Expression of cyclooxygenase-2 (COX-2) is involved in the chronic inflammation-related development of Barrett’s adenocarcinoma and the use of selective COX-2 inhibitors (coxibs) might provide new chemoprevention strategy for Barrett’s adenocarcinoma (BA). Despite an excellent gastrointestinal (GI) safety profile of coxibs, their use is limited because of the possible cardiovascular complications. The coupling of NSAIDs with a NO-donating moiety has led to the birth of a new class of anti-inflammatory drugs, called the COX-inhibiting nitric oxide donators (CINODs). The member of this group, NO-aspirin (NO-ASA) retains the anti-inflammatory properties of traditional aspirin (ASA), but the release of NO accounts for anti-thromboembolic effect and better GI safety profile. The role of NO-ASA in the prevention of Barrett’s adenocarcinoma (BA) has not been studied so far. Therefore, the aim of the present study was: 1) to analyse the expression of COX-2 in the biopsies obtained from BE; 2) to compare the effect of NO-ASA with that of ASA on proliferation rate in Barrett’s adenocarcinoma cell line (OE-33 cells); 3) to determine the effect of both compounds on the apoptosis rate using FACS analysis and expression of 32-kDa procaspase-3 and active proapoptotic 20-kDa caspase-3 in OE-33 cell line. The expression of COX-2 was assessed in biopsies obtained from the Barrett’s mucosa and normal squamous epithelial esophageal mucosa from 20 BE patients by RT-PCR and Western blot analysis, respectively. The BA cell line (OE-33) was incubated with NO-ASA or ASA (10-1000µM). The cell proliferation and apoptosis rate was measured by BrdU and FACS-analysis, respectively. The expression of caspase-3 (active and inactive form) was analyzed by Western blot. In Barrett’s mucosa a significant up-regulation of COX-2 was observed. Compared with traditional ASA, NO-ASA caused a significantly stronger induction of apoptosis (dose-dependently). Inhibition of cell proliferation in OE-33 cells observed under NO-ASA treatment was due to the apoptosis induction. The increase in apoptotic rate was accompanied by the upregulation of active 20-kDa caspase-3. At the highest concentration (1000µM), a necrotic death of OE-33 cells was observed under NO-ASA treatment. We conclude that: NO-ASA caused induction of apoptosis in BA cell line and slight growth inhibition. These results indicate that this compound may represent a promising chemopreventive agent for Barrett’s adenocarcinoma.
INTRODUCTION

Barrett’s esophagus (BE) is defined as the metaplastic conversion of normal esophageal squamous epithelium into columnar intestinalized epithelium with the development of goblet cells. BE is considered as a premalignant condition that may progress to adenocarcinoma called Barrett’s adenocarcinoma (BA). Over the past three decades we have observed dramatic increase in the incidence of BA while the incidence of esophageal squamous carcinoma remains unchanged. Meanwhile, BA represents the fastest growing cancer in the western world (1, 2).

Neoplastic progression in BE is a multi-step process in which the metaplastic columnar epithelium sequentially evolves through a metaplasia-dysplasia-carcinoma sequence. The process of carcinogenesis in the BE includes a number of molecular alterations including increased cell proliferation, abnormal expression of growth factors and oncogenes, aberrations in cell cycle control, dysregulation in apoptosis, changes in the expression of adhesion molecules and chromosomal abnormalities (aneuploids) (3).

Over the past few years, an increased evidence has been obtained showing that upregulation of cyclooxygenase-2 (COX-2) expression could be responsible for chronic inflammation-related cancer promotion. COX-2 is an inducible isoform of COX, a rate-limiting enzyme in the synthesis of prostaglandins (PG). PGE2 generated by COX-2 exerts several biological effects that may be advantageous for tumorigenesis such as: 1) promotion of angiogenesis; 2) inhibition of apoptosis; 3) stimulation of tumor metastasis by increasing matrix metalloproteinases and 4) decrease in immune surveillance (4).

Chemoprevention of cancers by selective COX-2 blockers (coxibs) has attracted great attention in recent years. The preclinical and clinical studies with the use of coxibs to inhibit carcinogenesis in gastrointestinal tract were very encouraging. Unfortunately, the use of these drugs is hampered by some severe cardiovascular complications including myocardial infarction, stroke and hypertension. This led to the withdrawal of important coxib called rofecoxib from the market and left some doubt about the safety of the entire class of these drugs (5).

