

Potential Involvement of the Cyclooxygenase-2 Pathway in the Regulation of Tumor-associated Angiogenesis and Growth in Pancreatic Cancer¹

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Abstract

Angiogenesis plays a crucial role in tumor development and growth. The present investigation was undertaken to test the potential involvement of the cyclooxygenase-2 (COX-2) pathway in the regulation of angiogenesis and growth in pancreatic cancer. We compared the angiogenic characteristics of a COX-2-positive human pancreatic tumor cell line, BxPC-3, with those of a COX-2-negative pancreatic tumor cell line, AsPC-1. Cultured BxPC-3 cells promoted a marked increase of endothelial cell migration in comparison with migration that occurred in the absence of cancer cells. Furthermore, BxPC-3 cell culture supernatants induced endothelial cell capillary morphogenesis *in vitro* and neovascularization *in vivo*. In contrast, cultured AsPC-1 cells elicited a modest effect on endothelial cell migration and neovascularization *in vivo*. Pretreatment of BxPC-3 cells with the selective COX-2 inhibitor NS-398 (50 μM) dramatically decreased angiogenic responses of endothelial cells. NS-398 (25–100 μM) caused inhibition of BxPC-3 cell proliferation but had no effect on AsPC-1 cell growth. SC-560, a selective COX-1 inhibitor, had no effect on growth of either cell lines. These results suggest an involvement of COX-2 in the control of tumor-dependent angiogenesis and growth in certain pancreatic cancers and provide the rationale for inhibition of the COX pathway as an effective therapeutic approach for pancreatic tumors.

Introduction

Carcinoma of the pancreas is the fourth leading cause of cancer-related death in the United States (1). Surgical resection in combination with radiation therapy and/or chemother-

apy is the standard treatment for this tumor (2). However, despite treatment, the majority of patients succumb to metastatic disease within the first year of diagnosis (3). The failure of conventional treatments to alter the course of this tumor underscores the importance of developing alternative approaches aimed at targeting molecular events involved in pancreatic tumorigenesis (4).

Prostaglandin endoperoxide synthase (COX³) is the rate-limiting enzyme involved in the oxidative transformation of arachidonic acid into prostaglandin H₂, which represents the precursor of several bioactive molecules, including prostaglandin E₂, prostacyclin, and thromboxane (5). Two different isoenzymes, COX-1 and COX-2, have been identified (6). COX-1 is constitutively expressed in most tissues, whereas expression of COX-2 is induced in response to a variety of stimuli (7, 8). Overexpression of COX-2 is detected in several tumors (9–12). In addition, COX-2 gene deletion in mice carrying deletion of the adenomatous polyposis gene markedly reduced intestinal polyposis (13). Moreover, recent work by Liu *et al.* (14) showed that overexpression of COX-2 leads to mammary tumors. These findings suggest a mechanistic link between COX-2 expression and tumorigenesis (8). In addition, nonsteroidal antiinflammatory drugs decrease the relative rate of human colorectal cancer, suggesting that COX-2 represents an attractive target for cancer prevention (15, 16).

One of the mechanisms by which COX-2 supports tumorigenesis is by stimulating angiogenesis, the formation of new blood vessels from existing vasculature, a crucial process for tumor growth and expansion (17, 18). Thus, tumor growth is markedly reduced in COX-2 (–/–) mice compared with wild-type or COX-1 (–/–) animals (19). Moreover, celecoxib, a selective COX-2 inhibitor, blocks neovascularization in the corneal micropocket assay (18).

COX-2 is overexpressed in the majority of human primary pancreatic carcinomas, irrespective of histological type and grade (20–23). In contrast, benign pancreatic tumors do not express COX-2 (23). These findings suggest that COX-2 represents a marker for the malignant potential of pancreatic cancer. However, the significance and therapeutic ramifications of COX-2 expression by pancreatic tumors remain unclear. In the present study, we compared COX-2-positive and COX-2-negative pancreatic tumor cell lines to evaluate the involvement of the COX-2 pathway in angiogenesis and growth of pancreatic cancer.

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³ The abbreviations used are: COX, cyclooxygenase; EC, endothelial cell; FBS, fetal bovine serum; BAEC, bovine aortic endothelial cell; PGE₂, prostaglandin E₂ enzyme; CM, conditioned medium; VEGF, vascular endothelial growth factor.

