Cell, Vol. 93, 705–716, May 29, 1998, Copyright ©1998 by Cell Press

Cyclooxygenase Regulates Angiogenesis Induced by Colon Cancer Cells

Masahiko Tsujii,[†‡] Sunao Kawano,[†] Shingo Tsuji,[†] Hitoshi Sawaoka,[†] Masatsugu Hori,[†] and Raymond N. DuBois*[‡]
*Departments of Medicine and Cell Biology
Vanderbilt University Medical Center
VA Medical Center
Nashville, Tennessee 37232
[†]First Department of Medicine
Osaka University School of Medicine
Osaka 565-0871
Japan

Summary

To explore the role of cyclooxygenase (COX) in endothelial cell migration and angiogenesis, we have used two in vitro model systems involving coculture of endothelial cells with colon carcinoma cells. COX-2-overexpressing cells produce prostaglandins, proangiogenic factors, and stimulate both endothelial migration and tube formation, while control cells have little activity. The effect is inhibited by antibodies to combinations of angiogenic factors, by NS-398 (a selective COX-2 inhibitor), and by aspirin. NS-398 does not inhibit production of angiogenic factors or angiogenesis induced by COX-2-negative cells. Treatment of endothelial cells with aspirin or a COX-1 antisense oligonucleotide inhibits COX-1 activity/expression and suppresses tube formation. Cyclooxygenase regulates colon carcinoma-induced angiogenesis by two mechanisms: COX-2 can modulate production of angiogenic factors by colon cancer cells, while COX-1 regulates angiogenesis in endothelial cells.

Introduction

Recent epidemiological studies document a 40%–50% reduction in mortality from colorectal cancer in individuals taking nonsteroidal antiinflammatory drugs (NSAIDs) (Smalley and DuBois, 1997). Since NSAIDs (like aspirin) are widely believed to have antiinflammatory effects due to their ability to inhibit cyclooxygenase enzyme activity, one possible mechanism for their antitumor properties is altered metabolism of arachidonic acid. Inhibition of cyclooxygenase leads to reduced conversion of arachidonic acid to proinflammatory prostaglandins and other bioactive lipids. In spite of their efficacy as anticancer agents, the precise mechanism(s) for the protective effect of NSAIDs remains unknown. An intense debate is underway that focuses on a wide range of mechanisms, some of which are unrelated to inhibition of cyclooxygenase activity (Giardiello et al., 1995; Prescott and White, 1996; Shiff and Rigas, 1997; Smalley and DuBois, 1997).

There are at least two isoforms of cyclooxygenase, COX-1 and COX-2. COX-1 is constitutively expressed in many tissues and cell types (Williams et al., 1997) but in some cases is increased during differentiation (Smith et al., 1993). By contrast, the expression of COX-2 is frequently up-regulated by mitogens, cytokines, and tumor promoters. Greater than 80% of colon cancers in humans have increased COX-2 levels when compared to adjacent normal tissue (Williams et al., 1997). COX inhibitors exhibit dramatic antineoplastic activity in a number of tumor model systems. These include colon cancer cells implanted into nude mice, tumor production in APC mutant mice, and carcinogen-induced tumors in rats (Oshima et al., 1996; Sheng et al., 1997; Kawamori et al., 1998). In one such study, Seed et al. demonstrated that topical administration of a nonselective COX inhibitor (diclofenac) reduced the growth of colon-26 cells, which express COX and produce PGE_2, when implanted into nude mice (Seed et al., 1997). In this study, the authors made the important observation that the antitumor effect of diclofenac was due to an antiangiogenic effect (Seed et al., 1997). This raises the possibility that elevated COX, especially COX-2, plays a role in the growth of certain colon cancer cells due to its ability to act as a tumor promoter via stimulation of angiogenesis. Such a mechanism might also explain the 90% inhibition of the growth of COX-2-positive HCA-7 tumors in vivo following COX-2 inhibitor treatment (Sheng et al., 1997).

Recent studies have confirmed the hypothesis that tumor growth, in general, is dependent on angiogenesis (Folkman, 1990). Any significant increase in tumor mass must be preceded by an increase in the vascular supply to deliver nutrients and oxygen to the tumor. The ability of a tumor to induce angiogenesis represents an essential step for tumor growth beyond 2–3 mm in size. Based on the work published by Seed et al. (1997) and our previous findings that NSAID treatment limits tumor growth in a number of different model systems, we hypothesized that COX is involved in angiogenesis.

In order to test this hypothesis, we have employed an experimental model in which endothelial and cancer cells are cocultured (Montesano et al., 1983). We evaluated two cell types: stably transfected COX-2-overexpressing Caco-2 cells because we had previously reported that COX-2 overexpression could modulate their phenotype (Tsujii et al., 1997) and HCA-7 cells because they express COX-2 constitutively (Sheng et al., 1997). We found that these cells produce high levels of angiogenic factors, which stimulate endothelial tube formation in the coculture model. A selective COX-2 inhibitor (NS-398) inhibits both the expression of angiogenic factors and endothelial tube formation by COX-2-positive Caco-2 or HCA-7 cells.

We also investigated the effect of COX inhibitors on endothelial tube formation induced by coculture with HCT-116 and DLD-1 cell lines, both of which lack COX-1 or -2 activity. HCT-116 or DLD-1 cells produce angiogenic factors in levels equivalent to those of COX-2 overexpressing Caco-2 or HCA-7 cells, and in coculture they stimulate endothelial tube formation. Aspirin (but not NS-398) markedly reduced endothelial tube formation induced by HCT-116 and DLD-1 cells but did not

[‡]To whom correspondence should be addressed.

