Cyclooxygenase-2 Expression in Human Esophageal Carcinoma


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ABSTRACT

On the basis of epidemiological observations that nonsteroidal anti-inflammatory drugs reduce the risk of esophageal carcinoma, we studied the expression of cyclooxygenase-2 (COX-2) in esophageal squamous cell carcinomas (SCCs; n = 172) and in esophageal adenocarcinomas (ADCs; n = 27). Using immunohistochemistry, we observed COX-2 expression in 91% of the SCCs and in 78% of the ADCs. Western blot analysis showed enhanced expression of the COX-2 protein in some tumors as compared with normal esophageal squamous epithelium, whereas similar amounts of the COX-1 protein were found in normal and cancerous tissues. COX expression was also studied in two esophageal cancer cell lines (OSC-1 and OSC-2) to evaluate the functional relevance of COX-2-derived prostaglandins (PGs). OSC-2 cells expressed COX-2 but not COX-1, whereas OSC-1 cells expressed high levels of COX-1 but showed only a very weak COX-2 expression. Accordingly, PGE2 synthesis was 600 times higher in OSC-1 cells expressed high levels of COX-1 but showed only a very weak COX-2 expression. In contrast, no effect of the COX-2 inhibitors was seen in OSC-1 cells. Our data demonstrate that COX-2 is expressed in the majority of esophageal SCCs and ADCs and that COX-2-derived PGs play an important role in the regulation of proliferation and apoptosis of esophageal tumor cells. It is concluded that inhibition of COX-2 may be useful in the therapy of esophageal cancer.

INTRODUCTION

Recent epidemiological studies indicate an inverse association between the risk of colorectal cancer and intake of NSAIDs such as aspirin, indomethacin, and sulindac (1–4). On the basis of these findings, subsequent studies have addressed the role of the COX enzyme as a target of these compounds. COX (PGH synthase) catalyzes the rate-limiting step in PG biosynthesis: the conversion of arachidonic acid to the PG endoperoxides PGG2 and PGH2. There are two different isoforms of COX, referred to as COX-1 and COX-2. They share over 60% identity at the amino acid level and have similar enzymatic activities, but although they catalyze the same reaction, the isoforms are suggested to have different biological functions (5–9). COX-1 is constitutively expressed in most mammalian tissues and is thought to carry out “housekeeping” functions such as cytoprotection of the gastric mucosa, regulation of renal blood flow, and control of platelet aggregation. In contrast, COX-2 mRNA and protein are normally undetectable in most tissues, but can be rapidly induced by proinflammatory or mitogenic stimuli including cytokines, endotoxins, interleukins, and phorbol ester (10–12). Enhanced expression of COX-2, but not of COX-1, has been found in colon cancer (13–16) and in gastric cancer (17). Increased levels of PGs, especially PGE2, were found in colon tumor tissues as compared with the surrounding normal tissue (18), suggesting that PGs may play a role in the development of tumors. Further evidence that COX-2 and PGs are involved in tumor growth comes from experimental studies. For example, overexpression of COX-2 in rat intestinal epithelial cells was found to reduce the rate of apoptosis and increased the expression of the antia apoptotic proto-oncogene Bcl-2 (19). Moreover, transfection of colon cancer cells with a COX-2 expression vector resulted in an increased metastatic potential of these cells (20). Recently, it has been shown that selective COX-2 inhibitors may significantly suppress experimental colon carcinogenesis (21, 22). There are no previous studies on the expression and role of COX-2 in esophageal carcinoma. Epidemiological studies have shown that intake of aspirin was associated with an up to 90% decreased risk of developing esophageal cancer (23, 24) and in experimental esophageal carcinogenesis, indomethacin had antitumor activity (25, 26).

In the present study, we have determined the expression of COX-2 in SCC and ADC of the esophagus. The functional role of COX-2-derived PGs for the regulation of cell proliferation and apoptosis was studied in two different esophageal cell lines.

