-2 and increased generation of prostaglandins around intestinal metaplasia may contribute to protection against apoptosis and increased proliferation.

1. INTRODUCTION

In inflammatory conditions, the transcription factor NF-κB is activated, resulting in the expression of NF-κB-regulated, inflammation-related genes. Exposure to pro-inflammatory cytokines in the absence of NF-κB activation will lead to apoptosis. Several genes have been reported to protect against apoptosis, e.g. inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), the Bcl-2 family member A1/Bfl1 and the IAP family member HIAP1. IAP family members block apoptosis by inhibiting caspase activity[1] and these genes therefore contribute to the survival off cells. Chronic inflammatory conditions are accompanied by constitutive activation of

NF-κB and hence, by the continuous expression of pro-survival genes. For chronic gastritis this continuous NF-kB expression in the gastric mucosa has been demonstrated[2]. Although beneficial for the survival of cells during chronic inflammation, the continuous activation of NF-κB may also pose a risk: cells with a pro-survival phenotype may give rise to proliferating cells and may thus be tumorigenic. Complete progression to a malignant phenotype of these cells will most likely also involve changes in the expression of non-NF-κB regulated genes and a shift in the balance of pro- and anti-apoptotic genes towards a more anti-apoptotic phenotype. Increased iNOS activity has been observed in chronic gastritis and gastric cancer[3-7]. iNOS synthesizes nitric oxide (NO)[8] and nitric oxide has been reported to inhibit caspase activity and to inhibit DNA repair enzymes. Therefore, prolonged exposure to NO produced by iNOS in inflammatory conditions may inhibit apoptosis and preserve DNA mutations, thus promoting tumorigenesis, as suggested for cholangiocarcinoma in primary sclerosing cholangitis[9]. Likewise, products of the COX-1 and COX-2 enzymes contribute to resistance against apoptosis. COX-1 is responsible for the production of prostaglandins (PGs) under normal conditions. COX-2 is expressed at low levels under normal conditions but is induced at sites of inflammation[10,11]. COX-2 is regulated by NF-kB and has been reported to be induced in gastritis and in gastric carcinoma[12,13] and may be a target for the chemoprotective effect of NSAIDs. Indeed, inhibition of COX enzymes in colon cancer cells induces apoptosis and causes regression of colorectal adenomatous polyps[14-20]. The aim of this study was to examine the expression of iNOS, COX-1 and COX-2 in gastritis and in intestinal metaplasia as a precursor lesion in the sequence to gastric carcinoma of the intestinal type. We also determined active caspase-3 as a marker for apoptosis. We investigated the differences in the expression of these apoptosis-related proteins between intestinal metaplasia and normal gastric mucosa.

2. MATERIALS AND METHODS

2.1 Patient selection and tissue collection

Endoscopic biopsies were obtained from patients referred for gastroduodenoscopy. Patients with malignancy, gastric surgery, pregnancy, active inflammatory disease (C-reactive protein greater than 3 mg/L), diabetes mellitus and patients previously treated for Helicobacter pylori (Hp) were excluded. Also excluded were patients using

NSAIDs, steroids, coumarin-derivatives, acetylsalicylic-acid, prostaglandins, antibiotics, bismuth, and patients revealing ulcers or erosions on endoscopy. The protocol was approved by the Medical Ethical Board of our institution and all patients gave written informed consent. At endoscopy, 10 biopsies were taken from gastric antrum and body. Rapid urease test, histological examination for Hp (hematoxylin-eosin and Giemsa staining) and bacterial cultures were performed on one antral for rapid urease test, two antral and two body for histology and two antral and two body biopsy samples for bacterial culture. Biopsies were fixed in formalin for histology. Histological sections were reviewed by a single experienced pathologist who was blinded for the other determinants of Hp status. Gastritis was graded, according to the updated Sidney classification, as acute inflammation (presence of neutrophils) or chronic inflammation (presence of mononuclear cells) and inflammation was semi-quantified on a 0-3 scale (0, none; 1, mild; 2, moderate; 3, marked).

2.2 Immunohistochemical analysis

2.2.1 Staining for iNOS, COX-2, COX-1 and active caspase-3

For antigen retrieval, sections were heated in a microwave (700W) or pressure cooker under conditions as described in table 1.

Table 1: Immunohistochemistry methods

Protein	Section	Antigen retrieval	Primary antibody
COX-1	Frozen	None	Goat polyclonal at 1:100 Santa Cruz Biotechnology; cat. nr. SC-1752
COX-2	Paraffin	2x15min at 98°C in 1mM EDTA; pH8.0	Mouse monoclonal at 1:50 BD-Transduction; cat. nr. 610203
INOS	Paraffin	2x15min at 98°C in 1mM EDTA; pH8.0	Mouse monoclonal at 1:100; BD-Transduction; cat. nr. 610431
Activated * Caspase-3	Paraffin	Microwave; 1x8min in 10mM citrate buffer; pH6.0	Rabbit polyclonal at 1:100 Cell Signaling Technology; cat. nr. 9661S

* As positive control for caspase-3 human coloncarcinoma and human ischemic heart tissue were used

After treatment sections were allowed to cool at room temperature for 15 minutes. Endogenous peroxidase activity was quenched by incubation in 0.3% H2O2 in

phosphate-buffered saline (PBS). Endogenous peroxidase activity for COX-1 staining on frozen sections was quenched in 0.075% H_2O_2 in phosphate-buffered saline.
For staining of iNOS and COX-2 monoclonal antibodies were used as primary antibody (table 1). Biotinylated goat anti mouse Ig (15µg/mL, Ventana Medical Systems, Tucson, Arizona, USA) and horse radish peroxidase conjugated avidin (Ventana Medical Systems) were used as secondary and tertiary reagents, respectively. All antibody incubations were performed for 1 hr at room temperature. Incubations were performed in a Ventana ES automated staining system, according to manufacturer's instructions. Peroxidase activity was detected using DAB as substrate. Slides were counterstained with hematoxyllin and mounted in mounting medium. For staining of active caspase-3 and COX-1 polyclonal antibodies were used as primary antibody (table 1). Horseradish peroxidase conjugated goat anti rabbit Ig (1:50, Dako,Glostrup, Denmark) and horseradish peroxidase conjugated rabbit anti goat Ig (1:50, Dako, Glostrup, Denmark) were used as secondary and tertiary antibodies. All antibody incubations were performed for 1 hr at room temperature. Staining was developed using DAB as chromogen. Slides were counterstained with hematoxyllin and mounted in mounting medium.

2.2.2 Scoring
The immunohistochemical sections were scored by 3 different observers for the percentages of cells stained. In case of differences in interpretation the sample was scored again and a consensus was reached.

3. RESULTS

3.1 Clinical and histological characteristics
Tissue samples from 42 patients were analyzed. Eight patients had normal gastric histology and were designated as controls. Ten samples out of 42 contained intestinal metaplasia.

3.2 Immunohistochemical analysis
iNOS staining was negative in controls. In Hp-positive gastritis patients iNOS staining was positive in endothelial cells and in some inflammatory cells. There was no relation between the intensity of iNOS staining and the grade of gastritis. Staining for

iNOS in intestinal metaplasia was highly positive. All samples containing intestinal metaplasia demonstrated staining for over 50% of the epithelial cells. There was a clear differentiation between gastric type epithelium which was consistently negative and metaplastic epithelium (fig. 1a,b,c).

COX-1 staining was positive in all samples. Staining was similar in normal mucosa and mucosa of patients with gastritis. Staining was present only in lamina propria immune cells (fig. 1d).

COX-2 staining was present at low level in normal gastric mucosa, was more intense in inflamed mucosa and was strongly increased in areas surrounding intestinal metaplasia. Staining was present only in lamina propria immune cells and myofibroblasts (fig. 1e,f,g). In control biopsies activated caspase-3 was negative. Hp-positive gastritis samples revealed positive staining, but only in lamina propria immune cells. In intestinal metaplasia activated caspase-3 demonstrated positive staining in lamina propria immune cells.

4. DISCUSSION

In this study we demonstrated high expression of iNOS in epithelium of intestinal metaplasia. iNOS staining was absent in normal gastric mucosa and in epithelium of patients with gastritis. iNOS staining was present in endothelial cells and in lamina propria immune cells in inflamed gastric mucosa of patients irrespective of the etiology of the gastritis. No relationship could be demonstrated between the grade of gastritis and the expression of iNOS in these cells. This is in contrast with a previous report of Fu et al [9] who demonstrated increased expression of iNOS in Hp-positive gastritis. In addition, Fu et al observed iNOS staining of gastric epithelium in the presence of gastritis. A possible explanation for these differences is that in our study patients with abnormalities seen during upper endoscopy were excluded whereas this was not an exclusion criterium in the report from Fu et al. Therefore their samples may have included biopsies of ulcers and erosions which probably influenced iNOS expression. Although induced iNOS expression in gastric mucosa has been demonstrated previously our series shows a high correlation between iNOS expression and intestinal metaplasia. The reason for this high expression remains to be elucidated. iNOS gene expression is dependent on the activation of the transcription factor NF-κB. This implicates the presence of inflammatory cytokines in

inflamed gastric mucosa but fails to explain why iNOS expression is absent in surrounding non-intestinal metaplastic gastric epithelium. Alternatively, NF-κB could be constitutively activated in intestinal metaplasia conferring a proliferation advantage to these cells, as recently demonstrated for gastric cancer cells[22]. Compared to normal gastric antral mucosa, NF-κB in Hp gastritis is translocated in the nuclei of epithelial cells[8, 23-26] and a relationship between NF-κB activity and iNOS expression in Hp associated gastritis has been demonstrated[27,28]. In these studies inhibition of NF-κB blocked iNOS expression and nitrite production. Intestinal metaplasia is considered as pre-malignant stage in the sequence inflammation-atrophy-metaplasia-gastric carcinoma. Expression of iNOS could confer a survival advantage to cells via different mechanisms: 1) Increased NO generation inhibits caspase activity and hence apoptosis, thus promoting inappropriate cell survival and carcinogenesis [7, 29]. High levels of iNOS have been demonstrated in gastric cancer[30] and in neoplasia of the colon[31]. To investigate whether a relationship exists between the highly induced expression of iNOS in epithelium of intestinal metaplasia and apoptosis we investigated the expression of activated caspase-3 as a marker for apoptosis. In normal gastric epithelium activated caspase 3 was negative whereas in inflamed gastric epithelium and in epithelium of intestinal metaplasia, activated caspase-3 was also negative. Only lamina propria immune cells demonstrated positive staining. This suggests that iNOS expression in epithelium of intestinal metaplasia does not lead to gross changes in apoptosis involving caspase-3 mediated pathways. 2) NF-kB activation allows proliferation of cells and iNOS knockout mice display impaired liver regeneration after partial hepatectomy[32]. Although we did not investigate proliferation in this study, it is possible that epithelial cells of intestinal metaplasia need NF-kB activation and iNOS expression in order to proliferate. 3) NO produced by iNOS inhibits DNA repair enzymes, and increased iNOS expression may therefore result in the appearance of potentially tumorigenic cells, containing DNA mutations, as recently suggested for the pathogenesis of cholangiocarcinoma[15]. Our results suggest that positive iNOS staining is a highly specific diagnostic criterium for intestinal metaplasia.

Increased expression of COX-2 surrounding intestinal metaplasia was also observed. This suggests a role of COX-2 in the development of gastric carcinoma. It has been reported that COX-2 is induced in gastric carcinoma[33]. Although COX-2 expression was detected in normal tissue samples, increased expression was observed in

samples with gastritis. COX-2 staining was expressed only in lamina propria immune cells and myofibroblasts and was especially strong in areas surrounding intestinal metaplasia. COX-2-mediated release of prostaglandins from lamina propria immune cells could promote proliferation of intestinal epithelial cells as recently described[34] .

In conclusion: iNOS expression was highly and selectively induced in metaplastic epithelium, suggesting an important role for NO in the sequence to gastric carcinoma of the intestinal type. The precise role of NO in the biology of the inflamed mucosa remains to be investigated, but appears to be linked to proliferation rather than inhibition of caspase-3 mediated apoptosis. Increased generation of prostaglandins around intestinal metaplasia may contribute to increased proliferation.

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CHAPTER 5

DIFFUSE AND INTESTINAL TYPE GASTRIC CARCINOMAS DIFFER IN THEIR EXPRESSION OF APOPTOSIS-RELATED PROTEINS

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SUMMARY

Background: Gastric carcinomas can be divided into intestinal and diffuse type, the latter 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 and in 8 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 carcinoma. Bcl-2 was expressed in lamina propria immune cells. iNOS was expressed in 6 of 11 intestinal type and in 4 of 8 diffuse type carcinomas and COX-2 was expressed in 8 of 11 intestinal type and in 6 of 8 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.

1. INTRODUCTION

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 tumor[3]. 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 carcinoma. Finally, because activation of the inflammation related transcription factor nuclear factor (NF-kB) contributes to resistance to apoptosis and facilitates proliferation, we also investigated the expression of the NF-kB-regulated proteins inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in the two types of gastric carcinoma.

2. MATERIALS AND METHODS

Biopsies from tumors and resected tumors 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.

2.1 Immunohistochemical Analysis

Sections, 4-micrometer-thick, were cut from paraffin wax embedded tissues, and dewaxed.

2.1.2 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 (700W) or pressure cooker under the conditions described in table 1. After treatment, sections were allowed to cool at room temperature for 15 minutes. Endogenous peroxidase activity was quenched by incubation in 0.3% H_2O_2 in phosphate-buffered saline. Table 1 lists the monoclonal antibodies used for Fas, iNOS, COX-2, Ki-67 and Bcl-xl immunohistochemistry. Biotinylated goat antimouse immunoglobulin (15µg/mL, Ventana Medical Systems, Tucson, Arizona, USA) and horseradish 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 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.The monoclonal antibodies used as primary antibodies for Bcl-2 and Bax staining are also listed in table 1. Horseradish peroxidase conjugated rabbit anti mouse immunoglobulin (1:50 dilution, Dako, Glostrup, Denmark) and horseradish peroxidase conjugated goat anti rabbit Ig (1:50 dilution, Dako, Glostrup, Denmark) were used as secondary and tertiary antibodies respectively. For staining of active caspase-3, a polyclonal antibody was used as the primary antibody (table 1) Horseradish peroxidase conjugated goat anti rabbit immunoglobulin (1:50 dilution, Dako, Glostrup, Denmark) and horseradish peroxidase conjugated rabbit anti goat immunoglobulin (1:50 dilution, Dako, Glostrup, Denmark) were used as secondary and tertiary antibodies 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.

2.2 Scoring of immunoreactivity

The immunohistochemical sections stained for iNOS, COX-2, Fas, Bax, Bcl-2 and Bcl-xl were scored separately by 3 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 staining-positive tumor cells. No staining was scored as 0; 0 to 10% was scored as 1; 11 to 50% was scored as 2 and 51 to 100% was scored as 3.

2.3 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, IL, USA).

A p value < 0.05 was considered significant.