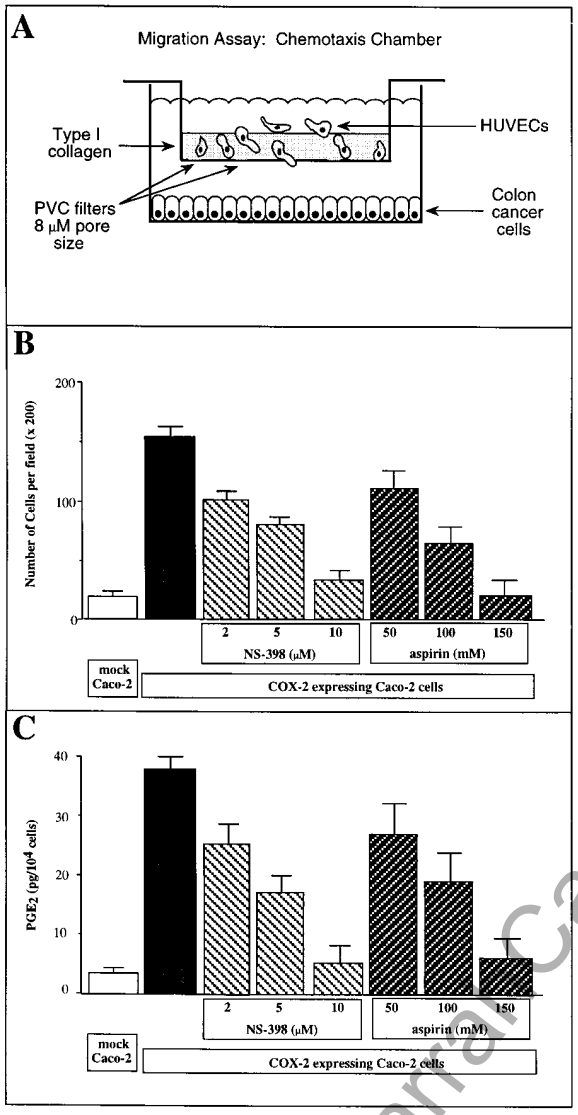

Figure 1. Effect of COX-2 Expression and COX Inhibitors on Prostaglandin Production and Migration of Cocultured HUVECs

(A) Schematic diagram of the migration assay chamber. Colonic carcinoma cells were grown to confluence in the lower chamber, the medium was changed, and an upper chamber containing a monolayer of HUVECs plated onto a layer of type I collagen was inserted. The cells were incubated at 37°C for 4 hr, and then the number of cells that had migrated through the collagen gel onto the filters in the bottom of the upper chamber were counted manually.

(B) Migration of HUVECs cocultured with COX-2-expressing Caco-2 cells. Cell movement was evaluated by the number of cells that migrated toward conditioned medium of mock or COX-2-overexpressing Caco-2 cells in the outer chambers. Incubation was performed at 37° for 4 hr, and at the end of the incubation period, nonmigrated cells on the upper surface of the filter were removed, and the migrated cells were stained and counted manually.

(C) PGE_2 production from mock and COX-2-expressing Caco-2 cells plus and minus aspirin and NS-398. COX-2-expressing Caco-2 cells were treated with NS-398 or aspirin at the indicated concentrations for 24 hr prior to harvest. Medium was taken from mock or COX-2-expressing Caco-2 cells and used for ELISA assay.

affect production of proangiogenic factors. Based on this result, we evaluated the potential role of endothelial COX activity on endothelial tube formation. We found that both a nonselective COX inhibitor (aspirin) and treatment with COX-1 antisense oligonucleotide inhibited endothelial tube formation in the HCT-116 coculture model, although a COX-2 selective inhibitor (NS-398) and COX-2 antisense oligonucleotide showed no inhibitory effect. These results demonstrate that COX-1 activity in endothelial cells plays an important role in the modulation of angiogenesis and may be a relevant target for cancer prevention or treatment in tumors lacking COX-2 expression.

Results

COX-2 Overexpression in Colon Cancer Cells Promotes the Motility of Cocultured Endothelial Cells

Since endothelial cell proliferation and migration are required early in the angiogenic response, our first series of experiments was designed to study whether COX-2 expression in colon cancer cells affected these properties of endothelial cells. To achieve this goal, we used the experimental set-up shown in Figure 1A. In brief, a chemotaxis chamber was employed in which the lower chamber contained colorectal carcinoma cells, and the upper chamber contained a layer of type I collagen over which human umbilical vein endothelial cells (HUVECs) were plated. The number of endothelial cells that migrated through the collagen gel to the bottom of the lower chamber was scored. As shown in Figure 1B, HUVECs cocultured with COX-2-transfected Caco-2 cells migrate 8-fold faster than cells cocultured with control Caco-2 cells (vector alone). This was not due to an effect on endothelial cell proliferation or to a difference in binding of HUVECs to collagen (data not shown). The mobilization of HUVECs in response to COX-2-overexpressing Caco-2 cells was reduced in the presence of a selective COX-2 inhibitor (NS-398) in a dose-dependent manner that correlated well with inhibition of prostaglandin production (Figure 1C). COX-2-expressing Caco-2 cells had a 9-fold increase in PGE_2 production compared to mock Caco-2 cells, and NS-398 or aspirin treatment inhibited PGE_2 production in a dose-dependent manner (Figure 1C). These results show that increased COX-2 expression in Caco-2 cells stimulates their ability to promote endothelial cell motility through a matrix and that inhibition of COX-2 enzyme activity reverses this effect.

