Apoptosis in (pre-) malignant lesions in the gastro-intestinal tract
Woude, Christien Janneke van der

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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 discriminate 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-κB-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.