Int J Colorectal Dis (1999) 14:212–218

ORIGINAL ARTICLE

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Cellular localization of cyclo-oxygenase isozymes in Crohn's disease and colorectal cancer

Accepted: 7 September 1999

Abstract Deregulation of cyclo-oxygenase isozyme expression has been shown to be a consistent feature of inflammatory bowel diseases and colorectal cancer in humans. This study investigated the cellular localization of aberrant cyclo-oxygenase expression in normal and diseased colon. Biopsies of seven normal colonic tissues, eight tissue samples from patients suffering from Crohn's disease, five polyps from patients with familiar adenomatous polyposis coli, and ten sporadic adenocarcinomas were analyzed using isozyme-selective immunoprecipitation, western blotting, and immunohistochemistry. Cyclo-oxygenase-1 expression was demonstrated in normal human colon, Crohn's disease, and colorectal tumors. In normal colon and also in adenomatous polyps, cyclo-oxygenase-1 specific immunosignals were localized to epithelial cells of the upper part of the crypts and endocrine cells of the lower part. In Crohn's disease cyclo-oxygenase-1 expression was restricted to cells of the inflammatory infiltrate. While barely detectable in normal colon, cyclo-oxygenase-2 protein was strongly increased in epithelial cells located in the uppermost part of the crypts, in surface epithelial cells, and in mononuclear cells of the lamina propria of Crohn's disease. The constitutive overexpression of cyclo-oxygenase-2 protein observed in the majority of the adenomatous polyps and all adenocarcinomas was attributed to both epithelial and interstitial cells in that the latter predominated in adenomas, and epithelial cells were the prevailing cyclo-oxygenase-2 expressing cell type in adenocarcinomas. In conclusion, both autocrine and paracrine effects of aberrant cyclo-oxygenase-2 expression may contribute to the development of Crohn's disease and colonic tumor development.

Key words Cyclo-oxygenase · Colon carcinogenesis · Crohn's disease · Familiar adenomatous polyposis coli · Immunohistochemistry

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Introduction

Inflammatory bowel diseases (IBDs) including Crohn's disease, ulcerative colitis, and genetically predisposed familial adenomatous polyposis coli (FAP) are known to be associated with an increased risk of colon cancer [1, 2]. Among the therapeutic regimens used for treating IBDs are inhibitors of prostaglandin biosynthesis, including nonsteroidal anti-inflammatory drugs (NSAIDs) [1, 3]. In fact, both epidemiological evidence and clinical studies have shown that NSAIDs reduce colon cancer mortality and dramatically prevent colonic polyp formation in FAP (for review see [2]). Among the molecular mechanism(s) of NSAID action the inhibition of cyclo-oxygenase (COX), i.e., of prostaglandin biosynthesis, is thought to play a major role in the antitumor effect [4]. The tissue level of prostaglandins has indeed been found to be strongly elevated in polyps and colorectal adenocarcinomas [5] as well as in the mucosa of patients suffering from IBDs, which corresponds to the clinical severity of the disease [6].

At least two COX isozymes are known to catalyze the oxygenation of arachidonic acid to prostaglandins [7]. COX-1 is constitutively expressed in many tissues, including the intestine and the colon, where it is thought to be responsible for the production of prostaglandin E_2 being critically involved in the maintenance of mucosal integrity [8]. As a rule, COX-2 is an inducible enzyme, the expression of which is upregulated in most cell types by proinflammatory cytokines, growth factors, and tumor promoters, indicating that prostaglandins generated by

this isozyme act as mediators of inflammation and cell proliferation [9]. Studies using reverse transcriptase polymerase chain reaction analysis and western blotting have reported COX-2 to be absent in normal human colon but strongly expressed in IBDs [10, 11] and to varying degrees in human adenomas and adenocarcinomas [12–15]. Expression of COX-1 protein is observed at comparable levels in normal colon and in the colon of patients suffering from IBD and cancer [11, 12, 14, 15]. The changes in COX isozyme expression that accompany IBDs and colon cancer suggest a critical role of COX-2 in the development or maintenance of the disease states. Less detailed information has been provided for the cellular localization of COX isozyme expression in normal and diseased colon or in colorectal tumors [12, 13, 15]. The aim of this study was to provide information on the cell-type expression of COX isozymes in these disease states.

