[CANCER RESEARCH 59, 987–990, March 1, 1999]

Advances in Brief

Cyclooxygenase-2 Expression Is Up-Regulated in Human Pancreatic Cancer[1]

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Abstract

A large body of evidence suggests that cyclooxygenase-2 (COX-2) is important in gastrointestinal cancer. The purpose of this study was to determine whether COX-2 was expressed in adenocarcinoma of the human pancreas. Quantitative reverse transcription-PCR, immunoblotting, and immunohistochemistry were used to assess the expression of COX-2 in pancreatic tissue. Levels of COX-2 mRNA were increased by >60-fold in pancreatic cancer compared to adjacent nontumorous tissue. COX-2 protein was present in 9 of 10 cases of adenocarcinoma of the pancreas but was undetectable in nontumorous pancreatic tissue. Immunohistochemical analysis showed that COX-2 was expressed in malignant epithelial cells. In cultured human pancreatic cancer cells, levels of COX-2 mRNA and protein were induced by treatment with tumor-promoting phorbol esters. Taken together, these results suggest that COX-2 may be a target for the prevention or treatment of pancreatic cancer.

Introduction

Adenocarcinoma of the pancreas is one of the most lethal malignancies. Its mortality rate is in excess of 95% of its incidence rate. In the United States, ~25,000 new cases of pancreatic cancer are diagnosed annually (1). Pancreatic cancer now ranks fourth and fifth as a cause of cancer death in men and women, respectively, in the United States (1). Unfortunately, >90% of pancreatic cancer patients present with metastatic disease or advanced local disease, precluding a curative surgical resection. Chemotherapy has not resulted in a significant survival benefit, and the 5-year survival rate is <1.3% in the United States (1), with a median survival of 4.1 months. On the basis of these observations, it is clear that new molecular targets are needed for the prevention and treatment of pancreatic cancer.

Results from recent studies have established the presence of two distinct COX[3] enzymes, a constitutive enzyme (COX-1) and an inducible form (COX-2). COXs catalyze the formation of prostaglandins from arachidonic acid. *COX-1* is thought to be a housekeeping gene with essentially constant levels of expression, whereas *COX-2* is an early response gene that, like c-*jun* and c-*fos*, is induced rapidly by growth factors, tumor promoters, oncogenes, and carcinogens (2).

Multiple lines of evidence suggest that COX-2 is important in carcinogenesis. For example, COX-2 is up-regulated in transformed cells (3) and in various forms of cancer (4–7), whereas levels of COX-1 are relatively constant. Moreover, a null mutation for COX-2 caused a marked reduction in the number and size of intestinal polyps in $APC^{\Delta 716}$ mice, a murine model of familial adenomatous polyposis (8). COX-2 knockout mice also developed ~75% fewer chemically induced skin papillomas than control mice (9). In addition to the genetic evidence implicating COX-2 in carcinogenesis, newly developed selective inhibitors of COX-2 protect against gastrointestinal tumor formation (8, 10). Here, we investigated whether COX-2 was up-regulated in pancreatic cancer. Our data show that levels of COX-2 are increased in adenocarcinoma of the pancreas and raise the possibility that selective inhibitors of COX-2 may be useful in the prevention or treatment of this disease.

Materials and Methods

Materials. RPMI 1640, DMEM, fetal bovine-serum, penicillin, streptomycin, COX-2, and β_2-microglobulin primers were from Life Technologies, Inc. (Grand Island, NY). RNeasy Mini kits were from Qiagen (Santa Clarita, CA). GeneAmp RNA PCR kits were from Perkin Elmer Corp. (Norwalk, CT). GenElute Agarose Spin Columns were from Supelco (Bellefonte, PA). Lowry protein assay kits, PMA, and secondary antibody to IgG conjugated to horseradish peroxidase were from Sigma Chemical Co. (St. Louis, MO). The COX-2 standard for immunoblotting was from Cayman Chemical Co. (Ann Arbor, MI). The COX-2 polyclonal antibody, PG-27, was from Oxford Biomedical Research, Inc. (Oxford, MI). Nitrocellulose membranes were from Schleicher & Schuell (Keene, NH). Western blotting detection reagents (ECL) were from Amersham Pharmacia Biotech. The tyramide signal and amplification kit was from NEN Life Science (Boston, MA). The Vector Blocking Kit was from Vector Laboratories (Burlingame, CA).

