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Advances in Brief

Cyclooxygenase-1 is Overexpressed and Promotes Angiogenic Growth Factor Production in Ovarian Cancer[1]

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Abstract

Inhibition of cyclooxygenase-2 (COX-2) catalytic activity has proven successful in restricting the growth of epithelial-derived cancers *in vivo*. Whether COX-2 inhibitor therapy would be beneficial in the prevention and/or treatment of ovarian cancer, the most lethal gynecological malignancy worldwide, is not known. Most patients with ovarian cancer undergo cytoreductive therapy. Because many of the cytotoxic drugs used to treat ovarian cancer induce COX-2 expression, samples from patients that had not undergone cytoreductive therapy were specifically chosen for COX isoform expression analysis. A majority of specimens exhibited elevated levels of COX-1, not COX-2, mRNA, and protein compared with normal ovarian tissue. Focal regions within the tumor expressing high COX-1 also had elevated levels of pro-angiogenic proteins. Selective inhibition of COX-1, not COX-2, inhibited arachidonic acid-stimulated vascular endothelial growth factor production, which could be reversed by cotreatment with prostaglandin E_2. Thus, COX-1 may contribute to carcinoma development in the ovary through stimulation of neovascularization. Clinical studies testing the efficacy of COX inhibition as adjuvant therapy for ovarian cancer may see more beneficial effects with adjuvant therapy with either a COX-1 selective or nonselective cyclooxygenase inhibitor as compared with a COX-2 selective drug.

Introduction

The COX[5] enzymes COX-1 and COX-2 catalyze the rate-limiting step in the biosynthesis of PGs derived from AA. Research over the last decade, primarily in studies focused on colorectal cancer, has established that NSAIDs are effective in both cancer prevention and as adjuvant therapy in the treatment of established tumors (1). These drugs are thought to inhibit colorectal cancer cell growth primarily through inhibition of COX-2, although other noncyclooxygenase biochemical targets may be involved. Recent experiments suggest that COX-2 is up-regulated in a range of extracolonic cancers, and selective COX-2 inhibitors have potent antineoplastic effects *in vivo* in preclinical models of a variety of solid malignancies. These data have led to the initiation of a number of clinical trials that are testing the efficacy of COX-2-specific inhibitor therapy in the primary or secondary prevention of cancer or as part of a combination therapy regimen for established tumors. Whether COX-2 selective inhibitor therapy will prove beneficial in the prevention and/or treatment of ovarian cancer is not known. Ovarian cancer is associated with a high mortality rate because of the absence of effective screening strategies to identify patients at high risk or who have already developed early neoplastic lesions still amenable to treatment (2, 3).

In addition, treatment options for patients diagnosed with advanced disease remain inadequate. Thus, ovarian cancer represents a potential candidate to target for chemoprevention. The results of epidemiological studies examining whether NSAIDs can prevent or delay the development of ovarian cancer have been mixed. Several population- and hospital-based case control studies have documented that exposure to several NSAIDs is associated with a time- and dose-dependent decrease in the risk for the development of ovarian cancer (4–6), whereas other studies detected either no statistically significant association or found the reduction in risk to be associated only with the use of particular NSAIDs (7, 8). There are also conflicting data available describing the expression of COX isotypes in ovarian cancer; Dore *et al.* (9) found COX-1 to be the predominant COX isoform expressed, whereas two other groups reported finding high levels of COX-2 (10, 11). However, these latter two expression studies did not specifically rule out samples that had undergone cytoreductive therapy, a treatment that is common in patients presenting with advanced ovarian malignancy. Because many compounds used in this type of therapy induce COX-2 (12, 13), it is unclear if COX-2 was found to be elevated because of the malignant process itself or simply secondary to the use of cytotoxic agents for treatment of primary disease. To investigate the role of the cyclooxygenase pathway in the pathogenesis of ovarian cancer and determine whether a scientific rationale exists for the use of COX inhibitors in the prevention and/or treatment of the disease, we evaluated COX-1 and COX-2 expression in ovarian cancer samples taken specifically from patients who had not undergone cytoreductive therapy.

Materials and Methods

Cell Culture. The SK-OV-3, OV90, and OVCAR-3 cells were purchased from American Type Culture Collection and grown in the recommended media under standard conditions.