MATERIALS AND METHODS

Materials. Reagents used for the experiment were as follows: flusulide (Schering AG, Berlin, Germany), NS-398 (Biomol, Hamburg, Germany), COX-1 monoclonal antibody (Cayman Chemicals, Ann Arbor, MI), COX-2 monoclonal antibody (Transduction Laboratories, Lexington, KY), COX-1 protein from ram seminal vesicle (Cayman Chemicals, and human recombinant COX-2 protein (Calbiochem, San Diego, CA). The antigen used for production of the monoclonal COX-2 antibody was a M, 26,600 COOH-terminal fragment of rat COX-2 protein. Electrophoresis reagents were from Bio-Rad Laboratories (München, Germany). All other reagents were of analytical grade and, if not otherwise mentioned, purchased from Sigma (Deisenhofen, Germany) or Merck (Darmstadt, Germany).

Patients. Tissue samples were collected retrospectively from 172 patients with SCC of the esophagus who underwent esophagectomy between January 1978 and December 1994. No preoperative radio- or chemotherapy had been performed. Of the patients, 133 were male and 39 were female. The median age was 58 years (range, 24–82). Additionally, 27 patients who underwent esophageal resection without prior radio- or chemotherapy between January 1987 and December 1997 due to ADC of the esophagus were included. All ADCs arose in the setting of Barrett’s esophagus, as shown by the histological demonstration of metaplastic and/or dysplastic Barrett’s mucosa adjacent to the ADCs. Of these patients, 26 were male and 1 was female. The median age was 62 years (range, 36–81).

Pathological Review of the Surgical Samples. The esophagectomy specimens were fixed in 4% buffered formaldehyde, embedded in paraffin, sectioned, and stained with H&E. The pT and pN categories were determined according to the criteria proposed by the Union Internationale Contre le Cancer (27). Tumor type and the grade of tumor differentiation were determined according to the criteria proposed by the WHO (28). Tumor size was defined as the largest diameter of the tumor. Of the 172 SCCs, 29 tumors were categorized as pT1 (16.9%), 33 as pT2 (19.2%), 105 as pT3 (61.0%), and 5 as pT4 (2.9%). Among all cases, 83 tumors were in category pN0 (48.3%) and 89 tumors were in category pN1 (51.7%). Of all tumors, 19 were graded as G1 (11.1%), 70 as G2 (40.7%), 52 as G3 (30.2%), and 31 as G4 (18.0%); one hundred eighteen tumors had a maximum diameter of ≤5 cm (68.6%), whereas 54 tumors were >5 cm (31.4%).
Of the 27 ADCs, 8 tumors were categorized as pT1 (29.6%), 5 as pT2 (18.5%), and 14 as pT3 (51.9%). Among all cases, 11 tumors were in category pN0 (40.7%) and 16 tumors were in category pN1 (59.3%). Of all tumors, 12 were graded as G2 (44.4%), 13 as G3 (48.2%), and 2 as G4 (7.4%); fourteen tumors had a maximum diameter of ≤5 cm (51.9%), whereas 13 tumors were >5 cm (48.1%).

**Cell Lines.** OSC-1 and OSC-2 are permanent cell lines derived from two poorly differentiated SCCs of the esophagus; they have been established and characterized in our laboratory (29). The cell lines were maintained in DMEM (Life Technologies, Inc., Eggenstein, Germany) supplemented with FCS (10%), penicillin (100 units/ml), and streptomycin (0.1 mg/ml).

**COX-2 Immunohistochemistry.** COX-2 protein expression was investigated in SCC, in ADC, and in the cell lines. For each of the carcinomas, one representative section containing central and peripheral portions of the tumor was selected. As control, five specimens of nonmalignant esophageal squamous epithelium from tumor-free resection margins of the esophagectomy specimens and five specimens of metaplastic Barrett’s mucosa situated adjacent to the ADC were selected for COX-2 immunohistochemistry. After microwave pretreatment in citrate buffer (pH 6.0) for 15 min at 750 W, slides were incubated overnight at 4°C with the monoclonal COX-2 antibody at a dilution of 1:50. After a second incubation with a biotin-conjugated antimonospecific antibody, slides were incubated with an avidin-biotin-peroxidase reagent (ABC Kit “Elite-Vectastain”; Vector Laboratories, Burlingame, CA). Reaction products were visualized by immersing slides in diaminobenzidine tetrachloride and finally counterstained with hemalaun. Positive staining of normal esophageal squamous epithelium and of smooth muscle cells within the esophageal wall provided an internal positive control for COX-2 staining. The immunohistochemical expression of COX-2 in the tumor cells was examined by one senior pathologist (M.S.) using light microscopy. The percentage of positive tumor cells was determined semiquantitatively by assessing the whole tumor section, and each sample was assigned to one of the following categories: 0 (0–4%), 1 (5–24%), 2 (25–49%), 3 (50–74%), or 4 (75–100%). The intensity of immunostaining was determined as 0 (negative), 1+ (weak), and 2+ (strong).