Patient Samples. Biopsy specimens were obtained at the time of surgery from 10 patients with adenocarcinoma of the exocrine pancreas. Tissue samples were taken from a nonnecrotic area of the tumor and from adjacent nontumorous tissue; samples were immediately frozen in liquid nitrogen and subsequently stored at −80°C. Informed consent was obtained from each patient. The study was approved by the Committee on Human Rights in Research at Weill Medical College of Cornell University.

Tissue Culture. Three human pancreatic adenocarcinoma cell lines (Su 86.86, BxPC-3, and Panc-1) were obtained from American Type Culture Collection (Manassas, VA). The Su 86.86 and BxPC-3 cell lines were maintained in RPMI 1640; the Panc-1 cell line was maintained in DMEM supplemented with 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were plated for experimental use in complete medium and allowed to attach and grow for 48 h in a 5% CO_2/water-saturated incubator at 37°C. The medium was then replaced with serum-free medium. Twenty-four h later, cells were treated with vehicle or PMA under serum-free conditions.

Western Blotting. Frozen tissue was thawed in ice-cold homogenization buffer containing 150 mM NaCl, 100 mM Tris-buffered saline (pH 8), 1% Tween 20, 50 mM diethyldithiocarbamate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml trypsin-chymotrypsin inhibitor, and 10 μg/ml pepstatin. Tissues were homogenized using a glass-on-glass tissue homogenizer. Homogenates were centrifuged at 11,750 × *g* for 10 min at 4°C to remove the particulate material.

Cellular lysates were prepared by treating cells with the same lysis buffer that was used for the tissue samples. Lysates were sonicated for 20 s on ice and centrifuged at 11,750 × *g* for 10 min to sediment the particulate material. The protein concentration of the supernatant was measured using the Lowry protein assay kit. Immunoblot analysis for COX-2 was performed as in previous studies (11).

Received 11/19/98; accepted 1/14/99.

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[1] This work was supported by the Alice Bohmfalk Charitable Trust to T. J. F.

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[3] The abbreviations used are: COX, cyclooxygenase; PMA, phorbol 12-myristate 13-acetate; RT-PCR, reverse transcription-PCR; nt, nucleotide(s).

Construction of a COX-2 Competitor Template Containing a nt Deletion. A competitive RT-PCR deletion construct (mimic) for COX-2 was synthesized using a mutant sense primer (nt 932–955 attached to nt 1111–1130; 5′-GGTCTGGTGCCTGGTCTGATGATGGAGTGGCTATCACTTCAAAC-3′) and an antisense primer (nt 1634–1655; 5′-GTCCTTTCAAGGAGAATGGTGC-3′), producing a 569-bp PCR product. The mutant sense primer contains the primer-binding sequence of endogenous target (from nt 932 to 955) attached to the end of an intervening DNA sequence (a 156-bp deletion from nt 956 to nt 1110). Thus, the mimic DNA has primer binding sequences that are identical to the target cDNA. The 569-bp mimic was further amplified using the sense primer (5′-GGTCTGGTGCCTGGTCTGATGATG-3′) and the antisense primer (5′-GTCCTTTCAAGGAGAATGGTGC-3′) in a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM dNTP, 2.5 units of AmpiTaq DNA polymerase, and 400 nM primers for 35 cycles consisting of denaturation at 94°C for 20 s, annealing at 60°C for 20 s, and extension at 72°C for 30 s in a Perkin Elmer 2400 thermal cycler. The PCR products were electrophoresed on 1% agarose gels and gel-purified using GenElute Agarose Spin Columns according to the manufacturer's protocol.

RNA Isolation and Reverse Transcription. Total RNA was isolated from pancreatic tissue (~50 mg) and cell monolayers using an RNeasy Mini Kits from Qiagen. One μg of total RNA was reverse-transcribed using the GeneAmp RNA PCR kit according to the manufacturer's protocol.

