INTRODUCTION

Barrett's esophagus (BE) is an acquired condition which is defined as an endoscopically-detectable changes of esophageal epithelium proven with biopsy of specialized intestinal-type glandular epithelium (SIM) (1). The current definition of BE is a combination of endoscopic and histological criteria. The BE diagnosis is confirmed when intestinal metaplasia is detected by histological analysis (2). The endoscopic techniques allowing the biopsy of esophageal mucosa in BE patients have documented that these patients are at higher risk of progression to esophageal dysplasia and esophageal adenocarcinoma (EAC) (3). The presence of BE increases the EAC risk by about 40 fold (4). Since 1970s, the incidence of EAC has increased dramatically (5).

Gastroesophageal reflux disease (GERD) is an established risk factor for BE (6). In clinical studies duodeno-gastroesophageal bile acid reflux is also identified as an important risk factor for the development of BE (7). The incidence of EAC has increased dramatically (8). Since 1970s, the incidence of EAC has increased dramatically (9).

JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2010, 61, 4, 409-418

www.jpp.krakow.pl

J. MAJKA1, K. REMBIASZ2, M. MIGACZEWSKI1, A. BUDZYNSKA1, A. PTAK-BELOWSKA1, R. PABIANCZYK1, K. URBANCZYK3, A. ZUB-POKROWIECKA2, M. MATLOK2, T. BRZOZOWSKI1

CYCLOOXYGENASE-2 (COX-2) IS THE KEY EVENT IN PATHOPHYSIOLOGY OF BARRETT'S ESOPHAGUS. LESSON FROM EXPERIMENTAL ANIMAL MODEL AND HUMAN SUBJECTS

1Department of Physiology, Jagiellonian University Medical College, Cracow, Poland;
2II Department of General Surgery, Jagiellonian University Medical College, Cracow, Poland;
3Department of Pathomorphology, Jagiellonian University Medical College, Cracow, Poland

Mixed reflux of the gastroduodenal contents induces the esophageal mucosal damage and inflammation progressing chronic esophagitis and premalignant Barrett's esophagus (BE). The role of cyclooxygenase-2 (COX-2) and chronic inflammation in the progression of BE toward adenocarcinoma of the esophagus has not been extensively studied in experimental models of BE in animals and in human subjects. We evaluated the expression of COX-2 in rat model of BE and examined the usefulness of COX-2 expression in determining the risk of malignant transformation in patients with BE treated with argon plasma coagulation (APC) that allows for effective ablation of metaplastic mucosa (group A) without or with proton pump inhibitors (PPI). In addition, the group B of patients was subjected to laparoscopic Nissen's fundoplication and group K that served as control, received PPI treatment only. Expression of COX-2 was evaluated in fresh-frozen biopsy specimens obtained from the distal esophagus in all 60 patients before and 12 months after treatment. In experimental studies, eighty rats were surgically prepared with esophagogastroduodenal anastomosis (EGDA) resulting in chronic esophagitis. At 4 months, the esophageal damage in EGDA rats was evaluated by macroscopic and histological index score, the plasma IL-1β and TNF-α levels was determined by ELISA and the mucosal expression of COX-2 mRNA and COX-2 protein were assessed by RT-PCR and Western Blot, respectively. Chronic esophagitis was developed in all EGDA animals followed by the rise in the plasma TNF-α and IL-1β levels. Histology revealed extensive esophageal ulcerations with development of columnar epithelium, formation of mucus glands in squamous epithelium, intestinal metaplasia distant to anastomosis consisting of goblet cells, infiltration of inflammatory cells including plasma cells and lymphocytes. COX-2 mRNA was absent in the esophageal mucosa of sham-control animals but strongly upregulated in metaplastic Barrett's epithelium. In BE patients, the overexpression of COX-2 was documented in patients with dysplasia. After APC (group A) or Nissen's fundoplication (group B), the expression of COX-2 mRNA was markedly reduced and these effects were positively correlated with histopathological findings. Controls failed to show significant alterations in COX-2 expression. We conclude that 1) EGDA rats serve as the suitable model of the chronic esophagitis by the gastrointestinal refluxate resembling many features of those observed in human Barrett's esophagus, as confirmed by severe morphology changes, excessive release of proinflammatory cytokines TNF-α and IL-1β and overexpression of COX-2, and 2) the significant correlation of the degree of COX-2 overexpression with histopathological findings indicates the usefulness of this inducible biomarker as a valuable indicator of the risk of malignant transformation in patients with BE.