Materials and methods

Materials

Goat anti-human COX-1 (SC1754) and COX-2 (SC1745) antisera, horseradish peroxidase conjugated donkey anti-goat IgG, and alkaline phosphatase conjugated donkey anti-goat IgG antibodies were obtained from Santa Cruz (Heidelberg, Germany). Alkaline phosphatase conjugated goat anti-rabbit IgG antibody was from Dianova (Hamburg, Germany).

Biopsies

After informed consent, colon biopsies were performed on patients in the Department of Gastroenterology at the University of Münster, Germany and the Pathology Department of the Institute "Pirogov," Sofia and Medical University, Pleven, Bulgaria. Immediately after surgery one part of each tissue sample was fixed in 4% paraformaldehyde for routine diagnosis, and the remainder was snap-frozen in liquid nitrogen and stored at –70°C. Histological diagnosis evaluation used hematoxylin-eosin stained paraffin sections according to standard diagnostic criteria. Clinical information including age, sex, symptoms, location of the lesion, treatment modality, and last follow-up was obtained from hospital charts. The study included seven normal colonic tissues [three control biopsies resected at a distance of 5 cm from the colorectal tumor (matched controls) and four nonmatched control biopsies; mean age of the patients: 63±34 years], eight tissue samples from patients with Crohn's disease (mean age 33±10 years), five adenomatous polyps from FAP patients (mean age 30±4 years), and ten sporadic adenocarcinomas (mean age 69±9 years). About half of the patients in each group were men (see Table 1).

Homogenization of tissue samples

Frozen biopsies were ground in a precooled steel mortar using a pistill and the ground tissue was pulverized in a ball mill (Retsch, Haan, Germany) for 10 s. All steps were performed at the temperature of liquid nitrogen.

Protein determination

Protein concentrations were determined by means of the Biorad DC Protein Assay using bovine serum albumin as standard.

Table 1 Characterization of patients

Patient no.	Sex	Age (years)	Histopathology[a]
1	F	24	Normal colon
2	M	83	
3	F	81	
4	n.a.	n.a.	
5	n.a.	n.a.	
6	n.a.	n.a.	
7	n.a	n.a.	
8	M	33	Adenomatous polyp
9	F	33	
10	M	24	
11	M	33	
12	M	34	
13	M	61	Adenocarcinoma
14	F	72	
15	F	71	
16	F	81	
17	F	72	
18	M	57	
19	F	59	
20	F	65	
21	M	83	
22	M	74	
23	F	24	Crohn's disease
24	F	28	
25	M	41	
26	M	35	
27	M	36	
28	M	17	
29	F	46	
30	F	40	

[a] For more details see Table 2

Immunoprecipitation and western blot analysis

Tissue powder was homogenized in buffer containing 50 mM Tris/HCl pH 7.4, 1 mM diethyldithiocarbamate, 2 mM EDTA, 1.0% Tween 20, 0.2 mg/ml α_2-macroglobulin, 1 mM phenylmethylsulfonyl fluoride, aprotinin and leupeptin, 10 µg/ml each. After centrifugation (4000 *g*, 4°C, 20 min), approximately 1–1.5 mg of the supernatant protein was used for immunoprecipitation of COX-2 according to the procedure described recently [16]. COX-1 was then immunoprecipitated from the supernatant of the COX-2 precipitation as described [17]. Immunoprecipitated proteins were electrophoresed using 7.5% sodium dodecyl sulfate polyacrylamide gels. The proteins were electroblotted onto polyvinylidene fluoride membranes as described previously [18]. Subsequently the membranes were handled according to the protocol recommended by the supplier of the Tropix-Western light chemiluminescence detection system (Perkin Elmer Applied Biosystems, Weiterstadt, Germany). Goat anti-human COX-2 (SC 1745) and rabbit anti-COX-1 antibodies were used at a dilution of 1:2000 and the anti-(goat/rabbit) IgG alkaline phosphatase antibodies at a dilution of 1:2000/5000. Protein sizes were estimated by comparison with coblotted molecular weight standard proteins (SDS-6H, Sigma, Deisenhofen, Germany).

