Growth Stimulation of COX-2–Negative Pancreatic Cancer by a Selective COX-2 Inhibitor

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Abstract
Cyclooxygenase 2 (COX-2) inhibitors are promising antiangiogenic agents in several preclinical models. The aim of the present study was to evaluate the effect of selective COX-2 inhibitors on vascular endothelial growth factor (VEGF) production in vitro and angiogenesis and growth of pancreatic cancer in vivo, focusing on putative differences between COX-2–negative and COX-2–positive tumors. VEGF production and angiogenesis in vitro were determined by ELISA and endothelial cell migration assay. To determine whether the effect of COX-2 inhibitors was mediated by peroxisome proliferator–activated receptor γ (PPAR-γ), we used a dominant-negative PPAR-γ and a pharmacologic inhibitor. In vitro findings were validated in a pancreatic cancer animal model. Microvessel density was assessed by CD31 immunostaining. Intratumoral prostaglandin and VEGF levels were measured by mass spectroscopy and ELISA. Selective COX-2 inhibitors had a concentration-dependent effect on VEGF production in vitro. Higher concentrations increased VEGF levels and stimulated angiogenesis by activating PPAR-γ. In vivo, nimesulide increased VEGF production by cancer cells in COX-2–positive and COX-2–negative pancreatic tumors. In COX-2–negative pancreatic cancer, this effect was associated with an increase in angiogenesis and growth. In COX-2–positive pancreatic cancer, the nimesulide-induced increase of VEGF production by the cancer cells was offset by a decrease in VEGF production by the nonmalignant cell types leading to reduced tumor angiogenesis and growth. Selective COX-2 inhibitors had opposite effects on growth and angiogenesis in pancreatic cancer depending on COX-2 expression. These findings imply that assessing the COX-2 profile of the pancreatic tumor is mandatory before initiating therapy with a selective COX-2 inhibitor. (Cancer Res 2005; 65(3): 982-90)

Introduction
Soon after the solution of the cyclooxygenase (COX) crystal structures in the early to mid-1990s (1, 2), an unparalleled structure-based drug development program was started by several pharmaceutical companies, ultimately culminating in the introduction of selective COX-2 inhibitors into the market. These drugs were and for the most part are still heralded as the ultimate weapon against a multitude of human diseases, including cancer, and are currently being tested in several clinical trials as antitumor agents. Targeting COX-2 provides an intriguing, multilayered opportunity to fight cancer, as COX-2 has been causally linked to the suppression of the host immune system, to conferring resistance to apoptosis, to stimulate cancer cell growth and invasion, and to promote angiogenesis (3–6). Most antitumor effects of COX-2 inhibitors are mediated by reducing prostaglandin levels. However, some effects of selective COX-2 inhibitors cannot be explained by simple inhibition of the COX-2 enzyme, as these drugs can also provoke responses in COX-2–negative cells (7–14). Because the pathways activated or inhibited by COX-2 inhibitors in COX-2–lacking cells are largely unknown, the phenotypic changes in these cells are unpredictable.

One putative COX-independent pathway includes peroxisome proliferator–activated receptor γ (PPAR-γ; ref. 15), which belongs to the nuclear hormone receptor superfamily and functions as a ligand-activated transcription factor (16). Recently, a possible role of PPAR-γ in human malignancies (prostate, breast, gastric, lung, and pancreatic cancer) has been implicated (17–21). Although the majority of the published studies imply an antitumor effect of PPAR-γ ligands, some authors have observed an opposite effect with PPAR-γ ligands promoting the development of colon and breast tumors in vivo (22–24). The effect of PPAR-γ activation on angiogenesis is also controversial in the literature. PPAR-γ ligands have been associated with either inhibiting or stimulating angiogenesis (25, 26).

The majority of human pancreatic cancers overexpress COX-2. However, 10% to 40% of pancreatic tumors are considered to be COX-2 negative (27–33). The effect of selective COX-2 inhibitors on the subset of COX-2–negative pancreatic cancer is poorly understood. As the COX-2 profile of the tumor is usually not known before treatment with selective COX-2 inhibitors, the definition of COX-2–independent pathways activated and/or inhibited by COX-2 inhibitors and the resulting phenotypic changes bears tremendous clinical importance. In this context, the aim of the present study was to evaluate the therapeutic effects of selective COX-2 inhibitors on pancreatic cancer angiogenesis and growth, with special emphasis on the putative difference between COX-2–positive and COX-2–negative tumors as well as on the possible involvement of PPAR-γ.

