

Aberrant cyclooxygenase isozyme expression in human intrahepatic cholangiocarcinoma

S Chariyalertsak, V Sirikulchayanonta, D Mayer, A Kopp-Schneider, G Fürstenberger, F Marks, K Müller-Decker

Abstract

Methods—Cellular localisation of the cyclooxygenase (COX) isozymes COX-1 and COX-2 was analysed in 24 cholangiocarcinomas, including 17 matched tissues originating from non-tumorous liver tissue adjacent to tumours and seven biopsies of normal human liver, by immunohistochemistry using isozyme selective antibodies.

Results—In normal liver, constitutive expression of COX-2 protein was a characteristic feature of hepatocytes whereas no COX-2 immunosignal was detectable in normal bile duct epithelium, Kupffer, and endothelial cells. In cholangiocarcinoma cells, COX-2 protein was strongly expressed at high frequency. The intensity, percentage of positive cells, and pattern of COX-2 expression were found to be independent of the stage of tumour differentiation. In hepatocytes of matched non-tumorous tissue, COX-2 expression was unaltered. In contrast, strong COX-1 expression was frequently localised to Kupffer cells, endothelial cells, and occasionally to hepatocytes, but not to bile duct epithelial cells. In approximately half of moderately and poorly differentiated but not well differentiated cholangiocarcinomas, weak to moderate COX-1 staining was found in tumour cells while COX-1 expression in Kupffer cells was much more pronounced.

Conclusion—Aberrant COX-2 expression occurs during the early stage while COX-1 over expression seems to be related to later stages of cholangiocarcinogenesis.

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Keywords: cyclooxygenase; immunohistochemistry; cholangiocarcinoma; bile duct; hepatocyte; Kupffer cell

Cyclooxygenases (COX) catalyse the biosynthesis of prostaglandin endohydroperoxides preferring arachidonic acid as substrate. Most cell types investigated to date express COX-1 constitutively whereas COX-2 is induced by a variety of stimuli.¹ Both COX isoforms are inhibited by non-steroidal anti-inflammatory drugs (NSAIDs). Evidence that COX might play a role in human carcinogenesis came from numerous epidemiological studies² which described a reduced risk of colorectal cancer in those with regular intake of aspirin or other NSAIDs. Clinical treatment with NSAIDs in patients suffering from familial adenomatous polyposis coli (APC) due to a germline

mutation in the APC tumour suppressor gene led to regression of existing colorectal adenomas.^{3,4} This chemopreventive effect has been confirmed in animal models of colon and skin cancer for both conventional NSAIDs, such as aspirin and sulindac,⁵⁻⁹ as well as for selective COX-2 inhibitors.¹⁰⁻¹²

A causal relationship between aberrant COX-2 expression and tumour development was shown by both genetic and pharmacological approaches: APC⁷¹⁶ mice carrying a truncation deletion in the tumour suppressor gene APC developed intestinal neoplasias, the number and size of which were found to be markedly reduced on gene knockout of COX-2 or treatment with a highly selective COX-2 inhibitor.¹²

In humans, aberrant COX-2 expression is not restricted to colonic adenocarcinoma¹³⁻¹⁵ but has also been found in squamous cell carcinoma of the skin,¹⁶ head and neck,¹⁷ oesophagus,¹⁸ and lung,¹⁹ in adenocarcinoma of the lung,¹⁹ stomach,²⁰ pancreas,²¹ and breast,²² as well as in hepatocellular carcinoma.^{23,24} Except for the liver, corresponding normal tissues expressed low to undetectable amounts of COX-2. In most studies expression of COX-1 protein was found not to be deregulated in the course of tumour development.

Here, we describe for the first time expression of both COX isozymes in human cholangiocarcinomas. This tumour is one of the most common cancers in South East Asian countries.

Materials and methods

MATERIALS

The goat antihuman COX-1 (SC1754) and COX-2- (SC1745) antisera, the corresponding blocking peptides, as well as the horseradish peroxidase conjugated donkey anti-goat IgG were obtained from Santa Cruz, Heidelberg, Germany. Goat IgG was from Dianova, Hamburg, Germany.