Intensity of immunostaining was judged relative to smooth muscle cells within the sample, which were designated arbitrarily as 2+. Additionally, an immunoreactive score was calculated by multiplication of the percentage of positive cells and the staining intensity, as proposed by Krajewska et al. (30). In the case of heterogeneous staining intensities within one sample, each component was scored independently, and the results were summed. For example, a specimen containing 25% tumor cells with strong intensity (1 × 2 = 2), 25% tumor cells with weak intensity (1 × 1 = 1), and 50% tumor cells without immunoreactivity received a score of 2 + 1 + 0 = 3.

When the primary antibody was replaced by an irrelevant isotype-matched monoclonal mouse antibody at the same dilution as the COX-2 antibody (1:50), no immunostaining was detectable in any of the investigated tissues.

**RT-PCR.** Total cellular RNA was prepared from cell lines OSC-1 and OSC-2 using a modified Trizol extraction technique (Life Technologies, Inc.). Total RNA (1 µg) was converted into cDNA with murine leukemia virus reverse transcriptase at 42°C for 15 min and denatured at 95°C for 10 min. PCR products were separated by 1.5% agarose gel electrophoresis and ethidium bromide staining. Fragments were identified by fragment size and restriction analysis using Clal, HaeIII, HinfI, NcoI, and PstI restriction enzymes.

Negative controls of COX-1 and COX-2 RT-PCR, which contained no reverse transcriptase, showed no PCR products.

**Western Blotting.** For Western blot analysis, tumor tissue from seven ADCs and five samples of metaplastic Barrett’s mucosa situated adjacent to the ADC were selected for COX-2 immunohistochemistry. After microwave pretreatment in citrate buffer (pH 6.0) for 15 min at 750 W, slides were incubated overnight at 4°C with the monoclonal COX-2 antibody at a dilution of 1:50. After a second incubation with a biotin-conjugated antimonospecific antibody, slides were incubated with an avidin-biotin-peroxidase reagent (ABC Kit “Elite-Vectastain”; Vector Laboratories, Burlingame, CA). Reaction products were visualized by immersing slides in diaminobenzidine tetrachloride and finally counterstained with hemalaun. Positive staining of normal esophageal squamous epithelium and of smooth muscle cells within the esophageal wall provided an internal positive control for COX-2 staining. The immunohistochemical expression of COX-2 in the tumor cells was examined by one senior pathologist (M.S.) using light microscopy. The percentage of positive tumor cells was determined semiquantitatively by assessing the whole tumor section, and each sample was assigned to one of the following categories: 0 (0–4%), 1 (5–24%), 2 (25–49%), 3 (50–74%), or 4 (75–100%). The intensity of immunostaining was determined as 0 (negative), 1+ (weak), and 2+ (strong). Intensity of immunostaining was judged relative to smooth muscle cells within the sample, which were designated arbitrarily as 2+. Additionally, an immunoreactive score was calculated by multiplication of the percentage of positive cells and the staining intensity, as proposed by Krajewska et al. (30). In the case of heterogeneous staining intensities within one sample, each component was scored independently, and the results were summed. For example, a specimen containing 25% tumor cells with strong intensity (1 × 2 = 2), 25% tumor cells with weak intensity (1 × 1 = 1), and 50% tumor cells without immunoreactivity received a score of 2 + 1 + 0 = 3.

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Negative controls of COX-1 and COX-2 RT-PCR, which contained no reverse transcriptase, showed no PCR products.