Key words: Barrett's esophagus, cyclooxygenase-2, esophageal gastroduodenal anastomosis, interleukin-1beta, tumor necrosis factor-alpha, argon plasma beam coagulation, laparoscopic Nissen fundoplication
factor in the etiology of BE and EAC. Exposure of esophageal mucosa to short episodes of bile acid in acidic gastric environment results in the disruption of cell membrane and tight cellular junctions and an increase in the process of cell proliferation (7, 8). This process allows gastric juice with activated pepsin to penetrate to the submucosal region causing more severe injury and promoting esophageal epithelium phenotypic differentiation to SIM (9). Chronic exposure of esophageal mucosa to gastric and bile acid first induces the esophagitis that is considered to predispose the esophageal structure to the development of BE and EAC (10). The factors and mechanism underlying the evolution from SIM to BE and EAC are still not fully understood.

An important enzymes involved in inflammation and tumor growth is cyclooxygenase (COX)-1 and COX-2. There are two isoforms of COX enzymes: constitutive COX-1 which is homeostatically expressed in most tissues and the inducible COX-2 stimulated by various cytokines and growth factors and detectable in the inflamed tissues (12). Recent investigation demonstrated the essential role of the COX-2 and prostaglandins (PG) in SIM-BE-EAC progression (13). Overexpression of COX-2 is associated with decreased apoptosis, cell to cell adhesion, increased proliferation and angiogenesis (14, 15) contributes to the increased immunosuppression and mediates carcinogenic effects (16). Epidemiological studies have shown that the treatment with non-steroidal anti-inflammatory drugs (NSAIDs), and the inhibitors of COX-2 enzyme activity are associated with decreased risk of the development of EAC, however, this issue is controversial because of the adverse effects of NSAIDs (17, 18).

BE is believed to be an end-stage of gastro-oesophageal reflux disease (GERD) as it is significantly more frequent among patients with reflux than in general population. Even though it is generally recognized as condition preceding development of adenocarcinoma of the gastroesophageal junction, the exact mechanisms of oncogenesis are not clear. Clinically, the BE must involve at least 3 cm to fulfill the diagnostic criteria - so called "classical" or "long segment" BE. Currently if the lesions are found in the segment shorter than 3 cm it is classified as "short segment" (19, 20). The cases of metaplasia presenting only as irregularities or patches of properly localized Z-line are referred to as "ultra short" BE by some authors (21).

Progression of Barrett's metaplasia into esophageal adenocarcinoma (EAC) is a result of chronic irritation of the mucosa by refluxate (22, 23). Even though BE is reversible the chronic inflammation may promote progression of BE into EAC. Progression of the BE is related to the chemical constituents of the refluxate. The correlation of bile salts concentration and the degree of mucosal damage has been proven (22-25). Identification of the mechanism of BE and early diagnostic procedure seem to be crucial for selection of the group of patients at particular risk.

Barrett's esophagus is undoubting a serious complication of GERD, and thus might be considered a consequence of malfunction of lower esophageal sphincter (LES). The management of BE is based on the treatment of esophageal reflux of gastroduodenal content. This can be achieved by chronic use of medications decreasing gastric acid secretion - mainly proton pump inhibitors (PPIs). Similarly anti-reflux surgery by restoration of high pressure zone in the gastroesophageal junction decreases exposure of the distal esophagus to irritating gastroduodenal refluxate (26). Both methods are used in combination with various techniques of endoscopic mucosal ablation. All those procedures, including argon plasma beam coagulation (APC), promote reepithelialization in the region of GE junction (27).

There are no uniformly accepted standards of management of BE so far. It might be however assumed that effective treatment of GERD combined with ablation of metaplastic mucosa should decrease the risk of carcinogenesis. Accepting expression of COX-2 as a valuable marker of this process, it may prove useful in monitoring the treatment results.

The aim of our study was to determine the effect of chronic gastric reflux on the microscopic status and histology of esophageal mucosa, the expression of mRNA for COX-2 and plasma proinflammatory cytokine levels and, in human study, to evaluate the contribution of expression of COX-2 mRNA to the surgical treatment of the patients with BE.

MATERIAL AND METHODS

Animal studies

Eight-week-old male Wistar rats weighting 200-250 g and fasted for 24 hours before surgery were used for all experiments. An anastomosis between the gastroesophageal junction and the duodenum (EDGA) on its anterior mesenteric border was created to induce mixed duodenogastroesophageal reflux according to the method introduced by Nishijima et al. (7). Briefly, first under general pentobarbital (60 mg/kg i.p.) anesthesia, midline laparotomy was performed and then the longitudinal incision extending approximately 7 mm was made along the lower part of anterior esophagus wall including the gastroesophageal junction area. The second incision also approximately 7 mm in length was performed 4 cm distally from Treitz ligament on the anterior mesenteric border of the duodenum. The esophageal and duodenal openings were side to side Anastomosed using 7-0 silk sutures on an a traumatic needle. The idea was to make a shortcut for the chronic mixed gastroduodenal contents reflux through the damaged lower esophageal sphincter.