COX-2 Expression in Colon Cancer Cells Stimulates Angiogenesis of Cocultured Endothelial Cells

Later stages of angiogenesis require morphological alterations of endothelial cells, which result in lumen formation (Yang and Moses, 1990). To examine this, we next employed an in vitro model of angiogenesis in which HUVECs are induced to invade a three-dimensional collagen gel where they form a network of capillary-like tubes when stimulated by the appropriate angiogenic factors (Figure 2A). Confluent monolayers of HUVECs cocultured in this assay chamber (Figure 2A) for 4 days with COX-2-overexpressing Caco-2 cells formed networks of cord-like structures within the gel (Figure 2B). By contrast, HUVECs cocultured with mock Caco-2 cells showed little invasion or network cord formation. Quantitative evaluation of cord formation revealed that

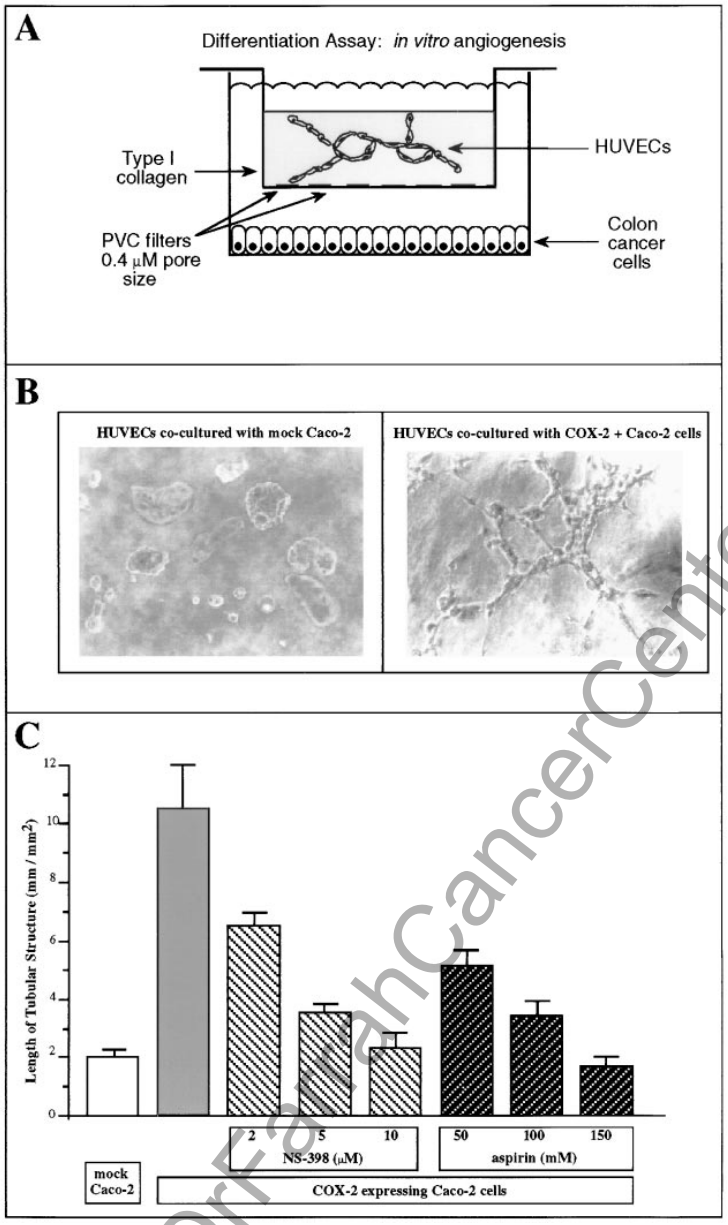

Figure 2. Effect of COX-2 Expression on Angiogenesis In Vitro

(A) Schematic diagram of the differentiation assay chamber. Colonic carcinoma cells were grown to confluence in the lower chamber, the medium was changed, and an upper chamber was inserted. The upper chamber was prepared by plating a monolayer of HUVECs onto a very thick layer of type I collagen followed by incubation at 37°C for 24 hr. The HUVECs were then cocultured with the colonic carcinoma cells at 37°C for 4 days, and following incubation, the number of tube-like structures that formed in the gel were measured by total length per field (X 200).

(B) Phase contrast micrographs of HUVECs grown in collagen gel. (Left panel) HUVECs cocultured with mock Caco-2 cells did not form tubular structure, although a few HUVECs invade into collagen gel. (Right panel) HUVECs cocultured with COX-2-expressing Caco-2 cells showed tubular structure in the gel.

(C) Quantitative analysis of angiogenesis in vitro by measuring the length of the tubular structures formed by HUVECs. The dose-dependent effect of NS-398 or aspirin treatment on endothelial tube formation was also assessed. The cell culture medium was changed daily with fresh addition of NS-398 or aspirin.

coculture with COX-2-overexpressing Caco-2 cells increased the length of the endothelial tubular structures by 5- to 6-fold compared to that seen with mock Caco-2 cells (Figure 2C). NS-398 or aspirin inhibited this enhanced endothelial tube formation in a dose-dependent manner that correlated well with inhibition of prostaglandin production (Figure 1C). These results demonstrate that COX-2 overexpression in Caco-2 cells leads to increased angiogenesis in vitro, which is inhibited by treatment with COX inhibitors.

COX-2 Expression in Colon Cancer Cells Stimulates Production of Angiogenic Factors

Gastrointestinal cancer cells are known to produce a wide variety of factors that may contribute to angiogenesis, including VEGF, bFGF, bFGF-binding protein, TBF-β, PDGF-B, endothelin-1, and iNOS (Abe et al., 1993; Warren et al., 1995; Koolwijk et al., 1996; Sankar et al., 1996; Yamazaki et al., 1996; Skobe et al., 1997; Papapetropoulos et al., 1997). Expression of these mRNAs was therefore measured in Caco-2 cells by Northern blot analysis. COX-2 overexpression was associated with increased levels of RNA for these angiogenic factors, and this effect was inhibited by NS-398 treatment (see Figure 3A). Using an enzyme-linked immunoassay (ELISA), we also measured levels of VEGF, bFGF, TBF-β, PDGF, and endothelin-1 in the culture medium of mock and COX-2-overexpressing Caco-2 cells. The results of these assays (shown in Figure 3B) indicate that COX-2-expressing Caco-2 cells secreted these angiogenic factors into the culture medium. Again, NS-398 treatment clearly inhibited their synthesis and secretion. We also measured the production of nitric oxide (NO) from

