Selective Inhibition of Cyclooxygenase-2 Suppresses the Growth of Pancreatic Cancer Cells in Vitro and in Vivo

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Cyclooxygenase-2 (COX-2), a prostaglandin synthetase, is involved in development of certain tumors. We therefore analyzed COX-2 expression in pancreatic cancer tissues (53 samples) and Panc-1 human pancreatic cancer cells by immunohistochemistry, RT-PCR and western-blotting analyses. Also, immunohistochemistry of proliferating cell nuclear antigen (PCNA) was performed. We found expression of COX-2 was dramatically upregulated in 36 of 53 cases (67.9%) and the expression of COX-2 was associated with the diameter (> 3 cm) of the tumors (p < 0.05), but not with the age, gender, tumor location, differentiation, lymph-node metastases and TNM stage. The positivity rate of PCNA expression in the pancreatic cancer cells of the COX-2 positive group (32.88 ± 13.26%) was significantly higher than that in the COX-2 negative group (24.56 ± 11.51%) (p < 0.05). Then we investigated the effect of selective inhibitors of COX-2 (NS398 and celecoxib) on proliferation of Panc-1 cells by 3-(4,5 dimethyl-2-thiazolyl)-2.5-diphenyl-2H-tetrazolium bromide (MTT) assay. Either NS398 or celecoxib suppressed proliferation of Panc-1 cells dose-dependently in vitro. Furthermore, Panc-1 cells were implanted into nude mice, and celecoxib was administrated orally with feed. The volume of the tumor xenografted into nude mice was decreased by 51.6% in the celecoxib group (p < 0.01). In conclusion, the increased expression of COX-2 may be responsible for rapid proliferation of pancreatic cancer, and specific inhibition of COX-2 suppresses proliferation of Panc-1 cells in vitro and in nude mice. The selective inhibitor of COX-2 may be an effectual agent for pancreatic cancer chemoprevention.

Keywords: pancreatic neoplasm; cyclooxygenase-2; proliferation; celecoxib; NS398.

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Pancreatic cancer is the forth to fifth leading cause of death from cancer in many different countries (Eckel et al. 2006). Because this disease is characterized by extensive local invasion and early lymphatic and hematogenous metastasis (Wray et al. 2005; Mann et al. 2006), only 1-4% of all patients with the pancreatic cancer survive 5 years after diagnosis (Eckel et al. 2006). Therefore, developing chemoprevention measures for pancreatic cancer is an important direction of research. Cyclooxygenase-2 (COX-2) has been proven an important promoter of tumor growth in
various cancer entities, and therefore has been considered a target for therapy (Antonakopoulos et al. 2007). It was frequently observed that COX-2 was over-expressed in a variety of gastrointestinal cancers including pancreatic cancer (de Maat et al. 2007; Nagahara et al. 2007; Buchanan et al. 2007). Recent studies proved that patients whose pancreatic cancer cells expressed COX-2 had a significantly worse prognosis than patients whose tumor cells did not express COX-2. Evidence exists that COX-2 plays an important role in development of tumor and its mechanisms involved in proliferation, apoptosis, angiogenesis and metastases (Tatsuguchi et al. 2004; Iwata et al. 2007; Ohno et al. 2007).

Although COX-2 has been studied in several cancer types, its relationship with pancreatic cancer has not been fully elucidated. Especially, we lack the enough research data resulting from animal experimentation. So far we can’t assure whether inhibition of COX-2 pathway is an effective chemoprevention measure for pancreatic cancer in vivo. In this study we have investigated the expression of COX-2 in primary human pancreatic cancer and then studied the regulation of COX-2 in the growth of human pancreatic cancer cell line in vitro and in nude mice tumor model.

MATERIALS AND METHODS

Patient samples

Banked paraffin-embedded tissue samples of 53 patients of pancreatic adenocarcinomas (from 28 men and 25 women, mean age 55.4 years, range 45-75 years) obtained from the tenth people’s hospital of Tongji university were analyzed by immunohistochemistry. All patients have signed a consent document, in which they agreed that the surgical excision of malignancy would be used in medical research before their operations.