The abdomen muscles and skin were closed in the two separate layers with 4-0 silk sutures. Directly after the end of surgery procedure, the animals were infused s.c. with 10-15 ml of isotonic sodium chloride and then were housed in the separate cages under standard laboratory conditions (room temperature 22±2% relative humidity 50±5% and 12 hours light/dark cycle), fasted and fed only with tap water for 48 hours.

Determination of gross and microscopic esophageal mucosal damage

After the termination of the experiment the animals were sacrificed by pentobarbital overdose and esophagus was removed. Immediately after removal it was opened longitudinally, rinsed with saline and pinned open for macroscopic examination. The lesion index score was calculated (macroscopic degree of injury 0-5) after gross inspection of the esophagus under dissecting microscope by a researcher blind to the experimental grouping (28). For microscopic evaluation, the entire esophagus, contiguous anastomosis site and 5 mm of jejunal mucosa were removed and the lumen was longitudinally opened by sectioning through the dorsal aspect of the esophageal wall and divided into proximal, middle and distal segments (28). These segments were embedded in paraffin, cut into 4 µm sections and stained by hematoxylin and eosin (H&E) for microscopic evaluation. The macroscopic and histological findings of the squamous epithelium were classified into index score (1 to 5) as follows: 1- presence of basal cell hyperplasia, hyperkeratosis and papillomatosis; 2- glandular metaplasia with mucous-secreting cells surrounded by squamous epithelium, 3- squamous cell carcinoma; 4- adenocarcinoma, carcinoma, and 5- adenocarcinoma.

Determination of plasma IL-1β and TNF-α levels

Immediately after the termination of experiment, a venous blood sample was drawn from the vena cava and placed into
EDTA-containing vials and used for the determination of plasma IL-1β and TNF-α. Blood was collected and placed into sterile, plastic syringes, kept in ice till centrifugation. The blood samples were centrifuged with the speed 1000 G for 10 minutes in 15°C temperature and the sera were stored in -80°C. The serum level of proinflammatory cytokines IL-1β and TNF-α were evaluated with high sensitive ELISA (Quantikine HS, R&D Systems, Minneapolis, Minn., USA) according to manufacturer's instructions and our study published before (29). Intensity of the colorful reaction was estimated in the spectrophotometer Stat Fax 2100 (Awareness Technology Inc, Pal City, FL, USA) at 490 nm. The intra-and inter-assay coefficients of variation were 8.5% and 10.6%, respectively, for TNF-α, and 10.2% and 10.4%, respectively, for IL-1β.

**Expression of COX-2 transcripts in the rat esophageal mucosa determined by reverse transcriptase-polymerase chain reaction (RT-PCR)**

The mRNA expression for COX-2 was determined by RT-PCR in the unchanged esophageal mucosa of intact rats or those with EDGA. Samples of the esophageal mucosa (about 200 mg) were scraped off into ice using glass slides and then immediately snapped frozen in liquid nitrogen and stored at -80°C. The total RNA was isolated from the esophageal mucosa according to the technique described by Chomczynski and Sacchi (1987) using Trizol Reagent (Invitrogen, Carlsbad, USA) and the manufacturer's protocol (30). The first strand of cDNA was synthesized from total cellular RNA (2 µg) using Reverse Transcription System (Promega, Madison, USA). The PCR was carried out in an automatic DNA thermal cycler, using 1 µg cDNA and Promega PCR reagents. For amplification of COX-2 cDNA, and gene-specific primers (SIGMA-Aldrich St. Louis, USA) were used. The COX-2 primer sequences were as follows: upstream: 5’-ACA ACA TTT CTT TTC TTC-3’, downstream: 5’-CCT TAT TTC CTT TCA CAC C-3’. The expected length of this PCR product was 201 bp. Primer annealing was carried out at 56°C for COX-2 and the amplification of the control rat β-actin was performed on the same samples to verify the RNA integrity. The β-actin primer sequences were as follows: upstream, 5’-TTG TAA CCA ACT GGG ACG ATA TGG-3’, downstream, 5’-GAT CTT GAT CTT CAT GGT GCT AGG-3’ and primers annealing temperature was 54°C. The expected length of PCR product was 764 bp. PCR products were separated by electrophoresis in 2% agarose gel containing 0.5 µg/ml ethidium bromide and then visualized with EDGA rats was made by determination of the COX-2/β-actin ratio of the immunoreactive area by densitometry (Gel-Pro Analyser, Fotodyne Incorporated, USA).