Table 1: Immunohistochemistry methods

Protein	Section	Antigen retrieval	Primary antibody
Fas	Paraffin	MW 3x10min at 98°C in 1mM EDTA; pH8.0	Mouse monoclonal at 1:400 Upstate Biotechnology, Lake Placid; cat. nr. 05-201
Bcl-xl	Paraffin	MW 2x15min at 98°C in 0,1M Tris HCl; pH9.0	Mouse monoclonal at 1:100 Zymed Laboratories, South San Francisco; cat. nr. 33-6300
Bax	Paraffin	MW 1x8 min boiling in 10mM citrate; pH6.0	Mouse monoclonal at 1:400 Santa Cruz Biotecnology P19, Santa Cruz ; cat. nr. SC-526
Bcl-2	Paraffin	MW 1x8 min boiling in 10mM citrate; pH6.0	Mouse monoclonal at 1:50 Dako Glostrup, Denmark; cat. nr. M 0887
COX-2	Paraffin	MW 3x10min at 98°C in 1mM EDTA; pH8.0	Mouse monoclonal at 1:50 Transduction Laboratories; cat. nr. C-22420
INOS	Paraffin	MW 3x10min at 98°C in 1mM EDTA; pH8.0	Mouse monoclonal at 1:50 Transduction Laboratories; cat. nr. N-39120
Active caspase-3	Paraffin	MW 1x8min in 10mM citrate buffer; pH6.0	Rabbit polyclonal at 1:100 Cell Signalling Technology, Beverly MA, USA; cat. nr. 9661S
Ki-67	Paraffin	3x15min at 115°C in 0.2% SDS in maleate buffer; pH 6.0	Mouse monoclonal MIB-1 at 1:400. Immunotech, Marseille, France

MW: microwave 700W
For the Fas staining human tonsil was used as positive control, for Bcl-xl and Bax staining Reed-Sternberg cells were used as positive control.

3. RESULTS

3.1 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 (range: 58-91) and for the diffuse type carcinoma 66 (range: 56-92) years. Tissue was obtained both from biopsies (seven diffuse type and eight intestinal type) and resected tumors (one diffuse type and three intestinal type).

3.2 Immunohistochemical staining of tumor cells of the diffuse and intestinal type carcinoma.

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.

Fas was expressed in all eleven intestinal type gastric carcinomas and in only one of the eight diffuse type carcinomas (fig. 1a and b). There was a significant difference between the staining intensity in the intestinal type and the diffuse type carcinomas: (r = -0.602 (SD.0.101)), p = 0.001. Bcl-xl was expressed in 10 of 11 intestinal carcinomas and in only 1 of 8 diffuse type carcinomas (fig. 1c and d). There was a significant difference between the staining intensity in the intestinal type and the diffuse type carcinomas: (r = -0.602 (SD 0.101)), p= 0.001. Bax was expressed in both types of carcinomas, and the difference between staining intensity in the intestinal type and the diffuse type carcinomas was not significant: (r = -0.244 (SD 0.218)), p =0.275. iNOS was expressed in 6 of 11 intestinal type carcinomas and in 4 of 8 diffuse type carcinomas. There was no significant difference between staining intensity in the intestinal type and the diffuse type carcinomas: (r = -0.144 (SD 0.180)), p= 0.532. COX-2 was expressed in 8 of 11 intestinal type and in 6 of 8 diffuse type carcinomas. There was no significant difference between staining intensity in the intestinal type and the diffuse type carcinomas: (r = 0.122 (SD 0.193)), p= 0.757. Bcl-2 was only expressed in lamina propria immune cells. Staining for active caspase-3, indicating apoptosis was seen in both intestinal and diffuse type carcinoma cells. However, not all tumors were positive for caspase-3 and not all tumor 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 figure 2. Ki-67 staining, which is a measure of proliferative cells was seen in both types of carcinoma in all tumor specimens and also in crypts. For all staining procedures, no differences were seen between sections derived from biopsy material and sections derived from resected tumor material.

Table 2: overview staining results for tumor cells

Type	COX-2	iNOS	Bcl-2	Bax	Bcl-xl	Fas
Intestinal	2	0	0	1	2	1
Intestinal	1	0	0	0	3	1
Intestinal	1	0	0	2	2	3
Intestinal	1	2	0	2	3	3
Intestinal	1	1	0	1	0	1
Intestinal	2	1	0	1	1	3
Intestinal	1	2	0	2	2	2
Intestinal	0	2	0	2	2	3
Intestinal	0	0	0	1	3	2
Intestinal	0	0	0	2	3	2
Intestinal	1	1	0	1	1	2
Diffuse	1	0	0	1	0	0
Diffuse	2	0	0	1	0	1
Diffuse	0	0	0	1	1	0
Diffuse	1	1	0	1	0	0
Diffuse	2	1	0	1	0	0
Diffuse	0	0	0	2	0	0
Diffuse	1	1	0	1	0	0
Diffuse	2	1	0	1	0	0

0 : 0% of tumor cells stained, 1 : 0-10% of tumor cells stained, 2 : 10-50% of tumor cells stained, 3 : >50% of tumor cells stained.

4. 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 tumor 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 Fas 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[4]. 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, 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 tumors compared to well-differentiated gastric tumors[5]. Our results are in contrast to those of a previous study from Vollmers et al who reported Fas expression in the diffuse type carcinoma but not in intestinal type carcinoma[6]. 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 recognized 3 different bands, indicating non-specific binding.

Bcl-xl is expressed in 10 of 11 intestinal carcinomas and in only 1 of 8 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, Bak) and anti-apoptotic members (Bcl-2, Bcl-xl). Bcl-2 proteins regulate the permeability of the mitochondrial membrane. Increased mitochondrial permeability allows leakage of cytochrome c from mitochondria into cytoplasm, triggering caspase activation and apoptosis. Pro-apoptotic Bcl-2 proteins increase mitochondrial membrane permeability, whereas anti-apoptotic members antagonise the effects of pro-apoptotic Bcl-2 proteins[7].

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 observed between diffuse type and intestinal type gastric carcinoma. The apoptosis seen in the tumor cells in these samples may be indicative of enhanced proliferation, because these cells were also positive in 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 tumor cells. This does not exlude the possibility that the differences in 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 tumor cells but only in lamina propria immune cells. Kyokane et al demonstrated Bcl-2 expression in early gastric cancer of the elevated type[8]. Others demonstrated Bcl-2 expression in tumor cells of both intestinal type carcinoma as well as diffuse type gastric carcinoma, but mostly in a small proportion of the tumor cells[9,10] . 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-kB is important in the resistance against apoptosis. NF-kB regulates the transcription of many inflammation-associated genes, including anti-apoptotic genes such as iNOS and COX-2. We have previously reported on the protective role of NF-kB activation and iNOS in liver cells[11]. We observed iNOS expression in both types of gastric carcinomas. Increased iNOS activity has been observed in gastric cancer[12,13]. iNOS synthesizes nitric oxide (NO). NO may promote the generation and proliferation of tumor cells by various mechanisms. First, NO may inhibit apoptosis by inhibiting caspase activity due to nitrosylation of essential cysteine residues in the catalytic site of caspases[14]. Secondly, 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[15]. 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 inhibtion of COX-2 for example, in colon carcinoma cell lines, induces apoptosis of these tumor cells[16,17]. However, there was no clear difference between COX-2 expression in the two carcinomas and this observation is in agreement with other reports[9,18].

In conclusion, intestinal type and diffuse type gastric carcinomas can be differentiated on 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.

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CHAPTER 6

ACTIVE CELIAC DISEASE INDUCES AN ANTI-APOPTOTIC PHENOTYPE WHICH LIMITS APOPTOSIS

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SUMMARY

Background: Epithelial cell loss in celiac disease (CD) could be due to a disturbed balance between proliferation and apoptotic or necrotic cell death. Increased apoptotic cell death by TUNEL assay has been reported in active CD. However, this assay frequently yields false-positive results. We re-addressed the issue of apoptosis in CD using two novel and more specific assays to detect apoptosis. Since increased cell loss in CD may also lead to adaptive changes in the expression of apoptosis-related proteins, we also investigated the expression of several apoptosis related proteins in patients with active CD and non-active CD.

Methods: intestinal biopsies of normal controls, patients with active CD and CD patients on gluten-free diet and after gluten challenge. Apoptotic bodies in CD were scored in hematoxylin eosin. Immunohistochemical staining for active caspase-3, cytokeratin 18, and apoptosis-related proteins Fas, iNOS, Bcl-2, Bcl-xl and Bax were performed.

Results: Very limited apoptosis of intestinal epithelial cells in most cases of active celiac disease was observed. Patients with active CD had increased epithelial expression of iNOS and decreased epithelial expression of Fas compared to control biopsies and CD patients on gluten-free diet. No changes in the expression of Bax, Bcl-2 and Bcl-xl were observed between the various groups.

Conclusion: In active celiac disease there is limited apoptotic cell death of intestinal epithelial cells. The lack of apoptosis could be due to adaptive changes resulting in decreased expression of pro-apoptotic Fas and increased expression of anti-apoptotic iNOS. Apparently, the epithelial cells in active celiac disease get lost in other ways, for example via necrosis or shedding.

1. INTRODUCTION

Celiac disease (CD) is characterized by malabsorption secondary to intestinal villous atrophy. The histologic lesions include an increased number of intraepithelial lymphocytes (IEL), crypt hyperplasia and villous blunting[1]. These changes are thought to be initiated by a T-cell mediated reaction to dietary gluten in genetically susceptible people. Despite marked proliferation, the mucosa in CD is flat but returns to normal on gluten withdrawal in most patients[2,3].

Increased villous epithelial cell loss in CD could be due to a disturbed balance between proliferation and apoptotic or necrotic cell death. In untreated CD, increased apoptotic cell death of intestinal epithelial cells has been reported. This observation was based on the detection of fragmented DNA using terminal uridine deoxynucleotidyl nick end labelling (TUNEL) in small intestinal biopsies. In these studies, apoptosis detected by TUNEL correlated with proliferation and returned to normal with gluten free diet[4]. Therefore, increased apoptosis may be responsible for villous atrophy in CD[5,6]. However the TUNEL assay, upon which these results are based, frequently yields false-positive results[7,8,9,10]. Positive TUNEL results are obtained in necrotic tissue, due to DNA fragmentation in these necrotic areas and in highly proliferating tissue. At present time two novel and more specific assays exist to detect apoptosis: immunohistochemical staining for active caspase-3 using an antibody recognizing only processed and active caspase-3 and Cytodeath, an antibody specific for caspase-3 cleaved cytokeratin 18. The decision for a cell to go into apoptosis depends on the balance between pro- and anti-apoptotic signals. Apoptotic stimuli include ligation of the Fas- and TNF-α receptors by their respective ligands. Disruption of mitochondrial integrity with leakage of cytochrome c into the cytoplasm constitutes another pro-apoptotic signal. The mitochondrial integrity is disrupted by pro-apoptotic members of the Bcl-2 family, such as Bax, Bad, Bid and Bak. The action of these pro-apoptotic members is antagonized by anti-apoptotic members like Bcl-2, Bcl-xl and A1/Bfl-1[11]. The relative levels of pro- and anti-apoptotic members of the Bcl-2 family determine whether a cell is resistant or sensitive to apoptotic stimuli. Overexpression of Bcl-2 or other anti-apoptotic proteins inhibits apoptosis, whereas overexpression of pro-apoptotic proteins promotes apoptotic cell death.

It is not known whether the increased epithelial cell loss in CD leads to adaptive changes in the expression of apoptosis-related proteins. In addition, the effect of gluten-free diet on the expression of apoptosis-related proteins in CD has not been investigated. Finally, it has been shown that the expression of inducible nitric oxide synthase (iNOS) is enhanced in epithelium and lamina propria immune cells in the small intestine of patients with untreated CD compared to patients with treated CD[12,13,14]. Since nitric oxide (NO) has been shown to have anti-apoptotic properties[15] we also investigated iNOS expression.

The goals of the present study were to re-investigate the extent of apoptosis in active CD using specific markers of apoptotic cell death and to investigate changes in the expression of the apoptosis-related genes Fas, Bax, Bcl-xl, Bcl-2 and iNOS.

2. MATERIALS AND METHODS

2.1 Patients

Samples were obtained from patients with celiac disease diagnosed in the period between january 1993 and december 2000. All patients underwent a second endoscopy and biopsy after one year of gluten-free diet. In 5 patients gluten-challenge was performed and 3 to 4 weeks after gluten-challenge these patients underwent a third endoscopy and biopsy. Biopsy samples from small bowel obtained during upper endoscopy from patients with diarrhoea were used as controls. CD was diagnosed according to the following criteria: increased intra-epithelial lymphocyte count, infiltration of the lamina propria with lymphocytes, crypt hyperplasia and villous atrophy (scoring according to Marsh criteria)[16].

2.2 Immunohistochemistry

Apoptosis was determined by counting apoptotic bodies in the hematoxylin-eosin (HE) and immunohistochemical staining for active caspase-3 and caspase-cleaved cytokeratin 18 (Cytodeath). These markers are recognized as specific markers for apoptosis[10,17,18,19]. Analysis of the expression of both anti-apoptotic (Bcl-2 and Bcl-xl) and pro-apoptotic (Bax) Bcl-2 family members was performed as described previously[20]. Finally, the expression of the Fas receptor and the inflammation-associated enzyme iNOS was analysed. Positive control for apoptosis was liver tissue obtained from rats treated with D-galactosamine and endotoxin[21] and human colon carcinoma.

In table 1, the details of the immunohistochemical procedure are presented.

Table 1: Immunohistochemistry methods

Protein	Section	Antigen retrieval	Primary antibody
Active caspase-3	Paraffin	Microwave (700W) 1x8min in 10mM citrate buffer; pH6.0	Rabbit polyclonal at 1:100 Cell Signaling Technology; cat. nr. 9661S
Cytokeratin 18	Paraffin	Microwave (700W) 1x15min in 10mM citrate; pH 6.0.	Mouse monoclonal antibody; Roche Diagnostics; Almere, Netherlands; cat. nr. 2140322
Fas Ventana	Paraffin	2x15min at 98°C in 10mM citrate; pH 6.0	Mouse monoclonal at 1:400 Upstate Biotechnology, Lake Placid; cat. nr. 05-201
Bax	Paraffin	MW (700W) 8 min in 10mM citrate; pH6.0	Mouse monoclonal at 1:200 Santa Cruz Biotecnology P19, Santa Cruz ; cat. nr. Sc-7480
Bcl-2	Paraffin	MW (700W) 8 min in 10mM citrate; pH6.0	Mouse monoclonal at 1:50 Dako Glostrup, Denmark; cat. nr. M 0887
Bcl-xl Ventana	Paraffin	2x15min at 98°C in 0,1M Tris HCl; pH9.0	Mouse monoclonal at 1:100 Zymed Laboratories, South San Francisco; cat. nr. 33-6300
INOS Ventana	Paraffin	2x15min at 98°C in 1mM EDTA; pH8.0	Mouse monoclonal at 1:100 BD-Transduction Laboratories; cat. nr. N-39120

For the Fas staining human tonsil was used as positive control, for Bax staining Reed-Sternberg cells were used as positive control, for active caspase-3 liver tissue from D-galactosamine/endotoxin-treated rats served as positive control and for cytodeath human colon carcinoma was used as positive control.

2.3 Quantitation of immunoreactivity

The immunohistochemical sections stained for Fas, Bcl-2, Bax, Bcl-xl and iNOS were scored for percentages of positive cells in 3 randomly chosen high power fields by 3 different observers. No staining was scored as 0; 0 to 10% was scored as 1; 11 to 50% was scored as 2 and 51 to 100% was scored as 3. In the hematoxylin-eosin-stained tissue sections, apoptotic bodies were identified and counted by an experienced pathologist.

2.4 Statistical analysis

Results of the staining scores (Fas, iNOS, Bcl-2, Bax and Bcl-xl) are given as mean ± S.D.