**Transmission Electron Microscopic Examination for Apoptotic Cells.** For transmission electron microscopy, OSC-1 cells and OSC-2 cells were seeded on glass coverslips and incubated for 48 h with flosulide in different concentrations. Subsequently, the cells were fixed in 3% paraformaldehyde and 1% NP40 and H&E stained. Mitotic and apoptotic tumor cells were counted (blinded for the treatment the cells had received) in 10 randomly selected microscopic fields (corresponding to a total of at least 1000 tumor cells) under high power magnification (×400), using a quadratic reticle with 25 squares of 4 mm² inserted in the ocular lens (33). Mitotic and apoptotic cells were identified by standard criteria (34, 35). The mitotic index and apoptotic index/case were expressed as percentages of the mean number of mitotic figures or apoptotic bodies/100 intact tumor cells.

**Statistical Analysis.** Statistical analysis of the correlation between COX-2 expression in the tumors and clinicopathological parameters was performed by means of a two-sided Fisher’s exact test. For the comparison of COX-2 expression between SCC and ADC, the Kruskal-Wallis test was used. The effects of exposure of esophageal cancer cell lines to COX-2-inhibiting compounds were analyzed by means of unpaired Student’s t test. Ps <0.05 were considered significant.

**RESULTS**

**Expression of COX-1 and COX-2 in Normal Esophageal Squamous Epithelium and in SCCs.** Immunohistochemically, normal esophageal squamous epithelium invariably showed cytoplasmatic staining of weak intensity for COX-2 in all cell layers (Fig. 1a). Additionally, COX-2 immunoreactivity was found in smooth muscle cells (Fig. 1b), in fibroblasts, and in endothelial cells. Among the
carcinomas, 156 of 172 SCCs (90.7%) exhibited cytoplasmic immunoreactivity for COX-2 in the tumor cells (Fig. 1c), whereas 16 carcinomas (9.3%) were completely negative. Among COX-2-positive carcinomas, the percentage and staining intensity of positive tumor cells varied markedly between different tumors. The immunoreactive score ranged between 1 and 8 (median, 3). The details of expression patterns regarding the percentage of positive cells, staining intensity, and immunoreactive score are presented in Table 1. The results of the immunohistochemical investigations were confirmed by Western blot analysis, where all samples of SCCs investigated showed expression of the COX-2 protein. The expression of COX-2 in some carcinomas was enhanced as compared with the normal mucosa (Fig. 2).

Regarding COX-1, Western blot analysis showed expression of similar intensity in all investigated samples of normal esophageal epithelium (n = 1) and esophageal SCC (n = 7; Fig. 2). Immunohistochemical investigation of COX-1 in paraffin-embedded tumor samples did not reveal reliable staining results (data not shown).

Expression of COX-2 in Metaplastic Barrett’s Mucosa and in ADCs. Metaplastic Barrett’s mucosa showed no immunoreactivity for COX-2 (Fig. 1d), whereas COX-2 immunostaining again was observed in smooth muscle cells, in fibroblasts, and in endothelial cells. Of the 27 ADCs, 21 carcinomas (77.8%) showed cytoplasmatic immunoreactivity for COX-2 (Fig. 1e) and 6 carcinomas (22.2%) were completely negative. As in the case of SCC, there was marked intertumoral heterogeneity in terms of staining intensity and percentage of positive cells among COX-2-positive ADC. The immunoreactive score ranged between 1 and 8 (median, 3). The details of expression patterns are presented in Table 1. No significant differences were found when COX-2 expression among SCC and ADC was compared in terms of staining intensity, percentage of positive cells, or immunoreactive score.