Materials and Methods

Chemicals. Growth factor-reduced Matrigel was purchased from Becton Dickinson Labware (Bedford, MA). Transwell chemotactic chambers were purchased from Corning Costar Corp. (Cambridge, MA). NS-398 and SC-58125 were purchased from Calbiochem (San Diego, CA). SC-560 was obtained from Pharmacia Corp. (St. Louis, MO). Indomethacin was purchased from Sigma Chemical Co. (St. Louis, MO). The colorimetric proliferative assay kit was from Promega (Madison, WI). RPMI 1640 tissue culture medium was purchased from Bio-Whittaker (Walkersville, MD). EC tissue culture medium and EC attachment factor were purchased from Cell Systems (Kirkland, WA). FBS was purchased from Hyclone (Logan, UT). VEGF and PGE₂ immunoassays were purchased from Neogen Corp. (Lansing, MI). Diff-Quick Stain kit was purchased from Dade Behring, Inc. (Newark, DE). Antivon Willebrand factor (Factor VIII-related antigen) antibody and the catalyzed signal amplification system were purchased from DAKO Corp. (Carpinteria, CA).

Cell Culture. BxPC-3 and AsPC-1 cell lines were both generated from patients with pancreatic adenocarcinoma (24, 25) and obtained from American Tissue Culture Collection (Rockville, MD). BxPC-3 and AsPC-1 cells were maintained in RPMI 1640 containing 100 μ g/ml penicillin G and 100 mg/ml streptomycin and supplemented with 10 and 20% FBS, respectively. Cells were used when confluent. BAECs and human umbilical vein endothelial cells were purchased from Cell Systems and maintained as described previously (26). Cells were used between passages 4 and 12. The CM was generated by the addition of the corresponding serum-free medium to 80–90% to confluent BxPC-3 and AsPC-1 cells. At 24 h of incubation, medium was collected, subjected to low-speed centrifugation to remove detached cells and debris, and kept at -70°C until further use.

In Vitro Angiogenesis. The ability of BxPC-3 and AsPC-1 cells to support angiogenesis was tested *in vitro* by assaying migration and morphogenesis into capillary-like structures of ECs when cocultured with cancer cells. Migration assays were performed using a modified procedure of the method that we described previously (27). Briefly, tumor cells were grown in the bottom compartment of the Transwell chamber. Before the migration assay, cell monolayers were washed, and supernatants were replaced with serum-free media. BAECs (2.5×10^5) were placed in the top compartment of the Transwell chamber on polycarbonate filters (8- μ m pore size) coated with attachment factor and allowed to migrate. After incubations, filters were removed, and nonmigrated ECs were scraped off from the top of the filter. ECs that migrated through the filter were visualized and quantitated as described previously (27).

Tumor-induced capillary morphogenesis of ECs was assayed using a modified coculture assay system described by Tsujii *et al.* (28). Tumor cells were seeded at a density of 4×10^6 on polycarbonate filters (0.4- μ m pore size) and allowed to grow for 24 h, at which time they covered the entire surface of the filter. Thereafter, the medium was removed, cell monolayers were gently washed, and serum-free medium was added to each filter. Filters were introduced on top of six-well plates containing a thin layer of Matrigel onto

which BAECs were seeded at a density of 5×10^5 /ml in serum-free medium. Cocultures were incubated at 37°C and 5% CO₂ for 72–96 h. The presence of tube-like structures was assessed by light microscopy.

In Vivo Angiogenesis Assay. Tumor-induced angiogenesis was assayed *in vivo* as described by Passaniti *et al.* (29). Growth factor-reduced Matrigel (0.4 ml) was premixed with vehicle or CM derived from BxPC-3 and AsPC-1 cells grown in the absence or presence of NS-398 and injected in the flank of 5–6-week-old C57/BL/6N mice (Harlan, Indianapolis, IN). At 7 days, animals were sacrificed, and plugs were harvested, fixed, and embedded in paraffin. Sections of 5- μ m thickness were deparaffinized, rehydrated, and stained with H&E (29). Selected sections were subjected to immunohistochemistry using standard techniques (29). The primary antibody consisted of a polyclonal rabbit antihuman von Willebrand factor or Factor VIII-related antigen IgG (1:50 dilution; Ref. 30). The secondary antibody consisted of a polyclonal donkey antirabbit IgG (1:100 dilution). For negative controls, the primary antibody was replaced by a non-specific IgG. Visualization of antigen–antibody complexes was performed with the streptavidin–peroxidase staining kit following the manufacturer's instructions. Slides were counterstained with H&E. Angiogenesis was quantified by direct counting of vessels containing RBCs. Each treatment group included three animals.