To test the differences of the staining scores (Fas, iNOS, Bcl-2, Bax and Bcl-xl) between patients (in active CD, on gluten-free diet and after gluten challenge) and controls the non-parametric Mann-Whitney Test with the exact test method was used. Since multiple comparisons are made (patients with active celiac disease versus controls, patients with gluten-free diet versus controls, and patients with gluten-challenge versus controls) the level of significance is adjusted to: $\alpha = 0.05/3 = 0.0167$.

3. RESULTS

3.1 Patients

Included in this study were: 6 controls, 18 patients with active celiac disease and 12 of these patients during gluten-free diet. Five of the patients on gluten-free diet were subjected to gluten-challenge. The ages of patients at first biopsy ranged from 3-45 years, with a mean of 11.6 years. The ages of the controls ranged from 23-45 (median 33).

3.2 Immunohistochemistry

3.2.1 Detection of apoptosis

Only 1/18 patients with active celiac disease and 1/5 patients after gluten challenge displayed weakly positive active caspase-3 in intestinal epithelial cells. Most intestinal epithelial cells were negative for active caspase-3 in all patient groups, including patients with active CD and after gluten challenge (fig. 1a). In contrast, the positive control, liver tissue from D-galactosamine/endotoxin-treated rats, was strongly positive. Positive staining for cytokeratin 18 was also limited (fig. 1b). Finally, in hematoxylin-eosin stained sections, only very few cells with apoptotic morphology were observed in the epithelial cell layer in active CD (fig. 1c). Collectively, these findings indicate the absence of extensive apoptosis of epithelial cells in active CD.

3.2.2 Expression of apoptosis-related proteins

Fas staining is positive in crypts and increases towards the villus tip in controls (fig. 2a). In active celiac disease Fas staining is significantly decreased (fig. 2b) compared to controls . This was confirmed by staining 6 additional biopsies from patients with active CD. During gluten-free diet Fas staining became normal (fig. 2c). After gluten challenge, Fas staining is decreased compared to controls, as in active CD. Means scores ± SD in the controls, active CD, gluten-free diet and gluten challenge were: 1.9 ± 0.5, 0.3 ± 0.5, 1.4 ± 0.5 and 0.6 ± 0.56 respectively.

In normal intestine the anti-apoptotic family member Bcl-2 is expressed in crypts but not in villi (fig. 3a). In active CD Bcl-2 is weakly positive in the base of crypts and negative in surface epithelium (fig. 3b). During gluten-free diet Bcl-2 staining resembles the pattern of the control group. After gluten-challenge Bcl-2 is weakly positive in crypts and is decreased compared to the other groups (fig. 3c). Mean scores ± SD in the controls, active CD, gluten-free diet and gluten challenge were: 1.5 ± 0.5, 1.3 ± 0.7, 1.8 ± 0.7 and 0.8 ± 0.8 respectively (table 2).

Table 2: Overview statistical analysis

Method	Group	Versus group	Significance
Fas	Control	CD	<0.001*
	Control	GFD	0.11
	Control	Challenge	0.002
Bcl-2	Control	CD	0.37
	Control	GFD	0.46
	Control	Challenge	0.11
Bax	Control	CD	0.34
	Control	GFD	0.16
	Control	Challenge	0.55
Bcl-xl	Control	CD	1.00
	Control	GFD	1.00
	Control	Challenge	0.66
INOS	Control	CD	<0.001*
	Control	GFD	0.06
	Control	Challenge	<0.001*

* significant

The pro-apoptotic Bcl-2 family member Bax stained positive in crypts and surface epithelium of controls (fig. 4a). During the different stages of CD Bax staining did not change (active CD: fig. 4b, gluten-free diet: fig. 4c, gluten challenge: fig. 4d). Mean

scores ± SD in the controls, active CD, gluten-free diet and gluten challenge were: 2.8 ± 0.4, 2.6 ± 0.5, 2.4 ± 0.5 and 2.6 ± 0.6 respectively.

Bcl-xl was weakly expressed in the crypt and surface epithelium of all samples. Mean scores ± SD in the controls, active CD, gluten-free diet and gluten challenge were: 0.9 ± 0.3, 1.0 ± 0.7, 1.0 ± 0.8 and 0.8 ± 0.8 respectively.

Expression of the inflammation-associated protein iNOS is weakly positive in crypt epithelium in all samples and at the tip of some villi in the control group (fig. 5a). In active CD iNOS staining is significantly increased in crypts and surface epithelium compared to controls (fig. 5b). During gluten-free diet iNOS staining is reduced compared to active CD and resembles the pattern in controls (fig. 5c). In 2/9 samples during gluten-free diet iNOS staining in villi is increased compared to the other samples and compared to controls. The villi in these samples were normal. After gluten-challenge iNOS expression is again increased and is as strongly expressed as in active celiac disease (fig. 5d). Mean scores ± SD in the controls, CD, GFD and challenge were: 1.0 ± 0, 3.0 ±0, 1.3 ± 0.5 and 1.8 ± 0.9 respectively.

4. DISCUSSION

In the present study we did only detect very limited apoptosis in intestinal epithelial cells in the majority of cases of active celiac disease using specific methods such as identification and counting of apoptotic bodies in hematoxylin-eosin-stained sections, immunohistochemistry for active caspase-3 and caspase-cleaved cyokeratin-18. Our results are at variance with previously reported data on apoptosis in active celiac disease. however these results were obtained with the TUNEL assay[4] and this method is known to yield false-positive results in rapidly proliferating or highly necrotic tissue[7,8,9,10].

The most striking observation in this study is the down-regulation of Fas in active celiac disease. Expression of Fas makes a cell vulnerable to apoptosis induced by Fas ligand, which is highly expressed on T-lymphocytes. In celiac disease there is a massive infiltration of cytotoxic Fas ligand expressing T lymphocytes. The lack of Fas expression on the remaining intestinal epithelial cells in active celiac disease could be an adaptation of these cells to escape apoptosis. The limited expression of markers of ongoing apoptosis in these cells, such as the expression of active

caspase-3 is in line with this hypothesis. An alternative explanation for the lack of apoptotic epithelial cells in active celiac disease is that cells become necrotic soon after reaching the surface or are shed into the lumen before or shortly after initiation of apoptosis by FasLigand expressing T lymphocytes. Shedding of apoptotic cells may be so rapid that these cells are not detected in the remaining epithelial cell layer in active celiac disease. Indeed, it has been reported that apoptosis is induced in intestinal epithelial cells, during or immediately following detachment[22]. At present, with the material at hand, we cannot distinguish between these possibilities. The Fas-expression in celiac disease reported by Maiuri et al[23] differs from our results in active celiac disease but could be explained by the difference in sampling. In our study we included only patients with flat mucosa in all biopsies whereas Maiuri et al included patients with patchy distribution of celiac disease.

The expression of iNOS is regulated by inflammatory cytokines such as interleukin-1 and tumor necrosis factor-alpha. The product of iNOS, nitric oxide (NO) is a reactive radical which can have both anti- and pro-apoptotic effects. Inhibition of apoptosis by NO is explained by the reaction of NO with essential sulfhydryl groups of cysteine residues in the catalytic site of caspases. This reaction yields nitrosylated sulfhydryl groups and this inactivates caspases. Furthermore, nitric oxide activates the enzyme soluble guanylate cyclase, which produces anti-apoptotic cyclic-GMP[15]. This action is in accordance with the role of NF-kB as a transcription factor which regulates the transcription of anti-apoptotic survival genes[24]. Induction of iNOS in epithelial cells in active celiac disease could act as a defense mechanism against apoptosis. Moreover, the spurious production of large amounts of NO could induce apoptosis in T-lymphocytes which are highly sensitive to NO induced apoptosis. Therefore, the increased expression of iNOS in active CD could provide another protective adaptation and may even explain the lack of active caspase-3 in intestinal samples of patients with active CD.

Finally, the expression of Bcl-2 family proteins does not change significantly in active celiac disease and therefore are probably of no importance in the apoptotic pathway during different stages of celiac disease.

In conclusion, our results demonstrate at least two significant changes in the epithelial expression of apoptosis-modifying proteins: down-regulation of Fas and induction of iNOS. These changes could increase the resistance of epithelial cells towards apoptosis in active celiac disease, which is in line with the absence of clear

indications of apoptotic cell death in active celiac disease. The lack of apoptosis could be explained because of necrosis or immediately shedding of epithelial cells just before or after the initiation of apoptosis.

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CHAPTER 7

EXPRESSION OF APOPTOSIS-RELATED PROTEINS DURING MALIGNANT PROGRESSION IN CHRONIC ULCERATIVE COLITIS

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Summary

Chronic ulcerative colitis is associated with an increased risk for developing neoplasia in the colon through a dysplasia-carcinoma sequence. In this sequence we investigated the expression pattern of proteins involved in apoptosis and inflammation (iNOS, COX-2, Bcl-xl, Fas, active caspase-3) and compared this pattern to that observed in sporadic colon carcinoma. COX-2 was negative in the epithelium of all samples. iNOS was clearly present in areas of inflammation in the epithelium of chronic ulcerative colitis (CUC), weakly expressed in dysplasia and absent or weakly present in tumor cells. Bcl-xl expression was absent in CUC, increased in dysplasia and was highly expressed in most carcinomas. Fas expression was positive in the surface epithelium of CUC, dysplasia and in most tumor cells. Activated caspase-3 was weakly positive in all samples indicating limited apoptosis. Compared to CUC-associated carcinoma, iNOS was consistently expressed in sporadic colon carcinoma cells, whereas Bcl-xl was absent in tumor cells of sporadic colon carcinoma and Fas was only weakly expressed in sporadic colon carcinoma cells. Activated caspase-3 was present in both normal mucosa samples and in some tumor cells. Conclusion: there is a distinct pattern in the expression of apoptosis-related proteins, in particular iNOS, Bcl-xl and Fas, in the sequence from chronic ulcerative colitis to carcinoma, which differs from the one observed in sporadic carcinoma but bears striking resemblance to the sequence observed during neoplastic progression in Barrett's esophagus. Our results support a causal role for chronic inflammation in the development of cancer in chronic ulcerative colitis and therefore we propose to minimize inflammation in ulcerative colitis.

1. INTRODUCTION

Chronic ulcerative colitis (CUC) is associated with an increased risk of malignancy that develops through a dysplasia-carcinoma sequence[1]. Dysplasia is present in more than 70% of chronic ulcerative colitis patients with cancer and it coincides with the location of cancer arising from chronically inflamed mucosa[2]. Dysplastic areas are often difficult to recognize on endoscopy. They may appear as flat or only slightly elevated above the level of the mucosa. Dysplasia may also occur within or near raised plaque-like lesions, nodules, polyps, or masses, in which case the term dysplasia associated lesion or mass (DALM) is used. Strong support exists for a link between chronic inflammation and development of cancer in the gastrointestinal tract[3,4]. In inflammation the activation of the transcription factor nuclear factor kappa B (NF-kB) induces the expression of many genes involved in cell survival, including anti-apoptotic genes. Continuously increased expression of anti-apoptotic proteins endows cells with a survival and proliferation advantage and may facilitate the appearance of tumorigenic cells. Previously, we have studied the expression of the inflammation-activated, NF-kB-regulated proteins inducible nitric oxide (iNOS) and cyclo-oxygenase-2 (COX-2) in the sequence gastritis-metaplasia-gastric cancer and in the sequence metaplasia-dysplasia-adenocarcinoma in Barrett's esophagus[5]. Nitric oxide (NO), produced by iNOS, has been demonstrated to inhibit apoptosis by inhibiting caspase activity, although chronic exposure to high levels of NO can also promote apoptosis[6]. COX-2 inhibition prevents cell proliferation and promotes apoptosis and is being considered as a chemopreventive strategy in colon cancer[7]. In addition to changes in the expression of NF-kB-regulated anti-apoptotic proteins, tumor cells frequently demonstrate more permanent changes in the expression of other apoptosis-related proteins, e.g. death receptors (Fas) and Bcl-2 family members. The Bcl-2 family of proteins play a key role in apoptosis by determining the susceptibility of the mitochondrial outer membrane to pore formation. Leakage of mitochondrial proteins through these pores into the cytoplasm constitutes a strong apoptotic signal.

The pathogenesis of colon cancer in chronic ulcerative colitis is poorly understood, but there are indications that the pathogenesis is different from that of sporadic colon cancer:

1. Dysplasia in UC is preceded by a long history of chronic inflammation and can be observed at sites distant from the cancer, whereas dysplasia in sporadic colon cancer is usually associated with a discrete polyp without inflammation.
2. Mutations in the ras protooncogene are present in 40-60 percent of sporadic colon cancers and are probably an early event; in contrast, in cancer associated with UC, these mutations are less frequently observed and are probably a late event[8-10].
3. Loss of heterozygosity (LOH) for the p53 gene and src activation occurs in UC non-dysplastic epithelium, UC-associated dysplasia and in UC-associated carcinoma compared to the absence of LOH for p53 in regions with negative, indefinite or low grade dysplastic histology[11].
4. LOH in the APC loci in UC was noted in dysplasia with associated carcinoma, but LOH of APC was not present either in cases of nondysplastic epithelium or in HGD alone. Conversely, LOH in APC was present in 4 of 19 colonic adenomas[12].
The aim of this study was to determine the expression pattern of the NF-kB-regulated anti-apoptotic proteins iNOS and COX-2, the death receptor Fas and the anti-apoptotic Bcl-2 family member Bcl-xl in the sequence chronic ulcerative colitis to chronic ulcerative colitis-associated carcinoma. Furthermore, this expression pattern was compared to the expression patterns observed in the sequence from Barrett's esophagus to Barrett's esophagus-associated adenocarcinoma and to the expression pattern observed in sporadic colon carcinoma.

2. MATERIALS AND METHODS

2.1 Patient selection and tissue collection

Tissue samples were studied of 5 patients who participated in an endoscopy surveillance program between january 1990 and december 2002 at the Erasmus MC University Hospital Rotterdam because of longstanding colitis. In addition, tissue samples were studied of 4 different patients who underwent colectomy because of sporadic colon carcinoma. Histology was performed on samples stained with haematoxylin-eosin and samples were scored for the presence and degree of inflammation and dysplasia and the presence of adenocarcinoma. The classification of dysplasia was performed as proposed by Riddell et al[13] and scored by a single pathologist. Low- and high-grade dysplasia were for this purpose grouped into one category.

2.2 Immunohistochemical analysis

All staining procedures were performed on four-micrometer-thick formalin-fixed, deparaffinized sections. For an overview see table 1.

