

Differential expression of cyclooxygenase 2 in human colorectal cancer

J Dimberg, A Samuelsson, A Hugander, P Söderkvist

Abstract

Background—Experimental, clinical, and epidemiological studies have implicated mitogenic metabolites of arachidonic acid such as prostaglandin E₂ (PGE₂) in colorectal carcinogenesis. Recently, cyclooxygenase 2 (COX-2) which catalyses the conversion of arachidonic acid to PGE₂, has displayed increased levels in human colorectal cancer.

Aims—To evaluate whether there is differential COX-2 expression from different locations (caecum, ascending, transverse, descending, or sigmoid colon, and rectum) in human colorectal cancer.

Methods—Protein levels of COX-2 were determined by western blot analysis in tumours and adjacent normal mucosa of 39 patients with colorectal cancer.

Results—There was a notable overexpression of COX-2 protein in tumours located in the rectum ($p < 0.001$) compared with other locations in the colon. Rectal tumours revealed elevated COX-2 protein levels in 18/20 cases compared with 4/19 colonic cases. No association between enhanced COX-2 protein expression in tumour tissue and Dukes's stages was found.

Conclusions—Results suggest that the differential COX-2 expression may be due to differences in gene regulatory factors affecting COX-2 expression and/or reflect secondary changes in tumour progression which may have clinical implications.

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in preventing colorectal cancer. NSAIDs inhibit COX-2 activity and PGE₂ synthesis both in vitro and in vivo.^{4,5} Recently, increased COX-2 gene expression has been shown in human colorectal adenocarcinomas and in carcinogen induced rat colonic tumours.⁶⁻¹⁰ These observations suggest that abnormal production is an early event in the postinitiation phase of colon carcinogenesis. Moreover, previous studies have reported elevated levels of PGE₂ in both human and rat colonic tumours.¹¹⁻¹³ These findings suggest that COX-2 is responsible for the increased PGE₂ production although no studies have indicated whether upregulation of COX-2 is a primary event or a consequence in the pathogenesis of colorectal cancer.

Methods

PATIENTS AND SAMPLING

Tissue samples were obtained from 39 patients undergoing surgical resections for primary colorectal adenocarcinomas diagnosed at the Department of Surgery, Ryhov County Hospital, Jönköping, Sweden. Sporadic tumours from 20 men and 19 women (mean age 73 years, range 25-93) were collected and classified into four stages according to the Dukes classification system; stage A (n=4), stage B (n=18), stage C (n=12), and stage D (n=5). The tumours were localised in the caecum (n=7), ascending colon (n=5), transverse colon (n=1), descending colon (n=1), sigmoid colon (n=5), and rectum (n=20).

From each patient, tumour tissue and adjacent normal mucosa (about 10 cm from the tumour) was excised and collected. All tissues were immediately frozen and kept at -70°C until analysis.

PROTEIN PREPARATION

Frozen tumour tissue and normal mucosa were thawed and homogenised in ice cold lysis buffer (160 mM NaCl, 10 mM HEPES, 2 mM CaCl₂, 0.5% sodium dodecyl sulphate (SDS), 0.5% Triton X-100, 100 µg/ml phenylmethylsulphonyl fluoride, 1 µg/ml leupeptin, and 2 µg/ml aprotinin) and placed on ice for 15 minutes. Following clarification the lysis solution was centrifuged at 13 000 g for 10 minutes. Protein content of homogenates was determined for each sample using the Lowry assay.¹⁴

Abbreviations used in this paper: APC, adenomatous polyposis coli; COX, cyclooxygenase; cPLA₂, cytosolic phospholipase A₂; NSAID, non-steroidal anti-inflammatory drug; PGE₂, prostaglandin E₂; PLA₂-II, group II phospholipase A₂; PPAR_γ, peroxisome proliferator activated receptor; TCF/LEF, T cell factor/leucocyte enhancing factor.

Department of Biomedicine and Surgery, Division of Cell Biology, Faculty of Health Sciences, Linköping, Sweden
J Dimberg
P Söderkvist

Department of Natural Science and Biomedicine, University College of Health Sciences, Jönköping, Sweden
A Samuelsson

Department of Surgery, Ryhov County Hospital, Jönköping, Sweden
A Hugander

Correspondence to:
Dr J Dimberg, Department of Natural Science and Biomedicine, University College of Health Sciences, Box 1038, S-551 11 Jönköping, Sweden.