**Western blot analysis**

For Western blot analysis, proteins were extracted from the same esophageal mucosal samples as mentioned above. Approximately 10 µg of total protein extracts was loaded on SDS-polyacrylamide gels and run at 40 mA, followed by transfer onto nitrocellulose membrane (Protran, Schleicher and Schuell, Germany) by electroblotting. Solution of 3% BSA (Sigma Aldrich, Germany) in TBS/Tween-20 buffer (137 mmol NaCl, 20 mmol Tris-HCl, pH 7.4, 0.1% Tween-20) was used to block filters for at least 1 hour at room temperature. Specific primary antibody against COX-2 (rabbit polyclonal, dilution 1:400; Santa Cruz, USA), or β-actin (mouse monoclonal, dilution 1:3000; Sigma Aldrich, Germany) was added to the membrane, followed by an anti-rabbit-IgG or anti-mouse-IgG HRP-horseradish peroxidase conjugated secondary antibody (dilution 1:40 000 or 1:20 000) dissolved in 1% non-fat milk in TBS-Tween-20 buffer (30). Incubation of primary antibody was followed by 3 washes with TBS-Tween-20 buffer for 5 min while the incubation of the secondary antibody was followed by 6 washes for 5 min according to manufacturer instructions. Immunocomplexes were detected by SuperSignal West Pico Chemiluminescent Kit (Pierce, USA). Thereafter, the developed membrane was exposed to an X-ray film (Kodak, Wiesbaden, Germany).

**Human study**

Study group consisted of 60 patients with BE treated at the 2nd Department of Surgery UJ CM in Cracow between 2005-2008. The inclusion criteria for this study comprised: endoscopic picture of BE confirmed by histologically proven glandular metaplasia in the distal esophagus. Patients were enrolled into three study groups: group A- treated by APC ablation of BE combined with PPI, group B- APC ablation and anti-reflux procedure (LNF) and group K- PPI used as a single method of management.

There were 30 patients treated by APC ablation of metaplastic mucosa followed by the administration of 80 mg of pantoprazole (group A). In group B, consisting of 20 patients, APC ablation was combined with laparoscopic Nissen's fundoplication (LNF). There were 10 controls (group K) who refused endoscopic ablation and were treated with pantoprazole (80 mg per day) only.

There were 19 (63.3%) males and 11 (36.7%) females in the group A. Mean age was 54.9 years. Group B consisted of 16 (80%) men and 4 (20%) women. Mean age was 47.3 years. There were 7 (70%) male patients and 3 (30%) females in control (K) group. Mean age was 50.1 years. The differences in sex ratio and age were statistically insignificant (NS). Detailed demographic data of the study subjects are presented in the Table 1.

All patients underwent gastroscopy with evaluation of the entire upper GI tract to exclude other pathology of the esophagus, stomach or duodenum. In all subjects with endoscopic suspicion of BE biopsy samples were obtained from gastroesophageal junction for histological evaluation and assessment of the expression of COX-2. Throughout the entire study during all endoscopies biopsies were taken from all 4 quadrants of the gastroesophageal junction every one centimeter starting from the line of gastric folds to the Z-line (Fig. 1).

Similarly as in case of animal study, the expression of COX-2 RNA was determined by RT-PCR as described above.

Patients with confirmed BE, who refused any invasive management were enrolled into a controls group (K) and received pantoprazole 80 mg per day for 12 months (Fig. 1). Remaining 50 patients were submitted to endoscopic ablation of the mucosa of distal esophagus with APC. During each session

**Table 1. Demographics of the study groups.**

<table>
<thead>
<tr>
<th>GROUP</th>
<th>n</th>
<th>Sex</th>
<th>Age (years)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>female</td>
<td>male</td>
<td>mean</td>
</tr>
<tr>
<td>A</td>
<td>30</td>
<td>11</td>
<td>19</td>
<td>54.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>36.7%</td>
<td>63.3%</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>20</td>
<td>4</td>
<td>16</td>
<td>47.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20.0%</td>
<td>80.0%</td>
<td>-</td>
</tr>
<tr>
<td>K</td>
<td>10</td>
<td>3</td>
<td>7</td>
<td>50.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30.0%</td>
<td>70.0%</td>
<td>-</td>
</tr>
</tbody>
</table>
of endoscopic procedure 1-2 quadrants of the mucosa was destroyed. In case of involvement of 3 or 4 segments endoscopic ablation of consecutive quadrants was performed after 4-6 weeks. After completion of endoscopic therapy follow-up endoscopy with biopsy was performed 6 weeks later. In case of histologically documented failure the procedure was repeated until full eradication of metaplastic mucosa was achieved. Patients with confirmed positive response to endoscopic treatment were assigned into two groups (A and B).

Patients from group A refused anti-reflux surgery and were treated with pantoprazole 80 mg per day for 12 months. Group B consisted of patients in whom endoscopic ablation was combined with laparoscopic Nissen's fundoplication. Patients from all groups (A, B and K) were submitted to follow-up endoscopy after 12 months.