Reagents

NS-398 and celecoxib were purchased from Sigma Chemical Company (St. Louis, MO, USA) and Searle & Co. (Caguas, PR, USA) respectively and the effects of celecoxib and NS398 on cell growth were determined using a cell proliferation assay. Both drugs were dissolved in 100% DMSO and then diluted with RPMI1640 for cell culture experiments. The final concentration of DMSO for all treatments (including controls, where no drug was added) was maintained at 0.1%. All drug solutions were prepared fresh on the day of testing. COX-2 rabbit polyclonal antibody was purchased from Cayman Chemical Company (Ann Arbor, MI, USA).

Cell Line, Culture Condition, and Proliferation

Human pancreatic cancer cell line Panc-1 was grown in RPMI1640 medium containing 2% FBS at 37°C in 5% CO₂ humidified incubator. Cell proliferation was determined by the MTT (3-[4,5 dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide) assay. Briefly, cells were plated in a 96-well plate at 10,000 cells per well, cultured for 24 h in complete growth medium, then treated with 10 μM, 60 μM, 100 μM and 140 μM of celecoxib and 50 μM, 100 μM, 150 μM, and 200 μM of NS398 for 3 days. Experimental controls were treated with DMSO only. And MTT solution was added to the culture medium, and after 2 h optical density was read with an ELISA reader.

PGE2 immunoassay

PGE2 was measured in the supernatant by RIA using commercial kits (prostaglandin E2 125I RIA kit Amersham Pharmacia Biotech, Uppsala, Sweden) at the ends of experiment of MTT.

Immunohistochemistry for COX-2 and PCNA

Paraffin-embedded tissues were used for identification of COX-2 and PCNA (proliferating cell nuclear antigen). Immunohistochemical procedures were performed. Briefly, Sections analyzed for COX-2 were treated with pepsin (Biomeda, Foster, CA, USA) for 15 min at 37°C and washed with PBS and for PCNA were microwaved for 5 min. The sections were incubated with the primary antibody (1:200 [v/v]) for COX-2 or PCNA in a humidified chamber for 15-18 h at 4°C and were then incubated with the corresponding HRP-conjugated secondary antibody (1:200 [v/v]) for 1 h at 37°C. This was followed by incubation with DAB (Sigma Chemical Co., St. Louis, MO, USA) solution. The intensity of COX-2 positive cancer cells were graded on a scale of 4 grades: 0, no staining of cancer cells; 1, weak staining; 2, moderate staining; 3, strong staining. The percentage of PCNA staining cancer cells were also graded on a scale of 4 grades: 0, none; 1, 1%-33%; 2, 34%-66%; 3, 67%-100%. The PCNA indices (PI) were calculated as the number of PCNA positive cells in 1,000 pancreatic cancer cells in field at x 200 magnification. The Immunohistochemical intensity and pathologic characteristics of all tumor spec-
imens used in this study were examined by an independent pathologist.

Reverse transcription-PCR (RT-PCR)

RNA extraction was carried out from the Panc-1 cell line, the pancreatic cancer tissue and normal pancreas tissue from the same patient with Trizol reagent, and the total RNA was used to analyze the expression of COX-2 mRNA by RT-PCR. Primer of COX-2: sense primer: 5′-TGA AAC CCA CTC CAA ACA CAG-3′, antisense primer: 5′-TCA CAG GCA CAG GAG GAA G-3′(232 bps, as described previously). PCR conditions were 94°C for 5 min, 94°C for 45 sec, 54°C for 90 sec, 72°C for 90 sec, 72°C for 90 sec, 30 cycles (Hull et al. 1999).

Western blot analysis

For western blotting, Panc-1 cell line, the pancreatic cancer tissue and normal pancreas tissue were homogenized in lysis buffer containing 150 mM NaCl, 100 mM Tris-buffered saline (pH 8.0), 1% Tween 20, 50 mM diethyldithiocarbamate, 1 mM EDTA, 1 mM phenemethyl-sulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml trypsin inhibitor, and 10 μg/ml peptatin. Immunoblot analysis for COX-2 was performed as in previous studies. Briefly, 100 μg aliquots taken from the total quantity of protein were size-fractionated to a single dimension by SDS-PAGE (10% gels) and transblotted to 0.45 μm PVDF membrane (Bio-Rad, Richmond, CA, USA) in a semidry electroblotting apparatus (Bio-Rad). Blots were preblocked overnight at 4°C in a blocking solution {TBST buffer (Tris-buffered saline [TBS] [10 mM Tris-HCl, 150 mM NaCl], 0.05% Tween 20) containing 5% (w/v) dried nonfat milk, 0.05% sodium azide, 2% bovine serum albumin, 1% normal goat serum}. After blocking, the blots were incubated for 1 h with anti-COX-2 antibody (diluted 1 : 400) at room temperature for 1 h, and then incubated for 1 h at room temperature with anti-mouse IgG-HRP (diluted 1 : 500). Thereafter, color development was achieved by incubation with DAB solution.