TISSUE SPECIMENS

Archival paraffin blocks of liver specimens from 24 patients with cholangiocarcinoma and seven apparently normal cases were obtained from the Surgical Pathology Section, Department of Pathology, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand. All cholangiocarcinoma cases were clinically and

Abbreviations used in this paper: COX, cyclooxygenase; NSAID, non-steroidal anti-inflammatory drug; TPA, 12-O-tetra decanoylphorbol-13 acetate, APC, adenomatous polyposis coli.

Research Division,
National Cancer
Institute, Bangkok,
Thailand
S Chariyalertsak

Surgical Pathology
Section, Department
of Pathology,
Ramathibodi Hospital,
Mahidol University,
Bangkok, Thailand
V Sirikulchayanonta

Division of Cell
Pathology, Deutsches
Krebsforschungszentrum,
Heidelberg, Germany
D Mayer

Central Unit of
Biostatistics, Deutsches
Krebsforschungszentrum,
Heidelberg, Germany
A Kopp-Schneider

Division of
Biochemistry of Tissue
Specific Regulation,
Deutsches
Krebsforschungszentrum,
Heidelberg, Germany
G Fürstenberger
F Marks
K Müller-Decker

Correspondence to:
Dr K Müller-Decker,
Deutsches
Krebsforschungszentrum,
Division of Biochemistry of
Tissue-Specific Regulation,
Im Neuenheimer Feld 280,
69120 Heidelberg, Germany.
K.Mueller-Decker@
DKFZ-Heidelberg.de

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pathologically proved. There were eight females and 16 males, mean age 54 years (range 35–71). The control group comprised two females and five males, mean age 44 years (range 22–73).

HISTOLOGICAL EXAMINATION

Sections (3 μm) were stained with haematoxylin-eosin. The tumours were graded according to their histological features into one of the following three groups: (1) well differentiated cholangiocarcinoma: tumour cells are arranged to form well developed gland-like structures mainly lined by a single layer of epithelial cells with regular size and space and uniform nuclei with indistinct nucleoli; (2) moderately differentiated cholangiocarcinoma: tumour cells are organised to form less well formed glandular structures which are often lined by stratified layers of epithelial cells with a higher degree of nuclear atypia; (3) poorly differentiated cholangiocarcinoma: tumour cells rarely form glandular structures and their nuclei are arranged with an irregular spacing pattern and with markedly pleomorphic shapes

and sizes. Tumour cells possess prominent nucleoli.

IMMUNOHISTOCHEMISTRY

Sections (3 μm) were stained for COX-1 and COX-2 using a modification of a protocol described previously.²⁵ Before blocking endogenous peroxidase, sections were placed in 0.01 M sodium citrate solution, pH 6.0, and heated in a microwave (Bosch, Germany) at the highest power setting (600 W) for two, five minute cycles. Specimens were buffered in phosphate buffered saline for five minutes after being cooled for 30 minutes at room temperature. To assess the specificity of the immunoreaction, control sections from each tissue were incubated either with primary antibodies adsorbed with the respective peptide antigen (500-fold molar excess) or with goat IgG instead of goat anti-COX antibodies. As additional controls, unspecific binding of the secondary antibody or diaminobenzidine to liver tissue was checked by omitting the primary and/or secondary antibodies, respectively.