Quantitative PCR for COX-2 in Human Pancreatic Tissue. Each PCR was carried out in 25 μl of a reaction mix, containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM dNTP, 2.5 units of Amplitaq DNA polymerase, and 400 nM primers (sense primer, 5′-GGTCTGGTGCCTG-GTCTGATGATG-3′; antisense primer, 5′-GTCCTTTCAAGGAGAATG-GTGC-3′). Five-μl aliquots of the reverse-transcribed cDNA samples and various known amounts of COX-2 mimic (between 0.0001 and 0.05 pg), adjusted to the abundance of the target cDNA, were added to the reaction mix and coamplified for 35 cycles: denaturation at 94°C for 20 s, annealing at 65°C for 20 s, extension at 72°C for 90 s, and final extension at 72°C for 10 min. Ten μl of PCR products, 724-bp fragments from endogenous target cDNA, and 569-bp fragments from mimic COX-2 were then separated by electrophoresis on 1% agarose gels and visualized by ethidium bromide staining.

Semiquantitative PCR for COX-2 and β_2-microglobulin in Pancreatic Cell Lines. The semiquantitative analysis for COX-2 was performed using the same COX-2 primers as listed above in a 25-μl reaction mixture containing 5-μl aliquots of reverse transcribed cDNA samples, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM dNTP, 2.5 units of AmpliTaq DNA polymerase, and 400 nM primers for 35 cycles consisting of denaturation at 94°C for 20 s, annealing at 65°C for 20 s, extension at 72°C for 30 s, and final extension at 72°C for 10 min. A constitutively expressed gene, *β_2-microglobulin*, was used as an internal control, generating a 266-bp PCR product. The primers for β_2-microglobulin (from nt 75 to nt 340) were 5′-AGCAGAGAAT-GGAAAGTCAAA-3′ (sense) and 5′-ATGCTGCTTACATGTCTCGAT-3′ (antisense). The PCR conditions for β_2-microglobulin were identical to that for COX-2, except for annealing at 55°C for 20 s.

Immunohistochemistry. Tissues from 10 patients with adenocarcinoma of the pancreas were fixed in formalin, embedded in paraffin, cut into 4-μm sections and mounted onto polylysine-coated slides. Sections were dewaxed in xylene, rehydrated in descending alcohols, and blocked for endogenous peroxidase (3% H_2O_2 in MeOH) and avidin/biotin (Vector Blocking Kit). The sections were permeabilized in TNB-BB [0.1 M Tris (pH 7.5), 0.15 M NaCl, 0.5% blocking agent, 0.3% Triton X-100, and 0.2% saponin) and incubated in primary antibody overnight at 4°C. The polyclonal antiserum to COX-2 (PG-27; Oxford Biomedical Research Inc.) was used at a 1:500 dilution in TNB-BB. Control sections were incubated with antisera in the presence of a 100-fold excess of human recombinant COX-2 protein or with isotype-matched IgG normal rabbit serum. Immunoreactive complexes were detected using tyramide signal and amplification (TSA-indirect) and visualized with the peroxidase substrate, AEC. Slides were then counter stained in aqueous hematoxylin, mounted in crystal mount, and coverslipped in 50:50 xylene/Permount.

Statistical Analysis. Results were analyzed by the Wilcoxon signed rank test. A difference between groups of $P < 0.05$ was considered significant.

Results

Levels of COX-2 mRNA and Protein Are Increased in Pancreatic Cancer. To analyze the expression of COX-2, we developed a sensitive competitive RT-PCR assay in which the amount of COX-2 mRNA could be measured from minute quantities of RNA (0.5 μg total RNA). This method relies on the coamplification in the same tube of known amounts of competitor DNA (Fig. 1*A*) with COX-2 cDNA, obtained after reverse transcription from total tissue RNA. The competitor and target use the same PCR primers but yield amplicons with a different size (Fig. 1*A*), allowing their separation on a gel at the end of the reaction. Increased amounts of COX-2 mRNA were detected in 10 of 10 pancreatic cancers compared with nontumorous tissue (Fig. 1*B*). Overall, there was a >60-fold increase in amounts of COX-2 mRNA in tumorous (mean, 135 fg/μg total RNA) *versus* nontumorous (mean, 2 fg/μg total RNA) tissue. To determine whether levels of COX-2 protein were also increased in pancreatic cancer,