Human pancreatic cancer cells in nude mice

We purchased 10 specific pathogen-free six-week-old female BALB/C-nu/nu mice from the Cancer Research Center of Shanghai. The animals were maintained under specific pathogen-free conditions using a laminar air-flow rack in germ-free animal research center of Fudan University and had continuous free access to sterilized food (gamma ray-irradiated food) and autoclaved water. Experiments were started after 1 week of acclimatization. To produce xenograft tumors, cultured human pancreatic cancer cells (Panc-1) were trypsinized, washed, and resuspended in PBS, then 10 × 10⁶ cells (Panc-1) were inoculated subcutaneously into the upper right of mice. Two weeks after inoculated later, 10 nude mice were classified into two groups randomly, the control group and the celecoxib group. Previous study has shown that administration of 1,500 ppm celecoxib significantly inhibited azoxymethane (AOM)-induced colon tumorigenesis in rats (Reddy et al. 2000). So in our study the celecoxib group was fed with the feed containing 1,500 ppm celecoxib and the control group was fed with common feed alone. The sizes of the growing tumors were measured using a caliper weekly. Tumors were harvested 3 months after drug treatment. The lengths (L), the widths (W), and the heights (H) of the tumors were measured using a caliper and the volumes of tumors were calculated by using the formula of V = π (LWH)/6.

Statistical analysis

Continuous variables were expressed as means ± S.E. and a non-paired Student’s t-test was used for statistical evaluation. Categorical variables were expressed as proportions, and comparisons between groups were by χ² tests. The criterion for statistical significance was taken as p < 0.05.

RESULTS

COX-2 expression in paraffin-embedded tissue samples

Expression of COX-2 protein was investigated in paraffin-embedded tissue samples of 53 patients of pancreatic adenocarcinomas by immunohistochemistry (Fig. 1). COX-2 immunoreactivity was detected in 36 of 53 cases (67.9%) of the pancreatic adenocarcinomas. All pancreatic ductal epithelial cells and acinar cells in the nearby normal pancreatic tissues apart from neoplastic tissues are negative for COX-2, but the normal islet cells display a weakly positive staining. COX-2 immunoreactivity localized almost exclusively to the neoplastic cells, whereas the stroma of the tumors was negative. The staining pattern of COX-2 was of diffuse cytoplasmic type with occasional perinuclear staining. PCNA-LI (PCNA labeling index) of the COX-2 positive group
(24.56 ± 11.51%) is different significantly from that of the COX-2 negative group (32.88 ± 13.26) (p < 0.05).

**COX-2 expression in frozen tissue samples and cell line**

COX-2 protein was examined in total protein from Panc-1 cell line, one pancreatic cancer tissue and normal pancreatic tissue by immunoblotting analysis. Undetectable levels of COX-2 protein were observed in the normal pancreas tissue. In contrast, the COX-2 protein was detected in pancreatic cancer tissue and Panc-1 cell line as a single band corresponding to a molecular size of 72 kDa, which is compatible with the molecular size of COX-2 (Fig. 2). To determine whether transcription of COX-2 gene was increased RT-PCR analysis was used to examine the relative levels of COX-2 mRNA in upper tissues and cell line. The cancer tissue and Panc-1 cell expressed a 232-bp band for COX-2, and the levels of COX-2

![Fig. 1. Immunohistochemistry staining for COX-2 or PCNA in paraffin-embedded human pancreatic cancer tissues. A: normal pancreatic tissues (islet cells display a weak staining, whereas epithelial cells of the pancreatic duct and acinar cells are negative for COX-2) (× 200). B: pancreatic adenocarcinoma cells show strong cytoplasmic staining for COX-2 (× 400). C: pancreatic adenocarcinoma cells display strong perinuclear cytoplasmic staining for COX-2 (× 400). D: immunohistochemistry staining for PCNA in pancreatic cancer tissues (× 400). COX-2, cyclooxygenase-2; PCNA, proliferating cell nuclear antigen.](image)

![Fig. 2. western blotting analysis of COX-2: M, mol-wt marker; A: normal pancreas tissue; B: pancreatic cancer tissue; C: Panc-1 cell line.](image)

![Fig. 3. RT-PCR analysis of COX-2 mRNA: M, DNA marker BL2000; A: Panc-1 cell line; B: pancreatic cancer tissue; C: normal pancreas tissue.](image)
mRNA were significantly elevated when compared with normal pancreatic tissue (Fig. 3).

