ORIGINAL RESEARCH

Localization of cyclo-oxygenase-2 in human recurrent colorectal cancer

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Abstract

Aim: The aim of this paper is to examine COX-2 expression in human recurrent colorectal carcinoma tissues using immunohistochemistry and quantitative real-time PCR (qPCR).

Methods: Colon and rectal specimens were obtained from 26 patients with recurrent colorectal carcinomas. We examined COX-2 expression in human recurrent colorectal carcinoma tissues using immunohistochemistry and quantitative real-time PCR (qPCR).

Results: In recurrent colorectal cancer a strong cytoplasmic and perinuclear staining of COX-2 was found. Moderate to strong immunosignals were detected in almost all of the carcinomas. We observed a strong specific staining of COX-2 in vascular endothelium. COX-2 immunoreactivity was also detected in stromal cells such as mononuclear cells, fibroblasts, and smooth muscle cells. The real-time PCR analyses demonstrated marked overexpression of the COX-2 gene in the cancer mucosa in concert with the immunohistochemistry data.

Conclusion: We investigated COX-2 expression at the level of its protein as well as its messenger RNA in a series of recurrent colorectal cancers. These observations give additional information about the possibility that COX-2 could be involved in tumor promotion during colorectal cancer progression.

Colorectal cancer is still an important cause of death, although the incidence and mortality rates are decreasing.¹ An important explanation for the unfavorable prognosis of colorectal cancer patients is the fact that cancer recurs in about 40% of patients.² Most tumors recur in the first two years following surgical resection and in many cases is ultimate cause of death.

During the past several years many studies have been conducted to determine the precise role of eicosanoids in colorectal carcinogenesis. Cyclooxygenase (COX) is a key enzyme in the production of the prostaglandins (PG) and other eicosanoids. These products may have multiple roles in modulating cell growth and immunosurveillance in cancer.^{3,4} Two COX isoenzymes have been identified.⁵ COX-1 is constitutively expressed, while COX-2 is inducible in a number of cell types by a variety of factors such as cytokines, growth factors, oncogenes and tumor promoter.^{6,7} COX-2 is undetectable in normal intestine and its levels are elevated in up to 85% of colorectal adenocarcinomas.⁸ Experimental knockout of COX-2

results in suppression of intestinal polyposis in animal models of familial adenomatous polyposis.⁹

Epidemiological studies have shown lower than expected rates of colorectal adenomas and carcinomas in subjects who have taken non-steroidal anti-inflammatory drugs (NSAIDs).¹⁰⁻¹⁴ This cancer chemopreventive effect has been confirmed by animal models of colon and skin cancer for both conventional NSAIDs¹⁵⁻¹⁹ as well as for selective COX-2 inhibitors.²⁰⁻²² Although the precise mechanism for the protective effects of NSAIDs are still unknown, the ability of these drugs to induce cell cycle arrest and apoptosis has received much attention.

Here, we examined COX-2 expression in human recurrent colorectal carcinoma tissues using immunohistochemistry and quantitative real-time PCR (qPCR). We speculate that COX-2 overexpression in colorectal recurrences could provide a rational basis for studying chemoprevention of advanced disease with the use of selective COX-2 inhibitors after surgical curative resection.

Materials and Methods

Materials

Goat anti-human COX-2 (SC 1745) antisera, the corresponding blocking peptides, 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).

Tissue Specimens

Archival paraffin blocks of colon and rectal specimens were obtained from 26 patients with recurrent colorectal carcinomas. Additional tissue samples were obtained via colonoscopy in 12 patients. Endoscopy biopsies were snap-frozen in liquid nitrogen and stored at -70°C. Matched controls from normal colon mu-

cosa adjacent to the tumor were also obtained with informed consent in 12 patients. 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. There were 13 males and 13 females, mean age 59 years (range 49-72). The control group comprised tissues from 12 of these patients, 7 males and 5 females, mean age 52 years (range 49-69).

Homogenisation of Tissue Samples

Frozen biopsies were ground in a pre-cooled steel mortar using a pestle 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.

Immunohistochemistry

Paraffin sections (3µm) were stained as described previously [23]. Briefly, after blocking endogenous peroxidase by incubation with 3% H₂O₂ in methanol for 10 min, specimens were blocked (2.5% skim milk powder in PBS, Fluka, Neu-Ulm, Germany) for 1h and incubated with goat anti-human COX-2 antiserum (SC1745, diluted 1:25 in block solution for 16h at 4°C). The peroxidase-conjugated second antibody (donkey anti-goat IgG horseradish peroxidase) diluted 1:200 in block solution was added for 1h at room temperature. Incubation with substrate (0.07% DAB, 0.16% hydrogen peroxide; Fast DAB tablets, Sigma, Munich, Germany) was performed 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 colon tissue 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. The extent and intensity of staining were graded on a scale of 0 to 4, with 4 implying strong staining which was maximally intense throughout the specimen, and 0 implying negative staining. This scoring method has previously been described by Sano et al. [26]. The specimens were evaluated by two independent pathologists in different institutions.