Materials and Methods
Reagents
The PPAR-γ ligands 15-PGJ2 and troglitazone, the irreversible PPAR-γ antagonist GW9662, the selective COX-2 inhibitors nimesulide and DuP697, the rabbit polyclonal antibodies against COX-2 and PPAR-γ were purchased from Cayman Chemicals (Ann Arbor, MI). The mouse monoclonal β-actin antibody was purchased from Sigma Chemical Co. (St. Louis, MO) and rat anti-mouse CD31 antibody was obtained from BD Biosciences Pharmingen

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(San Diego, CA). Gel shift oligonucleotides containing a consensus and mutant PPAR-γ response element (PPRE) and the PPAR-γ and RXRα antibodies for supershift assays were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The full-length PPAR-γ was kindly provided by Alex Elfbrecht (Merck Research Laboratories, Rahway, NJ; ref. 34), pCMV-Luc and prACO;3neo were kindly provided by Syngenta CTL Cell Biology Group (Macclesfield, Cheshire, United Kingdom) and pCMX-mPPARγ was a generous gift from R.M. Evans (Howard Hughes Medical Institute, The Salk Institute for Biological Studies, San Diego, CA). Other reagents were purchased from common commercial sources.

Cell Culture
The human pancreatic cancer cell lines BxPC-3 and MIA PaCa-2, human umbilical vein endothelial cells, and the embryonic mouse fibroblast cell line NIH/3T3 were obtained from the American Type Culture Collection (Rockville, MD). BxPC-3 cells were grown in RPMI 1640 containing 10% fetal bovine serum, penicillin G (100 units/mL), and streptomycin (100 μg/mL) in a humidified atmosphere of 5% CO2 at 37°C. MIA PaCa-2 cells were cultured in DMEM containing 10% fetal bovine serum, penicillin G (100 units/mL), and streptomycin (100 μg/mL) in a humidified atmosphere of 10% CO2 at 37°C. All experiments were carried out using cancer cell lines between passages 7 and 12. Human umbilical vein endothelial cells were grown in endothelial cell basal medium supplemented with 0.1% human epidermal growth factor, 0.1% hydrocortisone, 0.1% gentamicin, 0.1% amphotericin-B, 0.4% bovine brain extract, and 2% fetal bovine serum (Cambrex Bio Science Walkersville, Walkersville, MD) in a humidified atmosphere of 5% CO2 at 37°C. NIH/3T3 cells were grown in DMEM supplemented with 10% BCS, penicillin G (100 units/mL), and streptomycin (100 μg/mL) in 5% CO2 at 37°C.

Vascular endothelial growth factor (VEGF) and PGE2 levels in the culture medium were determined as previously described (33). Briefly, cells were incubated with the indicated inhibitors and ligands for 24 hours in serum-free medium and VEGF and PGE2 levels in the culture medium were measured using the Quantikine Human VEGF Immunoassay (R&D Systems, Inc., Minneapolis, MN) and the PGE2 competitive enzyme immunoassay (Cayman Chemical), respectively, as recommended by the manufacturer. Protein levels were normalized to cell number.

Endothelial Cell Migration
Human endothelial cell migration was examined in a 24-well cell culture insert system (Becton Dickinson Labware, Franklin Lakes, NJ) as described previously (35). Briefly, pancreatic cancer cells (4 × 105) were seeded into each well of a 24-well companion TC plate in complete cell culture medium. After serum starvation for 24 hours, cells were challenged for 24 hours with nimesulide. Human umbilical vein endothelial cells (8 × 103) were then added to the cell culture inserts containing gelatin-coated polyethylene terephthalate track-etched membranes (8-μm pore size, 1 × 103 pores/cm2) and allowed to migrate for 6 hours. Migrated cells on the lower membrane surface were fixed in ice-cold methanol, stained using the Diff-Quick Stain Set (Dade Behring, Newark, DE) and counted.

Dominant-Negative PPAR-γ
A dominant-negative form of PPAR-γ was constructed by mutating the Leu666 and Gln671 of the full-length PPAR-γ into Ala. Mutations at these sites create a dominant-negative form of PPAR-γ (36). This dominant-negative PPAR-γ was subcloned into recombinant type 5 adenovirus and designated as Adx-D/N-PPAR-γ. Recombinant type 5 adenovirus expressing the LacZ gene was generated similarly and used as a control vector (Adx-LacZ). The human pancreatic cancer cell line MIA PaCa-2 was infected with 75 plaque forming units per cell in DMEM containing 0.4% fetal bovine serum.

