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

SUMMARY

The NF-κB-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 and these genes therefore contribute to the survival of 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-κB 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-κB 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

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

* As positive control for caspase-3 human colon carcinoma 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% H2O2 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 hematoxylin 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 hematoxylin 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. 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. Compared to normal gastric antral mucosa, NF-κB in Hp gastritis is translocated in the nuclei of epithelial cells and a relationship between NF-κB activity and iNOS expression in Hp associated gastritis has been demonstrated. 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. High levels of iNOS have been demonstrated in gastric cancer and in neoplasia of the colon. 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-κB activation allows proliferation of cells and iNOS knockout mice display impaired liver regeneration after partial hepatectomy. Although we did not investigate proliferation in this study, it is possible that epithelial cells of intestinal metaplasia need NF-κB 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. 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. 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. 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.

REFERENCES