Tissue Samples. Tissue samples of 11 epithelial ovarian cancers that had not undergone cytoreductive therapy and nine normal human ovaries were obtained immediately after surgery from surgical pathology specimens (University of Kansas Medical Center and Vanderbilt University). The tissues obtained from the pathologist were flash frozen in liquid Histo-freeze (Fisher) and stored at −80°C.

RNA Isolation and Northern Blot Analysis. Total RNA was extracted from the tissue specimens using TRIzol Kit (Life Technologies, Inc.). Total RNA (20 μg) was denatured, separated by formaldehyde-agarose gel electrophoresis, transferred, and cross-linked to nylon membranes by UV irradiation. Northern blots were prehybridized, hybridized, and washed as described previously (14, 15). For Northern hybridization, antisense ^{32}P-labled cRNA probes for mouse COX-1, human COX-2, and β-actin were generated. After

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[5] The abbreviations used are: COX, cyclooxygenase; NSAID, nonsteroidal anti-inflammatory drug; PG, prostaglandin; AA, arachidonic acid; HIF, hypoxia-inducible factor; VEGF, vascular endothelial growth factor.

hybridization, the blots were washed under stringent conditions, and the hybrids were detected by autoradiography. Stripping of the hybridized probe before subsequent rehybridization was achieved. Each blot was hybridized sequentially to COX-2, COX-1, and β-actin probes.

***In Situ* Hybridization.** *In situ* hybridization followed the protocol described previously (14). Frozen sections (10 μM) from each tissue specimen were mounted onto poly-L-lysine-coated slides and stored at −80°C until used. Serial sections were obtained to detect localization of gene expression in similar areas. Sections were brought to room temperature, fixed in cold 4% paraformaldehyde solution in PBS, acetylated, and hybridized at 45°C for 4 h in 50% formamide buffer containing ^{35}S-labeled antisense or sense cRNA probes specific to mouse cRNA to COX-1 and human-specific cRNAs to COX-2, HIF-1α, VEGF, and Flk-1. After hybridization and washing, the slides were incubated with RNase A (20 μg/ml) at 37°C for 20 min, and RNase A-resistant hybrids were detected by autoradiography using Kodak NTB-2 liquid emulsion. Parallel sections hybridized with the sense probes served as negative controls. Slides were poststained with H&E.

Immunohistochemistry. Immunocolocalization of COX-1 and COX-2 was performed in 2% paraformaldehyde-fixed frozen sections using a Zymed-Histostain SP kit (Zymed). Rabbit antipeptide antibodies to mouse COX-1 and COX-2 were used as described previously (15). These antibodies were found to cross-react with human tissues. After immunostaining, sections were lightly counterstained with hematoxylin or fast green. Red deposits indicate the site of immunoreactive proteins.

Preparation of Tissue Lysates. Tissue samples were homogenized in lysis buffer [1% Triton X-100, 1% deoxycholate, 10 mM Tris (pH 7.2), 150 mM NaCl, 1 mM sodium vanadate, 50 mM sodium fluoride, 1 mM phenylmethey-sulfonyl fluoride, 50 mg/ml aprotinin, and 50 mg/ml leupeptin]. The samples were transferred to eppendorf tubes and centrifuged at 14,000 × *g* for 15 min in the cold. The supernatants were transferred to fresh tubes, and protein concentrations were determined. These extracts were used for Western blot analysis.

Western Blot Analysis. Lysates (50 μg of protein/lane) were analyzed by SDS-PAGE on 10% Tris-glycine gels. Protein was electrotransferred to nitrocellulose membranes and blocked with a solution of PBS containing 5% milk and 0.1% Tween 20. Bands were detected using chemiluminescent detection reagents (Pierce). Blots were probed with a goat polyclonal antibody against COX-1 or COX-2 (Santa Cruz Biotechnology) followed by a peroxidase-conjugated antigoat (Sigma) or donkey-antigoat (Jackson ImmunoResearch Laboratories), respectively. After incubation, antibodies were washed in PBS and 0.1% Tween 20. Bands were detected using chemiluminescent detection reagents (Pierce).

PG Measurements. PG profiles for the OVCAR-3 cells were measured and quantified using a gas chromatography/negative ion chemical ionization mass spectrometric assay and a PGE_2 immunoassay kit (R&D Systems).

Cell Growth Measurements. Cells were plated at a density of ~20%, and treatment was initiated the following day with 0.1% DMSO or the indicated dose of COX inhibitor. Fresh media and drug was added every 48 h, and the number of viable cells was determined using a Coulter counter after 7 days of treatment.