**The expression levels of COX-2 in pancreatic cancer correlate with clinicopathologic features**

As shown in Table 1, the expression of COX-2 was significantly increased in pancreatic cancer in tumor > 3 cm in size \((p < 0.05)\), but it was not associated with the age, gender, tumor location, differentiation and lymph-node metastases and TNM stage.

**Inhibition of cell growth by NS398 and celecoxib**

The effects of Celecoxib and NS398 on cell proliferation are in Fig. 4. Cell viability was assessed by MTT method. The results showed that 3 days of exposure to Celecoxib or NS398 greatly reduced the number of rapidly proliferating Panc-1 cells \((p < 0.05)\) and cell proliferation was suppressed dose-dependently. The concentrations of PGE2 in culture medium of Panc-1 cell line treated with Celecoxib or NS398 are shown in Fig. 4. The production of PGE2 in Panc-1 cell was reduced dose-dependently and the levels of PGE2 are correlated with the inhibition of cell proliferation significantly \((p < 0.05)\).

**Effect of celecoxib on tumor growth**

Fig. 5 shows the effect of celecoxib on the growth of human pancreatic cancer xenografts in nude mice. Female nude mice were implanted with Panc-1 cell on day 0, and the feed containing 1,500 ppm celecoxib was administrated from day 7. Treatment with celecoxib had no significant effect on the body weight of the animals (data not shown). In vivo, the results of the efficacy trials of celecoxib in Panc-1 cell xenografts growing in nude mice are shown in Fig. 6A and the tumor weights at the termination of treatment are shown in Fig. 6B. The results showed that oral adminis-

<table>
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* \(\chi^2\) tests, \(p < 0.05\).
Fig. 4. Effect of COX-2 inhibitors on the Panc-1 cell line. A: inhibition effect by celecoxib; B: inhibition effect by NS398; C: productions of PGE2 of Panc-1 cells by celecoxib; D: productions of PGE2 of Panc-1 cells by NS398.

Fig. 5. Nude mice xenograft tumor modal. A: BALB/C-nu/nu nude mice xenograft tumor modal; B: harvested Panc-1 cell xenograft tissues; (including control group [a] and celecoxib group [b]).
tration of celecoxib suppressed tumor growth in nude mice significantly ($p < 0.01$). On day 90, the volume of the tumor was significantly smaller in the group treated with celecoxib than in the control group treated with common food. Comparison to the control group, tumor volume was suppressed by 51.6% in the celecoxib group.

**DISCUSSION**

Incidences of cancer of digestion system organs have been reported to be lower in users of COX inhibitors, such as aspirin, than in control subjects not using the agents (Rayyan et al. 2002). Although nonselective COX-2 inhibitors have been found to inhibit growth of many types of cancer cells as well as selective inhibitors, accumulating evidence indicates that COX-2 plays more important role in carcinogenesis than COX-1. Inhibitors of this enzyme have been reported to lower the incidence of colon tumors induced by chemical carcinogens in rodent models (Oshima et al. 1996; Kanwar et al. 2007). Since COX-2 expression has been reported in human pancreatic cancer (Koshiba et al. 1999; Stoeltzing et al. 2007), here we describe a link between inhibition of pancreatic cancer growth and selective inhibition of the COX-2 pathway.