**Statistical analysis**

Results are expressed as means ±S.E.M. Statistical analysis was done using analysis of variance and two way ANOVA test with Tukey post hoc test where appropriate. Differences of p<0.05 were considered significant.

**RESULTS**

**Studies in experimental EGDA rats**

1. The alteration in gross and microscopic appearance of esophageal mucosa in rats with EGDA

The macroscopic appearance of esophagus in intact rat and that subjected to EGDA is presented in Fig. 2. In contrast to the intact esophagus which shows normal appearance, the representative EGDA rat shows very thickened inner surface with longitudinal folds extending along the entire length of the esophagus. In addition, whitish nodular patches of 1-2 mm covers the esophageal surface, giving it a cobblestone appearance (Fig. 2). All EDGA animals developed chronic esophagitis, which reached the highest lesion index and histological score in rats with anastomosis compared with intact animals (Fig. 3 upper panel). Chronic exposure to mixed reflux consisting of gastric acid and duodenal bile salts resulted in chronic esophagitis and replacement of esophageal squamous epithelium by intestinalized columnar epithelium (Fig. 4 A-C). The esophageal mucosa of EGDA rats was heavily infiltrated with inflammatory cells, predominantly with neutrophils but eosinophils, plasma cells and lymphocytes were also present in the esophageal mucosa and submucosa of these animals. By histology, intestinal metaplasia and areas of mucinous adenocarcinoma infiltrating the esophageal wall were recognized in the esophageal mucosa in >80% of rats (Fig. 4 A-C).

2. Plasma cytokine levels and expression of COX-2 mRNA and protein in EGDA rats

Experimental development of BE was accompanied with an increase in the plasma IL-1β and TNF-α levels (Fig. 3 lower panel). The expression of COX-2 mRNA and protein was almost negligible in the intact esophageal mucosa but a dramatic upregulation of mRNA for COX-2 and protein was detected in esophageal mucosa of EGDA animals (Fig. 5).

**Studies in BE patients**

1. The expression of COX-2 in human biopsy from BE patients

Before treatment, most patients presented with overexpression of COX-2 in biopsies from gastroesophageal junction. Distribution of high, moderate and low overexpression of COX-2 was comparable in all study groups (Table 2).

In subjects treated with combination of APC and PPI (group A), an increased expression of COX-2 was found in 21 out of 30 patients. High overexpression of COX-2 (++) was present in 5
(23.8%) patients with low-grade and moderate dysplasia. In 3 (14.3%) expression was only moderately enhanced, while in 13 (61.9%) overexpression was regarded low (+). In remaining 9 patients expression of COX-2 in biopsy specimens of metaplastic mucosa of the distal esophagus was normal (Table 2).

In subjects treated with combination of APC and LNF (group B), the overexpression of COX-2 was found in 13 out of 20 patients. In most of them COX-2 expression was low (+)-8 (61.5%) patients, or moderate (++)-4 (30.8%) patients. High overexpression of COX-2 (+++) was found in 1 (7.7%) patient with low-grade and moderate dysplasia (Table 2).

In controls, who received PPI only (group K), an overexpression of COX-2 was found in 7 out of 10 patients. Highest level of overexpression (+++) was found in one (14.2%) patient with low-grade and moderate dysplasia. In 3 (42.9%) patients overexpression was regarded as moderate (+++) and in next 3 (42.9%) considered as low (+). In three patients from the control group expression of COX-2 was normal (Table 2).

In the initial evaluation of biopsy specimens, the degree of COX-2 overexpression was related to histological type of glandular metaplasia in all groups studied (Fig. 6). In one patient with gastric metaplasia (GM), the level of COX-2 overexpression was low (+). In the largest group of 42 patients with complete intestinal metaplasia (IM (+)) without dysplasia low (+) overexpression of COX-2 was most frequent (47.6%). In 40.5% patients from this group expression of COX-2 was normal. Only 12% of patients had moderate overexpression (++). Among 6 patients with incomplete intestinal metaplasia (IM (-)) without dysplasia two (33.3%) had normal expression of COX-2, next two (33.3%) had low (+) and remaining two (33.3%) moderate (+++) level of overexpression of COX-2. In patients with IM (+) and low-grade or moderate dysplasia overexpression of COX-2 was high (+++) in 75% of patients or moderate (+++) in 25% of patients. Among 3 patients with IM (-) with low or moderate dysplasia each had different level of COX-2 overexpression (Fig. 6).