VEGF Measurements. VEGF concentrations in the media of OVCAR-3 cells treated with the indicated dose of AA, COX inhibitor, and PGE_2 were measured using the Quantikine Human VEGF Immunoassay kit (R&D Systems) according to manufacturer instructions.

Results and Discussion

COX-1 and COX-2 expression levels in 11 epithelial ovarian cancers and nine normal human ovaries were determined using Northern blot and immunoblot analysis. Significant levels of COX-1 or COX-2 mRNA and protein were not detected in any of the normal human ovarian tissue (Fig. 1, *A* and *B*). However, dramatic elevations of COX-1, not COX-2, protein and mRNA were detected in a majority of the ovarian cancer samples tested. In colorectal and other cancers, COX-1 and COX-2 protein levels have been determined in multiple cell types, including epithelial, fibroblast, macrophage, and endothelial cells. *In situ* hybridization and immunohistochemistry analysis were done to determine the cellular localization of COX-1 and COX-2 protein in ovarian cancer. A significant elevation in COX-1, not COX-2, mRNA was observed by *in situ* hybridization, and COX-1 mRNA was localized to the epithelial compartment of the tumors (Fig. 2, *A* and *B*). Immunohistochemical analysis of sections of both normal human ovary and ovarian epithelial cancers confirmed the elevated expression of COX-1, not COX-2, protein in ovarian tumors, and the immunoreactivity detected with a COX-1-specific antibody was localized primarily to ovarian carcinoma cells within the tumor (Fig. 2*C*).

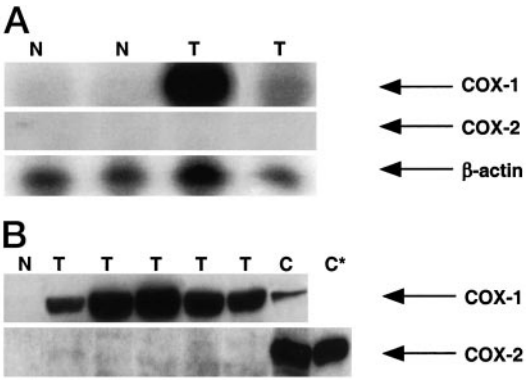

Fig. 1. COX-1, not COX-2, mRNA and protein levels are elevated in human ovarian cancer. *A*, Northern hybridization. Total RNA (20 μg) from normal human ovary and ovarian epithelial tumor tissue was fractionated on a formaldehyde-agarose gel and probed for COX-1 and COX-2 and β-actin mRNA expression. *B*, Western blotting. Total protein (50 μg) from normal human ovary and ovarian tumor tissue was fractionated on a 10% SDS-PAGE and probed with goat polyclonal antibodies specific for COX-1 and COX-2. The positive control lane labeled *C* consists of recombinant COX-1 and COX-2 protein, whereas the positive control lane *C** consists of an aliquot of cell lysate from the HCA-7 rectal adenocarcinoma line known to express high levels of COX-2 protein.

Angiogenesis, defined as the generation of new capillaries from preexisting vessels, is a critical factor in the sustained growth of solid tumors. Ovarian cancer is known to be highly vascular and is a primary cancer in which current antiangiogenic therapies are being tested (16, 17). VEGF and the VEGF receptor flk-1 are highly expressed in a majority of ovarian epithelial tumors, and VEGF expression is a negative prognostic factor for the disease (18, 19). The transcription factor HIF-1α is a dominant regulator of VEGF gene transcription and induces significant increases in VEGF mRNA copy number in response to various stimuli by binding to a hypoxia-responsive element within the VEGF promoter (20). There is also a strong link between the COX pathway and angiogenesis. Data from multiple groups suggest that a major mechanism by which COX-derived PGs promote polyp growth in the colon is through the stimulation of new blood vessel growth. To determine whether regions within ovarian tumors demonstrating high COX-1 expression correlate with foci of prominent angiogenic activity, *in situ* hybridization was done probing for COX-1, VEGF, Flk-1, and HIF-1α in serial sections. Regions of ovarian epithelial cells exhibiting high COX-1 also expressed significant levels of HIF-1α and VEGF (Figs. 2*A* and 3, *A* and *B*). High levels of flk-1 were seen in the endothelial cells located in the stroma adjacent to ovarian epithelial cells expressing COX-1, HIF-1α, and VEGF (Fig. 3*C*). The above data suggest that elevations in COX-1 expression are enhanced in regions of ovarian epithelial tumors undergoing extensive angiogenesis.