Immunohistochemistry

Paraffin sections were obtained from Prof. Dr. K.W. Schmid (Department of Pathology, University of Münster, Germany). Staining of specimens was performed as described [17]. Briefly, after blocking endogenous peroxidase by incubation with 3% H_2O_2 in methanol for 10 min, specimens were blocked (2.5% skim milk powder in PBS, Fluka, Neu-Ulm, Germany) for 1 h and incubated with goat anti-human COX-2 or goat anti-human COX-1 antise-

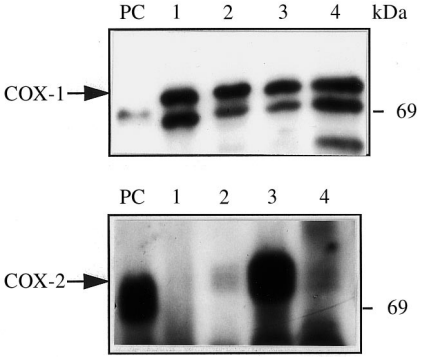

Fig. 1 Representative immunoblot analysis of COX-1 and COX-2 protein expression in normal human colon and in neoplasias. Homogenates from colon biopsies and the mouse keratinocyte cell line PDV (*PC*) were immunoprecipitated using rabbit anti-mouse COX-1 specific or rabbit anti-mouse COX-2 specific antibodies. The precipitates were electrophoresed and immunoblotted with the COX-1 specific (*above*) or COX-2 specific antiserum SC 1745 (*below*). The positions of the isozymes are indicated. Molecular weights of standard proteins are given in kilodalton (kDa): *sample 1* normal colon; *sample 2* adenomatous polyp; *sample 3* colon carcinoma; *sample 4* Crohn's disease. In total, four normal colon, five adenomatous polyps, ten carcinomas, and three Crohn's disease samples were analyzed

Table 2 Expression of COX-1 and COX-2 isoenzymes in adenomatous polyps and colorectal carcinomas [– no expression, (+) weak, + moderate, ++ high, +++ very high, ++++ extremely high expression]

	Histopathology	Grading[a]	COX-1	COX-2
Adenomas	Mild dysplasia		+	–
	Moderate dysplasia		++	–
			++	(+)
			++	(+)
			++	+
Carcinomas	II[a]	T1N0M0	+++++	+
		T3N0M0	++	+
		T3N0M0	+	+++
		T3N0M0	+	++++
		T3N1M0	+	+
		T4N2M0	+	
	II, III	T2N0M0	+	++
		T2N1M0	++	+
	III	T3N1M0	+	+++
		T3N2M0	++	+

Normal colonic mucosa was estimated to be ++++ for COX-1 and – to (+) for COX-2

[a] Classification according to the pTNM system [29]

rum (SC1745 or SC 1754, respectively, diluted 1:25 in block solution for 16 h at 4°C). The peroxidase-conjugated second antibody (donkey anti-goat IgG horseradish peroxidase) diluted 1:200 in block solution was added for 1 h at room temperature. Incubation with substrate (0.07% DAB, 0.16% hydrogen peroxide; Fast DAB tablets, Sigma, Munich, Germany) was for 15 min at room temperature. After counterstaining with hematoxylin, tissues were mounted in eukitt. To assess the specificity of the immunoreaction, control sections from each tissue were incubated with primary antibodies adsorbed with the respective peptide antigen (Santa Cruz; 500-fold molar excess). As additional controls, unspecific binding of the secondary antibody or DAB to skin structures was checked by omitting the primary and secondary antibodies, respectively. The sections were examined for staining using a Leitz orthoplan microscope. Photographs were taken on Fuji Color Super Gold films, 400 ASA, with 0.01 s exposure time.