Measurement of VEGF and PGE₂ Levels from Supernatants of Cultured Tumor Cells. VEGF and PGE₂ levels were measured in cell supernatants by enzyme immunoassay according to the manufacturer's instructions. Results are expressed as picograms of VEGF and PGE₂ released in the supernatant and normalized for protein concentration measured by the method of Bradford using BSA as a standard (31).

Cell Proliferation. Proliferation was determined by the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt] assay as described previously (32). Briefly, cells (2.5×10^4 /ml) were seeded in 24-well plates and allowed to grow overnight. Cells were incubated in medium containing 1% FBS. COX-1 and COX-2 inhibitors were dissolved in DMSO. The final concentration of DMSO was 0.1%. Equal volumes of DMSO were added to control cultures. Cells grown in medium supplemented with 10% FBS were used as internal positive controls.

Statistical Analysis. Data were analyzed using the InStat Software program (GraphPad Software, San Diego, CA). Student's *t* test or one-way ANOVA was used. Values are expressed as the mean \pm SD. *P*s \leq 0.05 were considered significant.

Results

Effect of COX-2 Expression and Inhibition on Pancreatic Tumor-associated Angiogenesis. To investigate the potential involvement of the COX-2 pathway in pancreatic cancer-induced angiogenesis, we used the BxPC-3 cell line, a human pancreatic tumor cell line, which constitutively expresses COX-2 (20, 33, 34). The COX-2-negative human pancreatic tumor cell line, AsPC-1, was used as a negative control (20, 33). We confirmed that BxPC-3 cells released

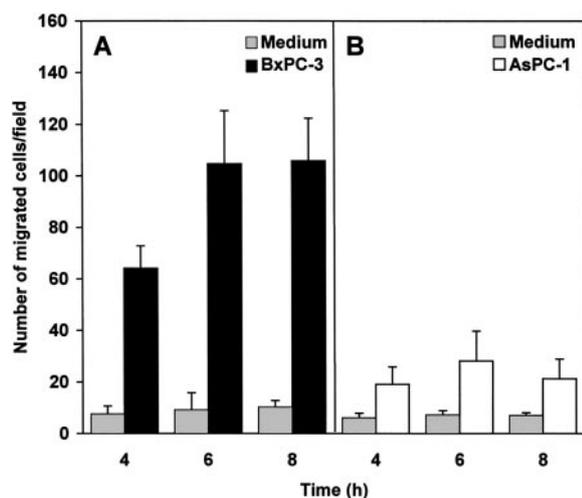


Fig. 1. Effect of COX-2 expression on pancreatic tumor-induced migration of ECs. BAECs were plated in the top compartment of the Transwell chamber and allowed to migrate for the indicated time intervals to the COX-2-expressing BxPC-3 cells (A) or the COX-2-negative cell line AsPC-1 (B), grown in the bottom compartment of the Transwell chamber. Migration assays were performed as described in "Materials and Methods." The data represent the mean number \pm SD of ECs migrated to the bottom side of the filter from triplicate determinations of a representative experiment.