To directly test the hypothesis that COX-1-derived PGs promote the development of ovarian cancer, a cell culture-based model system was developed and evaluated. The established ovarian cancer cell lines SK-OV-3, OV90, and OVCAR-3 were evaluated for COX-1 and COX-2 protein levels. Although none of the three cell lines expressed detectable levels of COX-2, the OVCAR-3 cells exhibited high levels of COX-1 protein and were chosen for further study (Fig. 4*A*). No

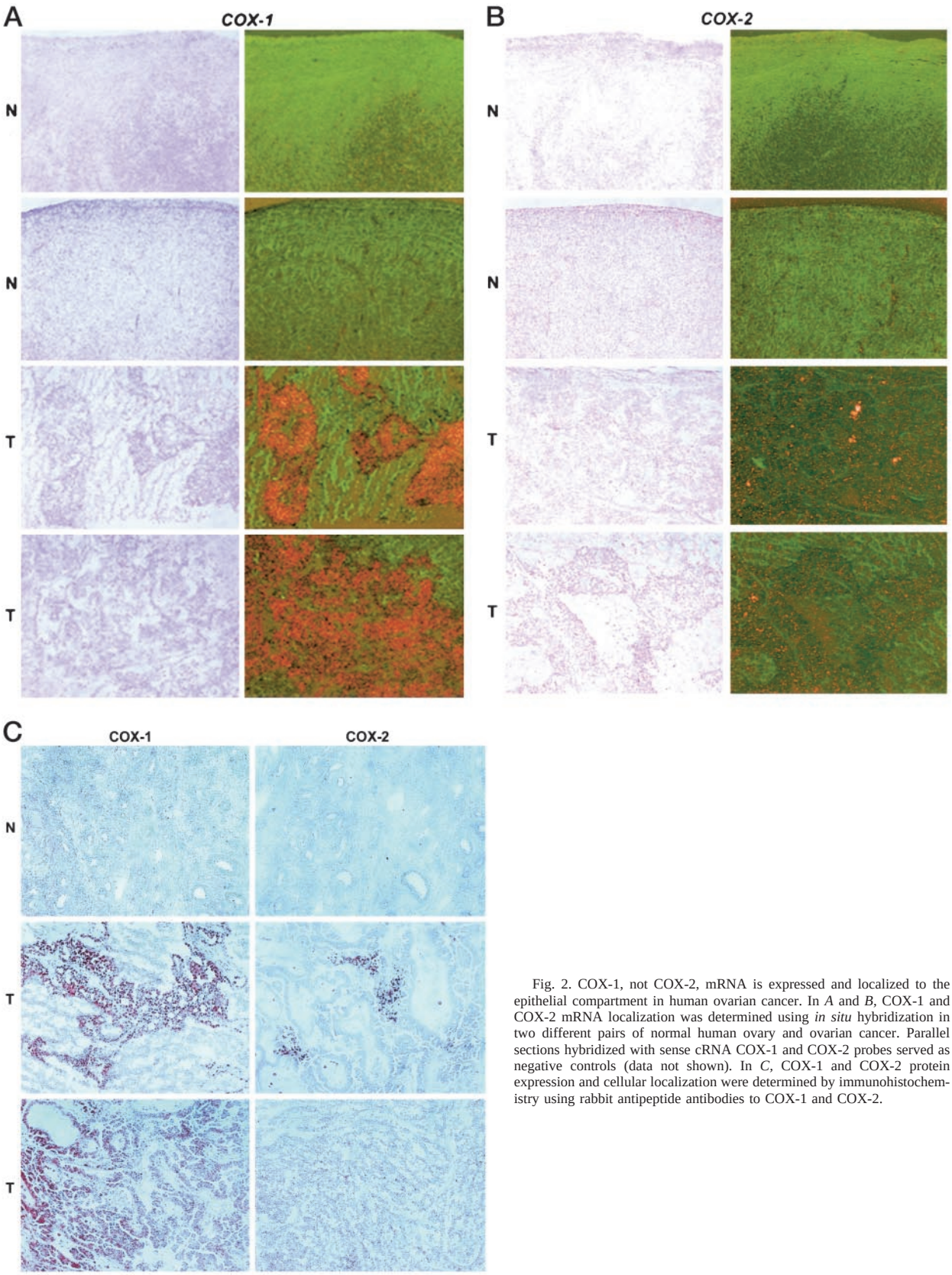

Fig. 2. COX-1, not COX-2, mRNA is expressed and localized to the epithelial compartment in human ovarian cancer. In *A* and *B*, COX-1 and COX-2 mRNA localization was determined using *in situ* hybridization in two different pairs of normal human ovary and ovarian cancer. Parallel sections hybridized with sense cRNA COX-1 and COX-2 probes served as negative controls (data not shown). In *C*, COX-1 and COX-2 protein expression and cellular localization were determined by immunohistochemistry using rabbit antipeptide antibodies to COX-1 and COX-2.

reports have been published documenting the types of PGs synthesized by ovarian carcinoma cells. Levels of the five major PG metabolites were measured in these cells in response to stimulation with AA, and significant levels of $PGF_{2\alpha}$, PGI_2, PGE_2, and PGD_2 were detected (Fig. 4*B*). Although most NSAIDs inhibit the enzymatic activity of both COX-1 and COX-2, isoform selective COX inhibitors have been developed. SC-560 is 700 times more selective for inhibiting COX-1 compared with COX-2, whereas celecoxib is ~3000-fold