Second gastroscopy with biopsy of gastro-esophageal junction for histology and assessment of the degree of COX-2 expression was performed after 12 months. Analyzing only the presence or absence of overexpression (omitting its severity) its incidence was statistically significantly lower in patients treated with APC ablation of metaplastic mucosa (group A and B) as compared to initial values. Overexpression of COX-2 was found

![Fig. 2. The gross appearance of the intact esophagus and that after 4 months of reflux due to surgical esophageal duodenogastroesophageal anastomosis.](image)

![Fig. 3. The macroscopic and microscopic index score of the damage induced in esophageal mucosa by the gastroduodenal refluxate (upper panel). The plasma of proinflammatory cytokines IL-1β and TNF-α in intact rats and for comparison, in those with gastroesophageal anastomosis (EDGA) (lower panel). Asterisk indicates a significant change as compared to intact esophageal mucosa of sham-control animal.](image)
in 6 patients from group A (combination of APC and PPI) and 3 from group B (combination of APC and LNF). The difference between group A and B was not significant (Table 3). The expression of COX-2 in the control group (K) did not change.

Fig. 4. Histopathology of rat esophagus and esophageal-duodenal junction in EGDA rats. Examples of experimental Barrett's esophagus (BE) which like in humans, is characterized by the replacement of esophageal squamous epithelium by intestinalized columnar epithelium due to chronic reflux disease. BE type of esophageal mucosa shows the presence of intestinal metaplasia (IM) in the oesophagus. (A) Development of mucinous adenocarcinoma form metaplasia in continuity, infiltrating the esophageal wall in depth. Multilayered epithelium were located mostly at the neosquamocolumnar junction, whereas fewer were located distant from the junction or in the mid-esophagus. (B,C) Metaplastic epithelium in detail with incomplete development of villi and crypts lined by absorptive cells with brush border and goblet cells (H&E, magn.x125).

Fig. 5. The expression of COX-2 mRNA (left upper panel) and protein (left lower panel) in esophageal mucosa of intact rats and those subjected to EGDA. The semi-quantitative ratio of mRNA and protein for COX-2 over β-actin mRNA and protein in esophageal mucosa of intact and EGDA rats. Asterisk indicates a significant increase as compared to that recorded in intact (sham-control) animals.
after 12 months of PPI treatment. The difference between both groups treated with APC (A and B) and controls (K) was statistically significant (Table 3).

The degree of COX-2 expression after treatment was the subject of further analysis. Among patients form group A (APC + PPI) low overexpression (+) was found in 4 (13.3%) and moderate in 2 (6.7%). Those last two patients initially had IM (+) without dysplasia. In the follow-up evaluation, after 12 months treatment none of the patients had high overexpression (+++) of COX-2. In 24 patient (80%) from this group expression of COX-2 was normal (Table 4).

Only 3 patients (15%) form group B (APC + LNF) showed increased expression of COX-2 in the follow-up evaluation performed after 12 months. In two of them (10%) the degree of overexpression was assessed as low (+) and in one (5%) as moderate (++). This last patient initially had IM (-) with low-grade dysplasia in the first evaluation. None of the patients had high overexpression of COX-2 (+++) in the follow-up examination (Table 4). Among controls (group K) treated for 12 months only with PPI excessive expression of COX-2 was found in 7 (70%) of patients. As in the initial evaluation high COX-2 overexpression (+++) has been demonstrated in one patient. In the follow-up examination overexpression of COX-2 was low (+) in 4 patients and moderate (+++) in 2 patients. In 3 patients at the beginning and after completion of study protocol expression of COX-2 was normal (Table 4).

**DISCUSSION**

Identification of the factors leading to the development of Barrett's esophagus and consequently adenocarcinoma remains the subject of basic and clinical research for many years. Chronic irritation of the mucosa of the distal esophagus by refluxate is crucial for the pathogenesis of this disease. It remains however unclear why some patients develop only reflux esophagitis, while others Barrett's metaplasia (5, 6).

We have demonstrated in this study that chronic esophageal mucosa inflammation as the secondary consequence to refluxed gastric and duodenal juice dramatically increased expression of mRNA and protein for COX-2 in rats with duodenogastroesophageal reflux. In addition, these effects were accompanied in these anastomosed animals by the rise in the plasma proinflammatory cytokines such as IL-1β and TNF-α. This is corroborative with previous observations (31, 32) suggesting that proinflammatory cytokines, i.e. TNF-α plays an important role in the gastrointestinal reflux-induced esophageal inflammation and progression of the normal esophageal epithelium into squamous epithelium. Our experimental study as well as clinical findings presented in this paper have demonstrated that inducible isofom of COX enzyme (COX-2) expression increased in BE, reaching the levels of this expression higher than