markedly high levels of PGE₂ than did AsPC-1 cells (3327 ± 634 pg/mg protein versus 212 ± 47 pg/mg protein; $P < 0.01$) using an enzyme immunoassay. We hypothesized that COX-2 expression conferred to BxPC-3 cells the ability to stimulate angiogenesis and furthermore that pharmacological inhibition of the COX-2 pathway was able to inhibit this response. To test this possibility, we investigated the ability of BxPC-3 cells to stimulate EC migration, a crucial step in the series of events leading to angiogenesis (35). We used a modified migration assay in which factors released by BxPC-3 cells, cultured in the bottom compartment of Transwell plates, functioned as chemoattractants for BAECs residing on filters in the top compartment of Transwell chambers (27). Exposure of ECs to BxPC-3 induced 8.5 ± 0.3 ($P < 0.001$), 11.4 ± 0.3 ($P < 0.001$), and 10.5 ± 0.2 ($P < 0.001$)-fold increase of migration compared with ECs exposed to serum-free medium containing no cancer cells at 4, 6, and 8 h, respectively (Fig. 1A). In contrast, the COX-2-negative cell line AsPC-1 stimulated EC migration by 3.1 ± 0.2 ($P > 0.05$), 3.8 ± 0.1 ($P > 0.05$), and 3 ± 0.1 ($P > 0.05$)-fold at 4, 6, and 8 h of stimulation (Fig. 1B). CM derived from BxPC-3 cells also stimulated EC migration (data not shown). Similar to BAECs, human umbilical vein endothelial cell migration was enhanced when cocultured with BxPC-3 cells (data not shown).

To determine whether the migratory response of ECs was linked to COX-2 expression, BxPC-3 cells were pretreated with NS-398, a selective COX-2 inhibitor (36). Before assessment of EC migration, we determined that NS-398 markedly attenuated the release of PGE₂ by BxPC-3 cells (3327 ± 634 pg/mg protein, untreated cells; 428 ± 71 pg/mg protein, NS-398-treated cells; $P < 0.01$). Pretreatment of BxPC-3 with NS-398 ($50 \mu\text{M}$) significantly ($P < 0.05$) decreased EC migration by $68\% \pm 17.6$ compared with migration of ECs

exposed to vehicle-treated BxPC-3 (Fig. 2A). In contrast, NS-398 had no effect on AsPC-1-dependent EC migration (Fig. 2B). The addition of 1, 2.5, and $5 \mu\text{M}$ PGE₂ to BxPC-3 cells treated with NS-398 ($50 \mu\text{M}$) restored 17, 43, and 46% of EC migration, respectively (Fig. 2C).

We next investigated the ability of BxPC-3 cells to promote EC differentiation. For this purpose, ECs seeded onto Matrigel were cocultured with cancer cells grown on a filter. This system allows factors produced by the cancer cells to diffuse and influence responses of underlying ECs. ECs cocultured with filters containing serum-free medium with no cancer cells failed to differentiate into tube-like structures (Fig. 2D1). In contrast, ECs cocultured with BxPC-3 cells differentiated into structures resembling blood vessels *in vitro* (Fig. 2D2). The ability of BxPC-3 cells to induce differentiation of ECs was completely abrogated by continuous exposure of BxPC-3 cells to NS-398 ($50 \mu\text{M}$; Fig. 2D3). Taken together, these findings suggest that expression of COX-2 in BxPC-3 cells is linked to their ability to promote migration and *in vitro* differentiation of ECs.

Pancreatic Tumor Cell-induced Angiogenesis *in Vivo*.

Next, we used the mouse Matrigel plug assay, an established *in vivo* angiogenesis model, to determine whether BxPC-3 cells stimulated neovascularization *in vivo*. Control plugs, containing Matrigel mixed with vehicle, showed few blood vessels (Fig. 3A). In contrast, a 10 ± 0.1 -fold increase of the number of blood vessels invading the plug was detected in Matrigel plugs containing 20% CM derived from BxPC-3 cells compared with control plugs ($P < 0.001$; Fig. 3A). Moreover, a 2.9 ± 0.2 -fold increase of the number of blood vessels was detected in plugs of Matrigel mixed with CM derived from AsPC-1 compared with control plugs ($P > 0.05$; Fig. 3A). Pretreatment of BxPC-3 cells with NS-398 ($50 \mu\text{M}$) completely abrogated the ability of CM to stimulate neovascularization (Fig. 3A). In contrast, NS-398 ($50 \mu\text{M}$) had no effect on the angiogenic response stimulated by CM derived from AsPC-1 cells (Fig. 3A). Representative sections of Matrigel plugs are shown (Fig. 3, B–D).