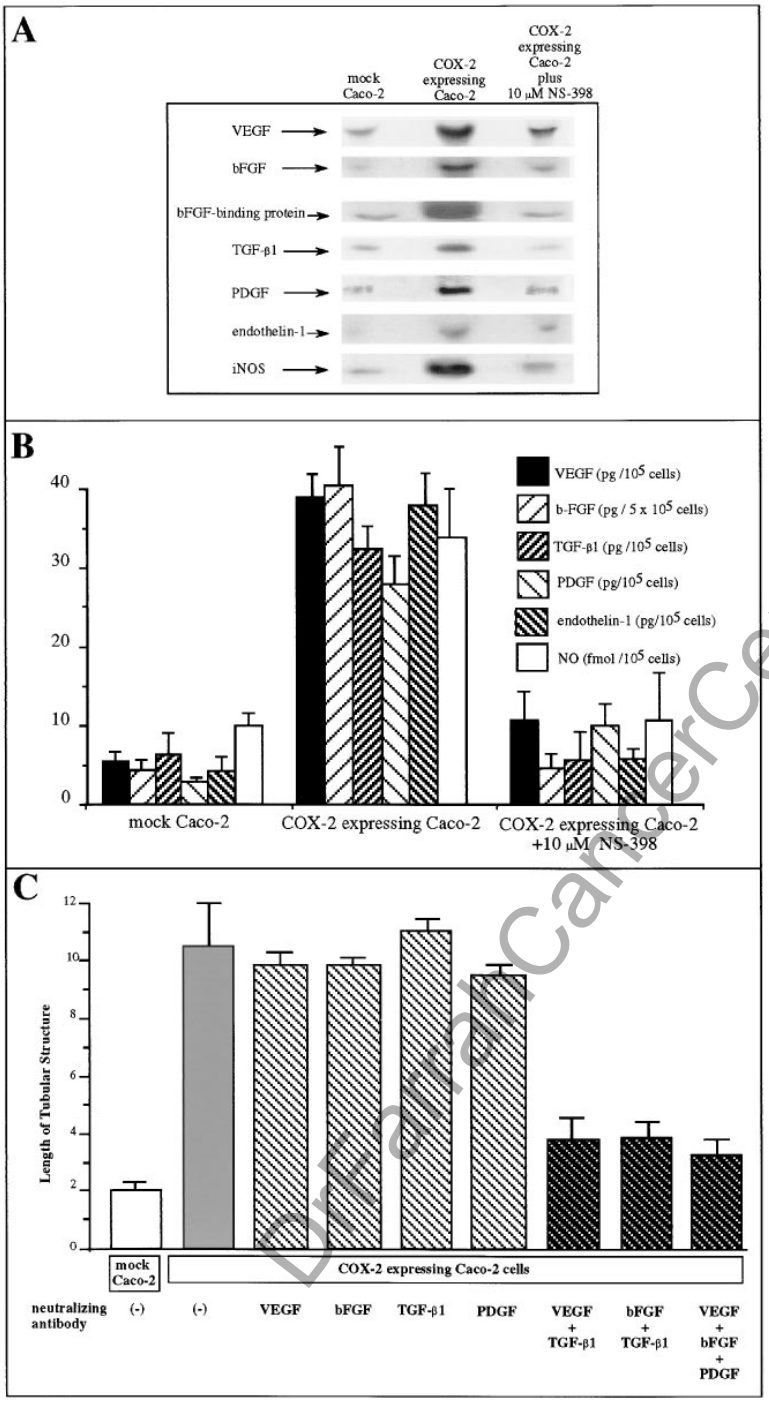

Figure 3. Effect of COX-2 Expression in Colon Cancer Cells on the Production of Angiogenic Factors

(A) Northern blotting analysis to determine mRNA levels of the indicated angiogenic factors in Caco-2 cells. Equal loading of RNA was verified by ethidium bromide staining of the gel and quantitation of the 18 S and 28 S bands.

(B) ELISA to determine the concentration of angiogenic factors in culture medium.

(C) The effect of neutralizing antibodies directed against angiogenic factors on endothelial tube formation. VEGF: 5 μg/ml of anti-VEGF antibody, 10 μg/ml of control rabbit IgG, 5 μg/ml of control goat IgG, and 5 μg/ml of chicken IgG. bFGF: 5 μg/ml of anti-bFGF antibody, 10 μg/ml of control rabbit IgG, 5 μg/ml of control goat IgG, and 5 μg/ml of chicken IgG. TGF-β1: 5 μg/ml of anti-TGF-β1 antibody, 10 μg/ml of control rabbit IgG, and 10 μg/ml of control goat IgG. PDGF: 5 μg/ml of anti-PDGF antibody, 10 μg/ml of control goat IgG, 5 μg/ml of control rabbit IgG, and 5 μg/ml of chicken IgG. VEGF + TGF-β1: 5 μg/ml of anti-VEGF antibody, 5 μg/ml of anti-TGF-β1 antibody, 10 μg/ml of control rabbit IgG, and 5 μg/ml control goat IgG. bFGF + TGF-β1: 5 μg/ml of anti-bFGF antibody, 5 μg/ml of anti-TGF-β-1 antibody, 10 μg/ml of control rabbit IgG, and 5 μg/ml of control goat IgG. VEGF + bFGF + PDGF: 5 μg/ml of anti-VEGF antibody, 5 μg/ml of anti-bFGF antibody, 5 μg/ml of anti-PDGF, 5 μg/ml of control goat IgG, and 5 μg/ml of control rabbit IgG. The cell culture medium was changed daily with addition of fresh antibodies at the concentrations given above. Conditioned medium from the COX-2-expressing cells has similar effects on endothelial migration and tube formation as coculture with COX-2-expressing cells. Adding the same combination of antibodies described above to the conditioned medium results in inhibition of endothelial tube formation (data not shown).

mock and COX-2-overexpressing Caco-2 cells to estimate nitric oxide synthase (NOS) activity. Cox-2-overexpressing Caco-2 cells showed a 3.6-fold increase in NO production compared with mock Caco-2 cells, and this was inhibited by treatment with NS-398 (Figure 3B).