A possible alternative to coxibs could represent a new class of anti-inflammatory drugs called the COX-inhibiting nitric oxide donators (CINODs) consisting of a traditional NSAID to which a NO releasing moiety is covalently attached. The release of NO from CINODs results in a wide spectrum of effects on different biological systems. NO plays a crucial role in maintaining the dilation of blood vessels and inhibits the aggregation of platelets, thereby significantly reducing ischemic cardiovascular events. Furthermore, NO released in the gastric mucosa plays an important protective role leading to the reduced
gastrointestinal toxicity (6 - 8). Based on these observations, NO coupled with aspirin (NO-aspirin) could represent a new promising chemopreventive agent (9, 10). However, there is no information on the effect of CINODs on these carcinogenesis in the Barrett’s mucosa.

The present study was undertaken: 1) to investigate the gene and protein expression of COX-2 in the Barrett’s mucosa; 2) to compare the effect of treatment with nitric oxide (NO)-releasing aspirin (NO-ASA) with that of conventional aspirin (ASA) on cell proliferation of BA cells (OE-33); 3) to examine the effect of NO-ASA and ASA on the apoptosis rate and the expression of 32-kDa procaspase-3 and 20-kDa caspase-3 in OE-33 cell line.

**PATIENTS AND METHODS**

The study was approved by the Institutional Ethics Committee of University Erlangen-Nuremberg. 20 patients with histologic record of BE who were undergoing a yearly follow-up to determine the progression of the disease were enrolled in this study. The endoscopies were performed using a video image endoscopes. For better localization of specialized intestinal metaplasia an enhanced magnification endoscopy combined with chromoendoscopy with the acetic acid was used. Endoscopic biopsies were obtained (4 biopsies) from BE and from normal squamous epithelium (4 biopsies) in the middle portion of esophagus. Large-forceps endoscopic mucosal samples from all epithelia were divided into 2 parts, 1 part fixed in formalin was used for histopathologic assessment and the other part was immediately frozen for the molecular analyzes. All specimens were analyzed independently by a pathologist to categorize normal esophageal mucosa, esophageal inflammation, metaplasia and/or dysplasia.

**RT-PCR**

For RT-PCR analysis, biopsy specimens were homogenized in 1mL of TRIZOL (Total RNA Isolation Reagent, Gibco BRL, Germany) and RNA was recovered according to the manufacturer’s instructions before resuspension in 10-20 µL of diethyl pyrocarbonate-treated water and quantification as described before (11). Single stranded cDNA was generated from 5 µg of total cellular RNA using StrataScript reverse transcriptase (Stratagene, Heidelberg, Germany) and oligo-(dT)-primers (Stratagene). The resultant cDNA was amplified in a 50 µl reaction volume containing 0.3 µl (2.5 U) Taq polymerase, 200 mM (each) dNTP (Pharmacia, Freiburg, Germany), 1.5 mM/l MgCl2, 5 µl 10x polymerase chain reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3), and primers used at final concentration of 0.5 µM. The PCR mixture was amplified in a DNA thermal cycler (Perkin-Elmer-Cetus, Norwalk, CT, USA) at the following specifications: 94°C for 45 s (denaturation), 60°C for 45 s (annealing), and 72°C for 2 min, for 32 cycles. The nucleotide sequences of the primers for COX -2 and β-actin were based on the published cDNA. Polymerase chain reaction products were analyzed by agarose gel electrophoresis. All primers were synthesized by GIBCO BRL/Life Technologies (Eggenstein, Germany).

**Western blot analysis**

Using TRIZOL reagent, proteins were extracted from the same biopsy samples as mentioned above. Approximately 10 µg of total protein extracts were loaded on SDS-polyacrylamide gels and run 40 mA, followed by transfer on nitrocellulose membrane (Protran, Schleicher and Schuell,
Germany) by electroblotting. 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 h at room temperature. Specific primary antibody against COX-2 (rabbit polyclonal, dilution 1:500), caspase-3 (mouse monoclonal dilution 1:1000) or β-actin (mouse monoclonal, dilution 1:5000; 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. Incubation of primary antibody was followed by 3 washes with TBS-Tween-20 buffer for 5 min. Incubation of the secondary antibody was followed by 6 washes for 5 min. Immunocomplexes were detected by the SuperSignal West Pico Chemiluminescent Kit (Pierce, USA). Thereafter, the developed membrane was exposed to an X-ray film (Kodak, Wiesbaden, Germany).