**Table 2.** The degree of overexpression of COX-2 in study groups before treatment.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>N</th>
<th>normal expression (- or +/-)</th>
<th>degree of COX-2 overexpression</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(+)</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>A</td>
<td>30</td>
<td>9</td>
<td>13</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>61.9%</td>
<td>14.3%</td>
</tr>
<tr>
<td>B</td>
<td>20</td>
<td>7</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>61.5%</td>
<td>30.8%</td>
</tr>
<tr>
<td>K</td>
<td>10</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>42.9%</td>
<td>42.9%</td>
</tr>
</tbody>
</table>

**Table 3.** Changes of the COX-2 expression after treatment compared to baseline.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>WHEN</th>
<th>expression of COX-2</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>absent</td>
<td>present</td>
</tr>
<tr>
<td>A</td>
<td>30</td>
<td>Before</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>After</td>
<td>24</td>
</tr>
<tr>
<td>B</td>
<td>20</td>
<td>Before</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>After</td>
<td>17</td>
</tr>
<tr>
<td>K</td>
<td>10</td>
<td>Before</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>After</td>
<td>3</td>
</tr>
</tbody>
</table>

A:B- NS; A:K- p<0.001; B:K- p<0.001

**Table 4.** The degree of COX-2 expression in the follow-up examination 12 months after treatment.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>normal expression (- or +/-)</th>
<th>degree of COX-2 overexpression</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(+)</td>
<td>++</td>
</tr>
<tr>
<td>A</td>
<td>30 24 (80%)</td>
<td>4 (13.3%)</td>
<td>2 (6.7%)</td>
</tr>
<tr>
<td>B</td>
<td>20 17 (85%)</td>
<td>2 (10%)</td>
<td>1 (5%)</td>
</tr>
<tr>
<td>K</td>
<td>10 3 (30%)</td>
<td>4 (40%)</td>
<td>2 (20%)</td>
</tr>
</tbody>
</table>
in intact esophageal tissue as reported before (33). Moreover, the clinical data revealed that COX-2 is overexpressed in the inflamed mucosa and this event may contribute to the cascade of sequence progression from the metaplasia to dysplasia and finally to adenocarcinoma (34). PG overexpression in BE has been shown to exert a number of effects including promotion of angiogenesis (35), increasing cell proliferation and differentiation, reduction in apoptosis and cell to cell adhesion (14, 15, 36).

In our study BE development was accompanied by a significant rise in the COX-2 mRNA and protein expression and elevated plasma levels of TNF-α and IL-1β levels. This supports the notion that endogenous PG derived from COX-2 enzymatic pathway and cytokines such as TNF-α and IL-1β that may stimulate the expression of COX-2, are linked with the inflammation and angiogenesis in BE (15, 37, 38). The significance of the overexpression of COX-2 in BE development and progression has not been fully clarified so far. Accumulated evidence (39-42) suggests that the overexpression of COX-2 leads to high tissue PG levels that facilitate metaplasia-dysplasia-adenocarcinoma sequence progression affecting growth and angiogenic factors, and generation of reactive oxygen species (ROS).

Long-term use of PPIs has been shown to be effective in GERD and BE patients. They provide pain relief, prevent stricture formation, are well tolerated and safe (43, 44). The common side effect of long-term PPI administration is hypergastrinemia, followed by COX-2 expression up regulation and PG overexpression in BE (45). This effect could be mediated directly by gastrin acting via CCK receptor (46) or via indirect pathway due to induction of EGF that in turn could upregulate the expression of COX-2 (47-49). We have clearly shown that the development of chronic reflux esophagitis in rodent model of BE is associated with severe morphological changes, increased expression of COX-2 and excessive release of proinflammatory cytokines. Further studies are needed to prove whether acid suppressive drugs (PPI-pantoprazole) and selective COX-2 inhibitors (celecoxib) could be useful as the therapeutic options in treatment of BE in experimental animals.

It is nowadays clear that identification of the presence of metaplasia (gastric or/and intestinal) and degree of dysplasia in the distal esophagus is not sufficient for proper evaluation of the stage of the disease in humans. The use of more precise markers of the risk of EAC is suggested. The value of more than 60 biomarkers is currently undergoing detailed studies, among them COX-2 is believed to be one of the most promising (23). It had been proven that chronic irritation caused by exposure to bile salts results in enhanced expression of COX-2 in the metaplastic mucosa (25). Numerous studies point out to increased activity of COX-2 and prostaglandin E2 (PGE2) as a factors promoting chronic inflammation and progression of carcinogenesis (22). Prostaglandins, the major metabolites of arachidonic acid cascade were proposed to act as the potent factors in carcinogenesis modulating cellular adherence, immune response, mutagenesis, cell proliferation, apoptosis and angiogenesis (22, 35, 49). Overexpression of COX-2 through mechanisms involving protein Bel-2 causes inhibition of apoptosis and concomitant enhancement of angiogenesis which promotes progression of cancer (30, 50). This is further supported by observation of excessive expression of COX-2 in human neoplasm of GI tract, breast, lung, uterus and prostate (25). The same overexpression was also found in patients with BE, especially in cases of high-grade dysplasia and adenocarcinoma (35, 51). It might be thus assumed that expression of COX-2 may be a useful marker of early stages of carcinogenesis.