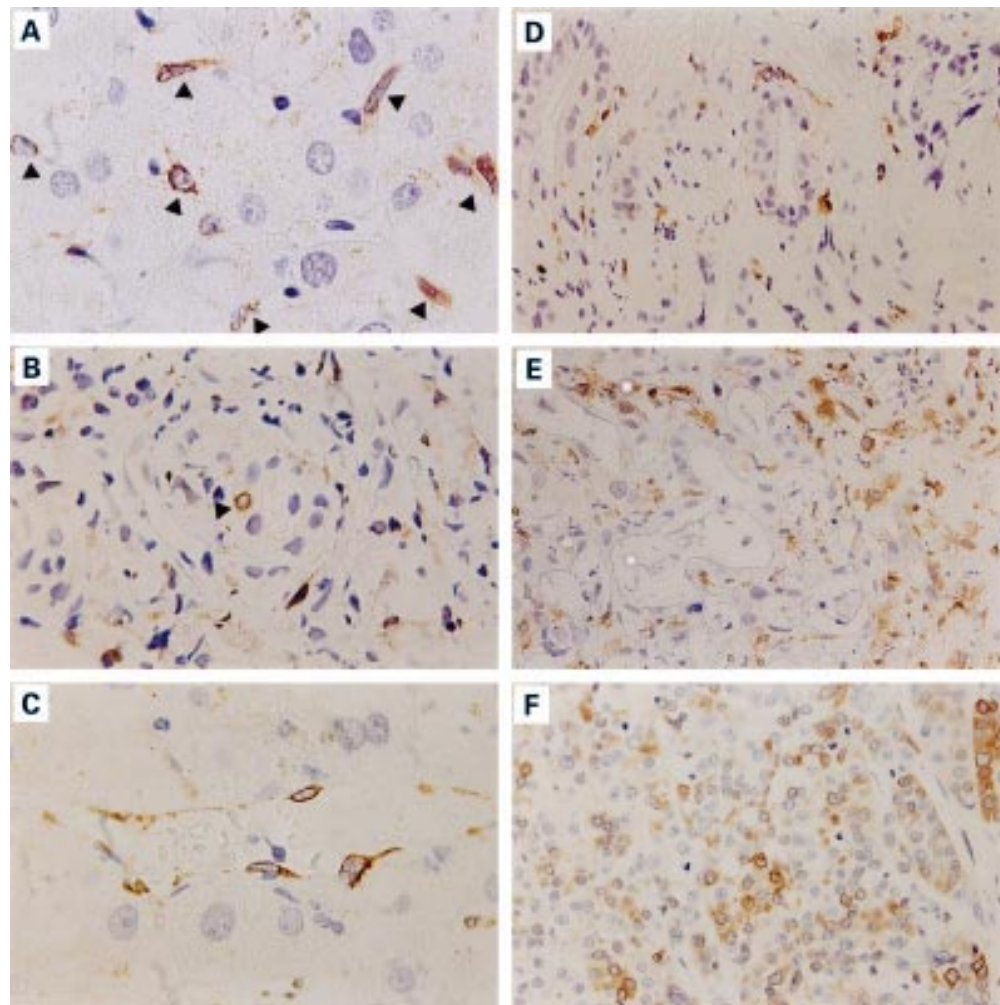


Figure 1 Immunohistochemical localisation of COX-1 in non-tumorous liver tissue and in intrahepatic cholangiocarcinomas at different stages of differentiation. Paraffin sections were stained using polyclonal goat anti-COX-1 antibodies (SC1754). The brown staining represents COX-1 protein. (A–C) Non-tumorous liver tissue. (A) Arrows indicate COX-1 positive Kupffer cells. (B) Arrow indicates a single epithelial cell of the bile duct expressing COX-1. (C) Blood vessel; endothelial cells express COX-1. (D) Well differentiated cholangiocarcinoma. (E) Moderately and (F) poorly differentiated cholangiocarcinomas showing COX-1 immunosignals in epithelial cells of the gland-like structures. Original magnifications: 1:375 (B, D, E, F); 1:725 (A, C).

Table 1 Expression of COX isozymes in normal liver

Case	COX-1						COX-2					
	Hepatocytes			Bile duct cells			Hepatocytes			Bile duct cells		
	Ratio of positive cells*	I	P	Ratio of positive cells	I	P	Ratio of positive cells	I	P	Ratio of positive cells	I	P
1	0	-	-	0	-	-	3	3	D	1	1	F
2	0	-	-	0	-	-	3	2	D	0	-	-
3	0	-	-	0	-	-	3	3	D	0	-	-
4	0	-	-	0	-	-	3	2	D	0	-	-
5	3	1	D	0	-	-	3	2	D	0	-	-
6	2	2	D	0	-	-	3	2	D	0	-	-
7	0	-	-	0	-	-	3	2	D	1	1	F

I, intensity; P, pattern; D, diffuse; F, focal.