Results

COX-1 and COX-2 protein expression in normal and diseased colon

Upon isozyme-selective immunoprecipitation and subsequent western blot analysis COX-1 specific signals were detected in protein extracts from normal colon specimens, tumor biopsy specimens, and tissue samples from patients with Crohn's disease. A representative result is shown in Fig. 1, upper panel. COX-2 specific signals were restricted to colon biopsies from patients with Crohn's disease and to tissue samples from neoplastic colon (see Fig. 1, lower panel). Among the tumor biopsies, pronounced interindividual variations in COX-1 and COX-2 expression were observed (Table 2). All 15 tumor biopsies were positive for COX-1. Compared to normal colon, COX-1 expression was significantly less in the adenomas and, with one exception, in the adenocarcinoma biopsy specimens. Regarding COX-2, two of the five adenomatous polyps were negative, and three showed weak to moderate immunosignals. A weak to extremely high COX-2 protein content was observed in all adenocarcinomas included in this study. However, no correlation of COX-2 expression with the histopathological classification of the adenocarcinomas was detected.

Immunolocalization of COX-1 and COX-2 in normal colon

Histologically normal areas of colon resected from patients with colon cancer or Crohn's disease were analyzed for expression of COX-1 and COX-2 using goat polyclonal antibodies raised against peptides from human COX-1 and COX-2 protein. Specific immunosignals for COX-1 were obtained in surface epithelial and in crypt epithelial cells (Fig. 2a–e). In addition to those located in the surface epithelium, colonocytes of the upper half of the crypts were found to be COX-1-positive (Fig. 2a). In the basal part of the crypts COX-1 expression was restricted to individual cells, which according to morphological criteria are endocrine cells, a specialized epithelial cell type of the lower crypt (Fig. 2a, d, e). Furthermore, mononuclear cells of the lamina propria mucosae and the regional lymphatic nodules as well as cells of the lamina muscularis mucosae showed COX-1 specific immunosignals (Fig. 2b, d). On the subcellular level COX-1 signals in colonocytes were found to be cytoplasmic and were concentrated in a region capping the nucleus towards the luminal side (Fig. 2c). In endocrine cells both cytoplasm and cell nucleus were COX-1 positive (Fig. 2e). COX-2-specific immunolabeling was oc-