We next undertook preliminary experiments to determine whether stimulation of angiogenesis by the COX-2-positive cells resulted from a direct effect of COX-2 products on ECs, rather than an indirect effect, caused by the release of angiogenic factors by the cancer cells. Previous studies showed increased expression of VEGF in pancreatic tumor cells (37). Hence, we measured VEGF protein levels in BxPC-3 and AsPC-1 cell supernatants. Supernatants from the COX-2-positive BxPC-3 cells had higher levels of VEGF protein compared with supernatants from the COX-2-negative cells, AsPC-1 ($P < 0.001$; Fig. 4). Moreover, treatment with NS-398 partially decreased VEGF release from BxPC-3 cells ($P < 0.05$) but had no effect on AsPC-1 cells.

Effect of Inhibitors of the COX Pathway on Growth of Pancreatic Cancer Cell Lines.

Next, we investigated the effect of pharmacological inhibition of COX-2 on BxPC-3 and AsPC-1 cell growth. Treatment of BxPC-3 cells with 25, 50, and $100 \mu\text{M}$ NS-398 induced $30\% \pm 7.2$ ($P < 0.01$), $33\% \pm 6$ ($P < 0.01$), and $50\% \pm 7.2$ ($P < 0.001$) inhibition of cell growth, respectively, but had no effect on AsPC-1 cell proliferation (Fig. 5A). PGE₂ did not restore NS-398-induced