PPAR-γ Protein Expression
PPAR-γ protein expression was determined as described previously (17). Briefly, total cell lysates were fractionated on 8% SDS-PAGE and transferred to nitrocellulose membrane. PPAR-γ protein was detected using a rabbit polyclonal PPAR-γ antibody (Cayman Chemical) and a horseradish peroxidase-conjugated anti-rabbit immunoglobulin as a secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ). Immunoreactive bands were visualized with the enhanced chemiluminescence system (Amersham Pharmacia Biotech).

PPAR-γ Transcriptional Activity
NIH/3T3 cells were transfected with the luciferase vector pCMV-Luc and the reporter plasmid prACO;3neo, which contains two functional PPREs of the acyl-CoA oxidase promoter (length: −1,273 to +20 of the promoter) and the PPAR-γ expression plasmid pCMX-mPPARγ. In some experiments, transfected cells were additionally infected with either Adx-D/N-PPAR-γ or Adx-LacZ for 24 hours or preincubated with GW9662 for 3 hours before challenged with the indicated ligands and inhibitors for 4 hours. Cell lysates were prepared with the Reporter Lysis Buffer (Promega Co., Madison, WI). β-Galactosidase activity was measured using the β-Galactosidase Enzyme Assay System (Promega) and normalized to luciferase activity measured with the Luciferase Assay System (Promega).

PPAR-γ Binding Activity
Electrophoretic mobility shift assays and supershift assays were done as described previously (37). Briefly, double-stranded oligonucleotides for consensus and mutant PPRE (Santa Cruz Biotechnology) were end-labeled with [γ-32P] ATP using T4 polynucleotide kinase. Nuclear extracts were isolated from MIA PaCa-2 cells incubated with the indicated ligands or inhibitors for 4 hours and from s.c. MIA PaCa-2 tumors treated with the indicated compounds for 4 weeks using a Nuclear Extraction Kit (Panomics, Redwood City, CA). For competition and supershift assays, nuclear extracts were preincubated at room temperature for 45 minutes with cold consensus or mutant PPRE (×200 excess) or antibodies against PPAR-γ (Santa Cruz Biotechnology) and RXRs (Santa Cruz Biotechnology) before addition of the labeled probe.

Pancreatic Cancer Animal Models
Animal studies were approved by the Chancellor's Animal Research Committee of the University of California, Los Angeles in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

s.c. Model.
Pancreatic cancer cells (2 × 105) were s.c. injected into the flanks of nude mice. After the tumor reached a size of ~2 × 2 mm animals were randomly allocated to receive either control vehicle (n = 8), low-dose nimesulide (150 μg/kg, n = 8), or high-dose nimesulide (1.5 mg/kg, n = 8). Drugs were prepared fresh each day and injected i.p. in a total volume of 50 μL for 2 weeks. In another series, animals were treated for 4 weeks with either control vehicle (n = 6) or high-dose nimesulide (1.5 mg/kg i.p.) in the absence (n = 6) or presence (n = 6) of the PPAR-γ inhibitor GW9662 (0.3 mg/kg by daily peritumoral injection).

Orthotopic Model.
Pancreatic tumors grown s.c. were harvested and tumor pieces (1 mm3) were transplanted into the tail of the pancreas of recipient nude mice (38). Animals were randomized and treated with either daily i.p. injections of nimesulide (1.5 mg/kg, n = 8) or control vehicle (n = 8) for 4 weeks. At the end of the study period blood samples and tumors were harvested. Plasma was obtained by centrifugation. Tumor volume was assessed using the formula for a hemi-ellipsoid or ellipsoid for the s.c. or orthotopic tumor, respectively. For histologic evaluation, the tumor was either fixed in formalin and embedded in paraffin or frozen in 2-methyl-butane/dry ice and embedded in optimum cutting temperature.