Real-time PCR (qPCR)

For determination of COX-2 (NM_000963) mRNA in normal and cancer mucosa, RNA was isolated by RNeasy Mini Kit 50 (Qiagen) following the manufacturer's instructions. 1 µg RNA was reverse transcribed in cDNA by SuperScript™ First-Strand Synthesis System and Oligo (dT)₁₂₋₁₈ primer (Invitrogen). The resulting cDNA was purified via QIAquick PCR Purification Kit (Qiagen). Analyses were done using the CHROMO4 System CFB-3240 (Bio-Rad) with Fast-Start SYBR Green Master mix (Roche Diagnostics) and COX-2 gene specific primers (forward 5'CTTCACGCATCAGTTTTTCAAG and reverse 5'TCACCGTAAATATGATTAAAGTCCAC). For normalization a second qPCR was performed with the primers for HMBS gene (hydroxymethylbilane synthase; NM_001024382) (forward 5' CGCATCTGGA GTTCAGGAGTA and reverse 5'CCAGGAT GATGGCACTGA). The program was; 95°C for 10min hold, 40 cycles of: 95°C for 5s, 57°C for 10s (annealing), 72°C for 10s (extension).

Results

Immunohistochemistry

Paraffin sections from 26 patients with recurrent colorectal cancer were analyzed for expression of COX-2 protein. Additionally normal matched mucosa from 12 of these patients was used as a control. Normal epithe-

TABLE 1. COX-2 expression intensity in recurrent colorectal cancer

Differentiation	Grade 4	Grade 3	Grade 2	Grade 1
Well	1	2	1	0
Moderate	10	6	0	0
Low	5	1	0	0

lium showed only weak staining (grade 0-1). Stromal mononuclear cells in normal colon mucosa were scarcely stained (Fig. 1A). In the tumor areas with dysplastic changes COX-2 immunostaining was significantly increased compared with normal mucosa (Fig. 1B). Tumor cells showed intense expression in 16 patients (grade 4) and moderate expression in 9 patients (grade 3) as shown in Table 1. Positive cells from moderate differentiated recurrent cancer displayed strong cytoplasmic and perinuclear staining (Fig. 1C). There was no relationship between degree of differentiation and intensity of COX-2 expression in analyzed recurrent colorectal cancers (Fig. 1C and E). The distribution pattern in low differentiated samples revealed uniform, but weaker intensity than dysplastic and highly differentiated tumors. We observed strong specific staining of COX-2 in vascular endothelium (Fig. 1F). This was in contrast to weak endothelial expression of this isoenzyme reported by Tomosawa et al. [26]. COX-2 positive stromal mononuclear cells were scattered diffusely throughout the analyzed regional lymph nodes (Fig. 1G). There was strong COX-2 signal in stromal cells with morphology of tumor-associated macrophages (Fig. 1H). The distribution of specific COX-2 expression showed higher intensity in epithelium of the middle and apical parts of the glands compared with the basic of the crypts (Fig. 2B). The specificity of the immunohistochemistry reactions was confirmed by peptide competition and omitting either the primary or secondary antibodies.

Real-time PCR

The real-time PCR analyses showed a significant increase (8 fold) in the COX-2 mRNA expression in

FIGURE 1. Immunohistochemical localisation of COX-2 in different structures of specimens of recurrent colorectal cancer. Paraffin sections were stained using the polyclonal goat anti-COX-2 antibodies (SC1745). (A) Transverse section of normal human colon glands showing weak predominantly stromal immunostaining for COX-2. (B) Cytoplasmatic, perinuclear and some stromal staining of COX-2 in moderate dysplastic epithelial cells. (C, D) COX-2 immunostaining in moderately differentiated cancer cells compared to peptide competition control. (E, F, G, H) Demonstration of different localisations of COX-2 overexpression: low differentiated carcinomatous cells (E), vascular endothelial cells (F), inflammatory mononuclear cells in lymph node (G) and in tumor-associated macrophages (H).

FIGURE 2. Immunohistochemical localisation of COX-2 in different parts of the colon crypts. (A) Non-tumorous tissue demonstrating weak positive immunosignals for COX-2 in stroma, but not in colonocytes. (B) COX-2 expression is distributed unevenly through the length of the longitudinal section of the crypts (more intensive staining in middle and apical area). (C,D) Higher magnification of the basis of the crypt (arrow from B) with predominantly perinuclear COX-2 immunostaining.

cancer mucosa tissue (n=14) compared to normal colon mucosa (n=8) (Fig.3).