*The ratio of positive cells per specimen was evaluated quantitatively and scored 0 for staining of $\leq 1\%$, 1 for staining of 2–10%, 2 for staining of 11–70%, and 3 for staining of $>70\%$ of cells examined.

The intensity of staining was graded as: 1, weakly; 2, moderately; and 3, strongly stained cells.

The pattern of staining was described as sporadic when stained cells were found occasionally; focal when stained cells were seen as a cluster(s) or group(s); and diffuse when stained cells were seen throughout.

-, no immunosignal.

IMMUNOHISTOCHEMICAL EVALUATION

Expression of COX-1 and COX-2 was evaluated according to the ratio of positive cells per specimen, intensity, and pattern of staining. The ratio of positive cells per specimen was evaluated quantitatively and scored 0 for staining of $\leq 1\%$, 1 for staining of 2–10%, 2 for staining of 11–70%, and 3 for staining of $>70\%$ of the cells examined. Intensity was graded as follows: no signal; 1, weakly positive; 2, moderately positive; and 3, strongly positive staining. The pattern of staining was described as sporadic when stained cells were found occasionally, as focal when stained cells were seen as a cluster(s) or group(s), and as diffuse when stained cells were seen throughout. Similar evaluations were applied for non-tumorous

bile duct cells and hepatocytes in the same sections of all specimens.

STATISTICAL ANALYSIS

The intensity of COX-1 and COX-2 expression in intrahepatic cholangiocarcinoma and matched non-tumorous tissue was compared by evaluating the sign of the difference in intensity of both tissues and using the sign test for statistical analysis. For comparison of normal tissue with differentially graded tumours or between groups of differentially graded tumours, COX-1 and COX-2 expression was classified as present or absent in the tissue (summarising intensity scores 1–3 into one category). Fisher's exact test was used for analysis of 2x2 tables. The Cochran-Armitage

Table 2 Expression of COX-1 in intrahepatic cholangiocarcinoma and matched non-tumorous tissue

Case No	Differentiation*	Matched non-tumorous tissue									
		Cholangiocarcinoma cells			Bile duct cells			Hepatocytes			
		Ratio of positive cells†	I	P	Ratio of positive cells	I	P	Ratio of positive cells	I	P	
8	Well	0	-	-	na	na	na	0	-	-	
9	Well	0	-	-	0	-	-	3	1	D	
10	Well	0	-	-	na	na	na	na	na	na	
11	Well	1	2	F	0	-	-	0	-	-	
12	Well	0	-	-	na	na	na	na	na	na	
13	Well	0	-	-	0	-	-	na	na	na	
14	Well	0	-	-	0	-	-	0	-	-	
15	Moderate	0	-	-	0	-	-	3	1	D	
16	Moderate	2	2	D	na	na	na	3	1	D	
17	Moderate	1	2	F	na	na	na	na	na	na	
18	Moderate	1	2	S	0	-	-	3	1	D	
19	Moderate	0	-	-	0	-	-	3	1	D	
20	Moderate	0	-	-	0	-	-	2	1	D	
21	Moderate	1	2	S	0	-	-	0	-	-	
22	Moderate	1	2	F	0	-	-	3	1	D	
23	Moderate	1	2	F	na	na	na	na	na	na	
24	Moderate	0	-	-	0	-	-	3	1	D	
25	Poor	0	-	-	0	-	-	0	-	-	
26	Poor	3	1	D	0	-	-	3	1	D	
27	Poor	0	-	-	0	-	-	3	1	D	
28	Poor	0	-	-	0	-	-	na	na	na	
29	Poor	2	2	D	na	na	na	1	2	F	
30	Poor	1	1	S	0	-	-	0	-	-	
31	Poor	2	2	D	2	2	F	na	na	na	

I, intensity; P, pattern; D, diffuse; F, focal; S, sporadic; na, not available.

*Well differentiated cholangiocarcinoma: tumour cells are arranged to form well developed gland-like structures mainly lined by a single layer of epithelial cells with regular size and space and uniform nuclei with indistinct nucleoli; moderately differentiated cholangiocarcinoma: tumour cells are organised to form less well formed glandular structures which are often lined by stratified layers of epithelial cells with a higher degree of nuclear atypia; poorly differentiated cholangiocarcinoma: tumour cells rarely form glandular structures and their nuclei are arranged with an irregular spacing pattern and with markedly pleomorphic shapes and sizes. Tumour cells possess prominent nucleoli.