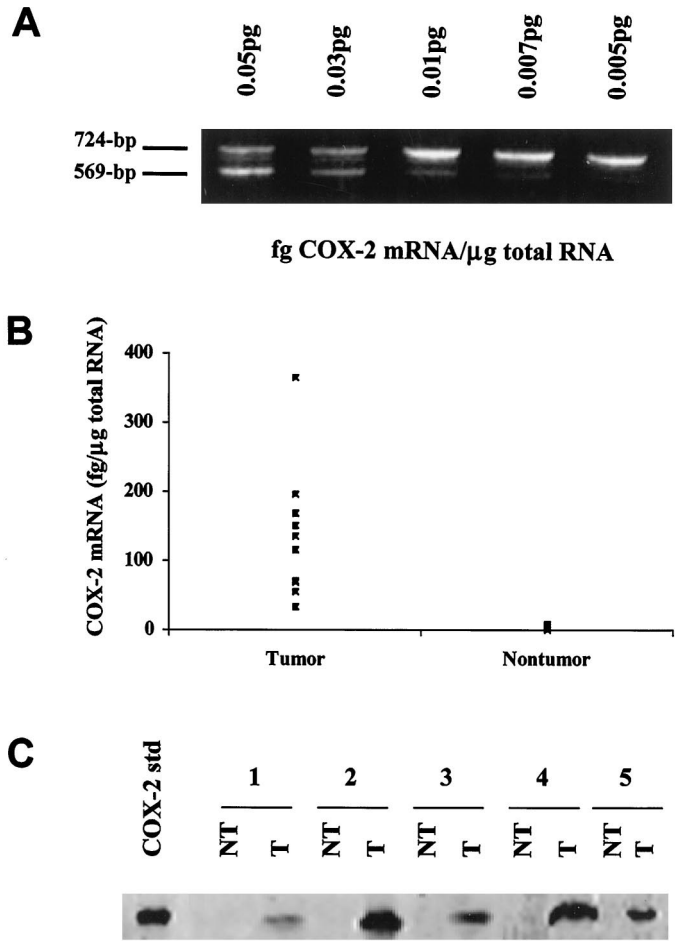

Fig. 1. Increased levels of COX-2 in human pancreatic adenocarcinoma. *A*, representative quantitative RT-PCR in a case of pancreatic adenocarcinoma. Five μl of endogenous cDNA and known concentrations (0.005–0.05 pg) of human COX-2 mimic are competing for a fixed amount of hCOX-2 primer in each reaction lane, giving rise to relative proportions of 569-bp mimic product and 724-bp target cDNA product. At 0.005 pg of mimic, there is more 724-bp target cDNA product than mimic product. At 0.03 pg, the amounts of mimic and cDNA product are similar. The amount of COX-2 present in this tumor was 150 fg/μg total RNA. *B*, quantitative RT-PCR was used to determine amounts of COX-2 mRNA in 10 pairs of tumorous and nontumorous pancreatic tissue. A >60-fold increase in amounts of COX-2 mRNA was detected in tumorous (mean 135 fg/μg total RNA, $n = 10$) *versus* nontumorous (mean, 2 fg/μg total RNA, $n = 10$) tissue ($P = 0.002$). *C*, immunoblotting was performed on paired tumorous (*Lanes T*) and nontumorous (*Lanes NT*) pancreatic tissue from five patients. Equal amounts of protein (100 μg/lane) were loaded onto a 10% SDS-polyacrylamide gel, electrophoresed, and subsequently transferred onto nitrocellulose. The immunoblot was probed with antibody specific for COX-2. Purified ovine COX-2 was used as a standard.