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The cyclooxygenase (COX) isozymes COX-1 and COX-2 catalyse the conversion of arachidonic acid to eicosanoids, namely prostaglandins and thromboxanes via endoperoxides.¹ Both isoforms are expressed in different cell types and tissues. COX-1 is a constitutively expressed isoform and is thought to carry out basal production of prostaglandins under homeostatic conditions. COX-2 is an inducible isoform and can be induced in response to mitogenic agents, growth factors, lipopolysaccharides, and cytokines. Both isoforms are also responsible for the synthesis of prostaglandin E₂ (PGE₂), and there is some evidence for a correlation between increased levels of PGE₂ and tumorigenesis.¹⁻³

Epidemiological and rodent studies have documented a protective effect of non-steroidal anti-inflammatory drugs (NSAIDs)

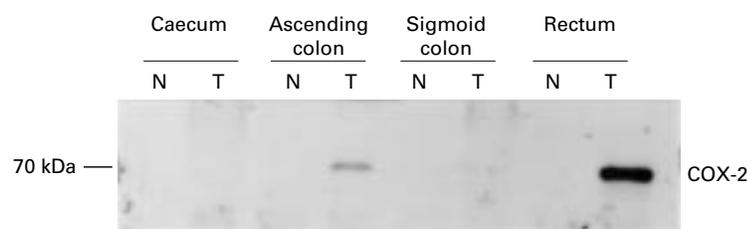


Figure 1 Western blot analysis of cyclooxygenase (COX) 2 protein expression in four representative specimens of human colorectal cancer. T, tumour tissue; N, matched normal mucosa from different locations. Molecular weight marker is indicated on the left.

SDS-PAGE AND WESTERN BLOTTING

Proteins (60 µg) in the lysates were separated by electrophoresis using 8% SDS-PAGE (polyacrylamide gel electrophoresis).¹⁴ Separated proteins were transferred onto nitrocellulose membranes (Amersham) in a transblot electrophoretic transfer cell (Bio-Rad Laboratories).

IMMUNODETECTION

After transblotting the blots were blocked for two hours with 5% (wt/vol) non-fat dry milk dissolved in TTBS (1× TTBS: 20 mM Tris, 150 mM NaCl, pH 7.5, 0.1% Tween 20). Blots were probed for two hours with an antihuman COX-2 antibody (Santa Cruz Biotechnology) diluted 1/250 in TTBS containing 3% (wt/vol) non-fat dry milk. After extensive washing in TTBS the blots were reincubated for one hour with horseradish peroxidase conjugated secondary antibody (Santa Cruz Biotechnology) diluted 1/4000 in TTBS containing 3% (wt/vol) non-fat dry milk. Blots were developed using an enhanced chemiluminescence (ECL) system (Amersham) and exposed to Hyperfilm ECL (Amersham). The optical density (OD) was measured by densitometry and the result is shown as relative expression (OD ratio) for tumour versus normal mucosa. All experiments were repeated in separate assays.

STATISTICAL ANALYSIS

Differences of COX-2 protein expression between rectum and colon in cancerous mucosa were examined by the χ^2 (Pearson) analysis. Significance was set at $p < 0.001$. The data were subjected to computerised statistical analysis using SPSS (Statistical Products and Services Solutions) for Windows, version 6.1.

Table 1 Distribution pattern of cyclooxygenase 2 protein expression in tissue from 39 patients with colorectal adenocarcinoma

Location	n	Relative expression* (tumour v normal mucosa)	
		≤2	>2 (7–48)
Rectum	20	2	18
Colon	19	15	4
Sigmoid	5	4	1
Descending	1	1	
Transverse	1	1	
Ascending	5	3	2
Caecum	7	6	1

*Relative expression >2 was considered to represent enhanced levels.

The differences in cyclooxygenase 2 protein expression between rectum and colon were significant.

Results

Western blot analyses of COX-2 protein in human colorectal tumour tissue were pairwise related to normal mucosa from 39 examined surgical specimens. Probing of tissue proteins immobilised on nitrocellulose membranes with antihuman COX-2 antibody and subsequent quantification by densitometry showed substantially higher levels of COX-2 in cancerous mucosa from the rectum (fig 1, table 1). In cancerous tissue of other parts, low or undetectable levels of COX-2 protein expression were usually found. The distribution pattern revealed a statistically significant prevalence ($p < 0.001$) of upregulated COX-2 in cancerous tissue originating from rectum in relation to tumours of colonic origin. Table 1 summarises results of the western analyses. By means of densitometric evaluation a 7–48 fold induction was found in 18/20 rectal tumour tissues in relation to COX-2 protein expression in normal tissue. Only 2/20 rectal tumours displayed no induction of COX-2, in contrast to 15/19 for tumours with colonic localisation.

