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Increased Expression of Cyclooxygenase-2 in Human Pancreatic Neoplasms and Potential for Chemoprevention by Cyclooxygenase Inhibitors

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BACKGROUND. Cyclooxygenase-2 (COX-2) is thought to be linked to carcinogenesis; however, very little is known about its expression in pancreatic neoplasms. The authors studied the expression of COX-2 in human pancreatic neoplasms and investigated the effect of COX inhibitors on the growth of human pancreatic carcinoma cells.

METHODS. Expression of COX-2 protein was immunohistochemically examined in 42 human pancreatic duct cell carcinomas (PDCs) and in 29 intraductal papillary mucinous tumors (IPMTs [adenomas, 19; carcinomas, 10]) of the pancreas that were resected surgically at the National Cancer Center Hospital in Tokyo. The growth of four human pancreatic carcinoma cell lines also was evaluated in the presence of COX inhibitors.

RESULTS. Marked COX-2 expression was observed in 57% (24 of 42) of PDCs, in 58% (11 of 19) of adenomas, and in 70% (7 of 10) of adenocarcinomas of IPMTs. However, there was no correlation between COX-2 expression and clinicopathologic indices of the patients. All four pancreatic cancer cell lines expressed COX-2 protein weakly or strongly, and the inhibitory effect of aspirin on cell growth was correlated with the expression of COX-2.

CONCLUSIONS. COX-2 was expressed in adenomas of IPMTs as well as in carcinomas and might have played a role in the development of pancreatic tumors. In this study, COX inhibitors, as nonsteroidal anti-inflammatory drugs, were shown to be possible preventive agents against pancreatic neoplasms. ***Cancer* 2001;91:333–8.**
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KEYWORDS: pancreatic duct cell carcinoma, intraductal papillary mucinous tumor, cyclooxygenase.

The incidence of pancreatic cancer has increased steadily and now ranks as the fifth most common cause of cancer-related death in Japan. For most patients, pancreatic cancer remains a lethal disease. At the time of diagnosis, approximately 40% of patients who have pancreatic cancer also have metastatic disease, 40–50% of patients have locally advanced disease that is not amenable to surgical resection, and only 10–20% of patients can be considered candidates for curative resection.[1] The survival of patients who have pancreatic cancer has changed little in the last 20 years despite advancements and innovations in surgical techniques and chemotherapy. Existing chemotherapy and radiation therapy do not improve the prognosis dramatically.[2,3] This poor outcome may be because of the biologically malignant behavior of the tumor, which easily invades surrounding tissues in its early stages. These findings suggest that greater effort should be focused on more effective prevention.

Cyclooxygenase (COX) is a rate-limiting enzyme in prostaglandin synthesis, and two isoforms have been characterized. The isoform designated COX-1 is expressed constitutively in most tissues.[4] Conversely, COX-2 is not present under physiologic onditions but is up-regulated by cytokines, growth factors, and tumor promoters.[5–9] Further, several recent investigations have demonstrated that the use of aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) reduces the incidence or mortality of colorectal cancer.[10–14]

Since NSAIDs are known to inhibit COX, the beneficial effect of NSAIDs in colon cancer may be related to the overexpression of COX, especially COX-2, which is inducible and implicated in epithelial tumor development.[15–18] These possibilities led us to examine whether COX is overexpressed in pancreatic cancers in correlation with clinicopathologic features.

MATERIALS AND METHODS

Tissue Specimens

Samples of formalin-fixed, paraffin-embedded, tumor tissue from 42 invasive pancreatic duct cell carcinomas (PDCs) and 29 (25 patients) intraductal papillary mucinous tumors of the pancreas (IPMTs) that had been resected surgically between 1990 and 1995 at the National Cancer Center Hospital, Tokyo, were studied. All of the patients had confirmed curative resection after intensive histologic exploration, and no patients had distant metastases at the time of surgery. The median age of the patients with PDC was 58.9 years (range: 40–76 yrs). Twenty-seven patients were male, and 15 were female. The patient distribution by stage according to the 1997 UICC classification[19] was Stage I, 5 patients; Stage II, 3 patients; Stage III, 27 patients; and Stage IV, 7 patients. Cases with curative resection included Stage IV cases in Category M1 with lymph node (LYM) factor. The median age of the patients with IPMT was 64.2 years (range: 40–77 yrs). Thirteen patients were male, and 12 were female. All of the patients with IPMT experienced a benign clinical course without metastasis and had no recurrence after surgical resection. These IPMTs were classified into 19 adenomas and 10 adenocarcinomas (no invasive tumors). In accordance with the World Health Organization (WHO) histologic typing of tumors of the exocrine pancreas,[20] we included both intraductal papillary mucinous adenoma (benign category) and intraductal papillary mucinous tumor with moderate dysplasia (borderline category) in the category "adenoma," and intraductal papillary mucinous carcinoma was described as "adenocarcinoma."