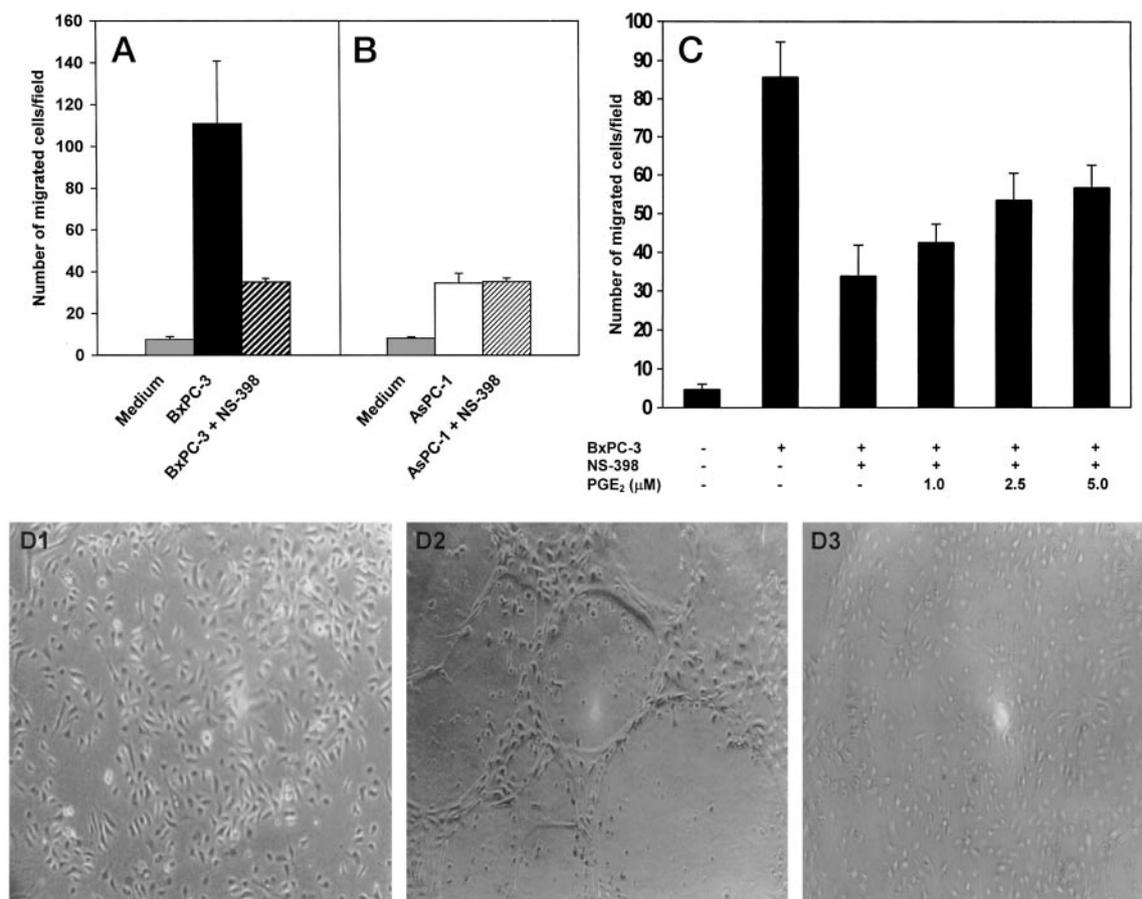


Fig. 2. Effect of COX-2 inhibition on pancreatic tumor-induced EC migration and *in vitro* differentiation. In **A**, BxPC-3 cells were grown in the bottom compartment of a Transwell chamber and treated with vehicle or NS-398 (50 μM). Migration of BAECs was evaluated by comparing the number of ECs migrated to medium alone with those migrated to BxPC-3 cells and quantified as described in "Materials and Methods." The data shown are mean \pm SD from triplicate determinations of a representative experiment. In **B**, BAECs were exposed to AsPC-1 cells, and migration was assayed as described above. Results are the mean \pm SD from triplicate determinations of a representative experiment. In **C**, increasing concentrations of PGE₂ were added to BxPC-3 cells treated with NS-398 (50 μM), and BAEC migration was assayed as described above. Results are the mean \pm SD of triplicate determinations of a representative experiment. In **D**, BAECs were plated on top of a thin layer of growth factor-reduced Matrigel in serum-free medium. BxPC-3 cells were cultured on polycarbonate filters in serum-containing medium. Thereafter, cells were incubated in serum-free medium with either vehicle or NS-398 (50 μM). BxPC-3 cells were then introduced on top of ECs plated on Matrigel and incubated at 37°C and 5% CO₂ for 96 h. Tube formation was assessed by light microscopy. **D1**, ECs cocultured with medium only. **D2**, ECs cocultured with BxPC-3 cells. **D3**, ECs cocultured with BxPC-3 cells in the presence of NS-398 (50 μM).

inhibition of BxPC-3 cell proliferation (data not shown). SC-560, a selective COX-1 inhibitor (38), had no effect on the growth of either cell lines (Fig. 5B).

Discussion

Pancreatic cancer remains a challenge in clinical oncology because of its poor prognosis and lack of effective treatment (1–3). A better understanding of the molecular events and biological behavior of pancreatic cancer cells should facilitate the development of novel strategies that hold the potential of improving the current survival rates.

Inhibition of angiogenesis has become a promising approach for the treatment or prevention of many human malignancies (35). Although pancreatic tumors appear to be hypovascular on radiological findings, there is indirect evidence suggesting a potential role for angiogenesis in pan-

creatic tumor growth and invasion (39). Studies by Kuehn *et al.* (40) detected up-regulation of angiogenic growth factor production in chronic pancreatitis and pancreatic cancer. Moreover, inhibitors of angiogenesis decreased metastasis in animal models of pancreatic cancer (41, 42). Furthermore, blockade of the COX-2 pathway was shown to interfere with the growth potential of pancreatic tumor cells (43–45). However, studies to directly assess the ability of pancreatic tumor cells to stimulate angiogenic responses of ECs and the involvement of the COX-2 pathway in these responses are lacking.

In the present study, using a combination of *in vitro* and *in vivo* assays, we show that pancreatic tumor cells stimulate angiogenesis. Moreover, we show that this response is linked to the expression of COX-2. Thus, EC migration and differentiation into tube-like structures as well as neovascu-