Discussion

Colorectal cancer (CRC) affects approximately 150,000 patients in the United States every year. Among all cancers, it is the second leading cause of death in the United States, with more than 52,000 deaths annually and affecting both men and women

almost equally.³⁷ The prognosis for patients with stage IV disease without specific therapy is poor, with a median survival of 5-6 months. However, a subset of patients with isolated sites of metastases can potentially be cured with surgery. Nevertheless, for the majority of patients with metastatic disease, the goal of therapy is palliation using systemic chemotherapy. For decades, standard first-line therapy consisted of fluorouracil (5-FU) plus leucovorin, with response rates of approximately 20% and a median survival of approximately 1 year. In the late 1990s and early 2000s, the addition of oxaliplatin and irinotecan to the backbone of 5-FU and leucovorin resulted in a dramatic improvement in median survival to nearly 24 months when patients received active first- and second-line therapy. Most recently, biologic agents, such as bevacizumab, cetuximab, and panitumumab, have further enhanced the efficacy of systemic medical therapy.³⁸

The current trend in cancer research is rational drug design, which begins with a molecular target initially identified by the basic scientist. The idea that targeted inhibition of the cyclooxygenase-2 pathway might be effective in the treatment of colorectal cancer was essentially generated by rational drug design in reverse. The first hints that non-steroidal anti-inflammatory drugs - compounds that inhibit the enzymatic activity of cyclooxygenase - had any efficacy against colorectal cancer came from both astute clinical observations and large population-based studies in cohorts of patients who were ingesting NSAIDs over a long period for other purposes. These studies provided the impetus for laboratory-based discoveries that led to the identification of a molecular target, cyclooxygenase-2, that has a tumor-promoting function in colorectal carcinogenesis.

The role of COX-2 has been defined by studies on colon carcinogenesis. Epidemiological studies have shown a decreased risk of colon cancer in individuals who take NSAIDs on a regular basis.²⁷ NSAIDs have been shown to decrease the size and number of adenomas in patients with a history of familial adenomatous polyposis.^{25,28,31}

FIGURE 3. The real-time PCR analyses showed a significant increase (8 fold) in the COX-2 mRNA expression in cancer mucosa tissue (n=14) compared to normal colon mucosa (n=8)

Eberhart et al. were the first to document significant elevations in COX-2 expression in 85% of human colorectal carcinomas and approximately 50% of colorectal adenomas.²⁹ In these samples, the levels of COX-1 were unchanged between normal mucosa and carcinoma. Two independent groups have confirmed these results.^{25,30} COX-2 is also overexpressed in adenomas from ApcMin mice⁴⁵ and carcinoma samples from the colon of AOM-treated rats.³⁹ There is no consensus at present on what cell types within a tumor express COX-2. Some groups have reported that COX-2 is primarily expressed in the epithelial cells of adenomas from ApcMin mice⁴⁵, carcinomas from AOM-treated rats⁴⁷ and sporadic human colorectal cancers.⁴⁰⁻⁴¹

In this study we have demonstrated marked expression of COX-2 in recurrent colorectal cancer. We focused our attention on advanced disease with the presumption that chemoprevention with use of COX-2 inhibitors could be applied after previous surgical treatment of the primary colorectal cancer. In recurrent colorectal cancer we found strong cytoplasmic and perinuclear staining of COX-2. Moderate to strong immunosignals were detected in almost all of

the carcinomas. Expression of COX-2 was independent of the degree of tumor differentiation. These results are in agreement with previously described lack of such correlation in oesophageal carcinoma³¹, colorectal adenocarcinomas^{32,33} and cholangiocarcinoma³⁴. We observed a strong specific staining of COX-2 in vascular endothelium. This was in contrast to weak endothelial expression of this isoenzyme reported from Tomozawa et al.²⁶ in recurrence of colorectal cancer and hematogenous metastasis. We have also detected COX-2 immunoreactivity in stromal cells such as mononuclear cells, fibroblasts, and smooth muscle cells, which is in agreement with other findings.²⁶ As noted earlier, PG is known to suppress the function of some immune cells, such as T lymphocytes.³⁵ Therefore, COX-2 may play an indirect role in the regulation of immunosurveillance against the growth of metastases or recurrences.³⁶ The real-time PCR analyses demonstrated marked overexpression of the COX-2 gene in the cancer mucosa in concert with the immunohistochemistry data. Thus, qPCR technique could theoretically be used as a fast and reliable method to predict the degree of the cancer development.

It is also not known whether COX-2 is involved in all colorectal tumors or whether COX-2 inhibitor therapy can be tailored to a specific subset of patients most likely to benefit from treatment. Most sporadic colorectal cancers arise in the setting of chromosomal instability associated with loss of heterozygosity.^{42,44} However, about 15–20% of colorectal tumors are associated primarily with microsatellite instability. Several reports indicate that colorectal tumors that arise in the setting of microsatellite instability have reduced or absent COX-2 expression, indicating that COX-2 inhibitors may be of limited use in these patients.⁴³

We have investigated COX-2 expression on the level of its protein as well as on its messenger RNA in a series of recurrent colorectal cancers. This observation give additional information about the possibility that COX-2 could be involved in tumor promotion during colorectal cancer progression.

Combining the evidence from humans, animal models and cell culture systems has helped to establish that targeted inhibition of COX-2 is a viable approach to the study of colorectal cancer prevention and/or treatment. But, despite these successes, many questions remain unanswered. Future studies examining susceptibility to intestinal polyps in mice with targeted deletions in specific PG synthases and PG receptors should help clarify the mechanisms by which COX-2 promotes tumorigenesis. These experiments might also lead to the discovery of novel and more potent inhibitors of colorectal cancer cell growth.

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