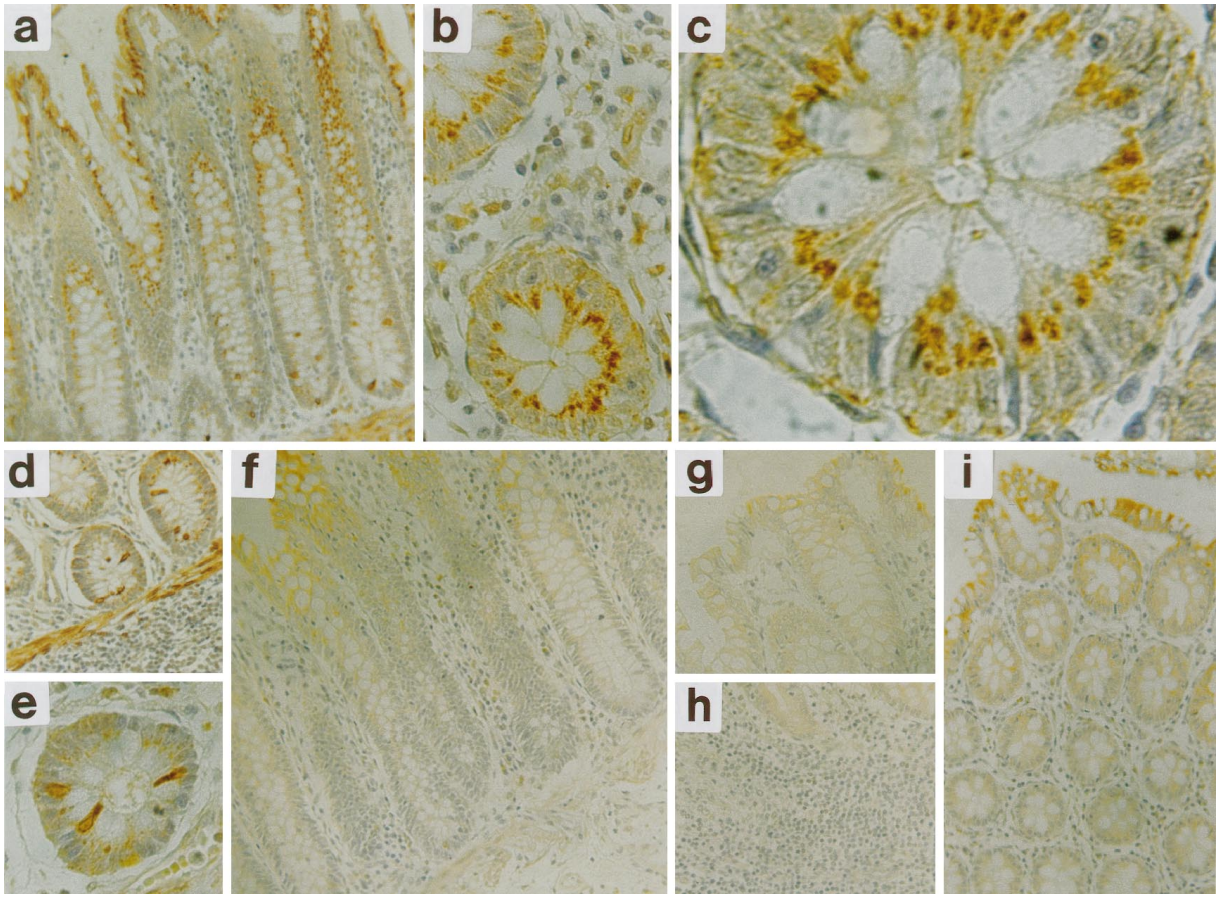

Fig. 2a–i Immunohistochemical localization of COX isoenzymes in sections of normal human colon. Paraffin sections were stained using the polyclonal goat anti COX-1 antiserum SC1754 (**a**–**e**) or the polyclonal goat anti-COX-2 peptide antiserum SC1745 (**f**–**i**). **a**,**d**,**f**–**i** ×125. **b**,**e** ×375. **c** ×725. One out three samples is shown

casionally observed in colonocytes of the normal surface epithelium of matched control colon as shown in Fig. 2f–i.

Immunolocalization of COX-1 and COX-2 in Crohn's disease and neoplastic colon

Compared with normal colon, significant changes in the cellular distribution of COX-1 and COX-2 were observed in Crohn's disease in that colonocytes of the surface and the crypt epithelium were only weakly decorated by the COX-1-specific antiserum, whereas prominent COX-1 expression was found in cells of the inflammatory infiltrate (data not shown). On the other hand, an intense COX-2 immunostaining was observed in cells of the surface epithelium (Fig. 3 a,b), while crypt epithelial cells were negative (Fig. 3d). COX-2 was also prominent in mononuclear cells of the inflammatory infiltrate (Fig. 3c).

Mononuclear cells of the lamina propria were identified as the prevailing COX-2 positive cell type of adenomatous polyps (Fig. 3e, f). Epithelial cells of the polyps were rarely COX-2-immunopositive and, if so, only weakly. In contrast, strong COX-1 specific immunoreactivity was observed in epithelial cells including colonocytes and endocrine cells of the polyps as well as cells of the lamina propria (data not shown).

In colon carcinomas COX-2 expression was predominantly found in the epithelial compartment. COX-2-specific staining occurred focally in about 50% of the crypts and was highly increased in the neoplastically altered cells (Fig. 3g). In addition, interstitial cells strongly expressed COX-2 protein (Fig. 3g–i). In epithelial cells COX-2 immunosignals were localized mainly to the cytoplasm and occasionally to the perinuclear region and nuclei (Fig. 3i). COX-1 expression in these samples showed a cellular and subcellular distribution similar to that of COX-2 (data not shown).