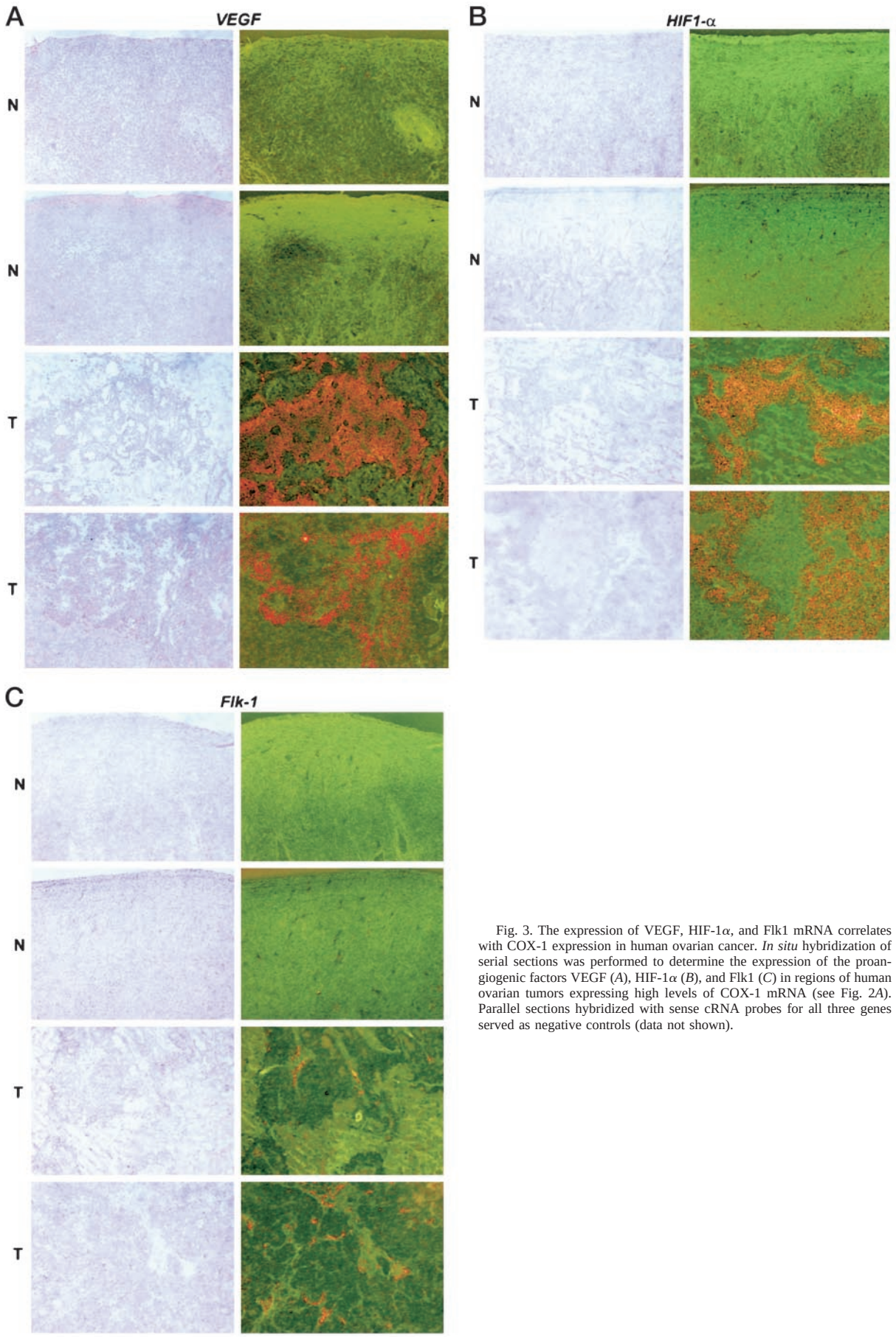

Fig. 3. The expression of VEGF, HIF-1α, and Flk1 mRNA correlates with COX-1 expression in human ovarian cancer. *In situ* hybridization of serial sections was performed to determine the expression of the proangiogenic factors VEGF (*A*), HIF-1α (*B*), and Flk1 (*C*) in regions of human ovarian tumors expressing high levels of COX-1 mRNA (see Fig. 2*A*). Parallel sections hybridized with sense cRNA probes for all three genes served as negative controls (data not shown).