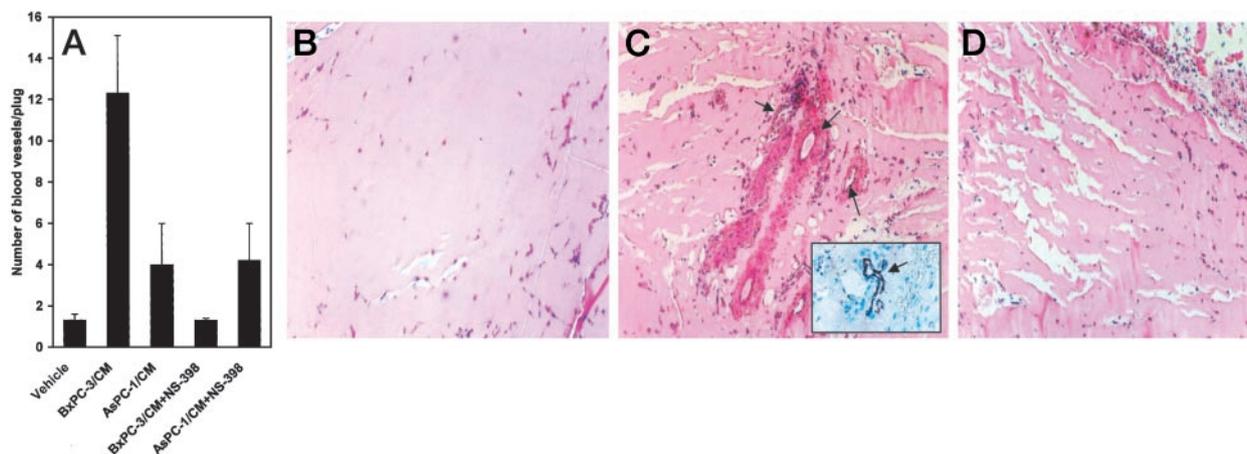


Fig. 3. Pancreatic tumor-induced *in vivo* angiogenesis: effects of COX-2 expression and COX-2 inhibition. **A**, quantification of neovessels in fixed Matrigel plugs. Sections of Matrigel plugs were stained with H&E, and the number of blood vessels from five high power fields was counted. Results are expressed as the mean \pm SD of the number of blood vessels detected in each plug from triplicate determinations of two independent experiments. **B**, representative section of Matrigel plugs mixed with vehicle. **C**, representative section of Matrigel plugs mixed with CM derived from BxPC-3 cells. *Inset* in **C** refers to high magnification of ECs lining blood vessels and stained positive with anti-Factor VIII antibodies. **D**, representative section of Matrigel plugs mixed with CM derived from AsPC-1 cells.

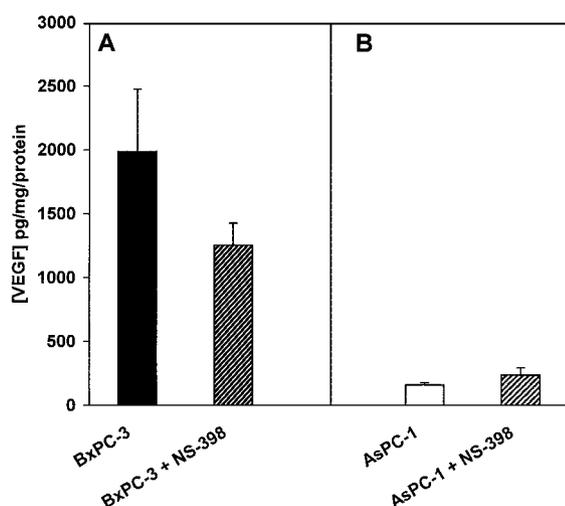


Fig. 4. Effect of COX-2 inhibition on production of VEGF protein in culture medium of BxPC-3 and AsPC-1 cells. Confluent cells were cultured for 24 h in serum-free medium containing NS-398 (50 μ M) or vehicle. Supernatants were collected, and VEGF protein levels were measured by enzyme immunoassay. Results are the mean \pm SD from triplicate determinations of a representative experiment.

larization *in vivo* were greatly stimulated by the COX-2-expressing pancreatic tumor cells compared with the angiogenic response of ECs elicited by the COX-2-negative AsPC-1 cell line. Selective inhibition of COX-2 enzymatic activity by NS-398 completely impaired the ability of the COX-2-expressing cells to stimulate angiogenesis. Taken together, these findings suggest a link between COX-2 expression and angiogenesis in pancreatic cancer.