Cell culture

The human cell line derived from esophageal adenocarcinoma (OE-33) (ECACC Salisbury, UK (ECACC No. 96070808) was purchased from the Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). Cells were cultured in RPMI 1640 (Biochrom AG, Berlin, Germany) medium containing 10% fetal calf serum (FBS) (Sigma-Aldrich Chemie GmbH Taufkirchen, Germany) and antibiotics (1% penicillin, 0.5% gentamycin) and at 37°C in a water-saturated atmosphere of 95% air and 5% CO₂.

Assessment of cell proliferation

For the assessment of cell proliferation, we used bromodeoxyuridine (BrdU) uptake which identifies cells in the DNA synthetic phase. The BrdU incorporation assay is a precise, fast and simple colorimetric test to quantitate cell proliferation. This technique is based on the incorporation of pyrimidine analog BrdU instead of thymidine into the DNA of proliferating cells. After its incorporation BrdU is detected by immunoassay.

BrdU incorporation was quantified by ELISA using BrdU colorimetric kit according to the manufacturer’s protocol (Roche, Mannheim, Germany). Cells were seeded at a density of 5’000 in 96-well MTP in RPMI medium without FCS. The OE-33 cells were cultured in the presence of aspirin and NO-aspirin (1-1000 µM) for additional 24 h. Subsequently, BrdU was added to the cells for the last 2 h of incubation, then centrifuged at 300xg for 10 min. After removing the culture medium the cells were fixed and DNA was denatured. Incubation with anti-BrdU-POD was performed for 1 h and after washing the immune complexes were detected by the subsequent substrate reaction. Reaction was stopped by adding 1 M H₂SO₄ and probes were measured in an ELISA reader at 450 nm.

Assessment of apoptosis by flow cytometric assay

To assess the effect of increasing doses of ASA and NO-ASA on the apoptosis rate in OE-33 cells and to exclude their possible toxic effects on these cells, FACS analyses were performed. DNA fragmentation was performed by DNA staining of isolated nuclei by flow cytometry. The cells were harvested by gentle scraping, washed, and lysed in a buffer containing of 0.1% sodium citrate, 0.01% Triton X-100, and 0.1 mg/ml propidium iodide. After incubation at 4°C, the nuclei were analyzed for DNA content by flow cytometry. Nonapoptotic nuclei were distinguished from apoptotic nuclei on the basis of their tight scatter profile. Flow cytometry was performed by the Flow Cytometry Facility in the Fiebiger Center at the University Erlangen-Nuremberg, Erlangen, Germany.
Statistical analysis

Data obtained from the densitometric analysis and cell proliferation determinations were analyzed using the paired *t* student test with a level of significance at *p*<0.05. Graphically, data are shown as bar graphs depicting the mean values and the standard errors of means (SEMs) (Sigmaplot Scientific Software; SPSS Inc., Chicago, IL).

RESULTS

In all biopsies obtained from Barrett’s esophagus a significant upregulation of COX-2 expression at mRNA and protein levels was observed. In contrast, in normal esophageal mucosa a low expression for mRNA and protein COX-2 expression was detected (Figs 1 and 2).

Fig. 3. demonstrates the effect of incubation of OE-33 cells with NO-ASA or ASA at increasing concentrations (10 µM-1000 µM) on cellular apoptosis by FACS. At the lowest concentration of 10 µM no significant changes in the apoptotic rate in the OE-33 cells was noticed after incubation with ASA. In contrast, incubation of the cells with NO-ASA induced a slight increase in apoptotic death. However, this change was not significant. At the higher NO-
ASA concentration of 100µM, a significant increase in the apoptotic rate was observed in OE-33 cells. In contrast, no significant changes in apoptosis were observed.

**Fig. 2.** Representative Western blot of COX-2 in biopsies obtained from Barrett’s mucosa and normal esophageal mucosa

**Fig. 3.** Apoptosis rate measured by FACS in OE-33 cells incubated with ASA or NO-ASA (10 vs. 100 µM). Asterisk indicates a significant change as compared to control cells. Cross indicates a significant change as compared to ASA-incubated cells.