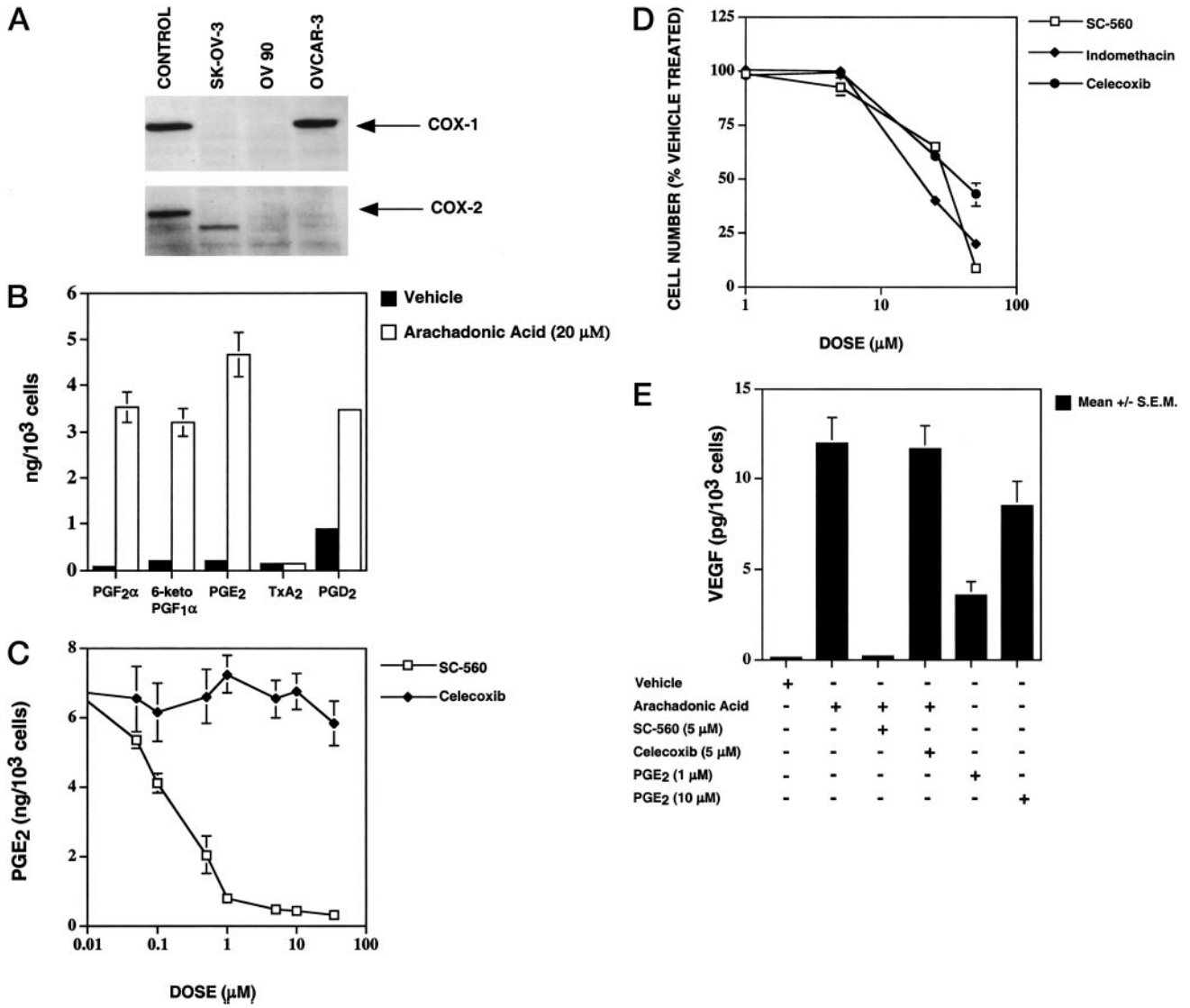

Fig. 4. The OVCAR-3 human ovarian epithelial cancer cell line expresses high levels of catalytically active COX-1, and COX-1-derived PGE_2 stimulates VEGF secretion in the OVCAR-3 human ovarian cancer cell line. In *A*, whole-cell lysates from the SK-OV-3, OV90, and OVCAR-3 human ovarian epithelial cancer cell lines were fractionated on a 10% PAGE and probed with goat polyclonal antibodies specific for COX-1 and COX-2. The control lane for the COX-1 blot is an aliquot of protein lysate from the MC-26 breast cancer cell line known to express high levels of COX-1, whereas the control lane for the COX-2 blot is an aliquot of protein lysate from the HCA-7 rectal adenocarcinoma line known to express high levels of COX-2 protein. In *B*, OVCAR-3 cells grown in serum-depleted media were treated with AA (20 μM) for 4 h, followed by measurement of different PG subtypes using a gas chromatography/negative ion chemical ionization mass spectrometric assay. In *C*, OVCAR-3 cells grown in serum-depleted media were treated with AA (20 μM) and increasing concentrations of a selective inhibitor against COX-1 (SC-560) or a selective inhibitor of COX-2 (celecoxib), followed by measurement of PGE_2 levels using a PGE_2 immunoassay. In *D*, OVCAR-3 cells were treated with increasing doses of a COX-1 selective (SC-560), non-COX isotype selective (indomethacin), or COX-2 selective (celecoxib) inhibitor for 7 days after the number of viable cells was counted and expressed as a percentage of control-treated cells (0.1% DMSO). Each experiment was done in triplicate, and each data point represents the mean of two