No association between enhanced COX-2 protein expression in tumour tissue and Dukes' stages was found (data not shown).

Discussion

Evidence that arachidonic acid and its metabolites may be mediators of tumorigenesis in colorectal carcinoma comes from experimental, clinical, and epidemiological studies in which inhibition of prostaglandin synthesis suppresses carcinogenesis.^{4,5} Group II phospholipase A2 (PLA2-II) and cytosolic phospholipase A2 (cPLA2) are enzymes which catalyse arachidonic acid release from the sn-2 position of glycerophospholipids and have been postulated to be the rate limiting step for PGE₂ production.¹ However, minor changes in PLA2-II and cPLA2 gene expression between tumour and non-cancerous tissue indicate that generation of arachidonic acid seems not to be the rate limiting step in PGE₂ formation via COX-2.¹⁵ Mutations of the human tumour suppressor gene *adenomatous polyposis coli* (APC) are prominent alterations in both sporadic and familial gastrointestinal tumours.¹⁶ It is interesting to note that it seems to be a link between the tumorigenic effect of APC mutations and arachidonic acid metabolism. Disruption of the COX-2 gene reduces the number of tumours in the Min mouse model of human familial adenomatous polyposis coli.¹⁷

Recent studies have indicated that enhanced expression of COX-2 and increased production of prostaglandins in intestinal epithelial cells protect the cells from apoptosis and stimulate production of angiogenic factors.^{18,19} In addition, the peroxisome proliferator activated receptor PPAR γ is a transcription factor that has regulatory functions in differentiation of cells and has recently been shown to be overexpressed in human colon cancer. Diverse fatty acids and prostaglandins can bind to PPAR γ and stimulate transcriptional activation of target genes.²⁰

One essential factor linked to colon cancer is β catenin which forms a complex with the APC protein. Mutations in APC and/or β catenin gene(s) result in dissociation of the APC- β catenin complex and excess free β catenin binds to TCF/LEF-1 transcription factor which controls certain target genes—for example *c-myc*, probably contributing to colon tumorigenesis.^{21,22} One might speculate that β catenin mediates activation of TCF/LEF-1 which, in turn, regulates COX-2 expression. This mechanism could link APC and/or β catenin mutation with increased COX-2 transcription. However, a recent study has shown that activation of PPAR γ in a human colon carcinoma cell line, HT-29, increased β catenin protein levels but produced no effect on COX-2 expression.²³

Several reports have shown that immunohistological staining of human colorectal tissue revealed COX-2 protein preferentially expressed in the cancer cells rather than infiltrating mononuclear cells or fibroblasts of the cancer stroma.^{7,9} In the present study we found a notable increase in COX-2 protein expression in tumour tissue compared with paired normal mucosa which is accompanied by increased mRNA levels determined by semiquantitative reverse transcriptase polymerase chain reaction.¹⁵ The enhanced COX-2 protein levels were preferentially localised to rectal tumours (18/20) while only 4/19 colonic tumours showed elevated COX-2. The explanation and interpretation of this finding is still uncertain but may be due to differences in genetic alterations in the tumour cells and/or differences in expression of COX-2 regulatory genes in the colon and rectum. Alternatively, our results may reflect secondary changes in tumour progression which differentially modulate the induction of COX-2 in different sites of the colon and rectum. A different pathway around COX-2 dependent oxidation of arachidonic acid to prostaglandins may be via another oxidase, for example, COX-1. Genetic analysis using COX-1 knockout mice can give further support to the proposal that COX-1 is a modifier for colorectal cancer.

Further investigations are necessary to determine the putative role of the COX-2 gene induction for tumour progression, apoptosis, and angiogenesis in human colorectal cancer. In conclusion, the present investigation showed that COX-2 protein is dramatically upregulated in a subset of human colorectal cancer

and that the distribution pattern of overexpressed COX-2 protein in tumours showed a pronounced prevalence for the rectum.

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