We next investigated which of the angiogenic factors produced by COX-2-positive Caco-2 cells stimulates angiogenesis in the coculture model (Figure 3C) using neutralizing antibodies or treatment with L-NAME (a nitric oxide synthase inhibitor). Each individual neutralizing antibody alone had no effect on endothelial tube formation, while the combination of at least two neutralizing antibodies (anti-VEGF and anti-TGF-β or anti-bFGF and anti-TGF-β) or at least three antibodies (anti-VEGF, anti-bFGF, and anti-PDGF) inhibited endothelial tube formation induced by COX-2-overexpressing Caco-2 cells. Endothelin-1 antibody or L-NAME (3 mM), which completely inhibits the increased production of nitric oxide by coculture with COX-2-overexpressing Caco-2 cells, had no significant effect on endothelial tube formation with any combination of other neutralizing antibodies (data not shown). These results indicate that VEGF,

bFGF, TGF-β, and PDGF can coordinately regulate angiogenesis in this system.

Aspirin Inhibits Angiogenesis Induced by Colon Carcinoma Cells Lacking COX Expression

To examine the specificity of the effects observed with a COX-2 inhibitor, we evaluated the effect of COX inhibitors on in vitro angiogenesis induced by coculture of HUVECs with HCT-116 cells. These colon carcinoma cells lack COX-1 or COX-2 activity (Sheng et al., 1997) but readily develop a vascular supply in the xenograft model and secrete angiogenic factors in vitro.

As shown in Figure 4A, coculture with HCT-116 cells increased the length of tubular structures formed by HUVECs in the collagen gel 7-fold compared to mock Caco-2 cells. HCT-116 cells secrete angiogenic factors including VEGF, bFGF, TGF-β, PDGF, endothelin-1, and NO (data not shown). Only the combination of at least two neutralizing antibodies (anti-VEGF and anti-TGF-β, or anti-bFGF and anti-TGF-β) or at least three neutralizing antibodies (anti-VEGF, anti-bFGF, and anti-PDGF) inhibited tube formation induced by coculture with HCT-116 cells (Figure 4A). Endothelin-1 antibody and L-NAME (3 mM) had no significant effect on tube formation induced by coculture with HCT-116 cells when given in any combination with other antibodies. These results confirm our earlier observations with COX-2-expressing Caco-2 cells that VEGF, bFGF, TGF-β, and PDGF are involved in angiogenesis in this system. We then investigated the effect of NS-398 and aspirin treatment on VEGF, bFGF, TGF-β, and PDGF production and endothelial tube formation. As shown in Figure 4B, neither NSAID had a significant effect on the production and secretion of these angiogenic factors by HCT-116 cells. Surprisingly, however, we found that aspirin effectively inhibits endothelial tube formation induced by HCT-116 coculture, while NS-398 had no effect (Figure 4C).

COX-1 Is Up-Regulated in Endothelial Cells by Angiogenic Factors

The finding that aspirin inhibited angiogenesis induced by COX-negative HCT-116 cells raised the possibility that the NSAID is acting on endothelial cells rather than on the colonic carcinoma cells. More specifically, angiogenic factors might induce COX expression in the endothelial cells. To test this hypothesis, we harvested HUVECs cocultured for 48 hr with either COX-2-expressing Caco-2 or HCT-116 cells and measured COX-1, COX-2, and Ets-1 protein levels by immunoblot analysis (Figures 5A and 5B). The *Ets-1* gene has been shown to play an important role in angiogenesis because abrogation of its expression can block angiogenesis (Iwasaka et al., 1996). COX-1, COX-2, and Ets-1 proteins were barely detectable in HUVECs cultured with mock Caco-2 cells. Significantly, coculture with COX-2-expressing Caco-2 cells or with HCT-116 cells induced the expression of COX-1, COX-2, and Ets-1 in HUVECs (Figures 5A and 5B). NS-398 (10 μM) and aspirin (150 mM) inhibited Ets-1 expression in HUVECs cocultured with COX-2-expressing Caco-2 cells (Figure 5A). On the other hand, as shown in Figure 5B, aspirin (but not NS-398) inhibited the expression of Ets-1 in HUVECs cocultured with HCT-116 cells (Figure 5B). The combination of neutralizing antibodies that suppressed in vitro angiogenesis also inhibited the induction of COX-1 and Ets-1 expression induced by coculture with COX-2-expressing Caco-2 or HCT-116 cells, while no combination of antibodies significantly inhibited COX-2 expression in endothelial cells.

We evaluated the effect of COX-1 and COX-2 expression in HUVECs on their ability to form tubes in collagen gel using either COX-1 or COX-2 antisense oligonucleotides. Immunoblot analysis of extracts from antisense-treated HUVECs confirmed that both COX-1 and COX-2 antisense oligonucleotides specifically suppressed COX-1 and COX-2 protein expression, respectively. As shown in Figure 5C, COX-1 antisense oligonucleotide, but not COX-2 antisense oligonucleotide, inhibited tube formation of HUVECs in the gel. COX-1 sense oligonucleotide or COX-2 antisense or sense oligonucleotide did not show an inhibitory effect on angiogenesis in vitro. In HUVECs cocultured with COX-2-expressing Caco-2 cells or with HCT-116 cells, COX-1 antisense oligonucleotide inhibited the expression of COX-1 and Ets-1, while COX-2 antisense oligonucleotide had no significant effect on COX-1 or Ets-1 expression (Figure 5B). These results demonstrate that COX-1 produced by endothelial cells plays an important role in angiogenesis in this in vitro assay.