ASA concentration of 100µM, a significant increase in the apoptotic rate was observed in OE-33 cells. In contrast, no significant changes in apoptosis were
observed in OE-33 cells incubated with ASA. The highest concentration of NO-ASA (1000 µM), was toxic for OE-33 cells. The FACS analysis showed increased necrotic cell death. In cells incubated with ASA, the apoptosis rate doubled as compared to control cells.

To evaluate the influence of ASA and NO-ASA on apoptosis the level of caspase-3 was determined. In basal conditions, OE-33 cells expressed procaspase-3, but no expression of 20 kDa active caspase-3 was detected. In the OE-33 cells incubated with NO-ASA Western blot analysis revealed a significant dose-dependent upregulation in the protein expression of active caspase-3 (20 kDa). The expression of cleaved caspase-3 was not detected in OE-33 cells incubated with ASA (Fig. 4).

In BrdU assay performed for the analysis cell proliferation, both ASA and NO-ASA have no significant effect for cell proliferation at the concentration of 10µM. At the higher concentration of 100 µM, NO-ASA caused a decrease in proliferation rate of OE-33 cells (~ 75% of control). In contrast, in OE-33 cells incubated with ASA at the same concentration (100µM) no significant change in cell proliferation was observed (Fig. 5).

**DISCUSSION**

The present study demonstrates for the first time the strong inhibitory effect of NO-aspirin on growth of Barrett’s carcinoma cells *in vitro*. This effect was
significantly stronger than that obtained with conventional aspirin. The growth inhibitory effect of NO-ASA was due to the activation of apoptosis as evidenced by FACS analysis and immunoblotting for caspase-3 expression.

It is of interest that NO-aspirin effect was not only due to increased apoptosis rate but also increased necrotic cell death. As postulated in previous reports NO released from NO-ASA not only induces apoptosis but also converts apoptosis into necrosis (12). It is of importance that NO-ASA was significantly more potent in inhibiting the growth of OE-33 cells compared with their traditional counterpart, aspirin.

The mechanism by which NO-ASA exerts its inhibitory effect on the tumor growth is still under investigation. Our results clearly provide an evidence for strong proapoptotic effect of this compound and these results are in agreement with the previous studies (13). In addition, other mechanisms were postulated such as effect on TCF/β-catenin signaling, modulation of mitogen-activated protein kinase (MAPKs) signaling, COX-2 inhibitory effect, induction of morphological changes in cancer cells (vacuolization of cytoplasm, loss of the integrity of the cell membrane), etc. (13, 14). Interestingly, the previous studies demonstrated that the presence of COX-2 is not required for the growth inhibitory effect of these compounds (“COX independence”) (13).

Fig. 5. Proliferation rate of OE-33 cells exposed to ASA or NO-ASA at doses 10, 100 or 1000µM expressed as a percent of control.
Several epidemiological, experimental and clinical studies established nonsteroidal anti-inflammatory drugs as promising cancer chemopreventive agents in Barrett’s cancer. This effect is due to direct inhibition of COX-2 activity and COX-2 independent effects. However, their clinical use is limited by their cardiovascular side effects (15). On the other side the use of traditional NSAIDs is associated with increased risk of gastrointestinal complications such as ulceration, bleeding and perforations. NO-NSAIDs were developed to overcome the limitations of traditional NSAIDs. A number of experimental and clinical studies, including our own, demonstrated that NO-NSAIDs protects gastric mucosa against various ulcerogens and accelerate ulcer healing (16).

Our present study demonstrated a significant overexpression of COX-2 at the mRNA and protein level in the Barrett’s epithelium indicating that COX-2 derived prostanoids play an important a role in the development of Barrett’s cancer and should be considered as a possible target of treatment using specific NO-NSAIDs. The previous in vitro and in vivo studies demonstrated that the selective COX-2 inhibitors inhibit carcinogenesis via decrease in cell proliferation and increase in apoptosis (17 - 19). The present data combined with recent evidence of their safety make a compelling argument for their further preclinical evaluation in animal models of esophageal carcinogenesis.

In summary, these data indicate that: 1) COX-2 is important target for chemoprevention in the Barrett’s carcinogenesis; 2) NO-aspirin significantly more potently inhibits growth of Barrett’s carcinoma cell line than traditional aspirin; 3) Growth inhibitory effect of NO-ASA is partly due to its strong proapoptotic and direct cytotoxic effect.

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