Immunohistochemistry

Tissue and cell immunohistochemical staining was performed with the Vectastain avidin-biotin peroxidase complex kit (Vector, Burlingame, CA). Pancreatic tissues were preserved in 10% formalin. Sections 3 μm in thickness were cut on silicone-coated glass slides (Muto Pure Chemicals, Tokyo), deparaffinized in xylene, dehydrated through a graded series of ethanol, and washed in running water. These sections then were treated with saponin solution (2 μg/mL) for 30 minutes at room temperature. Sections were rinsed in 3 changes of phosphate-buffered saline (PBS) and preincubated with 20% normal rabbit serum in PBS at 37 °C for 60 minutes. After they were washed with PBS, separate sections were incubated with the primary polyclonal antibodies (1 μg/mL, Santa Cruz, Arbington, UK) , which recognize epitopes of cyclooxygenase-2. Primary antibodies were diluted in 1% normal rabbit serum diluted with PBS at 4 °C overnight in a wet box. After they were treated with the primary antibody, endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol at RT for 45 minutes, and the sections were incubated with biotinylated antigout IgG. This procedure was followed by incubation with horseradish peroxidase-conjugated avidin-biotin complex at room temperature for 60 minutes. Immunostaining was observed with 3,3′diaminobenzidine (Sigma, St. Louis, MO) in Tris-buffered saline (TBS) for 5 minutes, and the reaction was stopped by washing with water. Finally, the sections were counterstained with Mayer's hematoxylin. Between all antibody incubations, the sections were washed 3 times for 5 minutes with PBS on a shaking platform.

Negative controls included sections that were treated with 1% normal rabbit serum alone in place of the primary antibody. Staining was defined as positive if more than 5% of tumor cells were stained clearly and the accompanying control, without primary antibody, was negative.

Staining results in primary tumors were compared with clinicopathologic features, including tumor-node-metastasis (TNM) staging, histologic type, tumor localization, tumor size, vessel invasion, lymphatic invasion, nodal status and gender. Since histology often varied within the same tumor, the histologic type of the tumor was based on the dominant pattern.

Human Pancreatic Carcinoma Cell Lines and MTT Assay

The four human pancreatic cancer cell lines KP-2, PNS-1, MiaPaca-2, and PANC-1 were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS). The cells were grown in 5% CO_2 in air as monolayers at 37 °C in 24-well, clustered, culture dishes.

TABLE 1
Incidence of the Expression of Cyclooxygenase-2 in Human Pancreatic Neoplasms

	No. of positive cases/ No. of tested cases	% positive
Duct cell carcinoma	24/42	57
IPMT	18/29	62
adenoma	11/19	58
adenocarcinoma	7/10	70

IPMT: intraductal papillary mucinous tumor

For the study of cell growth inhibition, 1 mL of a 5 $\times$ 10^4 cell suspension was incubated in a 96-well, clustered, culture dish (Coster, Cambridge, MA) with concentrations of 0 (control), 100, 250, 500, 1000 and 1500 μmol/L of aspirin (Sigma, Tokyo) or etodolac (1,8-diethyl-1,3,4,9-tetrahydropyrano-[3,4b] indole-1-acetic acid, kindly provided by Nippon Shinyaku Co., Ltd.), which is a new NSAID with high specificity to COX-2. Cells were cultured for 72 hours and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT tetrazolium bromide, Sigma, Tokyo) was added to the culture medium at 0.5 mg/mL. Culture medium was exchanged and tumor cells were incubated for 4 hours with MTT tetrazolium bromide at 37 °C. At the end of the incubation, the culture medium was aspirated. Formazon crystal precipitates were dissolved in 200 μL of dimethyl sulfoxide (Sigma, Tokyo). Solubilized formazon was quantified by obtaining absorption readings at 550 nm wavelength on an enzyme-linked immunosorbent assay (ELISA) reader. The MTT assay measures mitochondrial nicotinamide adenine dinucleotide phosphate (NADPH)-dependent dehydrogenase activity and is the most sensitive and reliable method for quantifying in vitro chemotherapy responses of tumor cells.[21] Reported numbers represent the average cell count in quintuplicate wells.