HCA-7 and DLD-1 Colon Cancer Cells Produce Proangiogenic Factors and Stimulate Angiogenesis of Cocultured Endothelial Cells

To test the generality of our finding with Caco-2 and HCT-116 cells, we have characterized two additional colorectal cancer cell lines with respect to promotion of endothelial tube formation and production of proangiogenic factors. We found that HCA-7 cells (which constitutively express COX-2) produced VEGF, bFGF, TGF-β, and PDGF, which is inhibited by treatment with NS-398 or aspirin (Figure 6A). Additionally, we found that both NS-398 and aspirin treatment inhibited endothelial tube formation induced by HCA-7 cells in a dose-dependent manner (Figure 6B). Since these cells constitutively express COX-2 in culture, these results are not likely due to a selection artifact of a particular cell type. We also examined another colon cancer cell line (DLD-1) that has undetectable COX-2 expression and found results similar to those seen with the HCT-116 cells (Figures 7A and 7B).

Cyclooxygenase Inhibitors Have Antineoplastic Activity

Our in vitro results have prompted us to evaluate the antineoplastic properties of NSAIDs in an in vivo tumor xenograft model system. For these experiments, we xenografted colon cancer cells in nude mice and then examined the growth rate of these tumors with and without cyclooxygenase inhibitor treatment. Figure 8A shows the results with Caco-2 cells. Interestingly, parental Caco-2 cells (lacking COX-2) do not grow well over the 28 day time period of this experiment. By contrast, the COX-2-expressing Caco-2 cells grow at a fairly rapid

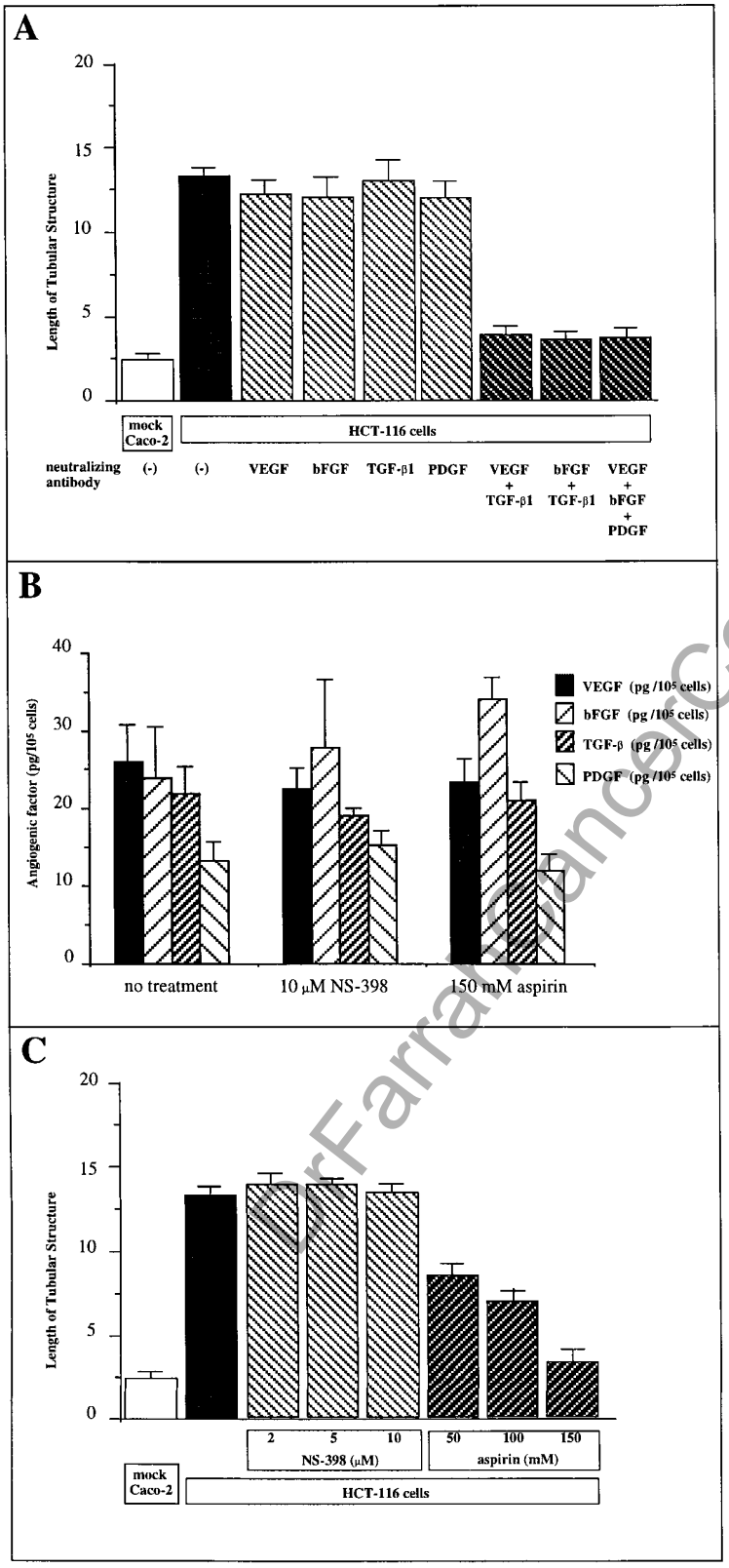

Figure 4. Production of Angiogenic Factors from HCT-116 and Endothelial Tube Formation Induced by Coculture with HCT-116 Cells

(A) In vitro angiogenesis induced by coculture with HCT-116 cells and the effect of neutralizing antibodies against angiogenic factors.