Table1: immunohistochemical staining methods

Protein	Section	Antigen retrieval	Primary antibody
iNOS	Paraffin	MW 2x15min at 98°C in 1mM EDTA; pH8.0	Mouse monoclonal at 1:100 BD-Transduction Laboratories; cat. nr. 610431
COX-2	Paraffin	MW 2x15min at 98°C in 1mM EDTA; pH8.0	Mouse monoclonal at 1:50 BD-Transduction Laboratories; cat. nr. 610203
Fas	Paraffin	MW 2x15min at 98°C in 10mM citrate; pH6.0	Mouse monoclonal at 1:400 Upstate Biotechnology, Lake Placid; cat. nr. 05-201
Bcl-xl	Paraffin	MW 2x15min at 98°C in 0,1M Tris HCl; pH9.0	Mouse monoclonal at 1:100 Zymed Laboratories, South San Francisco; cat. nr. 33-6300
Active caspase-3	Paraffin	Microwave (700W) 1x8min in 10mM citrate buffer; pH6.0	Rabbit polyclonal at 1:100 Cell Signaling Technology; cat. nr. 9661S

2.3 Quantitation of immunoreactivity

After staining, the immunohistochemical sections stained for iNOS, COX-2, Bcl-xl, and Fas were scored by 2 different observers for the percentage of positively stained epithelial cells and tumor cells. The intensity was not scored, due to the different immunostaining characteristics of the antibodies. In case of differences in interpretation the sample was scored again and a consensus was reached. No staining of epithelial/tumor cells was scored as 0; 0 to 10% of stained epithelial/tumor cells was scored as 1; 11 to 50% was scored as 2 and 51 to 100% was scored as 3. Active caspase-3 was scored as positive or negative in epithelial cells and tumor cells.

3. RESULTS

3.1 Patient characteristics of Ulcerative Colitis patients

All five patients (3 male) were subject of surveillance colonoscopies because of longstanding colitis (mean 15 years) and 4/5 were using immunosuppressives (3 cyclosporine and 1 methotrexate). Colectomy was performed in all patients because of cancer at a mean age of 50 years. The mean time to develop dysplasia/adenocarcinoma in this group was 16 years from the time of first diagnosis of colitis. In all cases the carcinoma was well differentiated.

3.2 Immunohistochemical staining of UC associated dysplasia and cancer

An overview of all the immunohistochemical results is presented in table 2 and 3.

In the epithelium of CUC, iNOS staining was present in areas of inflammation and negative in areas without inflammation (fig 1a). In dysplasia iNOS was weakly positive (score 1) in 3/5 patients and strong positive in 2/5 patients (score 2-3) (fig. 1b). iNOS staining in tumor cells was negative or weakly positive (score 0-1) (fig. 1c). COX-2 was negative in the epithelium of all samples but clearly positive in lamina propria immune cells and therefore these cells served as an internal control. Fas expression was positive in the surface epithelium of CUC (score 1). In dyplasia the epithelium revealed positive Fas expression (score 2) (fig. 1d). Also in 4 out of 5 cancers the tumor cells were strong positive for Fas (score 3) (fig. 1e).

Bcl-xl was not expressed in CUC, but there was positive expression in dysplasia (score 1-2) (fig. 1f) and strong expression in tumor cells of all but one cancer (score 3) (fig. 1g). Activated caspase-3 was weakly positive in all samples, indicating only limited ongoing apoptosis (fig. 1h).

3.3 Immunohistochemical staining of sporadic colon carcinoma

Patients were all male with a mean age of 63 years at time of diagnosis. All four sporadic carcinomas were well differentiated in patients. The TNM classification of the tumors is shown in table 3, which also includes the immunohistochemical staining results for the tumor samples and normal mucosa obtained from the resection material.

iNOS staining was weakly positive (score 1) in all normal mucosa samples and positive in tumor cells (score 1-2) (fig. 1i). In the normal mucosa of the resected large

bowel COX-2 staining revealed some positive staining in epithelial cells in addition to lamina propria immune cells. COX-2 was weakly positive in tumor cells (score 1). In the controls Fas staining was weakly positive in the surface epithelium (score 1). Also some tumor cells Fas revealed weakly positive Fas staining (score 1) (fig. 1j). Bxl-xl was negative to weakly positive in normal mucosa samples (score 0-1) and completely absent in tumor cells (score 0) (fig. 1k). Activated caspase-3 stained positive in both the normal mucosa samples and in tumor cells (fig. 1l).

Table 2: Immunohistochemical staining results of the ulcerative colitis group

		iNOS	Cox-2	Fas	Bcl-xl	Active caspase
Patient A	UC	0	0	1	0	Positive
	dysplasia	3	0	3	2	Positive
	Ca T3N2M0	1	0	3	3	Positive
Patient B	UC	0	0	1	0	Positive
	dysplasia	1	0	2	2	Positive
	Ca T3N1M0	1	0	3	2	Positive
Patient C	UC	0	0	1	0	Positive
	dysplasia	1	0	1	1	Positive
	Ca in situ TIS	0	0	0	1	Positive
Patient D	UC	0	0	1	0	Positive
	dysplasia	1	0	3	1	Positive
	Ca T1N0M0	1	0	3	3	Positive
Patient E	UC	0	0	1	0	Positive
	dysplasia	3	0	2	1	Positive
	Ca T2N1M0	0	0	3	3	Positive

0:0%, 1: 0-10%, 2: 10-50%, 3: 50-100%.
UC:ulcerative colitis
Ca: carcinoma

Table 3: Immunohistochemical staining results of the sporadic colon carcinoma group

		iNOS	Cox-2	Fas	Bcl-xl	Active caspase
Patient A	Normal mucosa	2	0	1	1	Positive
	Ca, T3N1M0	2	1	1	0	Positive
Patient B	Normal mucosa	1	1	1	1	Positive
	Ca, T3N0M0	2	2	1	0	Positive
Patient C	Normal mucosa	1	1	1	0	Positive
	Ca, T3N0M0	3	1	1	0	Positive
Patient D	Normal mucosa	1	1	1	1	Positive
	Ca, T3N2M0	1	0	1	0	Positive

0:0%, 1: 0-10%, 2: 10-50%, 3: 50-100%.
Ca : carcinoma

4. DISCUSSION

In the present study we observed increased expression of iNOS in UC associated dysplasia whereas iNOS expression was absent in UC-associated carcinoma. In contrast, Bcl-xl expression was absent in chronic UC, but was clearly positive in tumor cells. These patterns bears a striking similarity to the expression pattern observed for these anti-apoptotic proteins in Barrett's associated adenocarcinoma[5]. In the sequence leading to Barrett's associated adenocarcinoma, iNOS is expressed in the early stage of intestinal metaplasia, although the tumor cells themselves do not express iNOS. Likewise, the anti-apoptotic Bcl-2 family member Bcl-xl was nearly absent in early stages leading to Barrett's associated adenocarcinoma, but Bcl-xl was strongly expressed in tumor cells. Like the expression pattern observed in the Barrett's sequence, COX-2 expression was absent in the epithelium at any stage except that in Barrett's associated adenocarcinoma COX-2 expression was observed in a minority of the tumor cells. Both Barrett's associated adenocarcinoma and chronic ulcerative colitis associated carcinoma develop from inflamed mucosa and it is therefore tempting to speculate that common mechanisms, related to the continuous induction of NF-kB-regulated anti-apoptotic proteins, contribute to the malignant progression in these disorders. Therefore, a strong recommendation for the prevention of cancer in chronic ulcerative colitis could be to minimize inflammation in the colon of affected patients. According to our findings, the use of COX-2 inhibitors as a chemopreventive strategy will not be effective.

Interestingly, distinct differences were observed in the expression of Fas and Bcl-xl between tumor cells in UC-associated carcinoma and tumor cells in sporadic carcinoma. Fas was strongly expressed in most ulcerative colitis associated dysplasia and tumor cells, whereas Fas was weakly expressed in tumor cells of the sporadic carcinomas. Furthermore, Bcl-xl was clearly expressed in chronic ulcerative colitis tumor cells, but almost negative in sporadic colon cancer cells. Since non-adenoma-like DALM is an indication for colectomy whereas sporadic adenomas may be removed endoscopically even if they occur in an area histologically involved with colitis the differential expression of Fas and Bcl-xl could be used to distinguish chronic ulcerative colitis associated pre-malignant lesions from sporadic adenomas and thus prevent unnecessary colectomy. The differences in expression patterns of pro- and anti-apoptotic proteins did not result in differences in apoptosis. Activated caspase-3, used as a marker of apoptosis, was weakly present in both CUC associated colon cancer and sporadic colon cancer, and may be the result of increased apoptosis in the presence of increased (tumor) cell proliferation.

In conclusion: a distinct pattern in the expression of apoptosis-related proteins in the sequence from chronic ulcerative colitis to carcinoma was observed. This pattern differs from the one observed in sporadic carcinoma and bears more resemblance to the sequence of events observed in Barrett's adenocarcinoma. Our results support a causal role for chronic inflammation in the development of cancer in chronic ulcerative colitis and therefore we propose to minimize inflammation in ulcerative colitis.

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CHAPTER 8

SUMMARY, GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Chapter 1 provides a general introduction of this thesis starting with a brief overview of the actions of NF-kB-regulated, inflammation-related genes, such as inducible nitric oxide synthase (iNOS) and cyclo-oxygenase-2 (COX-2). The overall hypothesis of this thesis was that a distinct and maybe identical pattern of apoptosis exists in chronic inflammatory, pre-neoplastic and neoplastic disorders of the gastro-intestinal tract. To test this hypothesis the expression of the NF-kB-regulated anti-apoptotic genes iNOS and COX-2 in chronic inflammatory, pre-neoplastic and neoplastic disorders of the gastrointestinal tract was investigated, as well as the expression of the apoptosis-related genes Bcl-2, Bcl-xl and Bax in these disorders. Finally the extent of apoptosis, using activated caspase-3 as a specific parameter of apoptosis was determined.

In **chapter 2** an overview is provided of the existing literature on the activation of NF-kB and the expression of iNOS, COX-2 and apoptosis-related genes in (pre-) malignant lesions such as Barrett's esophagus (BE), BE-associated adenocarcinoma, intestinal metaplasia and adenocarcinoma of the stomach and inflammatory bowel diseases-related (IBD) neoplasia in the colon. As reported in this chapter the existing information on these parameters is often incomplete and contradictory. Moreover, the consequences of NF-kB activation and expression of NF-kB-regulated genes in the various stages of these sequences has not been thoroughly investigated. E.g. distinct changes in the expression of Bcl-2 family members occur in BE, but the consequences for the resistance against apoptosis are not clear. In the stomach NF-kB activation is involved in iNOS and COX-2 expression in Helicobacter pylori (Hp)-gastritis, intestinal metaplasia, dysplasia and in adenocarcinoma. Some data suggest that inhibition of NF-kB activation or NF-kB-regulated genes may sensitize gastric cancer cells to apoptosis or inhibit their proliferation. Only a limited amount of data concerning NF-kB activation and COX-2 and iNOS expression in IBD-related carcinogenesis has been published. Although these proteins are induced in IBD, their role in oncogenesis is not known. Data on the expression of Bcl-2 family members in inflammatory bowel diseases and associated neoplasia are conflicting.
In summary, existing data on sequential changes in the expression of NF-kB- and apoptosis-related proteins and their involvement in oncogenesis in the sequence

from (chronic) inflammation to cancer in the gastrointestinal tract is incomplete and conflicting.

To get more insight in the process of carcinogenesis in BE we studied several factors involved in the apoptotic and inflammatory pathway. The results of this study are described in **chapter 3.** In BE, iNOS is highly expressed in intestinal metaplasia and in 50% of the biopsies containing dysplasia, but not in BE-associated adenocarcinoma. All of our samples containing high-grade dysplasia were positive for iNOS. To test the hypothesis that iNOS expression protects against apoptosis, the extent of caspase-3 activation as a marker for apoptosis was determined, but no differences in the staining intensity of active caspase-3 was observed between iNOS-positive intestinal metaplasia in BE and iNOS-negative BE-associated dysplasia. COX-2 staining in this study was negative in the precancerous stages of BE. Most BE-associated adenocarcinomas were COX-2 positive but only in a minority of tumor cells. Fas staining was positive in epithelium of all biopsies from patients with BE, including gastric metaplasia and intestinal metaplasia, but negative in normal gastric mucosa. Therefore, Fas expression can be used to differentiate between normal gastric mucosa and esophageal epithelium. Our results suggest that Bcl-2 is not involved in the carcinogenesis of BE, because only lamina propria immune cells, not the epithelium, showed positive staining. On the other hand, the proapoptotic Bcl-2 family member Bax was positive in all samples. Although no significant differences in staining grade were observed among the different groups, there was a significant negative correlation between the intensity of Bax staining in each individual epithelial cell and the sequence from BE to BE-associated adenocarcinoma. We hypothesize that the epithelial cells transform into less Bax-positive cells and hence more apoptosis-resistant cells. Finally, the antiapoptotic Bcl-2 family member Bcl-xl, displayed increased expression from BE to BE-associated adenocarcinoma. Together, the reciprocal changes in the expression of Bax and Bcl-xl in the sequence from intestinal metaplasia to adenocarcinoma indicate that these cells become increasingly more resistant to apoptotic cell death, endowing these cells with a survival and proliferation advantage.

Conclusions from this chapter are:

1. The apoptotic balance in the transformation from intestinal metaplasia to adenocarcinoma switches to an anti-apoptotic phenotype due to increased Bcl-xl expression and decreased Bax expression.

2. Fas can be used as a marker for the differentiation of gastric mucosa and metaplasia in the esophagus.
3. iNOS is highly positive in BE-associated intestinal metaplasia.
4. COX-2 is not expressed in non-malignant BE. Therefore pharmacological inhibition of COX-2 activity is unlikely to be effective in the prevention of BE-associated adenocarcinomas. There was no clear correlation between iNOS expression and activation of pro- and anti-apoptotic genes.

Chapter 4 reports the results of COX and iNOS expression and apoptosis in normal gastric mucosa, Hp associated gastritis and intestinal metaplasia. iNOS is highly induced in epithelium of intestinal metaplasia but absent in normal gastric mucosa and in epithelium of patients with gastritis. The mechanism and consequences of the induction of iNOS expression in intestinal metaplasia remains to be elucidated. Expression of iNOS could confer a survival advantage to cells via different mechanisms as discussed in chapter 2. To investigate whether iNOS expression protects against apoptosis, we determined the expression of active caspase-3 as a marker for apoptosis. However, no differences were observed between iNOS-positive epithelium of intestinal metaplasia and iNOS-negative epithelium. Alternative survival advantages of increased iNOS expression may include proliferation advantages, as suggested by the observation that iNOS knockout mice display impaired liver regeneration after partial hepatectomy. Finally, increased iNOS expression and NO production may facilitate the appearance of tumorigenic cells: chronic inflammation is accompanied by exposure to reactive oxygen species. This exposure may induce DNA damage. NO inhibits DNA-repair enzymes and therefore, in chronic inflammation, DNA-damage in regenerating epithelium is not repaired and may contain tumorigenic DNA-mutations. This hypothesis needs to be further investigated since it implicates that iNOS positive cells in gastric mucosa should be eliminated. These results also demonstrate that iNOS expression is a highly specific diagnostic criterium for intestinal metaplasia. Increased expression of COX-2 in lamina propria immune cells and myofibroblasts surrounding intestinal metaplasia was also observed. COX-2 mediated release of prostaglandins from lamina propria immune cells could promote proliferation of intestinal epithelial cells as recently described.
In conclusion: iNOS expression was highly and selectively induced in metaplastic epithelium, suggesting an important role for NO in the sequence to gastric carcinoma

of the intestinal type. Increased expression of COX-2 and increased generation of prostaglandins around intestinal metaplasia may contribute to protection against apoptosis and increased proliferation.