independent experiments. Error bars = SE. In *E*, OVCAR-3 cells grown in serum-depleted media were treated with AA (20 μM) and the indicated concentrations of a COX-1 (SC-560) or COX-2 (celecoxib) selective inhibitor with or without the indicated dose of PGE_2 for 24 h, after which, VEGF levels in the media were measured using a human VEGF immunoassay.

more selective for COX-2 *versus* COX-1 (21). The ability of increasing doses of either SC-560 or celecoxb to inhibit AA-induced PGE_2 formation in OVCAR-3 cells was evaluated. SC-560, but not celecoxib, inhibited PGE_2 formation in a dose-dependent manner (Fig. 4*C*). These data confirmed our expression studies, indicating that COX-1 is the major source of PG production in these cells.

Finally, the ability of SC-560 to inhibit either DNA replication or VEGF secretion was tested to determine the functional relevance of COX-1 expression in ovarian cancer epithelial cells. Treatment of OVCAR-3 cells with increasing doses of either SC-560, celecoxib, or the non-COX isotype selective NSAID indomethacin resulted in significant decreases in cell number only at doses > 50 μM (Fig. 4*D*). Biological effects seen at such high drug concentrations are unlikely to be caused by inhibition of COX catalytic activity and have limited clinical relevance, because these levels are 50–100 times greater than the concentration of COX inhibitors that can be achieved *in vivo* (22). In contrast, treatment of OVCAR-3 cells with a low dose of SC-560, but not celecoxib, significantly inhibited AA-induced VEGF secretion, and this effect could be partially reversed by cotreatment with PGE_2 (Fig. 4*E*).