(B) The effect of COX inhibitors on production of angiogenic factors by HCT-116 cells.

(C) The effect of COX inhibitors on induction of endothelial tube formation by coculture with HCT-116 cells.

rate, and their growth is inhibited by treatment with both a selective COX-2 inhibitor (NS-398) and the nonselective NSAID (indomethacin).

Since HCT-116 cells lack cyclooxygenase-2 expression, a selective COX-2 inhibitor does not affect their growth as xenografts in nude mice (Figure 8B and Sheng

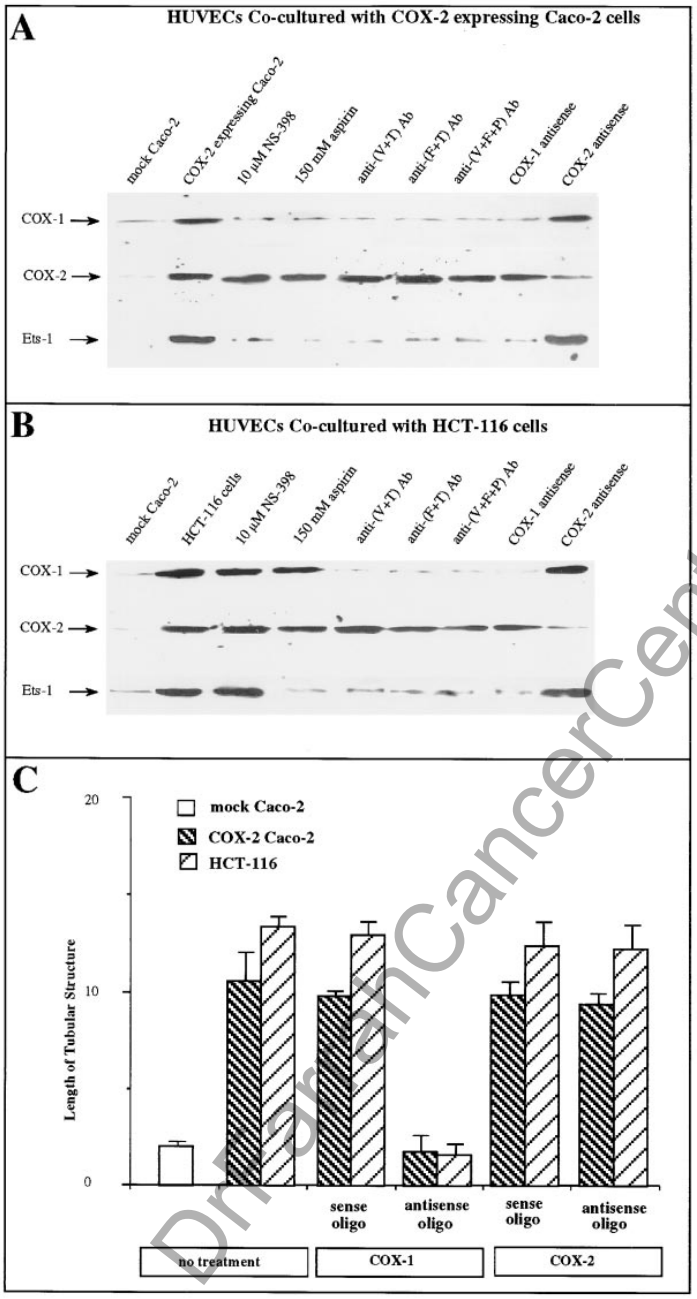

Figure 5. Western Blot Analysis of COX-1, COX-2, and Ets-1 in HUVECs and the Effect of COX-1 or COX-2 Antisense Oligonucleotides on Ets-1 Expression and Endothelial Tube Formation

COX-1, -2, and Ets-1 expression in HUVECs cocultured with mock and COX-2-expressing Caco-2 cells (A) or HCT-116 cells (B). Anti-(V + T) Ab: 5 μg/ml of anti-VEGF antibody, 5 μg/ml of anti-TGF-β1 antibody, 10 μ/ml of control rabbit IgG, and 5 μg/ml control goat IgG. Anti-(F + T) Ab: 5 μg/ml of anti-bFGF antibody, 5 μg/ml of anti-TGF-β-1 antibody, 10 μg/ml of control rabbit IgG, and 5 μg/ml of control goat IgG. Anti-(V + F + P) Ab: 5 μg/ml of anti-VEGF antibody, 5 μg/ml of anti-bFGF antibody, 5 μg/ml of anti-PDGF, 5 μg/ml of control goat IgG, and 5 μg/ml of control rabbit IgG. COX-1 or COX-2 antisense oligonucleotide (0.5 mM) was transfected into HUVECs using 10 μg/ml of lipofectin. (C) The effect of COX-1 or COX-2 sense or antisense oligonucleotide transfection into HUVECs on endothelial tube formation.

et al., 1997). However, a nonselective inhibitor with preference for COX-1 inhibition causes a significant reduction (3-fold) in tumor growth rate (Figure 8B). This result would have been predicted from the in vitro angiogenesis results shown in Figure 4C, further supporting the notion that endothelial COX-1 activity is important for tumor-induced angiogenesis.

Discussion

Cyclooxygenase-2, a key enzyme required for the conversion of arachidonic acid to prostaglandins, plays an important role in the promotion of intestinal tumorigenesis in animal models (Oshima et al., 1996; Sheng et al., 1997; Kawamori et al., 1998), but the underlying mechanism of action is still poorly understood. In this report, we have employed an endothelial cell/colon carcinoma coculture model system to explore the role of cyclooxygenase (COX) in tumor-related angiogenesis. We show that NSAIDs can inhibit angiogenesis by two mechanisms: by inhibiting COX-2 activity in colon carcinoma cells and downregulating production of angiogenic factors, and by inhibiting COX-1 activity in the endothelial cells themselves.