In **chapter 5** specific patterns in the expression of apoptosis-related proteins were determined to disriminate between intestinal and diffuse type gastric carcinoma. This study revealed striking differences in the expression of two apoptosis-related genes, Fas and Bcl-xl, between intestinal type and diffuse type gastric carcinoma. Fas expression was positive in all intestinal type carcinomas, but in only one diffuse type carcinoma. The diffuse type carcinoma has a poor prognosis compared to the intestinal type gastric carcinoma. This might be explained at least in part by the lack of Fas expression in diffuse type gastric carcinoma, resulting in less vulnerability to apoptosis induced by FasLigand expressing cells. Bcl-xl is expressed in 10/11 intestinal type gastric carcinoma and in only 1/7 diffuse type gastric carcinomas. However, no differences in markers for apoptosis (active caspase-3) and proliferation (Ki67 staining) were observed between diffuse type and intestinal type gastric carcinoma. This does not exclude the possibility that the differences in expression of Fas and/or Bcl-xl influence metastatic potential or susceptibility to immune surveillance. E.g. the poor prognosis of diffuse type gastric carcinoma, which lacks Fas, could be due to less susceptibility to immune surveillance by FasL-expressing T-lymphocytes and subsequently to increased metastatic potential. The NF-kB-regulated proteins iNOS and COX-2 are expressed in gastric carcinoma and their expression is similar in intestinal type and diffuse type gastric carcinoma.

In **chapter 6** we studied apoptosis and the expression of apoptosis-related proteins in celiac disease. The expression of the Bcl-2 family proteins Bax, Bcl-2 and Bcl-xl does not differ significantly between active celiac disease and inactive celiac disease or controls. However, our results demonstrate at least two significant changes in the epithelial expression of apoptosis-modifying proteins: a strong reduction of Fas and a strong induction of iNOS in active celiac disease compared to normal intestinal epithelium or inactive celiac disease. These changes could increase the resistance of epithelial cells towards apoptosis in active celiac disease, which is in line with the absence of clear indications of apoptotic cell death in active celiac disease. In conclusion: In active celiac disease there is no apoptotic cell death of intestinal

epithelial cells. The lack of apoptosis could be due to adaptive changes resulting in decreased expression of pro-apoptotic Fas and increased expression of anti-apoptotic iNOS. Apparently, epithelial cells in active celiac disease get lost in other ways, for example necrosis or shedding.

Finally in **chapter 7** we investigated alterations in the expression of proteins involved in apoptosis and inflammation (iNOS, COX-2, Bcl-xl, Fas, active caspase-3) in the sequence from chronic ulcerative colitis to ulcerative colitis-associated adenocarcinoma. In addition, we compared ulcerative colitis-associated carcinoma to sporadic carcinoma. COX-2 was negative in the epithelium of all samples. iNOS staining was positive in areas of inflammation in the epithelium of chronic ulcerative colitis (CUC), but iNOS was absent in the non-inflamed areas. In dysplasia iNOS was weakly to clearly positive. iNOS staining in tumor cells was negative or weakly positive. Bcl-xl was absent in CUC, moderately expressed in dysplasia and highly expressed in most carcinomas. Fas expression was positive in the surface epithelium of CUC. Epithelial Fas expression was positive in dysplasia and in most tumor cells. Activated caspase-3 was weakly positive in all samples indicating limited apoptosis. A different staining pattern was observed in sporadic colon carcinoma: iNOS staining was weakly positive in normal mucosa and moderately to clearly positive in tumor cells. Bxl-xl was negative to weakly positive in normal mucosa and absent in tumor cells. Fas staining was positive in normal surface epithelium and weakly positive in some tumor cells. Activated caspase-3 stained weakly positive in both the normal mucosa samples and in some tumor cells. We conclude that there is a distinct pattern in the expression of apoptosis-related proteins in the sequence from chronic ulcerative colitis to carcinoma. This pattern is distinct from the one observed in sporadic carcinoma and bears more resemblance to the sequence of events observed in Barrett's adenocarcinoma. According to our results the development of cancer in chronic ulcerative colitis is inflammation-based and therefore we make a strong recommendation for minimizing inflammation in ulcerative colitis.

PERSPECTIVES

Our studies have filled in some of the gaps in our knowledge on sequential changes in the expression of apoptosis-modifying genes in the sequences of (chronic) inflammation to cancer in the gastrointestinal tract. We have observed striking

similarities in these sequences between e.g. esophagus and the colon. Moreover, we have observed that NF-kB-regulated anti-apoptotic proteins such as iNOS are particularly strongly induced in intestinal metaplasia, an intermediary stage between inflammation and cancer. The most striking changes in protein expression in gastrointestinal cancer and normal epithelium are observed among members of the Bcl-2 family and the death receptor Fas. Our findings may have important implications for both diagnosis and treatment in BE, gastric cancer and ulcerative colitis. With regard to diagnosis, we have identified several markers for specific stages in the sequence of (chronic) inflammation to cancer. E.g. iNOS is a very specific marker for intestinal metaplasia, but not for chronic inflammation or cancer. Fas and Bcl-xl can be used as diagnostic criteria for the discrimination between intestinal type and diffuse type gastric carcinoma. COX-2 expression does not appear to be a very specific marker for any condition. Sporadic colon cancer and carcinoma in CUC can be distinguished on basis of the expression of Fas and active caspase-3. Active celiac disease is characterized by increased iNOS expression and reduced Fas expression. This knowledge can be used for monitoring adherence to gluten-free diet. With respect to treatment, our studies indicate limited value of chemopreventive strategies based on inhibition of COX-2 in BE adenocarcinoma and gastric carcinoma since the epithelial cells in intestinal metaplasia lack COX-2 expression. Strategies based on inhibition of iNOS are also probably of limited value in gastrointestinal cancer but may be useful in precursor stages, such as intestinal metaplasia in BE and gastritis. For ulcerative colitis we recommend to minimize inflammation because ongoing inflammation could be a risk factor for the development of UC-associated adenocarcinoma. An important remaining issue remains the elucidation of the functional consequences of the alterations in the expression of apoptosis-modifying proteins. E.g. although we have observed very specific changes in the expression of several apoptosis-related proteins in various gastrointestinal (pre-) malignant disorders, we were not able to correlate these changes with differences in the extent of apoptosis. One explanation is that caspase-3 activation is not a very specific or sensitive marker to detect small differences in (susceptibility to) apoptosis. Another explanation is that not the vulnerability to apoptosis is modified but rather the proliferation potential of (DNA-damaged) cells, their vulnerability to immune surveillance and/or their metastatic potential. Elucidating these remaining issues

requires follow-up studies including animal studies and in vitro studies, which were outside the scope of the investigations described in this thesis.

Addendum 1

NEDERLANDSE SAMENVATTING

Hoofdstuk 1 bevat een de introductie van dit proefschrift, beginnende met een kort overzicht van de functie van NF-kB gereguleerde en ontstekingsgerelateerde genen zoals induceerbaar stikstofoxide synthase (iNOS) en cyclooxygenase-2 (COX-2). De hypothese van dit proefschrift was dat er een karakterisitiek en mogelijk identiek expressie-patroon van apoptose-gerelateerde genen bestaat in chronische ontsteking, pre-maligne en maligne afwijkingen in het maagdarmkanaal. Om deze hypothese te onderzoeken is de expressie van de NF-kB gereguleerde genen iNOS en COX-2 en de apoptose-gerelateerde genen Bcl-2, Bcl-xl en Bax onderzocht in bovenstaande afwijkingen.
Ook werd de mate van apoptose onderzocht waarbij gebruik werd gemaakt van geactiveerd caspase-3 als specifieke marker voor actieve apoptose.

In **hoofdstuk 2** wordt een overzicht gegeven van de bestaande literatuur op het gebied van de activatie van de transcriptie factor NF-kB en de expressie van iNOS, COX-2 en apoptose-gerelateerde genen in (pre-)maligne afwijkingen zoals Barrett oesophagus (BO), BO-geassocieerde adenocarcinomen, intestinale metaplasie, adenocarcinomen van de maag en inflammatoire darmziekte gerelateerde maligniteiten in de dikke darm. Zoals aangegeven in dit hoofdstuk is de bestaande literatuur hierover vaak incompleet en tegenstrijdig. De consequenties van NF-kB activatie en de expressie van NF-kB gereguleerde genen in de verschillende stadia van de hierboven genoemde aandoeningen zijn onvoldoende onderzocht. Zoals bijvoorbeeld in het geval van BO, waar wel duidelijke afwijkingen in de expressie van genen uit de Bcl-2 familie zijn gevonden, maar waarbij de consequenties van deze afwijkingen voor de resistentie tegen apoptose nog onduidelijk zijn. In de maag is NF-kB activatie van belang voor de expressie van iNOS en COX-2 tijdens ontstekingen veroorzaakt door de Helicobacter pylori bacterie en in intestinale metaplasia, dysplasie en adenocarcinomen. Een deel van de gepubliceerde data doet vermoeden dat remming van NF-kB of NF-kB gereguleerde genen maagtumorcellen vatbaar maken voor apoptose en de proliferatie van deze tumorcellen tegengaat. Zeer weinig publicaties handelen over de activatie NF-kB en de expressie van iNOS en COX-2 bij inflammatoire darmziekten gerelateerde maligniteiten. Bekend is wel dat de expressie van deze genen toeneemt bij inflammatoire darmziekten, maar een eventuele rol van deze genen in de carcinogenese is onbekend. Literatuur over de expressie van leden van de Bcl-2 familie in het darmepitheel bij inflammatoire

darmziekten en inflammatoire darmziekten geassocieerde carcinomen is tegenstrijdig.
Samenvattend: de bestaande gegevens over veranderingen in de expressie van NF-kB- en apoptose gerelateerde genen en de deelname van deze genen aan de sequentie van (chronische) ontsteking tot uiteindelijk carcinoom in het maagdarmkanaal zijn incompleet en tegenstrijdig.

Om meer inzicht te verkrijgen in het proces van carcinogenese in Barrett oesophagus (BO) hebben we de expressie van verscheidene genen van belang voor apoptose en ontsteking onderzocht. De resultaten van deze studie worden in **hoofdstuk 3** beschreven. In BO en in 50% van de dysplastische gebieden in BO (100% in hooggradige dysplasie) is er een zeer duidelijke expressie van iNOS. Echter in BO-geassocieerde adenocarcinomen is er geen iNOS expressie in het epitheel. De caspase-3 activiteit werd gemeten om de hypothese te testen dat toegenomen iNOS expressie zou beschermen tegen apoptose, maar er werden geen verschillen gevonden in caspase-3 activiteit tussen iNOS-positief en negatief (dysplasie) BO epitheel. In BO was de expressie van COX-2 negatief, COX-2 positiviteit werd wel gevonden in BO geassocieerde adenocarcinomen, echter slechts in een klein aantal tumorcellen. De expressie van Fas was positief in het epitheel van alle biopten van patiënten met BO, ook in gastrische metaplasie, daarentegen was de expressie van Fas zeer laag tot negatief in biopten van normaal maagepitheel. Op grond hiervan menen wij dat Fas expressie kan worden gebruikt als differentiatie tussen normaal maagepitheel en esophagus gastrische metaplasie. Bcl-2 expressie was alleen positief in lamina propria immuun cellen, maar niet in het epitheel, derhalve speelt Bcl-2 geen rol in de carcinogenese in BO. Het pro-apoptotische Bcl-2 familielid Bax was wel positief in het epitheel van BO en BO-geassocieerde adenocarcinomen. Alhoewel we geen significantie verschillen in het aantal Bax-positieve cellen konden detecteren in de verschillende groepen, was er een significant negatieve correlatie tussen intensiteit van de Bax-expressie in de afzonderlijke epitheel cellen en de sequentie van BO tot BO-geassocieerde adenocarcinomen. Wij vermoeden dat de epitheelcellen gedurende deze sequentie transformeren tot minder Bax-positieve cellen en daardoor tot meer apoptose-resistente cellen. Als laatste bleek er een toegenomen expressie van het anti-apoptotisch Bcl-2 familielid Bcl-xl tijdens de transformatie van BO naar BO-geassocieerd adenocarcinoom. Samengevoegd laten

deze reciproke veranderingen in de expressie van Bax en Bcl-xl tijdens de sequentie van BO naar BO-geassocieerd adencarcinoom zien dat de epitheelcellen in toenemende mate weerstand opbouwen tegen geprogrammeerde celdood, waardoor deze cellen kunnen overleven en zelfs voortwoekeren.
De conclusies van dit hoofdstuk zijn: 1) De balans tussen pro- en anti-apoptotische factoren tijdens de evolutie van BO naar BO-geassocieerd adenocarcinoom verschuift naar een anti-apoptotisch fenotype ten gevolge van toegenomen Bcl-xl expressie en afgenomen Bax expressie in epitheelcellen. 2) Fas expressie kan worden gebruikt om te differentiëren tussen maagepitheel en BO-geassocieerde gastrische metaplasie. 3) Er is een zeer hoge iNOS expressie in BO-epitheel. 4) In BO is COX-2 expressie afwezig in het epitheel. Door deze bevinding lijkt het ons onwaarschijnlijk dat COX-2 remming kan worden gebruikt als preventie voor het ontwikkelen van BO-geassocieerd adenocarcinoom. 5) Er was geen correlatie tussen iNOS expressie en de mate van apoptose.

Hoofdstuk 4 handelt over de resultaten van onderzoek naar COX en iNOS expressie en apoptose in normaal maagepitheel, Helicobacter pylori geassocieerde maagontsteking en intestinale metaplasie. iNOS expressie is duidelijk aanwezig in intestinale metaplasie, maar afwezig in normaal maagepitheel en ook afwezig in het epitheel van patiënten met maagontsteking. De consequenties van deze bevindingen moeten verder worden uitgezocht. Onder andere zou dit kunnen betekenen dat de expressie van iNOS voor deze epitheelcellen een overlevingsvoordeel oplevert. Om te onderzoeken of iNOS eventueel zou beschermen tegen apoptose hebben we actief caspase-3 als een marker voor apoptose gemeten. Echter, er waren geen verschillen in apoptose tussen iNOS positief en iNOS negatief epitheel. Alternatieven voor een eventueel overlevingsvoordeel ten gevolge van toegenomen iNOS expressie zouden kunnen berusten op proliferatievoordelen zoals gesuggereerd door het feit dat iNOS-deficiënte muizen verminderde levercel-regeneratie vertonen na partiële hepatectomie. Verhoogde iNOS expressie en productie van NO zou ook het ontstaan van tumorigene cellen kunnen faciliteren: chronische ontsteking gaat gepaard met de blootstelling aan reactieve zuurstofradicalen. Deze blootstelling kan DNA-schade veroorzaken. NO remt DNA-herstel enzymen en daardoor wordt in chronische ontsteking DNA-schade minder efficiënt gerepareerd wat kan leiden tot tumorigene DNA-mutaties. Deze hypothese moet verder worden bestudeerd omdat

het impliceert dat iNOS positieve cellen in het maagepitheel moeten worden geëlimineerd. Wat deze resultaten laten zien is dat iNOS een zeer specifieke marker is voor de detectie van intestinale metaplasie. De expressie van COX-2 is toegenomen in lamina propria immuun cellen en in myofibroblasten rondom intestinale metaplasie. COX-2 gemediëerde productie van prostaglandinen vanuit de lamina propria immuun cellen zouden verantwoordelijk kunnen zijn voor proliferatie van intestinale epitheelcellen zoals eerder beschreven.
Concluderend: iNOS komt hoog en selectief tot expressie in intestinale metaplasie, wat een belangrijke rol suggereert voor stikstofoxide in de sequentie van ontstoken epitheel naar maagcarcinoom. Verhoogde expressie van COX-2 en productie prostaglandinen rondom intestinale metaplasie kunnen een rol spelen in de bescherming tegen apoptose en de toegenomen proliferatie.