In the present study we found that COX-2 is overexpressed in 67.9% of human pancreatic adenocarcinomas as detected by immunohistochemistry, which is accompanied by increased mRNA levels determined by RT-PCR. The pattern of the COX-2 immunoreactivity in pancreatic adenocarcinomas was of diffuse cytoplasmic type with occasional perinuclear staining. The explanation and interpretation of COX-2 expression in pancreatic cancer is still uncertain but may be due to stimulation of some inflammatory factors from infiltrating mononuclear cells or fibroblasts, some tumor promoters and genetic alterations during the carcinogenesis of pancreas, such as $ki-ras$, $APC$ and $P53$ whose mutation are often found in pancreatic cancer (Almoguera et al. 1988; Yamaguchi et al. 2005). Recent studies have suggested that Ras activation, $APC$ absence, dysfunctional $p53$ or insulin-like growth factor-1 receptor system may induce COX-2 expression in several systems (Gilhooly et al. 1999; Dobbie et al. 2002; Stoeltzing et al. 2007). The mutation of $ki-ras$ is an early event during the carcinogenesis of pancreas, so the results suggest the COX-2 protein might participate in the carcinogenesis of pancreas.

There are several mechanisms by which COX-2 participates in the growth of malignant tumors including promotion of proliferation, inhibition of apoptosis, increased metastatic potential and promotion of angiogenesis (Elder et al. 2002; Nagatsu et al. 2002; Lev-Ari et al. 2007). But proliferation of tumor cells causes expansion of
tumor volume at initial stage. In the present study, we found the expression of COX-2 was associated with tumor size, which suggests that COX-2 may stimulate cell proliferation in pancreatic cancer. Immunohistochemical detection of PCNA is a convenient and commonly used method to grade proliferative activity in various tissues. PCNA expression is closely correlated with the S-phase of the cell cycle. Our study shows the PCNA-LI of COX-2 positive group is significantly higher than COX-2 negative group, which also supported the hypothesis that COX-2 protein may promote proliferation of pancreatic cancer cells. To study whether inhibition of COX-2 pathway can block the proliferation of pancreatic cancer cells in vitro, we investigated the effects of NS-398 and celecoxib on PANC-1 cell line, which was proved to be COX-2-positive in this study in advance. NS-398 and celecoxib as selective inhibitors of COX-2 have been demonstrated to inhibit activity of COX-2 strongly and have little effect on COX-1 (Banu et al. 2007; Frampton and Keating 2007). The results showed that cell proliferation was inhibited by treatment with celecoxib and NS-398. The PANC-1 cell line was more sensitive to celecoxib compared with NS-398, suggesting that celecoxib may be more selective for COX-2 than NS-398. There is no enough clinical data on the optimum concentration of celecoxib required to show an antitumor effect. In prior studies, celecoxib inhibited cancer cell dose-dependently and high dose of celecoxib was still safe to patients (Steinbach et al. 2000). In present study, we found celecoxib above 20 μmol/l in vitro could inhibit the growth of pancreatic cancer cell evidently, which suggested the celecoxib concentration could reach the level of inhibiting tumor without unacceptable side effects in vivo. At the same time, the concentrations of PGE2 in culture medium of Panc-1 cells were reduced dose-dependently. Thus, PGE2 produced in Panc-1 cells is likely to be derived from COX-2, because this production can be decreased by selective inhibition of COX-2. And the inhibition of COX-2-derived PGE2 production was closely associated with inhibition of proliferation (Ito et al. 2004). Previous studies implicated that PGE2 enhanced pancreatic cancer invasiveness through an Ets-1-dependent induction of matrix metalloproteinase-2 (Mutoh et al. 2002), and overexpression of COX-2 increased tumor cell EP4 receptor expression and thus may increase sensitivity to the autocrine and paracrine effects of PGE2 itself (Dohadwala et al. 2002). The present results suggest the COX-2-derived prostaglandins production might stimulate the proliferation of Panc-1 cell line by autocrine and paracrine, which must be demonstrated in further study. Our study also showed that celecoxib could induce apoptosis on Panc-1 cells in vitro at 100 μmol/l concentration, which was proved by using electromicroscopy (data not shown). So we used celecoxib to research whether inhibition of COX-2 can suppress tumor growth during expansion of tumor volume at initial stage of pancreatic cancer xenografts in vivo. Our results indicate that oral administration of celecoxib significantly suppresses the growth of xenografts of Panc-1. In comparison to the control group, tumor volume was suppressed by 51.6% in the celecoxib group. The data indicate that COX-2 expression plays an important role in the growth of pancreatic cancer xenografts.

In conclusion, COX-2 gene is dramatically upregulated in human pancreatic adenocarcinomas, and specific inhibition of COX-2 suppressed cell proliferation and growth of tumor volume of the human pancreatic cancer in vitro and in nude mice.

References


Cyclooxygenase-2 in Pancreatic Cancer