Measurement of Nimesulide Levels in Animal Plasma
Nimesulide extraction was done using the single step organic extraction method with 1mL dichloromethane added to 100 μL of each plasma sample. The chromatographic separation and quantitation were achieved using a Hewlett-Packard high-performance liquid chromatography instrument (HPLC 1100, Palo Alto, CA). The mobile phase consisted of acetonitrile (100% v/v) flowing through a Phenomenex Kromasil (5 μm, 100 A, C18 × 250 × 2 mm, 5 μm beads) column at 0.2 mL/min. Nimesulide peaks were monitored using the UV detector at 230 nm wavelength against the 360 nm reference wavelength with 16 and 100 bandwiths, respectively. The obtained chromatograms were analyzed using Quantity Browser of the Xcalibur software package.
COX-2 Immunohistochemistry

Paraffin-embedded tissue samples were sectioned (4 μm), deparaffinized in xylene, and rehydrated in a graded ethanol series. Sections were subjected to heat-induced epitope retrieval using boiling citrate buffer (pH 6.0). Endogenous peroxidase activity was quenched by 0.3% H2O2 and unpecific binding sites were blocked with 5% bovine serum albumin. Immunostaining was done with a COX-2 antibody (5 μg/mL in blocking buffer) for 1 hour and a biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) at 5 μg/mL in blocking buffer for 1 hour. Antibody binding sites were finally visualized by avidin-biotin peroxidase complex solution (Vectorstain Elite ABC, Vector Laboratories) and 3,3′-diaminobenzidine (Sigma Chemical) as a chromogen. Sections were counterstained with Hematoxylin QS (Vector Laboratories). As a control, nonspecific antibodies at a concentration corresponding to that of the COX-2 antibody were used to stain all sections using the identical protocol.

Microvessel Density

Cryostat sections (5 μm) were fixed in acetone and blocked with 2.5% bovine serum albumin for 1 hour. Immunostaining was done with a rat anti-mouse CD31 monoclonal antibody (Pharmingen) at 1:75 dilution or a rabbit anti-human Von Willebrand Factor antibody (DAKO Co., Carpintera, CA), which strongly cross-reacts with the murine isofrom, at 1:200 for 2 hours and a biotinylated secondary goat anti-rat or goat anti-rabbit antibody, respectively, (Pierce Biotechnology, Inc., Rockford, IL) at 1:200 dilution for 30 minutes. Slides were then immersed in 0.3% H2O2 and antibody binding sites were visualized with the Vectastain Elite ABC (Vector Laboratories) and 3,3′-diaminobenzidine (Sigma Chemical) as a chromogen. Sections were counterstained with Hematoxylin QS (Vector Laboratories). As a control, nonspecific antibodies at a concentration corresponding to that of the COX-2 antibody were used to stain all sections using the identical protocol.

Intratumoral VEGF Levels

Tumor extracts were prepared by homogenizing and sonicating non-necrotic areas of pancreatic tumors in anti-protease lysis buffer (Roche Applied Science, Indianapolis, IN). Tumor homogenates were cleared by centrifugation and filtration. Mouse VEGF levels were determined using the mouse VEGF DuoSet ELISA Development System (R&D Systems). Briefly, a 96-well microplate was precoated with goat anti-mouse VEGF (0.4 μg/mL) overnight and mouse VEGF levels in the tumor homogenates were determined using a biotinylated goat anti-mouse VEGF (100 ng/mL) and Streptavidin-horseradish peroxidase. Human VEGF levels were measured using the Quantikine Human VEGF Immunoassay (R&D Systems) as recommended by the manufacturer. Mouse and human VEGF levels were normalized to total protein content.

Statistical Analysis

Data are presented as mean ± SD. Statistical comparisons were made using the Student’s t test for paired observations or by one-way ANOVA with post hoc Bonferroni’s correction for multiple comparisons. P < 0.05 was considered statistically significant.

Results

Concentration-Dependent Effects of Selective COX-2 Inhibitors on VEGF Production in Pancreatic Cancer Cells. In previous experiments, we have found that PGE2 stimulates VEGF production in COX-2–positive pancreatic cancer cells in an autocrine fashion by binding to the EP2 receptor (35). Based on these findings, we hypothesized that blocking basal PGE2 production by selective COX-2 inhibitors decreases VEGF levels. Incubation of the COX-2–positive pancreatic cancer cell line BxPC-3 with the structurally unrelated selective COX-2 inhibitors nimesulide and DuP697 for 24 hours almost completely inhibited PGE2 production. COX-2–positive BxPC-3 and COX-2–negative MIA PaCa-2 cells were barely detectable and did not change upon treatment with selective COX-2 inhibitors (data not shown). The COX-2 inhibitor–induced decrease in PGE2 production in COX-2–positive pancreatic cancer cells correlated with a reduction of VEGF levels in the culture medium (Fig. 1 A and B). However, this effect was seen only with lower concentrations of the COX-2 inhibitors (≤10 μmol/L).