†For definition see table 1.

-, no immunosignal.

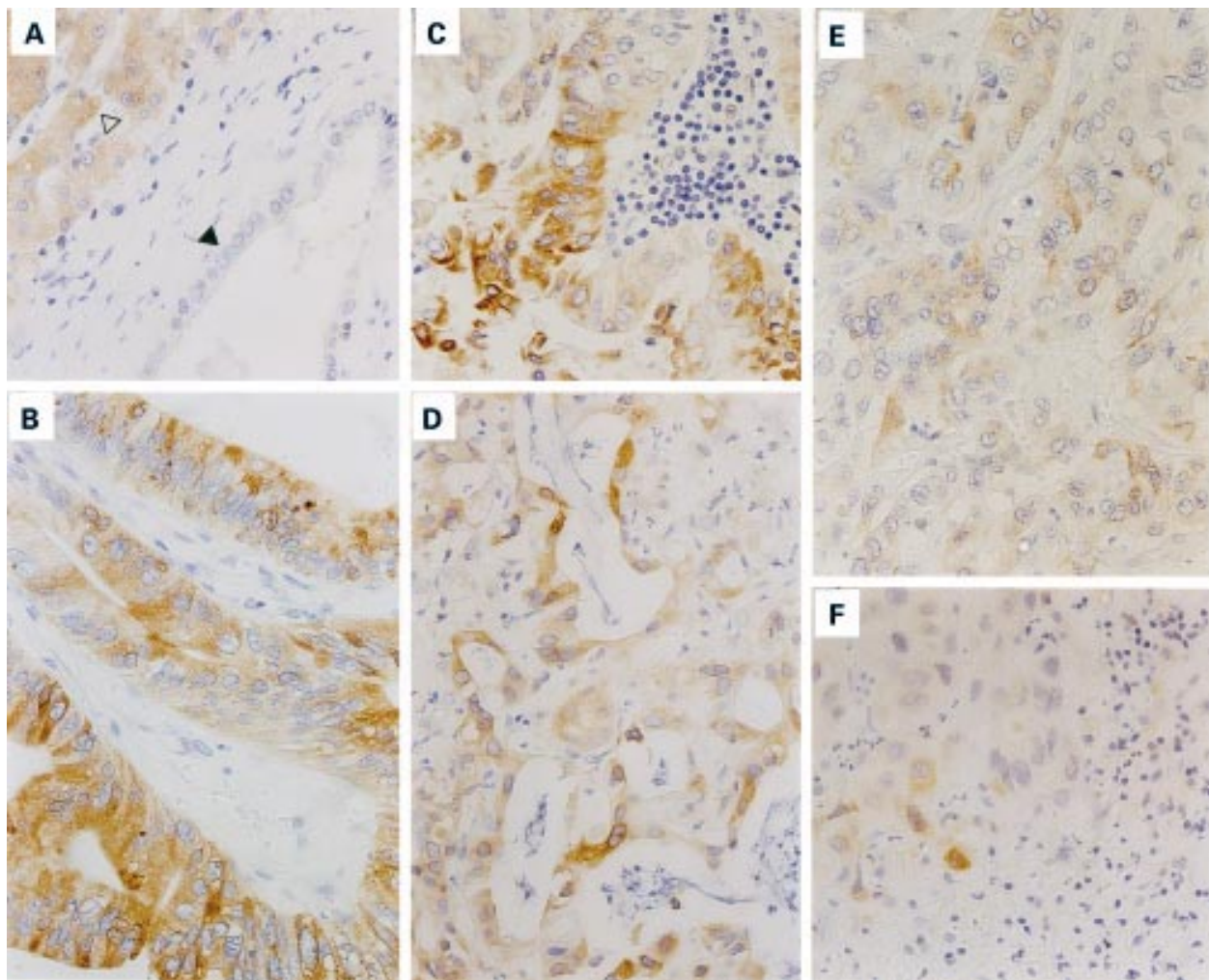


Figure 2 Immunohistochemical localisation of COX-2 in non-tumorous liver tissue and in intrahepatic cholangiocarcinomas at different stages of differentiation. Paraffin sections were stained using the polyclonal goat anti-COX-2 antibodies (SC1745). (A) Non-tumorous tissue demonstrating positive COX-2 immunosignals in hepatocytes (open arrow) but not in bile duct epithelial cells (filled arrow). (B, C) Well differentiated, (D) moderately differentiated, and (E, F) poorly differentiated cholangiocarcinomas showing COX-2 immunosignals in epithelial cells of the gland-like structures. Original magnifications: 1:375 (A–F).

trend test was used to test for a trend in the proportion of COX-1 positive specimens in relation to differentiation of the tumours. The Wilcoxon rank sum test was used to compare intensity scores of COX-2 expression of well differentiated versus moderately and poorly differentiated carcinomas. All statistical tests were performed using SAS (SAS Release 6.12, SAS Institute Inc., Cary, North Carolina, USA).

Results

IMMUNOLocalISATION OF COX-1 AND COX-2 IN NORMAL LIVER

Expression of COX-1 and COX-2 in human liver was analysed by immunohistochemistry using goat polyclonal antibodies raised against peptides from human COX-1 and COX-2 protein. The study included paraffin sections from seven histologically normal livers and 17 liver specimens originating from areas adjacent to the corresponding tumour regions with a histologically normal appearance (matched non-tumorous controls).

All seven normal liver sections showed specific immunosignals for COX-1, predomi-