Correlation between COX-2 Immunostaining in SCC and ADC and Other Pathological Parameters. For correlation analysis, SCC and ADC were dichotomized at the median immunoreactive score (3 in both groups of tumors) into one group with weak or no COX-2

Fig. 1. Examples of cytoplasmatic COX-2 immunoreactivity (brown reaction product) in all cell layers of normal esophageal squamous epithelium (a), in smooth muscle cells of the esophageal wall (arrow) together with unreactive cells of a SCC (⁎; b). Focal COX-2 immunoreactivity in a moderately differentiated SCC of the esophagus (arrow; c). No COX-2 immunoreactivity in metaplastic Barrett’s mucosa (positive reaction in fibroblastic stromal cells; ⁎; d), in contrast to a moderately differentiated ADC of the esophagus (e). COX-2 immunostaining in cells of the permanent esophageal carcinoma cell line OSC-2 (f).
expression and in one group with strong COX-2 expression. Among SCC, local lymph node metastasis was found more frequently among tumors with low COX-2 expression (58.5%) than among tumors with strong COX-2 expression (37.0%; \( P = 0.013 \)). No correlations were found between COX-2 expression and \( pT \) category or tumor differentiation. Among ADC, no significant correlations were found between COX-2 immunoreactivity and \( pT \) category, \( pN \) category, or tumor differentiation.

**Expression of COX-1 and COX-2, PG Synthesis, and Its Inhibition in Tumor Cell Lines.** The two tumor cell lines (OSC-1 and OSC-2) showed a different expression of the COX isoforms. OSC-2 expressed COX-2 mRNA (Fig. 3A) and COX-2 protein (Fig. 3B), but showed no COX-1 mRNA and protein expression. In contrast, OSC-1 expressed high levels of COX-1 protein, but showed only a very weak COX-2 protein expression (Fig. 3B). Additionally, immunostaining for COX-2 was found only in OSC-2 cells (Fig. 1F). To evaluate the functional activity of cyclooxygenases in the cell lines, we measured PG production by RIA. The cell line OSC-2 synthesized high amounts of PGE\(_2\) (143 ± 11 ng/ml) and also 6-keto-PGF\(_{1\alpha}\) (761 ± 9 pg/ml), whereas OSC-1 showed considerable lower PG synthesis (248 ± 13 pg/ml PGE\(_2\) and 34 ± 6 pg/ml 6-keto-PGF\(_{1\alpha}\); Fig. 4, A and B). To study the relevance of COX-2 for PG synthesis, we measured the inhibition of PGE\(_2\) generation by the COX-2-selective inhibitors flosulide (36) and NS-398 (37). Flosulide (1 nM -100 \( \mu \)M) caused a concentration-dependent and finally complete inhibition of PGE\(_2\) production in the OSC-2 cell line, whereas no effect on PG formation was observed in the OSC-1 cells (Fig. 5). Similar results were obtained with NS-398 (1 nM-100 \( \mu \)M), which inhibited PG synthesis less potently than flosulide, but also significantly at a concentration of 1 nM in the OSC-2 cells (Fig. 5).

**Effect of Flosulide and NS-398 on Proliferation and Apoptosis.** The effect of COX-2 inhibitors on proliferation of the tumor cells was studied by means of the XTT assay. Tumor cells were treated with flosulide and NS-398 in various concentrations, and the effect on proliferation were determined after 48 h of treatment. The selective COX-2 inhibitors flosulide and NS-398 suppressed only the proliferation of the tumor cell line OSC-2 with significant inhibition at 1 nM for flosulide and 100 nM for NS-398, respectively, whereas no effect on proliferation was observed in OSC-1 cells (Fig. 6). The results of

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\(^a\) Percentage of positive cells: 0, 0–4%; 1, 5–24%; 2, 25–49%; 3, 50–74%; 4, 75–100%.

\(^b\) Staining intensity: 0, negative; 1+, weak; 2+, strong.

\(^c\) Immunoreactive score: combination of \(^a\) and \(^b\) (see "Materials and Methods").
the XTT assay were confirmed by light microscopic examination of flosulide (1 nM–100 μM)-treated OSC-1 and OSC-2 cells. Because of the higher specificity for COX-2 and the stronger antiproliferative effect of flosulide, the effect on apoptosis was examined only with this compound. Treatment with 1 nM flosulide completely suppressed mitotic activity of OSC-2 cells, whereas mitotic activity of OSC-1 cells remained unchanged (data not shown). Additionally, a marked induction of apoptosis among OSC-2 cells, due to treatment with flosulide, was observed (Figs. 7 and 8). Transmission electron microscopic examination of OSC-1 cells and OSC-2 cells, treated by various concentrations of flosulide (1 nM–100 μM), confirmed massive induction of apoptosis among OSC-2 cells as compared with untreated controls, whereas no effect on apoptosis was observed in OSC-1 cells (data not shown).