Figure 1. Concentration-dependent effects of selective COX-2 inhibitors on VEGF production and angiogenesis in vitro. COX-2–positive BxPC-3 and COX-2–negative MIA PaCa-2 cells were treated with the selective COX-2 inhibitors nimesulide (A) and DuP697 (B) before assaying the cell culture supernatant for VEGF. The COX-2 inhibitory effect of nimesulide and DuP697 was confirmed by measuring PGE2 levels in the supernatant of BxPC-3 cells (insets). The effect of nimesulide on endothelial cell migration was determined by a double-chamber assay (C) and expressed as a fold-increase over control vehicle. VEGF and PGE2 measurements were done on duplicate samples of three independent experiments. The endothelial cell migration assay was repeated twice. Columns, mean value; bars, ±SD. *, P < 0.05 versus 0 μmol/L.
At higher concentrations, starting at around 30 μmol/L (data not shown) and being maximal at 100 μmol/L, both inhibitors markedly increased VEGF levels in both COX-2–positive and COX-2–negative pancreatic cancer cells (Fig. 1A and B), suggesting a COX-2–independent effect.

To delineate whether the COX-2 inhibitor–induced increase in VEGF bears biological relevance, we used an endothelial cell migration assay. Incubation of COX-2–positive BxPC-3 but not COX-2–negative MIA PaCa-2 cells with nimesulide at concentrations ≤10 μmol/L slightly reduced endothelial cell migration, whereas treatment of both COX-2–positive and COX-2–negative pancreatic cancer cells with nimesulide at 100 μmol/L significantly stimulated endothelial cell migration (Fig. 1C).

The Role of PPAR-γ in Mediating the Effect of High-Dose Nimesulide on VEGF Production. To investigate whether activation of the nuclear receptor PPAR-γ affects VEGF levels in pancreatic cancer cells, we used the endogenous and exogenous PPAR-γ ligands 15-PGJ2 and troglitazone, respectively. Both ligands dose-dependently stimulated VEGF production in COX-2–positive and COX-2–negative pancreatic cancer cells (Fig. 2A and B). To evaluate whether the effect of high-dose nimesulide on VEGF levels was mediated by PPAR-γ, we constructed a dominant-negative PPAR-γ mutant. This PPAR-γ mutant retains ligand and DNA binding but exhibits markedly reduced transactivation due to impaired coactivator recruitment (36). MIA PaCa-2 cells were infected with adenoviruses encoding for the mutant PPAR-γ. Successful infection was confirmed by Western blotting, which showed marked overexpression of PPAR-γ in Adx-D/N-PPAR-γ infected cells (Fig. 3A). The compound mutant almost completely abrogated the effect of nimesulide and 15-PGJ2 on VEGF levels (Fig. 3B). We confirmed these findings using the irreversible PPAR-γ inhibitor GW9662, which dose-dependently attenuated the effect of nimesulide and 15-PGJ2 on VEGF production in MIA PaCa-2 cells (Fig. 3C and D). To determine whether high-dose nimesulide enhances PPAR-γ binding activity, we did gel shift assays using consensus and mutant PPREs. Treatment of MIA PaCa-2 cells with nimesulide (100 μmol/L), DuP697 (100 μmol/L), or troglitazone (10 μmol/L)
for 4 hours stimulated binding of nuclear complexes to the consensus PPRE (Fig. 4A). Supershift assays confirmed the presence of PPAR-γ and RxRα in the bound complexes. PPAR-γ binding activity was inhibited by cocompetition using unlabeled wild-type oligonucleotides but was unaffected by unlabeled mutant PPRES. No PPAR-γ binding to radiolabeled mutant PPRE was detectable (Fig. 4A).

Having shown PPAR-γ binding activity, we then sought to investigate whether nimesulide activates PPAR-γ transcriptional activity using reporter assays. NIH/3T3 cells, which lack wild-type PPAR-γ (40), were transiently transfected with PPAR-γ and a PPARE-containing reporter construct. 15-PGJ2 dose-dependently and nimesulide at 100 μM stimulated PPAR-γ transcriptional activity (Fig. 4B), which was inhibited by the dominant-negative PPAR-γ and by preincubation with GW9662 (Fig. 4C). Lower concentrations of nimesulide (<10 μM/L) had no effect on PPAR-γ transcriptional activity.