nantly localised to Kupffer and endothelial cells. Representative stainings are shown in fig 1A and C. In only two of seven cases was weak to moderate COX-1 expression observed in more than 10% of hepatocytes per specimen (table 1). COX-1 positive hepatocytes were scattered diffusely throughout the specimens. In bile duct cells of normal livers, COX-1 signals were not detectable except occasionally in single cells, as shown in fig 1B. These rare cases were scored 0 (table 1). Compared with normal liver samples, no significant changes were observed in expression of COX-1 in Kupffer, endothelial, or bile duct cells of matched non-tumorous tissues. However, the percentage of cases that showed COX-1 positive hepatocytes was increased from 29% to 65% (table 2). In the majority of biopsies, the COX-1 signal was weak in intensity but was present in approximately 70% of hepatocytes and was distributed diffusely throughout the tissue.

All normal livers and matched non-tumorous tissues were found to be COX-2 positive (tables 1, 2). In most cases more than 70% of hepatocytes were stained and the

Table 3 Expression of COX-2 in intrahepatic cholangiocarcinoma and matched non-tumorous tissue

Case No	Differentiation*	Matched non-tumorous tissue								
		Cholangiocarcinoma cells			Bile duct cells			Hepatocytes		
		Ratio of positive cells†	I	P	Ratio of positive cells	I	P	Ratio of positive cells	I	P
8	Well	1	2	F	na	na	na	3	2	D
9	Well	3	3	D	0	-	-	3	3	D
10	Well	2	3	D	na	na	na	na	na	na
11	Well	3	3	D	0	-	-	3	3	D
12	Well	3	3	D	na	na	na	na	na	na
13	Well	3	3	D	1	1	F	3	3	D
14	Well	3	2	D	0	-	-	3	1	D
15	Moderate	2	2	F	0	-	-	3	3	D
16	Moderate	3	3	D	na	na	na	3	2	D
17	Moderate	3	3	D	na	na	na	na	na	na
18	Moderate	3	2	D	0	-	-	3	2	D
19	Moderate	0	-	-	0	-	-	3	2	D
20	Moderate	1	2	F	1	1	F	3	2	D
21	Moderate	1	2	F	0	-	-	3	3	D
22	Moderate	2	2	D	0	-	-	3	2	D
23	Moderate	3	2	D	na	na	na	na	na	na
24	Moderate	2	2	D	1	1	F	3	3	D
25	Poor	1	3	F	2	1	D	3	2	D
26	Poor	1	3	F	3	1	D	3	2	D
27	Poor	2	1	F	0	-	-	3	2	D
28	Poor	0	-	-	0	-	-	na	na	na
29	Poor	2	2	F	na	na	na	3	3	D
30	Poor	3	2	D	0	-	-	3	2	D
31	Poor	3	2	D	1	1	F	na	na	na

I, intensity; P, pattern; D, diffuse; F, focal; na, not available.