In the coculture model used in this study, cancer cells and ECs are not in direct contact. Therefore, the angiogenic response induced in ECs by the overlying cancer cells can be attributed to the release of angiogenic factors from cancer

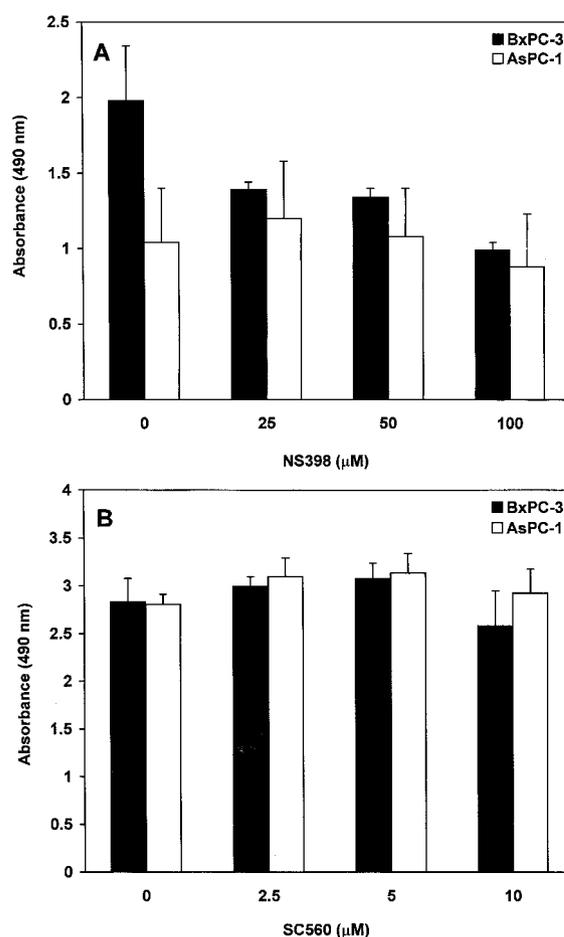


Fig. 5. Effect of COX inhibitor on pancreatic tumor cell growth. Cells were plated (2.5×10^4 /ml) in 24-well plates in serum-containing medium. After overnight incubation at 37°C and 5% CO₂, monolayers were incubated with NS-398 (**A**) or SC-560 (**B**) at the indicated concentrations. Proliferation was evaluated at 96 h by the MTS assay. Results are the mean \pm SD of triplicate determinations from a representative experiment.

cells. Studies by Yip-Schneider *et al.* (33) showed that BxPC-3 cells produce PGE₂ under basal conditions of growth and that treatment with NS-398 reduced PGE₂ release. Thus, PGE₂ represents a likely candidate for the angiogenic response detected in ECs exposed to the COX-2-positive pancreatic tumor cell line. Accordingly, we observed that the addition of PGE₂ to NS-398-treated BxPC-3 partially restored BxPC-3 cell-dependent EC migration. Moreover, a greater production of PGE₂ was detected in the COX-2-expressing BxPC-3 cells compared with AsPC-1 cells. Although additional mechanisms are involved in mediating the angiogenic effects of COX-2, our results suggest that COX-2 influences BxPC-3-induced angiogenic responses of ECs, at least in part, by enhancing the release of VEGF. Additional investigations are needed to fully elucidate the complex events involved in COX-2-mediated angiogenesis in pancreatic cancer. The present results wherein responses of cells that naturally expressed levels of COX-2 protein were compared with those of cells lacking COX-2 must be interpreted with caution because of the potential limitation of the system. Thus, the tumor cell lines used in the present study are not isogenic and therefore may possess differences in addition to COX-2 expression, which may influence their effects on the angiogenic responses of ECs.

In this study, we found that NS-398 (50 μM) completely abrogated tumor-induced neovascularization *in vivo* but had a marginal inhibitory effect on tumor growth, suggesting that COX-2 is preferentially involved in mediating the angiogenic effects of BxPC-3 rather than affecting directly their growth. It is possible that the antiproliferative effect of NS-398 on BxPC-3 cells is independent of COX-2 expression or prostaglandin production as suggested by other studies (33, 46). The extent and mechanism of the divergent effects of COX-2 on angiogenesis and cell growth in BxPC-3 cells remain to be investigated.

In summary, we demonstrated that expression of COX-2 in pancreatic tumor cells is linked to angiogenesis. Moreover, pharmacological inhibition of COX-2 decreases tumor-induced angiogenic responses of ECs *in vitro* as well as *in vivo*. These findings suggest an involvement of the COX-2 pathway in pancreatic tumor-associated angiogenesis and provide the rationale for clinical studies aimed at examining the efficacy of COX-2 inhibitors for the treatment or chemoprevention of certain pancreatic tumors.

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