**Opposite Effects of the Selective COX-2 Inhibitor Nimesulide on Pancreatic Cancer Growth and Angiogenesis in vivo.** Based on these *in vitro* findings, we employed a s.c. and an orthotopic pancreatic cancer model in nude mice (38) to evaluate whether selective COX-2 inhibitors could also stimulate VEGF production by cancer cells in *in vivo* and therefore promote angiogenesis and tumor growth. Animals were treated with daily injections of a low (150 μg/kg) or high (1.5 mg/kg) dose of nimesulide for 2 weeks (s.c. model) or 4 weeks (orthotopic model). Treatment of animals with nimesulide at 1.5 mg/kg for 4 weeks resulted in plasma concentrations of nimesulide of 32 ± 8 μM/L as determined by high-performance liquid chromatography.

Immunohistochemistry with an antibody recognizing human and murine COX-2 revealed the presence of the COX-2 protein only in BxPC-3 tumors (Fig. 5A). Importantly, COX-2 was detected exclusively in cancer cells. The low dose of nimesulide had no effect on the growth of COX-2–positive and COX-2–negative tumors (data not shown). In contrast, the high dose of nimesulide decreased the growth of COX-2–positive pancreatic tumors by ~50%, but stimulated the growth of COX-2–negative pancreatic cancers ~2-fold in both models (Fig. 5B, C, and D). To determine whether the growth-promoting effect of high-dose nimesulide on MIA PaCa-2 tumors *in vivo* was mediated by activating PPAR-γ, we used the PPAR-γ inhibitor GW9662 at a concentration which has been successfully used in recent studies (41). Peritumoral injection of GW9662 (0.3 mg/kg) significantly attenuated the effect of high-dose nimesulide on the growth of s.c. MIA PaCa-2 tumors (Fig. 5E). Gel shift analysis of tumor nuclear extracts showed enhanced DNA binding of PPAR-γ in high-dose nimesulide-treated MIA PaCa-2 tumors (Fig. 5F). This effect was partially inhibited by GW9662, indicating that nimesulide at this concentration stimulates PPAR-γ binding to its response elements *in vivo.*

To further characterize the *in vivo* effects of nimesulide, we measured intratumoral PGE2 levels. Liquid chromatography/mass spectroscopy analysis revealed higher levels of PGE2 in the BxPC-3 tumors than in the MIA PaCa-2 tumors (Fig. 6A). Nimesulide (1.5 mg/kg) reduced PGE2 levels in BxPC-3 tumors, whereas it had no effect in MIA PaCa-2 tumors (Fig. 6A), demonstrating that tissue concentrations of nimesulide sufficient for inhibiting COX-2 activity were achieved.

Macroscopic evaluation of the tumors revealed a greater number and a more complex network of blood vessels on the surface of nimesulide-treated as compared with untreated COX-2–negative tumors (Fig. 5D). Microscopic analysis confirmed that nimesulide (1.5 mg/kg) increased the number of microvessels in COX-2–negative pancreatic tumors as determined by CD31 immunostaining. In contrast, the COX-2 inhibitor reduced microvessel density in COX-2–positive tumors (Fig. 6B and C). These findings were
confirmed by staining the tumors for the Von Willebrand Factor, which showed similar changes in microvessel density (data not shown). Because our in vitro findings showed an effect of the COX-2 inhibitor on VEGF levels, we sought to evaluate whether the differences in microvessel density correlate to changes in intratumoral VEGF content. We separately measured human (generated by pancreatic cancer cells) and mouse (secreted by nonmalignant host cells) VEGF levels in tumor homogenates. Mouse VEGF levels were significantly lower in the COX-2–negative compared with COX-2–positive tumors. Nimesulide (1.5 mg/kg) decreased mouse VEGF levels only in the COX-2–positive pancreatic cancers. In contrast, nimesulide increased human VEGF in both COX-2–negative and COX-2–positive pancreatic tumors (Fig. 6D and E). Combining mouse and human VEGF levels, nimesulide (1.5 mg/kg) decreased total VEGF in COX-2–positive pancreatic cancers but increased total VEGF in COX-2–negative pancreatic cancers (Fig. 6D and E, insets).