Neoplasms of the ovary rarely produce symptoms that lead to prompt medical attention until the disease is highly advanced and often incurable. Currently, no effective detection strategies exist to identify patients at high risk or who have localized lesions that can be effectively treated. These facts make ovarian cancer account for a disproportionate number of lethal cancers and thus a primary candidate to target for prevention. Given the effectiveness of NSAID therapy in the chemoprevention of colorectal cancer, as well as several other types of solid tumors, it is important to determine the role, if

any, that NSAID treatment may have in the management of ovarian cancer. Our results here suggest that: (*a*) the COX-1 enzyme is overexpressed in a significant number of ovarian cancers; (*b*) COX-1 may promote ovarian cancer development via stimulation of angiogenesis; and (*c*) COX-1 or non-COX selective inhibitors should be further evaluated for their ability to inhibit ovarian cancer cell growth.

Our results indicate that COX-1, not COX-2, mRNA and protein levels are elevated in ovarian cancers from patients not exposed previously to cytotoxic chemotherapy. Similar results were obtained by Dore *et al.* (9), who used immunohistochemistry to demonstrate strong expression of COX-1, not COX-2, protein in human ovarian cancer specimens. Both of these results are in contradiction to data generated by two other groups that report elevated COX-2 levels in ovarian cancer (10, 11). However, neither of these two groups determined the status of COX-1 expression in their samples. The reason for the discrepancy with regard to COX-2 expression is not known but may be related to differences in clinical treatment regimens of patients before tissue collection, detection methods, tissue processing, and/or antibody cross-reactivity.

A majority of epithelial-derived tumors with elevated PG levels overexpresses COX-2 and not COX-1. This is consistent with the hypothesis that COX-1 is constitutively expressed and responsible for basal PG production, whereas COX-2 is highly inducible and responsible for the elevations in PG production that occur in response to pro-inflammatory cytokines and growth factors, both of which are likely to be highly concentrated within the microenvironment of a tumor. The basis for the divergent expression patterns of COX-1 and COX-2 in ovarian cancer is not known. The simplest explanation may be that the development of ovarian cancer is associated with the dysregulation of a unique combination of signaling pathways not found in other tumor types that converge to cause activation of COX-1, not COX-2, gene transcription. The COX-1 promoter is relatively uncharacterized, and transfection experiments using COX-1 and COX-2 promoter–reporter constructs in the OVCAR-3 cells may help determine the molecular basis for why COX-1 and not COX-2 is up-regulated ovarian cancer.

The role of COX-1 in neoplasia is not clear. COX-1 and COX-2 catalyze identical biochemical reactions. It is thus possible that the elevated COX-2 seen in many types of cancer is selected to simply increase total PG levels within the tumor microenvironment. Alternatively, COX-2 may exhibit unique substrate utilization, cellular or subcellular localization, and/or coupling with downstream PG synthases, resulting in a pro-oncogenic effect that cannot be substituted by COX-1. There are published reports using genetically modified mice to support both theories. Chulada *et al.* (23) demonstrated an equivalent reduction in intestinal polyposis in Min/+ mice with a genetic disruption of COX-1 or COX-2, and both genes were found to contribute equally to PGE_2 levels within polyps. In contrast, Williams *et al.* (24) found that xenografts of Lewis lung carcinoma cells grew more slowly only in mice genetically null for COX-2 but not COX-1. Future experiments using the OVCAR-3 cells may help clarify whether COX-1 has a unique pro-tumorigenic role in ovarian cancer or if it is simply an issue of generating a threshold level of PGs that can be derived from either COX isotype, *e.g.*, mRNA antisense or RNAi could be used to inhibit COX-1 expression (and hence VEGF secretion) in these cells and rescue experiments performed to determine whether transfection with COX-2 can substitute for COX-1 to stimulate angiogenic growth factor production.

Our results imply that PGE_2 positively regulates VEGF production in ovarian epithelial cells perhaps via the H1F1-α pathway. Future experiments designed to determine the expression levels of all four PGE_2 receptor (EP) subtypes in human ovarian cancer, as well as the ability of EP receptor subtype-specific agonists to stimulate angiogenic growth factor production, will be important.

In summary, our current results suggest that use of COX-1 or non-COX isotype selective NSAIDs may be more plausible than COX-2 inhibitor therapy in either the primary or secondary prevention of ovarian cancer or as a component of a cancer treatment for advanced tumors. However, it will be important to test this hypothesis *in vivo* using COX inhibitors that preferentially inhibit COX-1 or COX-2, and such experiments using preclinical models of ovarian cancer cell growth are currently underway.

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