Discussion

The aim of this study was to immunohistochemically investigate the cellular distribution of COX-1 and COX-2 in colon biopsy specimens from patients suffering from Crohn's disease and cancer. Compared to normal colon, significant changes in the expression pattern of COX isozymes appeared with manifestation of the chronic inflammatory state in Crohn's disease and during colon

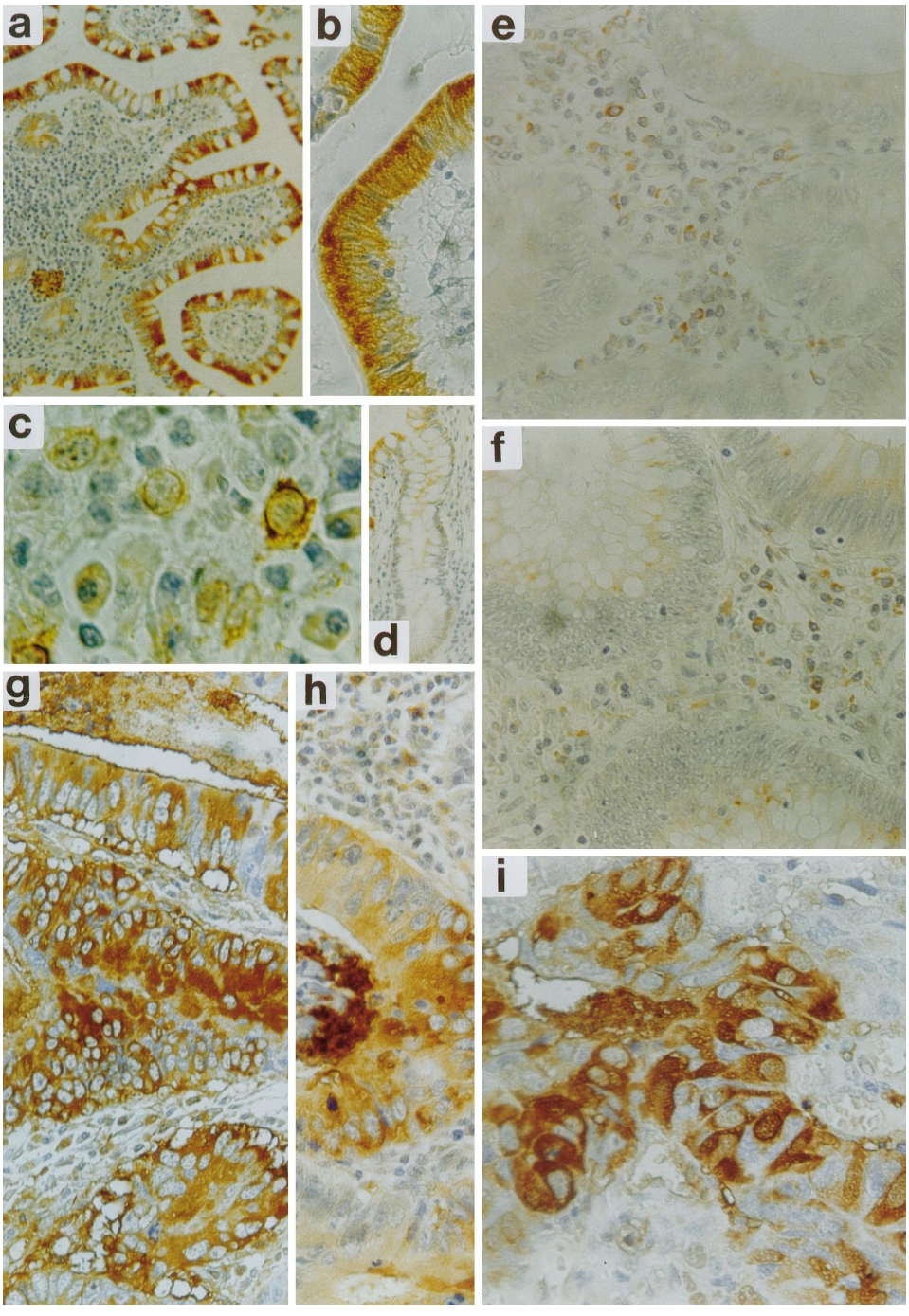

Fig. 3a–i Immunohistochemical localization of COX isoenzymes in inflamed and neoplastic colon. Paraffin sections were stained using the polyclonal goat anti-COX-2 antiserum SC1745. **a**–**d** Crohn's disease. **e**,**f** Adenomatous polyps. **g**–**i** Carcinoma. **a**,**d** ×125. **b**,**e**–**h** ×375. **c**,**i** ×725. In total, five Crohn's disease samples, four polyps, and three carcinomas were analyzed

carcinogenesis, the most significant effect being the constitutive overexpression of COX-2. The pathological alterations relate not only to the isozyme expression pattern but also to the COX-expressing cell types.