The molecular background for the sequence metaplasia-dysplasia-carcinoma may be the key to understanding the details of the pathogenesis of Barrett's esophagus. Defects of apoptosis appear to be particularly important in the development of dysplasia and adenocarcinoma in these patients. One of the proposed mechanisms of distorted pathways of programmed cell death is physiologically increased synthesis of the factors limiting this process. COX-2 inhibits apoptosis in vitro (14, 52). In the process of carcinogenesis overexpression of COX-2 is interfering with regulation of metabolism of arachidonic acid. This leads to the increased production of prostaglandins which influences the proliferation of the cells, enhances angiogenesis, reduces apoptosis, stimulates the tumor's growth, modulates immune response and reduces the adhesion of the cells leading in consequence to progression of neoplasm (53, 54). In this study overexpression of COX-2 was predominantly documented in majority of BE patients. The correlation of increased expression of COX-2 and the degree of dysplasia was also documented. This is in keeping with observation of Schirvani et al. (25) who reported on the synergistic role of acid and bile contained in the refluxate as factors responsible for increased expression of COX-2.

The effective suppression of gastric acid secretion can lead not only to reepithelialization of the squamous mucosa, but also to reduction of the expression of several biomarkers characteristic for the process of proliferation (54). In this study only one patient from the control group, in whom overexpression of COX-2 was not present both before and after treatment, the metaplasia disappeared completely after one year of PPI administration. Highest expression in this group was observed in a patient with moderate grade dysplasia associated with complete intestinal metaplasia. In this case however, both the baseline and the final expression of COX-2 did not change after 12-month therapy with pantoprazole administered daily in a dose of 80 mg. It seems likely that the monotherapy with PPI remained without a significant effect on the degree of expression of COX-2 in patients with BE. However, the relatively smaller group of patients and the short time of observation do not allow for the definite conclusions and further studies are definitely needed. Perhaps the combination of PPIs with selective inhibitors of COX-2 can be more beneficial in chemoprevention the adenocarcinoma of the esophagus.

In patients undergoing the argon plasma ablation procedure, the effective eradication of metaplastic mucosa resulted in marked reduction of COX-2 expression. Initially overexpression of COX-2 was found in 21 (70%) patients treated with the combination of APC and PPI (group A) and 13 (65%) patients in whom APC ablation was followed by antireflux procedure (group B). After 12 months of follow up, the increased expression of COX-2 has been observed only in 6 patients of group A and 3 group B (Table 3). The effect of ablative therapy supplemented by administration of pantoprazole or laparoscopic Nissen's fundoplication was significantly better compared to the control group with respect to the level of expression of COX-2.

Taking into consideration documented role of cellular signaling pathway involving COX-2 in the carcinogenesis it might be concluded that argon plasma ablation might be a superior method in prevention of progression of Barrett's metaplasia as compared to antisecretory treatment. The study, however, failed to demonstrate advantage of laparoscopic Nissen fundoplication over high doses of pantoprazole therapy as a supplementary method after APC ablation. Further studies are needed to fully document the diagnostic value of COX-2 expression as a prognostic factor in the development of more advanced forms of Barrett's esophagus and adenocarcinoma. It seems however apparent that level of expression of COX-2 correlates well with the histopathologic findings in Barrett's esophagus, particularly with the degree of dysplasia. This notion is supported by the association of different stages of metaplasia with various degrees of COX-2 expression. Therefore, we conclude that 1) the overexpression of COX-2 can be documented in most patients with Barrett's esophagus, particularly those with associated
dysplasia, and 2) in most patients pharmacological and/or surgical treatment seem to correlate with the degree of the overexpression of COX-2, which suggests that this parameter is very useful as a possible biomarker of the development of esophageal adenocarcinoma related to Barrett's metaplasia.

Acknowledgment: This study was in part supported by the state scientific grant from Jagiellonian University Medical College (K/ZDS/001524 to J.M.).

Conflict of interests: None declared.

REFERENCES


Received: September 4, 2009
Accepted: July 15, 2010

Author's address: Prof. Dr. Tomasz Brzozowski, Department of Physiology, Jagiellonian University Medical College, 16 Grzegorzecka Street, 31-531 Cracow, Poland; E-mail: mpbrzozo@cyf-kr.edu.pl

Assoc. Prof. Dr. Kazimierz Rembiasz, II Department of General Surgery, Jagiellonian University Medical College, 21 Kopernika Street, 31-501 Cracow, Poland. Phone: (+4812) 424-82-09