Statistical Analysis

The SAS program (SAS Institute. Inc., SAS Campus Drive, Cary, NC) was used for all analyses. The association of COX-2 expression with clinicopathologic variables was assessed by using Fisher's exact test and Student's *t* test. A *P* value of less than 0.05 was taken as significant.

RESULTS

COX-2 Expression in Pancreatic Neoplasms

Fifty-seven percent (24 of 42) of PDCs showed enhanced expression of COX-2 protein (Table 1). Immunohistochemically, COX-2 was stained diffusely in cytoplasm of the tumor cells. In contrast, no apparent expression was observed in adjacent normal pancreatic tissue, or only weak reactivity was found in vascular endothelial cells (Fig. 1).

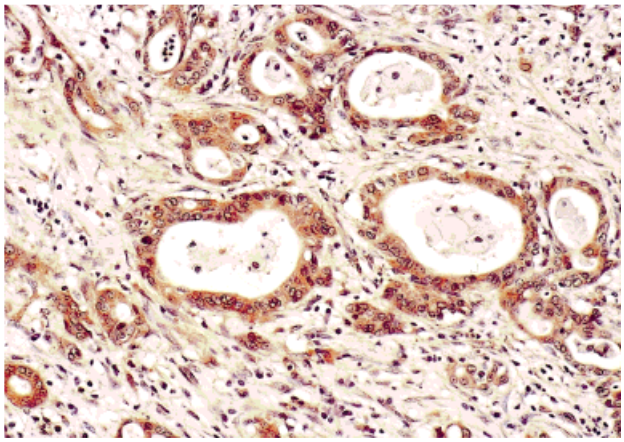

FIGURE 1. COX-2 is expressed in malignant epithelial cells in pancreatic duct cell carcinomas (PDCs). PDC shows diffuse, strong, perinuclear cytoplasmic immunoreactivity with anti-COX-2 antibody ($\times$200).

Positive reactions also were observed in IPMT (Fig. 2), which is believed to take an adenoma–carcinoma sequence in its carcinogenesis. Increased expression was detected not only in carcinoma but also in adenoma, and 70% (7 of 10) of adenocarcinomas and 58% (11 of 19) of adenomas were positive for COX-2.

Association between COX-2 Expression and Clinicopathologic Characteristics

There was no significant correlation between COX-2 expression and clinicopathologic indices of PDC and IPMT (Table 2, 3). COX-2 was overexpressed in both adenomas and carcinomas of IPMTs, suggesting that this protein may contribute to an early stage of pancreatic carcinogenesis.

Effects of COX Inhibitors on the Growth of Human Pancreatic Cancer Cell Lines

Expression of COX-2 was found in all four pancreatic cell lines. When the staining intensity was subclassified into two grades, KP-2 and PNS-1 cells were stained weakly and MIAPaCa-2 and Panc-1 cells were stained strongly.

The antitumor growth effects of aspirin or etodolac in the cell lines were examined by MTT assay (Fig.3), and compared with the results of their COX-2 expression. Pancreatic cancer cell lines with weak COX-2 expression showed a significantly lower IC_{50} for aspirin than those with strong COX-2 expression (Table 4).