*For definition of tumours and †scores see tables 1 and 2.

-, no immunosignal.

intensity of the immunosignal was at least moderate and was diffusely distributed throughout the tissue. A representative example is shown in fig 2A. Only in two of seven normal liver specimens and in six of 17 matched non-tumorous tissues was COX-2 specific immunolabelling seen in bile duct cells. The signal was weak in intensity and mostly appeared focal (not shown). COX-2 protein was undetectable in Kupffer and endothelial cells.

IMMUNOLocalISATION OF COX-1 AND COX-2 IN INTRAHEPATIC CHOLANGIOCARCINOMA

Compared with matched non-tumorous bile duct epithelium, a significant change in COX isozyme expression was observed in cholangiocarcinomas from 24 individual patients (evaluation of 17 matched pairs in the sign test resulted in $p=0.03$ for COX-1 and $p=0.0001$ for COX-2). In 11 of 24 cases the tumour tissue was stained by COX-1 specific antiserum of weak (2/11) or moderate (9/11) intensity and with varying numbers of positive cells and different patterns of staining (table 2). Among the well differentiated tumour samples only one of seven cholangiocarcinomas showed moderate COX-1 expression arranged focally in large gland-like structures of the tumour which were surrounded by extended necrotic areas. The proportion of COX-1 positive specimens among moderately and poorly differentiated cholangiocarcinomas was 6/10 and 4/7, respectively. Staining was mostly moderate in intensity (table 2). Thus the proportion of COX-1 positive specimens of cholangiocarcinoma (46%) was markedly different from that of normal bile duct (0%; $p=0.03$, Fisher's exact test), significantly increasing from normal bile duct tissue (0%) to well (14%), moderately (60%), and poorly

(57%) differentiated tumours ($p=0.005$, Cochran-Armitage trend test). COX-1 expression in well, moderately, and poorly differentiated tumours is shown in fig 1D-F.

In 22 of 24 (92%) cholangiocarcinomas, COX-2 expression was present in tumour cells (table 3). Thus the proportion of COX-2 positive tumour specimens differed significantly from that of the normal bile duct (29%; $p=0.0001$, Fisher's exact test). The pattern of COX-2 specific staining was focal (8/22 cases) or diffuse (14/22 cases) and the number of positive cells varied among individual tissue specimens (table 3). Remarkably, in 11 of 22 samples more than 70% of tumour cells were COX-2 positive. The proportion of COX-2 positive specimens increased from normal bile duct tissue (29%) to well differentiated cholangiocarcinoma (100%; $p=0.02$, Fisher's exact test). The incidence of COX-2 positive specimens was similar between differentially graded groups of cholangiocarcinomas ($p=1$, Fisher's exact test). However, the intensity of COX-2 expression seemed to be more pronounced in well differentiated than in moderately and poorly differentiated cholangiocarcinomas ($p=0.03$, Wilcoxon rank sum test). Representative patterns of COX-2 staining of cholangiocarcinomas are shown in fig 2B-F. Lymphocytes of inflammatory infiltrates were negative for COX-2 (fig 2C). In epithelial cells, COX-2 immunosignals were localised mainly to the cytoplasm, occasionally to the perinuclear region, and rarely to the nuclei (fig 2 B-F).

The specificity of the staining reactions was confirmed by complete quenching of the immunosignal on preadsorption of the antiserum with antigen. Furthermore, no immunosignals were obtained using goat IgG instead of goat anti-COX antibodies or by omitting either the primary or secondary antibodies.