In **hoofdstuk 5** werden de patronen in de expressie van apoptose-gerelateerde genen in maagcarcinomen onderzocht om een onderscheid te kunnen maken tussen intestinaal type maagcarcinoom en het diffuse type maagcarcinoom. Dit onderzoek liet opmerkelijke verschillen zien in de expressie van twee apoptose-gerelateerde genen: Fas en Bcl-xl. Fas expressie was aanwezig in alle carcinomen van het intestinale type en in 1 carcinoom van het diffuse type. Vergeleken met het intestinaal type carcinoom lijken patiënten met het diffuse type maagcarcinoom een slechtere prognose te hebben. Deels kan dit worden verklaard door de afwezigheid van Fas expressie in deze carcinomen, resulterend in een verminderde gevoeligheid voor apoptose geïnduceerd door cellen die FasLigand tot expressie brengen. In 10 van de 11 intestinaal type carcinomen en in 1 van de 7 diffuus type carcinomen komt Bcl-xl tot expressie. Ondanks bovenstaande bevindingen werden tussen de intestinaal type en diffuus type maagcarcinomen geen verschillen gezien in apoptose (actief caspase-3) en proliferatie (Ki67). Hierdoor is het nog steeds niet uitgesloten dat de gevonden verschillen in Fas en Bcl-xl expressie het vermogen van de tumor om uit te zaaien dan wel de gevoeligheid voor immuun surveillance beïnvloedt.
De NF-kB-gereguleerde genen iNOS en COX-2 komen tot expressie in beide type maagcarcinomen.
Concluderend: De expressie van Fas en Bcl-xl kan differentiëren tussen intestinaal type en diffuus type maagcarcinomen. Echter ondanks deze verschillen worden er

geen verschillen gevonden in apoptose en proliferatie tussen het intestinaal type en diffuus type maagcarcinoom.

In **hoofdstuk 6** worden de resultaten weergegeven van het onderzoek verricht naar apoptose en de expressie van apoptose-gerelateerde genen bij coeliakie. De expressie van leden van de Bcl-2 familie, Bax, Bcl-2 en Bcl-xl lieten geen significante verschillen zien tussen actieve coeliakie, niet-actieve coeliakie en controlebiopten. Wel vonden we verschillen in de expressie in het epitheel van Fas en iNOS. Fas expressie was afgenomen en de iNOS expressie was toegenomen in actieve coeliakie vergeleken met normaal epitheel en niet-actieve coeliakie. Deze veranderingen zouden de weerstand van epitheelcellen tegen apoptose kunnen verhogen in actieve coeliakie. Dit is in overeenstemming met de afwezigheid van duidelijk aantoonbare apoptose in actieve coeliakie. Conclusie: in actieve coeliakie lijkt apoptose van epitheelcellen beperkt te zijn. De afwezigheid van apoptose zou verklaard kunnen worden door aanpassingen in de epitheelcel resulterend in verminderde expressie van pro-apoptotisch Fas en toegenomen expressie van anti-apoptotisch iNOS. Zeer waarschijnlijk verdwijnen epitheelcellen bij actieve coeliakie op andere manieren dan via apoptose: mogelijk via necrose of uitstoting van de cellen in het lumen.

Tot slot beschrijven we in **hoofdstuk 7** de resultaten van ons onderzoek naar veranderingen in de expressie van verschillende genen betrokken bij apoptose en ontsteking (iNOS, COX-2, Bcl-xl, Fas, actief caspase-3) in epitheelcellen tijdens de sequentie van langer bestaande chronische colitis ulcerosa naar colitis ulcerosa geassocieerd adenocarcinoom. Bovendien hebben wij het colitis ulcerosa geassocieerd adenocarcinoom vergeleken met sporadisch coloncarcinoom. COX-2 was negatief in het epitheel van alle biopten. iNOS expressie was aanwezig in gebieden met ontsteking in het epitheel van chronische colitis ulcerosa, maar afwezig in uitgebluste colitis. In dysplasie was er duidelijk iNOS expressie aanwezig, echter in tumorcellen van colitis ulcerosa geassocieerd adenocarcinoom was de expressie van iNOS niet of nauwelijks aanwezig. Expressie van Bcl-xl was afwezig in chronische colitis ulcerosa, aanwezig in dysplasie en duidelijk aanwezig in de meeste carcinomen. Fas expressie was aanwezig in het oppervlakte epitheel van chronische colitis ulcerosa. Ook in dysplasie en in de meeste tumorcellen was er sprake van Fas

expressie. Actief caspase-3 was matig aantoonbaar in alle biopten wat wijst op een apoptose in deze sequentie. In de sporadische colon carcinomen werd een ander expressiepatroon geobserveerd: iNOS expressie bleek matig aanwezig in normaal epitheel en duidelijk aanwezig in tumorcellen. Bcl-xl expressie was voornamelijk afwezig in normaal epitheel en totaal afwezig in de tumorcellen van het sporadische type. Fas kwam tot expressie in oppervlakte epitheel van normaal colon en was zwak aanwezig in enkele tumorcellen. Actief caspase-3 was zwak aanwezig zowel in normaal colonepitheel als in enkele tumorcellen.

Wij concluderen dat er een specifiek expressie patroon bestaat van apoptose-gerelateerde genen in de sequentie van colitis ulcerosa naar colitis ulcerosa gerelateerde adenocarcinomen. Dit patroon is afwijkend van het patroon zoals geobserveerd in sporadisch coloncarcinoom en is meer in overeenstemming met het patroon gevonden tijdens de sequentie van Barrett oesophagus naar Barrett-geassocieerd adenocarcinoom. Volgens onze resultaten is het carcinoom wat ontstaat in chronische colitis ulcerosa gebaseerd op ontsteking en daarom zal zoveel mogelijk moeten worden getracht ontsteking in chronische colitis ulcerosa te beperken.

TOEKOMSTPERSPECTIEF

Onze onderzoeken hebben enkele hiaten opgevuld in de kennis over veranderingen in de expressie van apoptose-modificerende genen tijdens de sequentie van (chronische) ontsteking naar carcinoom in het maagdarmkanaal. Duidelijke overeenkomsten zijn waargenomen in deze sequentie tussen oesophagus en het colon. Verder hebben wij aangetoond dat NF-kB gereguleerde anti-apoptotische genen zoals iNOS sterk aanwezig zijn in intestinaal metaplasie, soms een tussenstation tussen ontsteking en carcinoom. De meest in het oog springende veranderingen hebben we gezien in de expressie van Bcl-2 familieleden en Fas. Deze bevindingen kunnen van belang zijn voor zowel de diagnose als de behandeling van Barrett oesophagus, maagcarcinoom en colitis ulcerosa.

Diagnose: iNOS is een specifieke marker voor intestinaal metaplasie, maar niet voor chronische ontsteking of carcinoom. Fas en Bcl-xl expressie kan worden gebruikt voor de discriminatie van intestinaal type en diffuus type maagcarcinoom. Daarentegen lijkt COX-2 geen specifieke marker te zijn in de door ons onderzochte ziektebeelden. Sporadisch coloncarcinoom en carcinoom ontstaan in colitis ulcerosa

kunnen worden onderscheiden op grond van Fas expressie. Actieve coeliakie geeft een duidelijke toename van de expresie van iNOS en een afname van de expressie van Fas. Deze wetenschap zou kunnen worden gebruikt om dieet-trouw bij coeliakie te monitoren.

Behandeling: onze studies laten weinig basis zien voor chemopreventieve behandelingen door COX-2 te remmen. Zowel in Barrett oesophagus geassocieerd adenocarcinoom als in maagcarcinoom is er geen expressie van COX-2 in het epitheel van intestinaal metaplasie. Waarschijnlijk helpt de remming van iNOS ons ook niet in de behandeling van gastro-intestinale tumoren, maar mogelijk kan de remming van iNOS nog van enige betekenis zijn tijdens de voorloperstadia van carcinomen. Voortdurende ontsteking bij patiënten met colitis ulcerosa lijkt een risico factor te zijn voor colitis ulcerosa geassocieerd adenocarcinoom en daarom adviseren wij om eventuele voortdurende ontsteking zoveel mogelijk te minimaliseren.

Een belangrijke onderwerp voor vervolgonderzoek is de functionele consequenties van de veranderingen in de expressie van apoptose-modificerende genen. Wij hebben namelijk zeer specifieke veranderingen geobserveerd in het voorkomen van deze genen in verschillende gastro-intestinale (pre-)maligne condities, maar wij waren niet in staat om deze bevindingen te correleren met veranderingen in de mate van apoptose. Mogelijk is actief caspase-3 als marker voor apoptose niet voldoende specifiek of gevoelig om kleine veranderingen in apoptose te detecteren. Ook is het mogelijk dat niet de gevoeligheid voor apoptose wordt gemodificeerd maar de proliferatie van (DNA-beschadigde) cellen, de gevoeligheid voor immuunsurveillance en/of het vermogen om te metastaseren. Om deze vragen te kunnen beantwoorden zullen vervolgstudies moeten plaatsvinden, met inbegrip van dier-experimentele en *in vitro* benaderingen.

Addendum 2

Dankwoord

Waar te beginnen met een dankwoord en waar te eindigen? Door het opschrijven van namen schieten mij nog zoveel andere mensen in gedachten die niet direct met het proefschrift te maken hebben gehad, maar wel indirect een duwtje hebben gegeven in de goede richting en zonder wie ik nu niet de MDL-arts was geweest die ik nu ben. Dus ook al noem ik niet alle namen, in mijn gedachten is mijn dankwoord een stuk langer.

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Leden van de promotiecommissie Prof. dr. C.G. Kallenberg, Prof. dr. P.M. Kluin en Prof. dr. E.J. Kuipers ben ik zeer erkentelijk voor het beoordelen van mijn proefschrift. Beste Ernst, even een apart woordje voor jou natuurlijk. Toen je mij (tijdens een borrel) vroeg om in Rotterdam te komen werken dacht ik aanvankelijk

aan te veel alcohol, maar later bleek het toch een serieuze vraag te zijn geweest. Alhoewel de gedachte aan Rotterdam mij niet bijzonder optimistisch stemde ben ik wegens jouw stimulerende optreden uiteindelijk wel in het Erasmus MC terechtgekomen. Nog steeds zonder spijt en nu met een extra titel mede dankzij jouw steun in het tot stand komen ervan.

Tijdens de 5 jaren van dit onderzoek hebben verscheidene analisten hun talenten gebruikt voor een eindeloze serie aan kleuringen. Bedankt: Alie de Jager-Krikken, Alexandra Beuving en in het bijzonder Manon Buist-Homan, die door de jaren heen de meest stabiele factor is geweest om maar weer de coupes op te zoeken en opnieuw kleuringen uit te voeren en zelf ook altijd zeer kritisch is gebleken over het uiteindelijke resultaat. Verder keek ik telkens met veel bewondering naar de systematiek waarmee alles werd uitgevoerd en opgeslagen, waardoor wanneer ik weer iets op een verfrommeld blaadje ergens achter had gelaten, altijd de resultaten uit de netjes gerangschikte mappen kon achterhalen. Zonder jullie geen onderzoek.

Ton Tiebosch en Herman van Dekken: een MDL-arts kan niet zonder patholoog!!!

De endoscopie-verpleegkundigen in Groningen: tijdens mijn eerste onderzoek wel 10 biopten uit de maag!! En dat naast het programma ingepland, zonder morren waren jullie altijd weer bereid om te helpen, geweldig.

De mede opleidingsassistenten: Rob de Knegt, Gerard Dijkstra en Laurens van der Waaij. Mede dankzij jullie is de tijd in Groningen een goede geweest.

Hendrik van Dullemen, Frans Peters, Bram Limburg, Els Haagsma, Aad van den Berg en Ids Klompmaker: veel van jullie geleerd.

Dan de overstap naar Rotterdam: als eerste wil ik Carla Capel enorm danken voor het vele werk verricht voor het tot stand komen van dit boekje, maar ook danken voor de hele organisatie van de promotiedag. Ik ben heel blij dat je onze afdeling bent komen versterken en hopelijk blijf je nog lang.

Verder natuurlijk mijn mede stafleden van de afdeling MDL, ik noem maar niet alle namen. Maar zeer erkentelijk ben ik voor het feit dat mij de mogelijkheid is geboden naast mijn werk ook nog de tijd te nemen voor het boekje.

Dan mijn geliefde ouders: papa en mama, jullie houding in onze opvoeding dat je alles kan bereiken als je het maar gewoon probeert is ongekend!! Ook al heb ik nooit veel prijs gegeven over de hele gang naar mijn promotie toe, jullie steun was er onvoorwaardelijk. Ik hoop dat ik voor mijn kinderen ook altijd een zelfde steun zal zijn.

Mijn paranimfen, mijn kleine grote zussen, Marieke en Tanja: onze verbondenheid kenmerkt zich altijd in het er voor elkaar zijn wanneer het nodig is en ik hoop dat het nog lang zo zal duren.

Bijna aan het einde van dit dankwoord gekomen wil ik toch mijn levensgezel noemen. Lieve Wilfred, je wilde absoluut niet genoemd worden in dit dankwoord, ik vind dat jij de grootste ondersteuning bent geweest in het tot stand komen ervan. Vanaf het moment dat wij elkaar hebben leren kennen heeft alles in het teken gestaan van mijn opleiding en mijn werk. Zelfs zover is het gegaan dat je als Amsterdammer nu in Rotterdam woont en je eigen carrière aan de kant hebt gezet voor het wel en wee van ons gezin. Hoe ik daar over denk is niet in woorden uit te drukken.

Als laatste het belangrijkste waar alles de afgelopen jaren om heeft gedraaid, onze lieve en bijzondere wereldkinderen: Lian en Tomas. Lieverds, ik hou van jullie tot aan de maan en weer terug!!

FIGURES CHAPTER 3

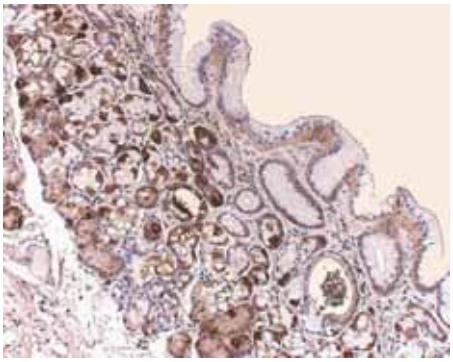

Fig. 1a: Fas staining is clearly positive in epithelium of GM.

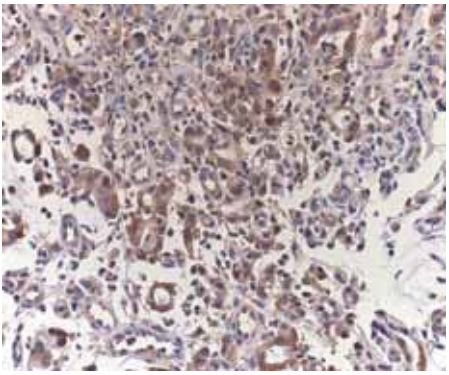

Fig. 1b: Fas staining is positive in tumor cells.

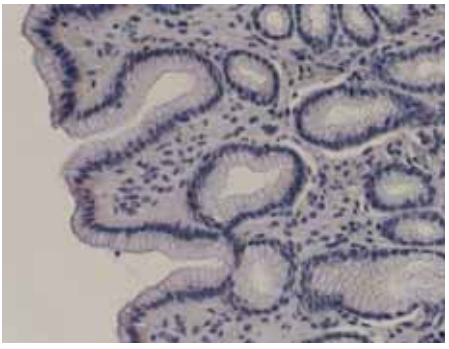

Fig. 1c: Fas-staining in normal gastric mucosa is negative.