DISCUSSION

This study shows that the COX-2 protein is present in the majority of esophageal SCCs and esophageal ADCs. Immunohistochemical analysis allowed to localize COX-2 within the cancer cells. COX-2 was also present in cells of the tumor stroma (e.g., in smooth muscle cells, vascular endothelium, and fibroblasts). Weak COX-2 expression was also found in the tissue of origin of esophageal SCC (i.e., normal squamous epithelium), but not in the tissue of origin of esophageal ADC (i.e., metaplastic Barrett’s mucosa). The results of our immunohistochemical analyses are supported by additional Western blot analysis of COX-2 expression in a limited number of esophageal malignant and nonmalignant tissues.

Our immunohistochemical analysis showed marked intertumoral heterogeneity in COX-2 expression, ranging from tumors with few weakly positive cancer cells to tumors with apparent COX-2 overexpression. Intertumoral heterogeneity has also been reported in previous studies on COX-2 expression in colon cancer (13–15). However, in these studies, the interest has not focused on this phenomenon, either because very small numbers of tumor samples have been investigated, or because tissue extracts with a mixture of neoplastic cells and nonneoplastic stromal cells were analyzed. In this type of analysis, however, it is hardly possible to determine whether the detected signal actually stems from the neoplastic tumor cells. The
shown that elevated PGE2 levels in COX-2 overexpressing cells act as differentiation and growth factors, as immunosuppressors, and as angiogenic agents (39–41). Additionally, it has been observed that exposure of colon carcinoma cells to NSAIDs induced hypophosphorylation of the retinoblastoma tumor suppressor protein as well as a down-regulation of multiple proliferation-promoting cyclins and cyclindependent kinases (45).

Apart from the demonstration of the presence of COX-2 in esophageal cancer, the present study suggests an important function of COX-2 in this tumor type. We have shown that selective inhibition of COX-2 in esophageal cancer cells induces apoptotic cell death and reduces proliferative activity, and that these effects correlate with the inhibition of PG synthesis. The specificity of this effect is underlined by the finding that only in the cell line OSC-2, which shows constitutively high expression of COX-2, the selective inhibitors exert their antiproliferative and apoptosis-inducing effect, whereas no effect was observed in the cell line OSC-1, which is characterized by high levels of COX-1 but only weak expression of COX-2. The finding that flosulide and NS-398 did not inhibit production of prostanooids in OSC-1 cells points to their high specificity for COX-2. Similar results have been reported by other authors, who found no inhibition of COX-1 by flosulide or NS-398 even at concentrations of 100 µ M (46, 47, 36).

The apparent difference between the cell lines correspond to our immunohistological analysis of COX-2 expression in esophageal SCC and ADC, which also showed marked intertumoral heterogeneity. Therefore, the significance of COX-2 probably varies between different tumors. We cannot conclude from our in vitro data whether the antiproliferative and proapoptotic effect of COX-2 inhibitors on esophageal cancer cells is exclusively mediated through the inhibition of PG synthesis or if other mechanisms are also involved. However, our data may provide an explanation for epidemiological studies indicating that the long-term intake of COX inhibitors substantially reduces the incidence of esophageal cancer. The results of our immunohistological analyses indicate that COX-2 is equally expressed in esophageal SCCs and ADCs. The pharmacological inhibition of COX-2 may, therefore, be effective in the two main types of esophageal cancer. The response of individual tumors to exposure of COX-2 inhibiting compounds in vivo, however, awaits further evaluation, especially because the level of COX-2 expression varies substantially between different tumors.

In conclusion, our study provides evidence that COX-2 is expressed in the majority of esophageal SCCs and ADCs and that COX-2-derived PGs are involved in the progression of esophageal cancer. By analogy to the situation in colorectal cancer, COX-2 may be a new target in the chemoprevention and chemotherapy of esophageal carcinoma.

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