Discussion

An increasing body of evidence from experimental animal and clinical studies has documented a critical role for COX-2 in the early stages of development of cancer in the colon,¹³⁻¹⁵ skin,^{16, 26} and possibly other epithelial tissues.¹⁸⁻²⁴ With the exception of basal cell carcinoma,¹⁶ epithelial cancer tissues investigated to date have shown elevated levels of COX-2 protein while levels of COX-1 mRNA and/or protein were found to be unchanged,^{13, 18, 20, 23} reduced,¹⁴ or increased²² compared with matched controls or normal tissue biopsies. Increased expression of COX-1 protein in breast cancer tissue was localised to stromal rather than tumour cells.²²

In intrahepatic cholangiocarcinoma we found aberrant over expression of both COX isozymes although deregulation with regard to incidence, intensity, and ratio of positive cells and pattern was more pronounced for COX-2 than for COX-1. Thus moderate to strong COX-2 immunosignals were found in more than 90% of cholangiocarcinomas whereas normal bile duct epithelial cells of matched non-tumorous controls or normal liver samples showed only weak COX-2 expression in a few individual cells in approximately 30% of samples. Expression of the COX-2 isozyme was independent of the degree of tumour differentiation. Differentiation independent upregulation of COX-2 expression was also described for oesophageal carcinoma¹⁸ and colorectal adenocarcinomas,²⁷ whereas in adenocarcinomas of the lung¹⁹ and hepatocellular carcinomas^{23, 24} COX-2 expression was found to be prominent in well differentiated tumours compared with low or undetectable expression in poorly differentiated tumours.

With some exceptions²⁸ the COX-2 gene is not constitutively expressed but transiently induced in a wide variety of cell types by appropriate stimuli, while in general COX-1 is expressed constitutively in most cell types. In our immunohistochemical study, COX-2 protein was the predominant COX isoform expressed in hepatocytes of normal liver. In contrast, endothelial and Kupffer cells were both negative for COX-2 protein but frequently expressed COX-1 protein, whereas in hepatocytes only a weak COX-1 immunosignal was noted in a few cases and bile duct epithelium was COX-1 negative. These results are in agreement with a previous study documenting expression of COX-1 and COX-2 mRNA in adult human liver²⁹ and with a recent study that clearly demonstrated COX-2 protein in normal human hepatocytes but not in endothelial or Kupffer cells.²³

Moderate COX-1 expression was seen in one of seven well differentiated cholangiocarcinomas. The incidence of positive cases was higher in moderately and poorly differentiated cholangiocarcinomas, suggesting that COX-1 expression is related to late rather than early stages of cholangiocarcinogenesis while COX-2 was also found in earlier stages of tumour development. The frequency of COX-1 signals in Kupffer and endothelial cells

was similar in normal and neoplastic liver specimens.

While an increasing body of evidence indicates a critical relationship between COX-2 expression and epithelial tumour development, only a few reports suggest a similar role for COX-1 in tumorigenesis. For example, tumorigenic transformation of immortalised endothelial cells in vitro was achieved by COX-1 over expression.³⁰ Moreover, the multiplicity and incidence of squamous cell carcinomas generated by the initiation-promotion protocol in mouse skin³¹ were found to be reduced in COX-1 null compared with wild-type mice.³²

Cholangiocarcinoma is common in South East Asia in regions endemic for the liver fluke *Opisthorchis viverrini*.³³ There is evidence for a significant relationship between liver fluke infection and cholangiocarcinoma in Thailand. Depending on the study, up to 70–90% of intrahepatic cholangiocarcinoma cases were found to be associated with *O. viverrini* infection.^{34, 35} Infection with this liver parasite as a result of nutritional habits is regarded as a promoting agent in bile duct tumorigenesis.^{36, 37} In fact, in hamsters initiated by dimethylnitrosamine, *O. viverrini* infection was effective in promoting the development of cholangiocarcinoma.³⁷ The tumour promoting effect may be due to permanent irritation of the liver by the parasite. Our study shows for the first time aberrant expression of COX isozymes in human cholangiocarcinogenesis. Given that experimentally induced cholangiocarcinoma in hamsters reflects this aberrant COX isozyme expression, selective COX-1^{38, 39} and COX-2 inhibitors⁴⁰ can be tested for their chemopreventive activity in this animal model. This should provide information on the possible applicability of such inhibitors in the treatment of patients already chronically infected with *O. viverrini* and thus predisposed to cholangiocarcinoma.

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