**Discussion**

In the present study, we found that two structurally unrelated selective COX-2 inhibitors had concentration-dependent effects on VEGF levels in the culture medium of pancreatic cancer cells. At lower concentrations (≤10 μmol/L), both inhibitors decreased VEGF levels only in COX-2–positive pancreatic cancer cells, an effect which is most likely to be caused by inhibiting the autocrine effects of COX-2–derived prostaglandins. This is in accordance to our previous experiments (35), in which PGE₂...
produced by COX-2–positive pancreatic cancer cells stimulated VEGF production in an autocrine fashion. These data are also consistent to Chu et al. (42), showing antiangiogenic properties of a selective COX-2 inhibitor associated with a reduced VEGF production. However, at higher concentrations, starting at around 30 μmol/L and being maximal at 100 μmol/L, both inhibitors increased VEGF levels in COX-2–positive and COX-2–negative pancreatic cancer cells, indicating possible COX-independent effects. At higher inhibitor concentrations, VEGF levels decreased again, presumably caused by the proapoptotic properties of the COX-2 inhibitors at these concentrations (7). In the present study, using COX-2 inhibitors at concentrations of maximal 100 μmol/L for 24 hours, no induction of apoptosis was detectable (data not shown).

In recent years, an increasing number of studies have reported COX-independent effects of selective COX-2 inhibitors (7–14). However, as these findings are mostly single reports in specific cellular systems, a well-defined general COX-independent pathway activated and/or inhibited by selective COX-2 inhibitors has not emerged yet. Earlier studies have shown that nonselective COX inhibitors can activate the nuclear receptor PPAR-γ (15). Intriguingly, the transcriptional activation was seen only at higher concentration of the inhibitors. Based on these findings and our own studies showing that pancreatic cancer cells express PPAR-γ (17), we sought to determine whether the effect of selective COX-2 inhibitors (at higher concentrations) on VEGF levels in pancreatic cancer cells was indeed mediated by PPAR-γ. The exact role of PPAR-γ in tumor angiogenesis and growth is still ambiguous. Whereas some studies attribute PPAR-γ ligands antiangiogenic and antiproliferative properties, others showed proangiogenic and growth-stimulating effects of PPAR-γ activation. The overall effect of PPAR-γ activation may depend on the pharmacologic properties of the specific ligand, the cellular equipment of coactivators and corepressors, the timely pattern of activation, and the presence of other cellular pathways interacting with PPAR-γ (24, 43–45).

In our study, two different PPAR-γ ligands dose-dependently increased VEGF levels in pancreatic cancer cells, supporting the proangiogenic and protumorigenic role of PPAR-γ activation. This observation seems to be contrary to our previous study, in which PPAR-γ ligands inhibited proliferation of pancreatic cancer cells by induction of apoptosis (17), advocating the antitumorigenic properties of PPAR-γ activation. However, the proapoptotic effects in vitro were clearly seen only at relatively high concentrations of the PPAR-γ ligands (≥10 μmol/L). In the present study, concentrations of PPAR-γ ligands (≤1 μmol/L) sufficient for increasing VEGF levels did not induce apoptosis (data not shown). Importantly, an increase in VEGF levels, neglecting autocrine pathways, confers protumorigenic properties dominantly in vivo and may not affect proliferation in vitro. Using a dominant-negative PPAR-γ mutant and a pharmacologic PPAR-γ inhibitor, we were able to

Figure 6. Effect of nimesulide on intratumoral PGE2 and VEGF levels and on tumor angiogenesis. Intratumoral PGE2 levels were measured by liquid chromatography/mass spectroscopy and standardized to PGE2 in control-vehicle treated BxPC-3 tumors (A). *, P < 0.05 versus control. Microvessel density in vehicle-treated and nimesulide (nime)-treated BxPC-3 and MIA PaCa-2 tumors was assessed by immunohistochemical staining of CD31 (B) and quantified using ImagePro 5.0 software (C). *, P < 0.05 versus control. Intratumoral human and mouse VEGF levels were measured using species-specific ELISA kits in BxPC-3 (D) and MIA PaCa-2 tumors (E) and standardized to total tumor protein content. The effect of nimesulide on overall intratumoral VEGF levels was determined by adding murine and human VEGF together (D and E; insets). *, P < 0.05 versus control. Columns, mean value; bars, ±SD. For VEGF and PGE2 quantification, each tumor sample was measured in triplicate.
show that the effect of high-dose COX-2 inhibitor on VEGF production was mediated by PPAR-γ. Gel shift and reporter assays showed that the selective COX-2 inhibitor stimulated PPAR-γ binding and transcriptional activity, respectively. However, only high concentrations of the selective COX-2 inhibitor increased PPAR-γ transcriptional activity, an effect that agrees with earlier studies (15). The concentrations of the COX-2 inhibitor needed to stimulate PPAR-γ transcriptional activity correlated thereby with the concentrations needed to increase VEGF levels, supporting the causal relationship. For the reporter studies, we used murine NIH/3T3 fibroblasts, which lack wild-type PPAR-γ (40). This allowed us to clearly delineate the contribution of PPAR-γ to the observed effect of the COX-2 inhibitor. However, the exact mechanism by which the COX-2 inhibitor activates PPAR-γ still remains unknown. Nimesulide may activate PPAR-γ indirectly through accumulation of the COX-2 substrate arachidonic acid (46). However, the finding that the dominant-negative receptor and GW9662 abrogate the effect of nimesulide also in COX-2-negative cells makes this an unlikely explanation.