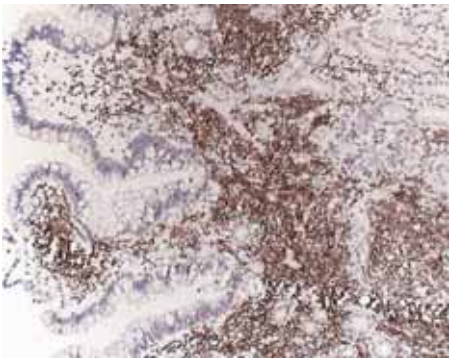

Fig. 1d: Bcl-2 staining only positive in lamina propria immune cells.

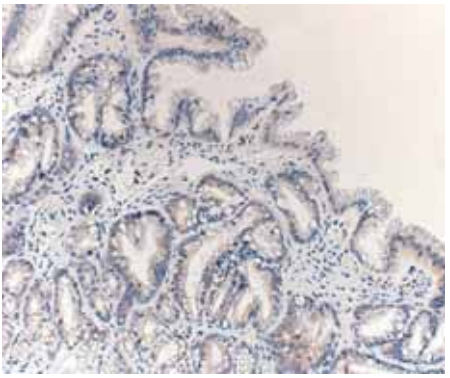

Fig. 1e: Bax staining is positive in epithelium of all groups: shown here IM.

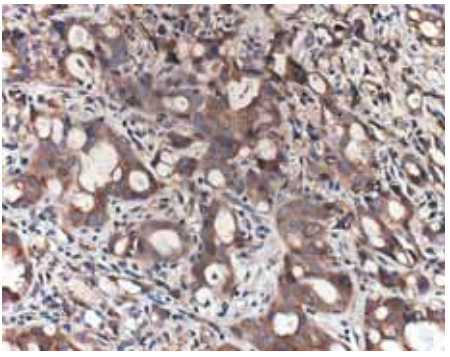

Fig. 1f: Bax-positivity in tumor cells.

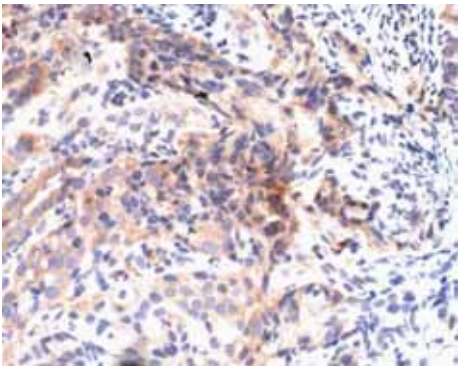

Fig. 1g: Bcl-xl staining is positive in tumor cells.

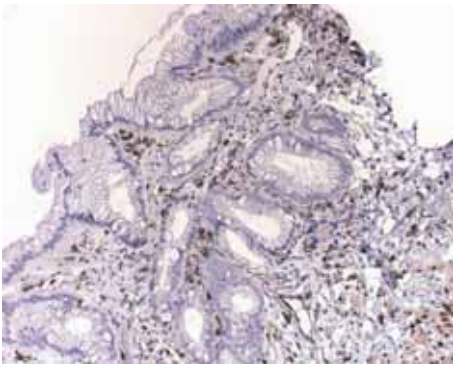

Fig. 1j: COX-2 positivity in lamina propria immune cells and myofibroblasts.

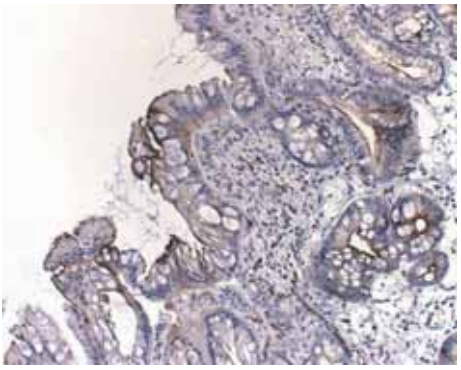

Fig. 1h: iNOS staining strongly positive in IM.

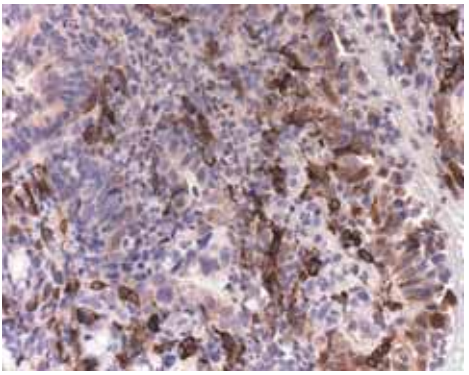

Fig. 1k: COX-2 positivity in only a minority of tumor cells.

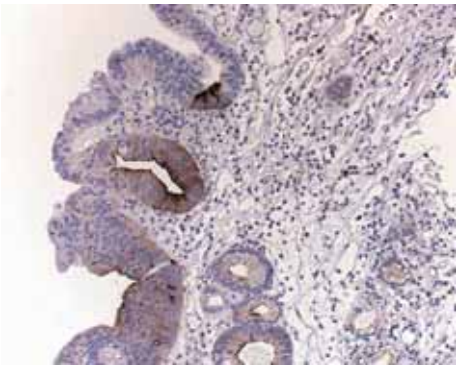

Fig. 1i: iNOS positive in epithelium of high-grade dysplasia.

FIGURES CHAPTER 4

Figure 1: immunohistochemical staining of iNOS (a,b,c) in controls, gastritis and intestinal metaplasia note in fig. c the difference of staining in intestinal metaplasia and normal gastric mucosa

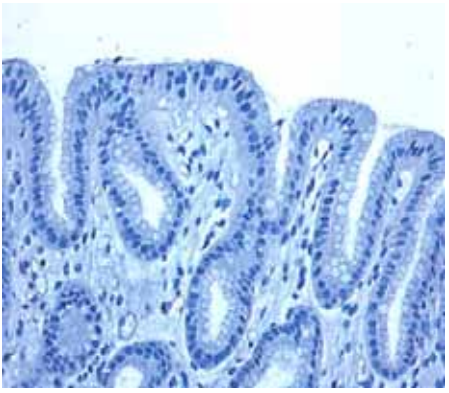

Fig. 1a

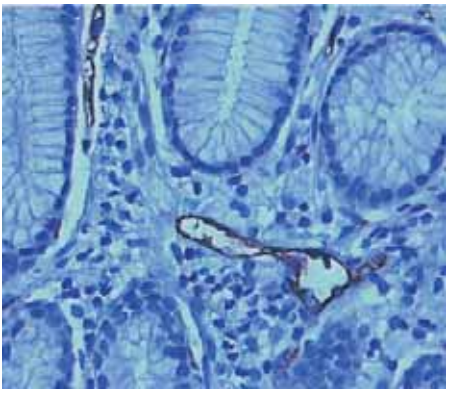

Fig. 1c

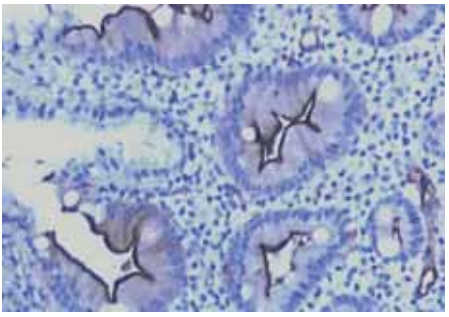

Fig. 1b

COX-1 (d) in intestinal metaplasia and COX-2 (e,f,g) in controls, gastritis and intestinal metaplasia, note in fig. g only lamina propria immune cells and myofibroblast surrounding intestinal metaplasia demonstrate COX-2 positive staining.

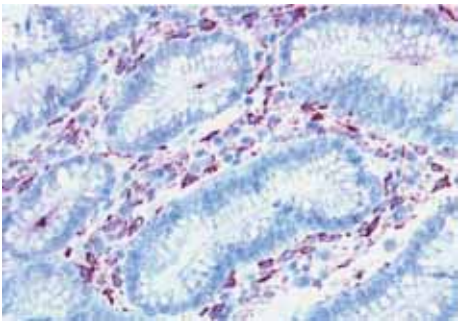

Fig. 1d

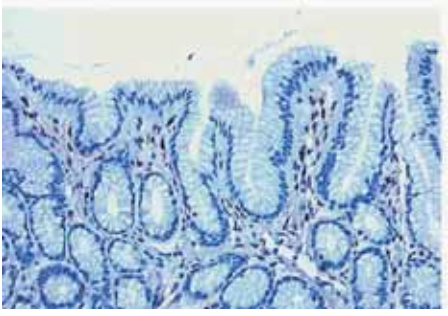

Fig. 1e

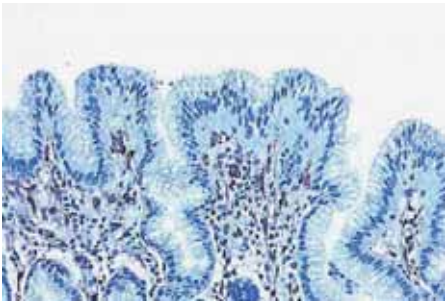

Fig. 1f

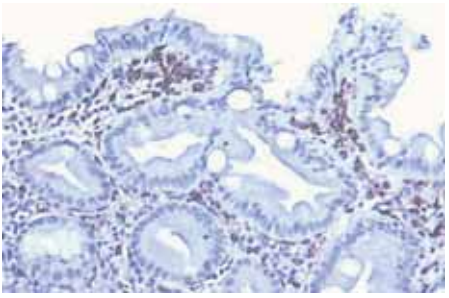

Fig. 1g

FIGURES CHAPTER 5

Figure 1: Fas and Bcl-xl immunohistochemistry in intestinal (a and c) and diffuse (b and d) type gastric cancer. Note the positive staining for Fas and Bcl-xl in the intestinal type gastric cancer. Only 1/7 diffuse type carcinomas stained positive for Fas. Shown here is a Fas-negative sample. Also, only 1/7 of diffuse type carcinomas stained positive for Bcl-xl. Shown here is a Bcl-xl negative sample.

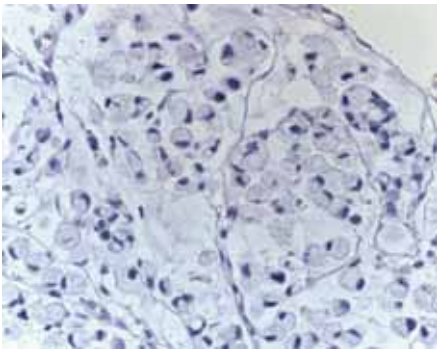

Fig. 1a

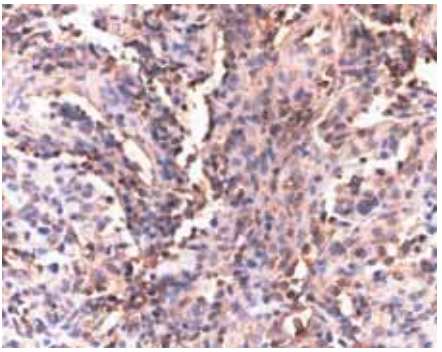

Fig. 1b

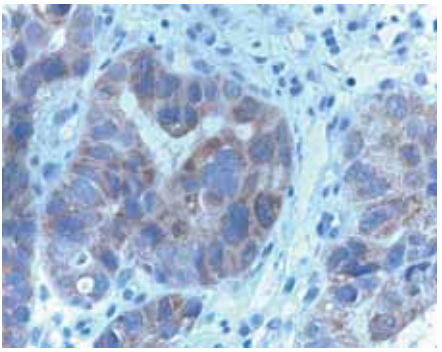

Fig. 1c

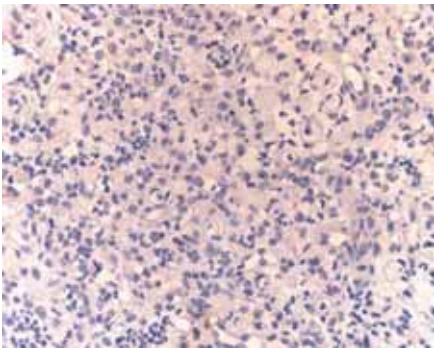

Fig. 1d

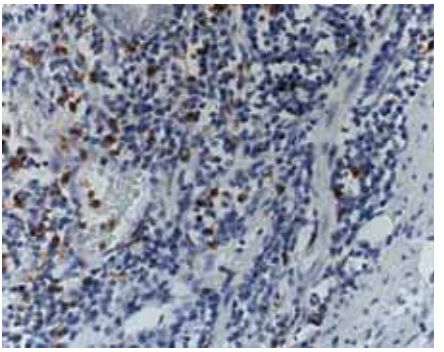

Fig. 2: Example of active caspase-3 staining in a diffuse type carcinoma. Note that not all tumor cells stained positive for active caspase-3.

FIGURES CHAPTER 6

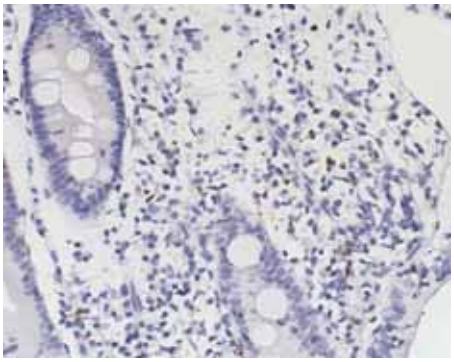

Fig. 1a: Active caspase-3 is negative in intestinal epithelial cells of active CD.

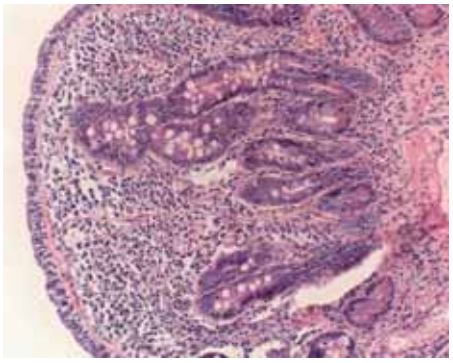

Fig. 1b: No apoptotic bodies in the epithelial layer of the small intestine in active CD.

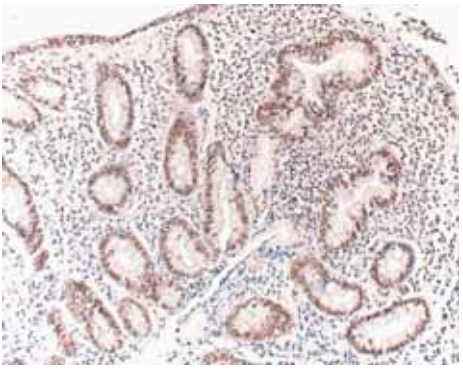

Fig. 1c: Lack of expression of cyto-keratin in active CD.

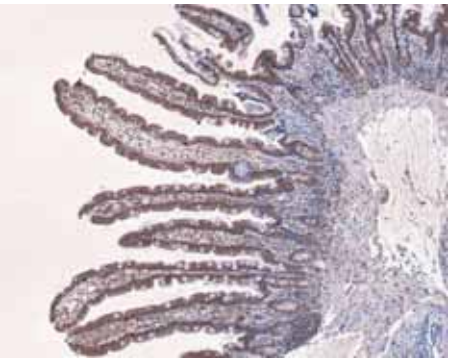

Fig. 2a: Fas staining in controls.