In normal colon, COX-1 protein was found to be constitutively expressed, whereas COX-2 was barely detected, confirming previously published data [12, 14, 15]. With regard to cellular distribution we found COX-1 positive colonocytes to be concentrated in the upper part of crypts, indicating COX-1 expression to be related to differentiation rather than proliferation. A similar differentiation-dependent COX-1 expression pattern has also been observed in human skin [19]. Our results are in contrast to those of Singer et al. [11], who reported COX-1 positive colonocytes to be restricted to the lower part of the crypts. We also observed COX-1 specific immunosignals in the lower part of the crypts. Morphological criteria suggest these signals to be attributable to endocrine cells [20], which have not yet been shown to express COX-1. Both colonocytes and endocrine cells exhibit various intracellular distributions of COX-1, which was predominantly cytoplasmic

in the former and both cytoplasmic and nuclear in the latter cell type.

In contrast to the strong expression of COX-1 in normal colonocytes, only weak COX-1-specific immunosignals were observed in colonocytes of biopsies from Crohn's disease, while Singer et al. [11] found COX-1 equally expressed in epithelial cells of normal and diseased colon. This difference most probably reflects interindividual variations. On the other hand, our data on the cellular localization of COX-2 expression in Crohn's disease patients are in good agreement with those published by Singer et al. [11] in that we observed a strong expression of this isozyme not only in epithelial cells of the upper part of the crypt and on the surface of the colon but also in mononuclear cells of the lamina propria. It remains to be analyzed whether the expression of COX isozymes is restricted to discrete subpopulations of mononuclear cells which in IBD have been recently characterized by cell surface markers [21–23].

In the course of polyp formation the extent of COX-1 expression and number of COX-1 positive cell types (differentiating colonocytes, endocrine, and mononuclear cells) did not differ from the controls. This observation confirms and extends previously published data [13–15, 24]. Biopsies from adenocarcinomas showed a similar cellular and subcellular distribution of COX-1, except for endocrine cells, which were not detected in the tumor tissue. This may be due to a disturbance of the colonic endocrine system, i.e., a decrease in the number of endocrine cells, as has recently been shown to occur in patients with adenocarcinomas [25]. COX-2 protein in adenomatous polyps was localized predominantly in mononuclear cells of the lamina propria, with only a few weakly decorated epithelial cells. A strong COX-2 specific staining of both epithelial and interstitial cells was a characteristic feature of adenocarcinomas. The intensity of COX-2 expression was subject to strong interindividual variations, which were not correlated with the histopathological grading of the tumors. Similar observations have been made by Hao et al. [24]. Aberrant COX-2-expression in colorectal tumors has been attributed either to cells of the interstitium or to epithelial cells [14, 15, 24, 26]. Here we have shown that both interstitial and epithelial cells are COX-2 positive in colorectal tumors, with interstitial cells predominating in adenomatous polyps and epithelial cells in adenocarcinomas.

The constitutive overexpression of COX-2 in epithelial cells of the crypt and the surface epithelial cells may represent a mechanistic link between IBDs, such as Crohn's disease, ulcerative colitis, and colon cancer. Pharmacological and genetic evidence points to a critical function of aberrant COX-2 expression in the development of colon cancer. Selective inhibitors of COX-2 as well as gene knock-out experiments clearly indicate that elimination of COX-2 expression and activity leads to suppression of colon cancer development [27, 28]. Thus the aberrant expression of COX-2 in IBDs may be associated with the increased cancer risk of these patients. Moreover, the inhibition of the enzymatic activity of COX-2 by isozyme-selective inhibitors may help to reduce the risk of developing colon cancer, particularly in genetically predisposed FAP patients and those suffering from IBDs.

Acknowledgements We deeply appreciate the cooperation of Prof. Dr. K.W. Schmid, Institute of Pathology, University of Münster, Germany and Prof. Dr. Kratzev, Pathology Department of the Institute "Pirogov," Sofia and Medical University, Pleven, Bulgaria.

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