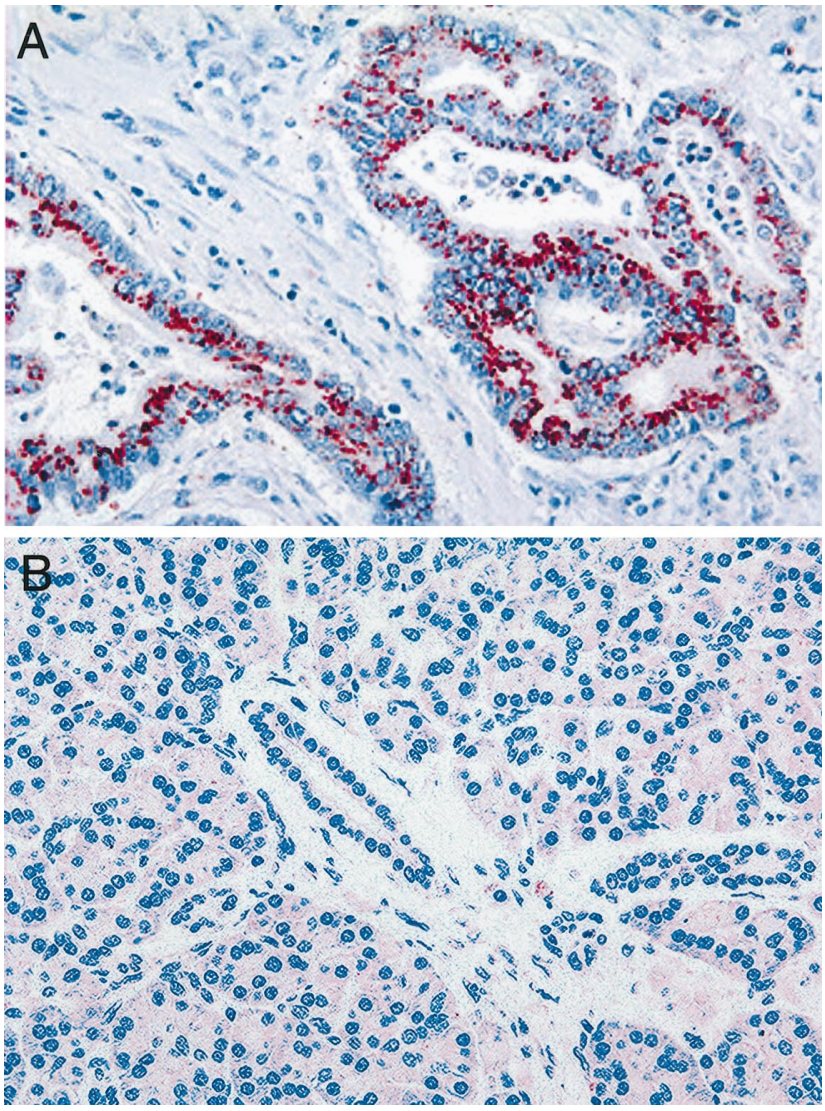

Fig. 2. COX-2 is expressed in malignant epithelial cells in pancreatic adenocarcinoma. *A*, pancreatic adenocarcinoma shows diffuse, strong, granular, perinuclear cytoplasmic immunoreactivity with anti-COX-2 antibody (×200). *B*, benign ductule surrounded by histologically unremarkable pancreatic acini showing no COX-2 immunoreactivity (×200).

Western blot analysis of paired tumorous and nontumorous tissue was performed (Fig. 1*C*). COX-2 protein was detected in tumor tissue from 9 of 10 patients but was undetectable in nontumorous tissue in the same patients.

Immunohistochemical analysis of 10 cases of pancreatic cancer revealed that COX-2 expression was multifocal and moderate to strong in the majority of cases. Expression of COX-2 was localized to tumor cells, not to surrounding stromal cells or infiltrating inflammatory cells (Fig. 2*A*). The staining pattern was predominantly granular, perinuclear cytoplasmic. This staining was specific for COX-2 because immunoreactivity was lost when the antiserum to COX-2 was incubated with human recombinant COX-2. Staining for COX-2 was negligible in nontumorous tissue (Fig. 2*B*) with the exception of pancreatic endocrine cells. Interestingly, moderate COX-2 immunoreactivity was detected in metastatic pancreatic adenocarcinoma in regional lymph nodes from two patients.

COX-2 Is Induced by Tumor-promoting Phorbol Esters in Human Pancreatic Carcinoma Cell Lines. To determine whether the up-regulation of COX-2 mRNA and protein seen in pancreatic tumors was reproducible *in vitro*, we analyzed three human pancreatic cancer cell lines: Su 86.86, Panc 1, and BxPC-3. As shown in Fig. 3, COX-2 mRNA and protein were detected under basal conditions in the Su 86.86 cell line. Treatment with PMA, a tumor promoter, induced COX-2 mRNA and protein in as little as 3 h. Similar results were obtained in the Panc 1 and BxPC-3 cell lines (data not shown).