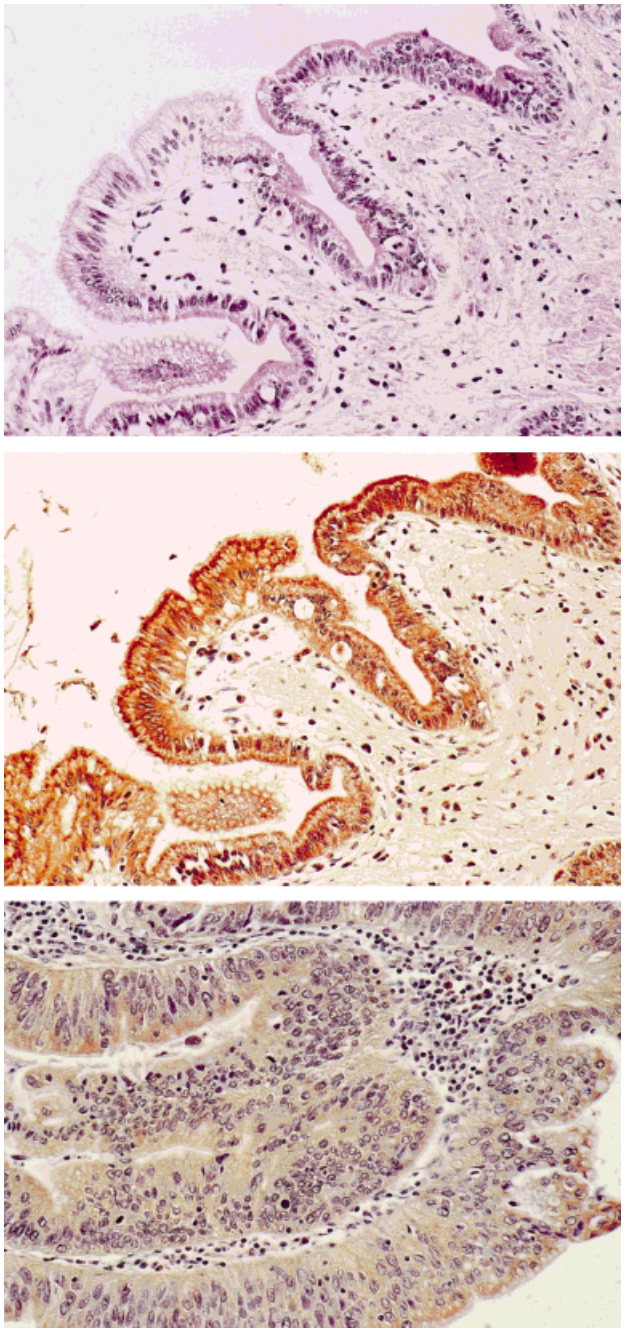

FIGURE 2. (A) Intraductal papillary mucinous adenoma (H&E, ×200): The normal duct epithelium is replaced by papillary proliferations of columnar epithelium which contain some goblet-like cells. Epithelial cells are tall and columnar in shape and have increased nuclear size and chromaticity. There is no severe dysplasia change, and mitoses are absent. (B) Intraductal papillary mucinous adenoma (immunostaining for COX-2, ×200): COX-2 is expressed with a diffuse cytoplasmic pattern. (C) Intraductal papillary mucinous adenocarcinoma (immunostaining for COX-2, ×200): A papillary proliferation of severely dysplastic cells with various-sized hyperchromatic nuclei and conspicuous nucleoli. COX-2 is expressed with a diffuse cytoplasmic pattern.

TABLE 2
Correlation between COX-2 Expression and Clinicopathologic Factors of Pancreatic Duct Cell Carcinoma

	COX-2 positive	COX-2 negative	*P* value
Mean age	62.2	60.5	0.5129
Gender			
Male	15	12	> 0.9999
Female	9	6	
Tumor size (cm)	3.9	3.7	0.7612
Location			
Head	16	13	0.9616
Body and tail	8	5	
Histologic type			
Differentiated	19	17	0.3397
Undifferentiated	5	1	
Lymphatic invasion			
Positive	17	17	0.1257
Negative	7	1	
Venous invasion			
Positive	14	14	0.3211
Negative	10	4	
Lymph node metastasis			
Positive	19	15	> 0.9999
Negative	5	3	
TNM stage			
Stage I	4	1	0.5359
Stage II, III, IV	20	17	

TABLE 3
Correlation between COX-2 Expression and Clinicopathologic Factors of IPMT of the Pancreas