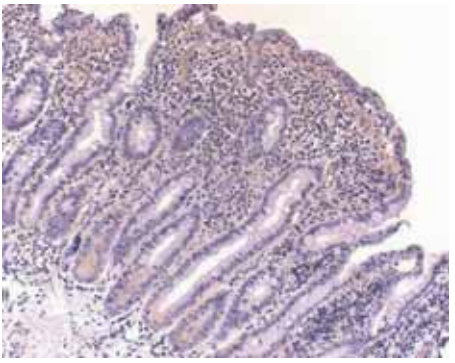

Fig. 2b: Decreased Fas staining in active CD.

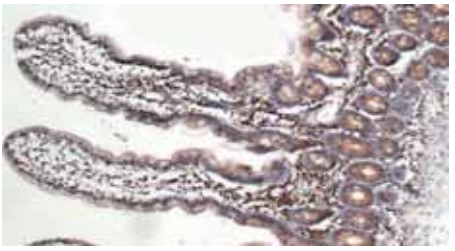

Fig. 2c: Fas staining in gluten-free diet.

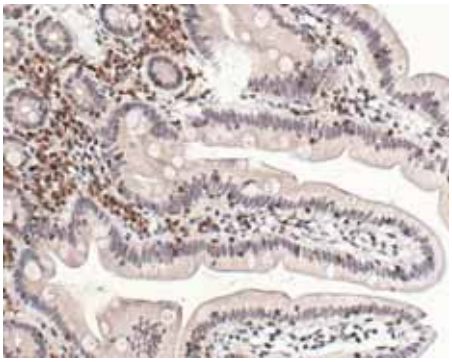

Fig. 3a: Bcl-2 weakly positive in the base of crypts and negative in surface epitheliumin controls.

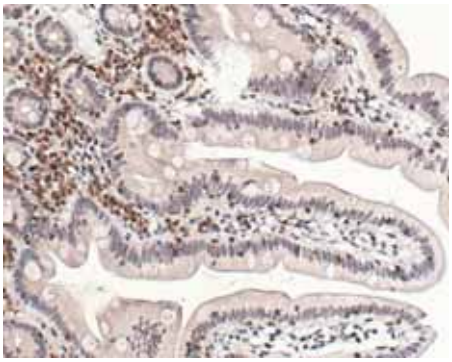

Fig. 3b: Bcl-2 staining in active celiac disease.

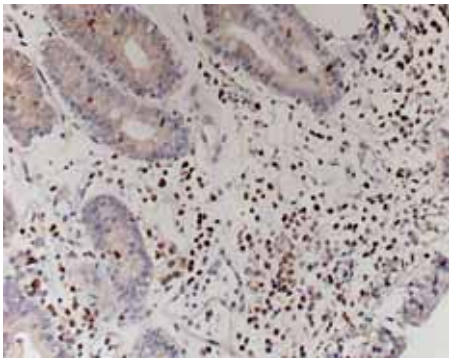

Fig. 3c: Bcl-2 staining during gluten-challenge.

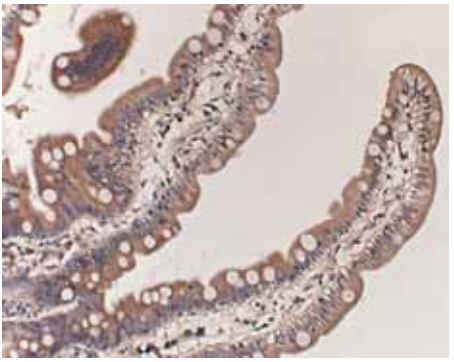

Fig. 4a: Bax staining in controls.
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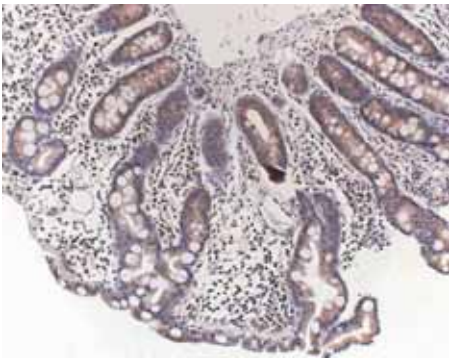

Fig. 4b: Bax staining in active CD.

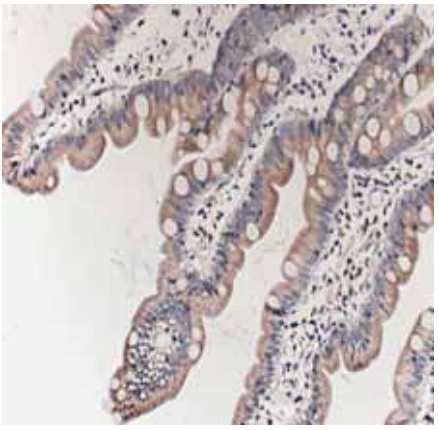

Figure 4c: Bax staining in gluten-free diet

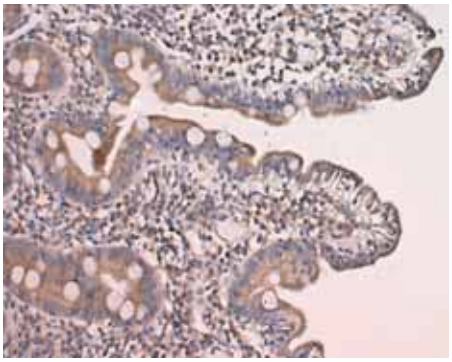

Fig. 4d: Bax staining during gluten-challenge.

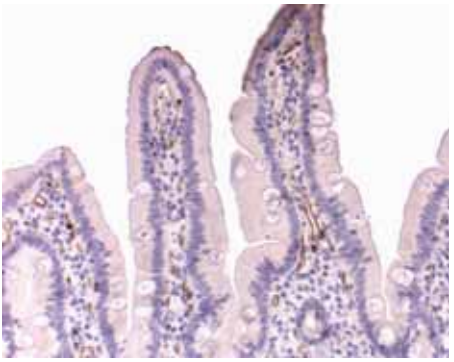

Fig. 5c: iNOS staining in gluten-free diet.

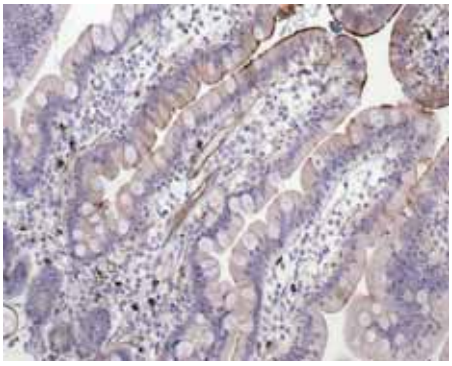

Fig. 5a: iNOS staining in controls.

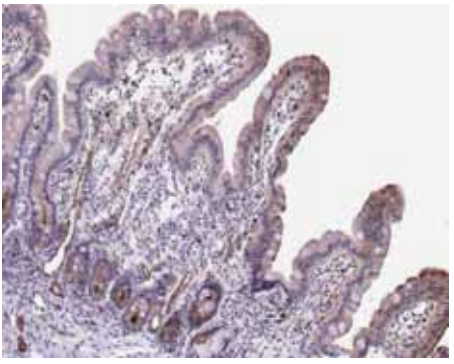

Fig. 5d: iNOS staining during gluten-challenge.

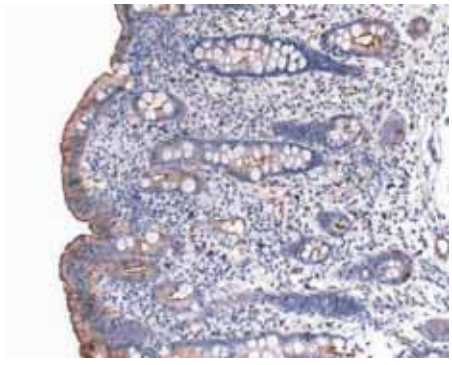

Fig. 5b: iNOS staining is strongly increased in active CD.

FIGURES CHAPTER 7

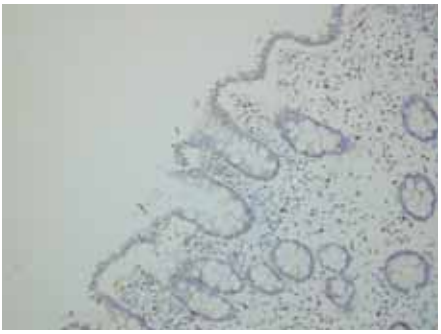

Fig.1a: Absence of iNOS expression in chronic ulcerative colitis.

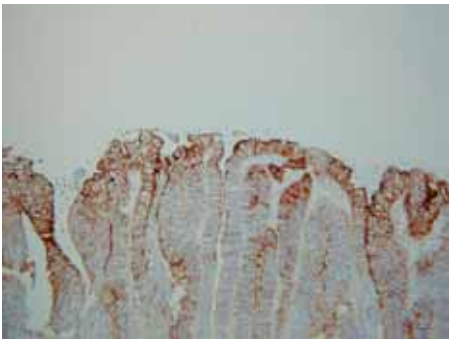

Fig. 1b: iNOS expression is present in epithelial cells in dysplasia

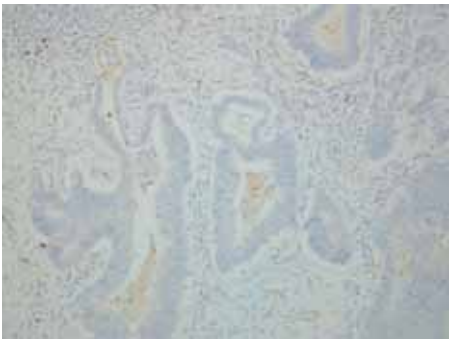

Fig. 1c: Absence of iNOS expression in tumor cells.

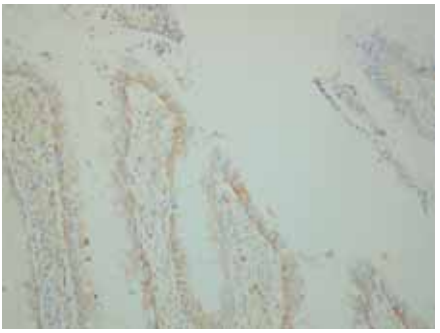

Fig. 1d: Fas is expressed in epithelial cells in dysplasia.

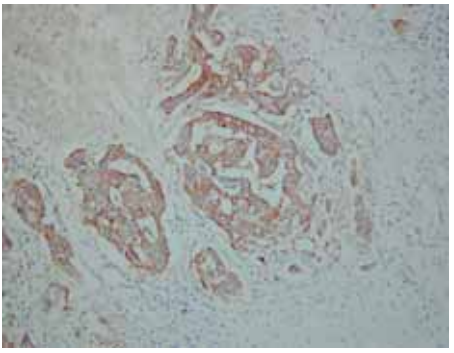

Fig. 1e: Fas is strongly expressed in tumor cells.

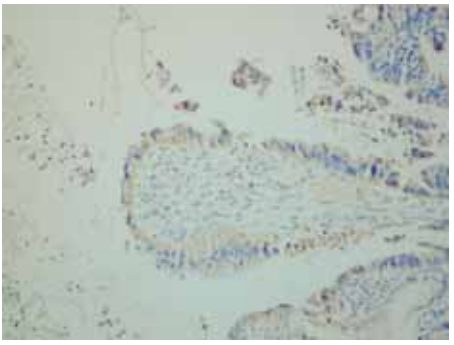

Fig. 1f: Bcl-xl is expressed in epithelial cells in dysplasia

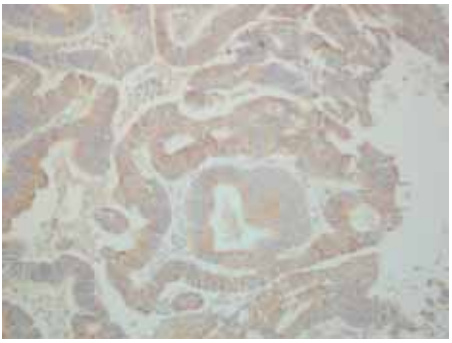

Fig 1g: Bcl-xl is expressed in tumor cells.

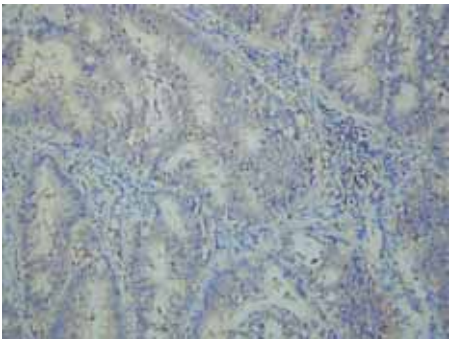

Fig. 1j: Fas is weakly expressed on sporadic colon carcinoma tumor cells
.

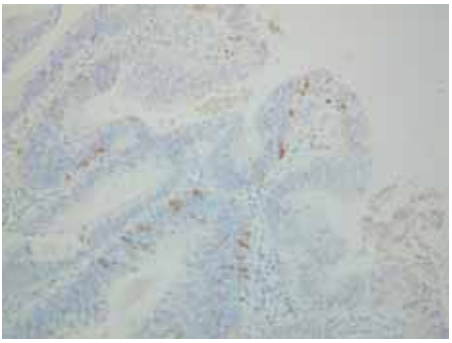

Fig. 1h: Caspase-3 is activated in epithelial cells in dysplasia.

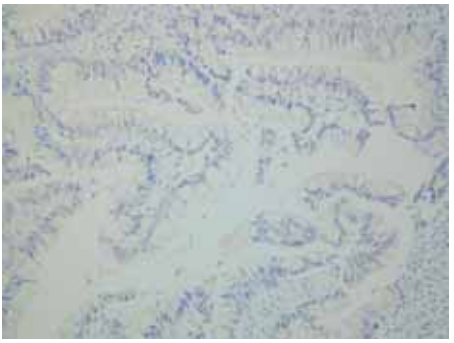

Fig. 1k: Absence of Bcl-xl expression in sporadic coloncarcinoma cells.

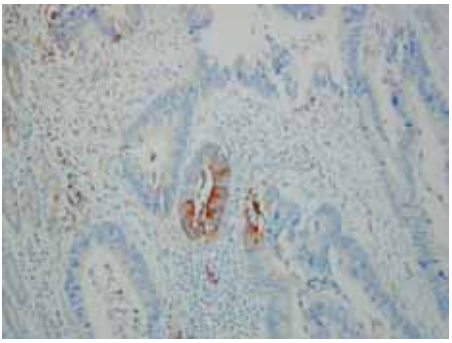

Fig. 1i: iNOS is expressed in tumor cells of sporadic adenocarcinoma.

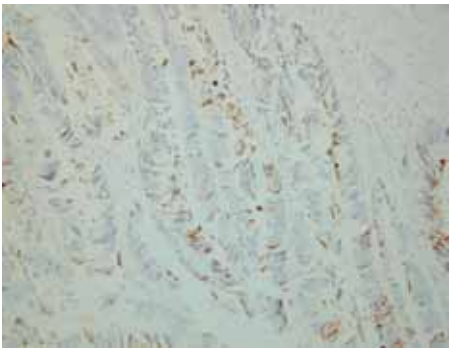

Fig. 1l: Caspase-3 is activated in sporadic coloncancer tumor cells.