Discussion

In this study, we discovered that amounts of COX-2 mRNA and protein are increased in pancreatic cancer. Immunohistochemistry revealed that COX-2 was expressed in tumor cells. COX-2 was also induced by PMA in cultured human pancreatic cancer cell lines. To our knowledge, this is the first evidence that COX-2 is up-regulated in pancreatic cancer.

COX-2 can potentially predispose to carcinogenesis via multiple mechanisms. In extrahepatic tissues in which cytochrome P450 content is low, COX may be important for metabolism of carcinogens. For example, several classes of chemical carcinogens, *e.g.*, dihydrodiol derivatives of polycyclic aromatic hydrocarbons, aromatic amines, and heterocyclic amines, are activated to mutagenic derivatives by COX (12). The metabolism of carcinogens by COX-2 may be important, therefore, for understanding the link between cigarette smoking (1) or consumption of grilled or fried meat (1) and pancreatic cancer. Additionally, enhanced synthesis of prostaglandins, a consequence of up-regulation of COX-2, favors the growth of malignant cells by increasing cell proliferation (13), promoting angiogenesis

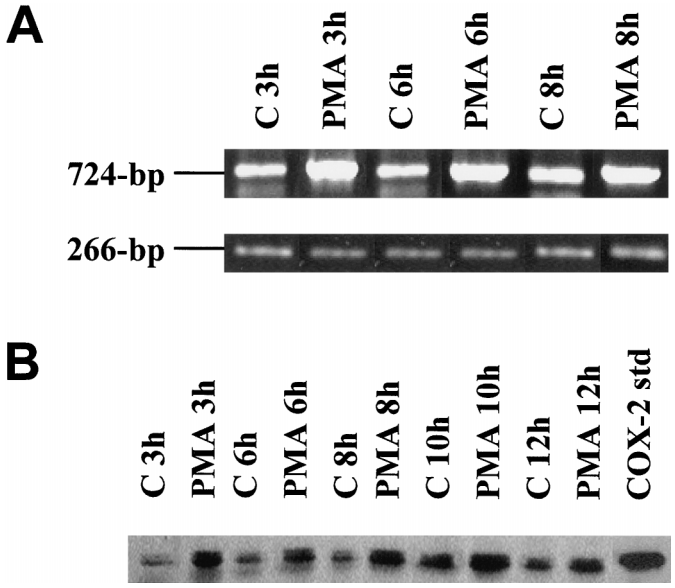

Fig. 3. COX-2 is induced by treatment with phorbol esters. Su 86.86 cells were treated with PMA (50 ng/ml) or serum-free medium (*C*) for the indicated time periods. In *A*, total cellular RNA was isolated. Each lane contained 1 μg of RNA. PCR resulted in 724- and 266-bp products representing human COX-2 and β_2 microglobulin, respectively. In *B*, lysate protein (30 μg/lane) was loaded onto a 10% SDS gel, electrophoresed, and subsequently transferred to a nitrocellulose membrane. The immunoblot was probed with antibody specific for COX-2. Ovine COX-2 was used as a standard (*std*).

(14), and inhibiting immune surveillance (15). In intestinal epithelial cells, overexpression of COX-2 inhibits apoptosis (16) and increases the invasiveness of malignant cells (17). Additional studies are needed to determine which of these mechanisms are important in adenocarcinoma of the pancreas.

It also is interesting to consider the possible link between the known genetic alterations in pancreatic cancer and COX-2. Mutations in the Ki-*ras* oncogene (18) are common in pancreatic cancer. Levels of COX-2 are increased in Ras-transformed epithelial cells (3, 19). It is reasonable to postulate, therefore, that activation of the Ras pathway contributes to the up-regulation of COX-2 in pancreatic cancer. Mutations of the *Apc* gene also occur in pancreatic cancer (20). The potential significance of the link between COX-2 and Apc was highlighted by the finding that COX-2 deficiency protects against tumor formation in mice carrying a defective *Apc* gene (8).

Recently, selective inhibitors of COX-2 have been developed. These compounds possess anticancer properties (8, 10) and appear to be safer than traditional nonsteroidal anti-inflammatory drugs. On the basis of results of this study, it will be important to establish whether inhibiting COX-2 will be useful alone or in combination with chemotherapy or radiotherapy as a novel treatment for pancreatic cancer.

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