	COX-2 positive	COX-2 negative	*P* value
Mean age	62.1	67.4	0.1311
Gender			
Male	7	6	0.7937
Female	8	4	
Tumor size (cm)	3.6	4.0	0.6298
Location			
Head	11	10	0.1839
Body and tail	7	1	
Histologic type			
Adenoma	11	8	0.8027
Adenocarcinoma	7	3	

IPMT: intraductal papillary mucinous tumor

DISCUSSION

In the current study, we found that COX-2 protein was expressed in 57% of PDCs and in 62% of IPMTs. Many reports already have documented overexpression of COX-2 in malignant tumors;[6,22–30] however, we believe that there has been no study with a large number of PDCs and IPMTs focusing on a relation with clinicopathologic features. Further, to our knowledge, this is the first study on inhibition of human pancreatic cancer cell growth by COX inhibitors.

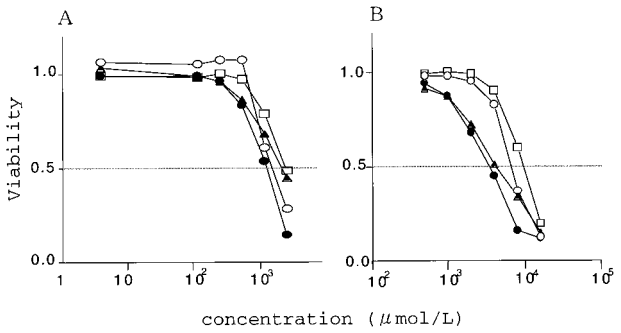

FIGURE 3. Dose-dependent growth inhibition of human pancreatic cancer cell lines following 72 hours of treatment with (A) etodolac and (B) aspirin. □: KP-2; ○: PNS-1; ▲: PANC-1; ●: MiaPaca-2.

TABLE 4
Intensity of COX-2 Expression and IC_{50} for Etodolac and Aspirin in Human Pancreatic Cancer Cell Lines

		IC_{50} (μmol/L)	
Cell line	**COX-2 expression**	**Etodolac**	**Aspirin**
KP-2	weak	1900	13,300
PNS-1	weak	1240	6600
MiaPaca-2	strong	1050	3500
PANC-1	strong	1680	4200

We found no significant relation between COX-2 expression and clinicopathologic indices in our study, a finding that was compatible with previous reports on other organs. Further investigation is required to clarify the clinical meaning of COX-2 protein, although it has been reported to activate the metastatic potential of tumor cells in vitro.[31]

Since most of the specimens of PDC that were examined in this study were from patients who were in advanced stages of their disease, it was unclear to us whether COX-2 protein was involved in early events in tumorigenesis of PDC. We demonstrated that adenoma of IPMT, which is thought to develop to carcinoma as colorectal cancer,[32,33] overexpressed COX-2 at a high rate. Therefore, COX-2 might have played a role in tumor progression of PDC in our study patients. Recent studies have revealed that COX-2 protein is involved in several mechanisms, such as prostaglandin synthesis, promotion of angiogenesis,[34] inhibition of immune surveillance,[35] and inhibition of apoptosis.[36] Additional studies are needed to determine which of these mechanisms are important to pancreatic tumorigenesis.

It is well known that when patients with familial adenomatous polyposis (FAP) are treated with sulindac, they show a significant decrease in the number and size of polyps.[37,38] Because adenomas in IPMTs showed increased COX-2 expression and have had no useful less invasive treatment, COX-2 inhibitor therapy for patients with IPMT may be of clinical benefit to reduce tumor progression. In vitro, both aspirin and etodolac showed concentration-dependent inhibitory effects on four pancreatic cell lines. In particular, the IC_{50} of etodolac, a COX-2-specific inhibitor, was much lower than that of aspirin, which was consistent with previous findings in colon cancer cell lines.[39,40] Although the reason remains unclear, there was a negative correlation between the intensity of COX-2 expression and the IC_{50} of aspirin but not of etodolac.

In conclusion, we have shown that COX-2 is expressed in human PDC and IPMT of the pancreas and that aspirin and etodolac each inhibited the proliferation of pancreatic cancer cells. These results suggest that COX-2 inhibitors may be possible preventive agents against PDC and IPMT of the pancreas.

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