Our in vitro findings raised the hypothesis, that a selective COX-2 inhibitor may also stimulate VEGF levels and as a result tumor growth of pancreatic cancer in vivo. Nimesulide at plasma concentration, which can also be achieved in humans (47), reduced angiogenesis and growth of the COX-2–positive pancreatic tumor but stimulated microvessel density and growth of the COX-2–negative pancreatic tumor. To our knowledge, this is the first report of a stimulatory effect of a selective COX-2 inhibitor on angiogenesis and growth of any human cancer. The nimesulide-induced decrease in microvessel density in COX-2–positive pancreatic cancer was accompanied by a reduction in total intratumoral VEGF. Exploiting the characteristics of our xenograft model, in which human cancer cells grow in a mouse host, further analysis showed that treatment with nimesulide increased cancer cell generated human VEGF in both COX-2–positive and COX-2–negative tumors: an effect which correlates to our in vitro finding. In COX-2–positive pancreatic cancer this effect was offset by a marked reduction in host cell generated murine VEGF, leading to a decrease in total VEGF and a reduction in the number of microvessels. Because nimesulide had no effect on murine VEGF in COX-2–negative pancreatic cancer, the increase of human VEGF dominated, which led to stimulated angiogenesis.

Our study strongly implies that PGE_2 generated by COX-2 in pancreatic cancer cells is a driving force for angiogenesis in pancreatic carcinomas. This conclusion is supported by the following observations: (1) Using an antibody which recognizes murine and human COX-2 isoforms, immunohistochemical analysis detected COX-2 exclusively in pancreatic cancer cells; a notion which has also been described by others (30, 33). (2) PGE_2 levels were significantly higher in COX-2–positive pancreatic tumors compared with COX-2–negative pancreatic tumors. (3) Expression of COX-2 and high intratumoral PGE_2 levels positively correlated to the number of tumor microvessels. (4) Treatment with a selective COX-2 inhibitor decreased PGE_2 levels only in COX-2–positive pancreatic cancer, which was accompanied by a reduction in angiogenesis. The importance of the COX-2/PGE_2 system in tumor angiogenesis has also been recognized by others. PGE_2 is capable of stimulating VEGF production in various cell types, which are all found in the tumor stroma, including fibroblasts, endothelial cells, and inflammatory cells (48–50). This highlights the potentially critical role of PGE_2 as a paracrine mediator of VEGF stimulation and hence angiogenesis. In addition, in a sarcoma model Amano et al. (51) found that angiogenesis was attenuated in prostaglandin receptor knockout animals. Although we did not study the pathways by which PGE_2 stimulates VEGF production, recent evidence suggests that hypoxia inducible factor-1 might be involved (52).

Although our study implies that the growth-inhibitory effect of nimesulide in COX-2–positive pancreatic cancer was primarily caused by a reduction of tumor angiogenesis, a direct antiproliferative effect on the cancer cells seems also plausible. However, despite the evidence in vitro showing induction of apoptosis in cancer cells treated with COX-2 inhibitors, we strongly favor the antiangiogenic explanation. Our preference is mainly based on the histologic examination of the tumors. Nimesulide-treated COX-2–positive pancreatic tumors had a prominent central necrotic area with a reduction in microvessels predominantly in the tumor periphery. This pattern indicates that the reduction in blood supply is the primary cause of cancer cell death. Furthermore, staining with the proliferation marker Ki-67 did not detect changes in pancreatic cancer cell proliferation in non-necrotic tumor regions (data not shown).

In conclusion, our studies provide the first evidence of growth-stimulating properties of selective COX-2 inhibitors in a well-defined subset of COX-2–negative pancreatic cancers. In COX-2–positive pancreatic carcinomas, the selective COX-2 inhibitor reduced angiogenesis and growth. Molecular profiling of the pancreatic tumor seems to be warranted, especially in the light of several ongoing clinical trials using selective COX-2 inhibitors as a therapeutic agent in pancreatic cancer patients.

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