COX-2-Overexpressing Colon Cancer Cells Promote Angiogenesis

We show that colon carcinoma cells (Caco-2) that are forced to express COX-2 stimulate endothelial motility

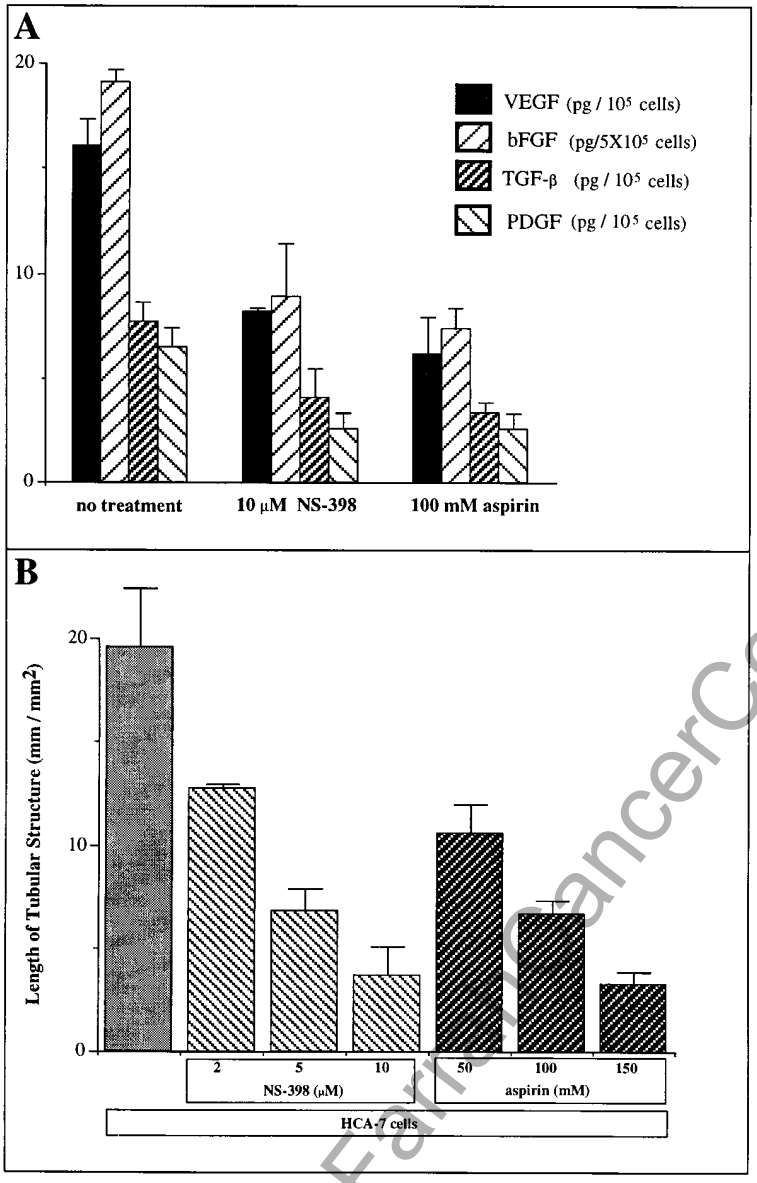

Figure 6. Production of Angiogenic Factors from HCA-7 Cells and Endothelial Tube Formation Induced by Coculture with HCA-7 Cells

(A) The effect of COX inhibitors on production of angiogenic factors by HCA-7 cells.

(B) The effect of COX inhibitors on induction of endothelial tube formation by coculture with HCA-7 cells. Quantitative analysis of angiogenesis in vitro by measuring the length of the tubular structures formed by HUVECs. The dose-dependent effect of NS-398 or aspirin treatment on endothelial tube formation was also assessed. The cell culture medium was changed daily with fresh addition of NS-398 or aspirin.

and tube formation by increased production of proangiogenic factors. Similar activity is seen in untransfected HCA-7 cells which constitutively express COX-2, suggesting that these results are not an artifact due to forced overproduction of the enzyme. Since these effects can be reversed by inhibition of COX-2, the production of prostaglandins seems to play an important role in regulating the production of angiogenic factors by Caco-2 and HCA-7 cells. The array of prostaglandins produced by Caco-2 cells and their cellular targets is currently under investigation. We have previously reported that HCA-7 cells produce a wide array of eicosanoids (Coffey et al., 1997). These bioactive lipids can have both autocrine and paracrine effects but usually act in the microenvironment of the cells from which they are produced. Two classes of prostaglandin receptors exist to transduce signals upon binding of ligand: the G-coupled cytoplasmic receptor class (i.e., EP1–4 for PGE_2) (Breyer et al., 1996) and the nuclear PPAR receptor class (i.e., PPARα, PPARγ, and PPARδ), which acts directly as a transcription factor upon ligand binding (Forman et al., 1996). We are currently evaluating the prostaglandin-mediated transcriptional regulation of genes encoding key angiogenic factors (VEGF, TGF-β1, and PDGF) to further elucidate the mechanisms involved in the regulation of angiogenesis by COX-2. We have previously reported that some colon cancer cells and rodent intestinal tumors have increased expression of PPARγ (DuBois et al., 1998).

Endothelial Cells Upregulate COX-1 in Response to Angiogenic Factors

COX-1 is thought to be expressed constitutively in many tissues; however, in some cell types it has been reported that COX-1 expression is inducible and that its induction coincides with cell differentiation (Hoff et al., 1993; Murakami et al., 1995; Ueda et al., 1997). One such cell type is the endothelial cell. In pulmonary artery endothelial

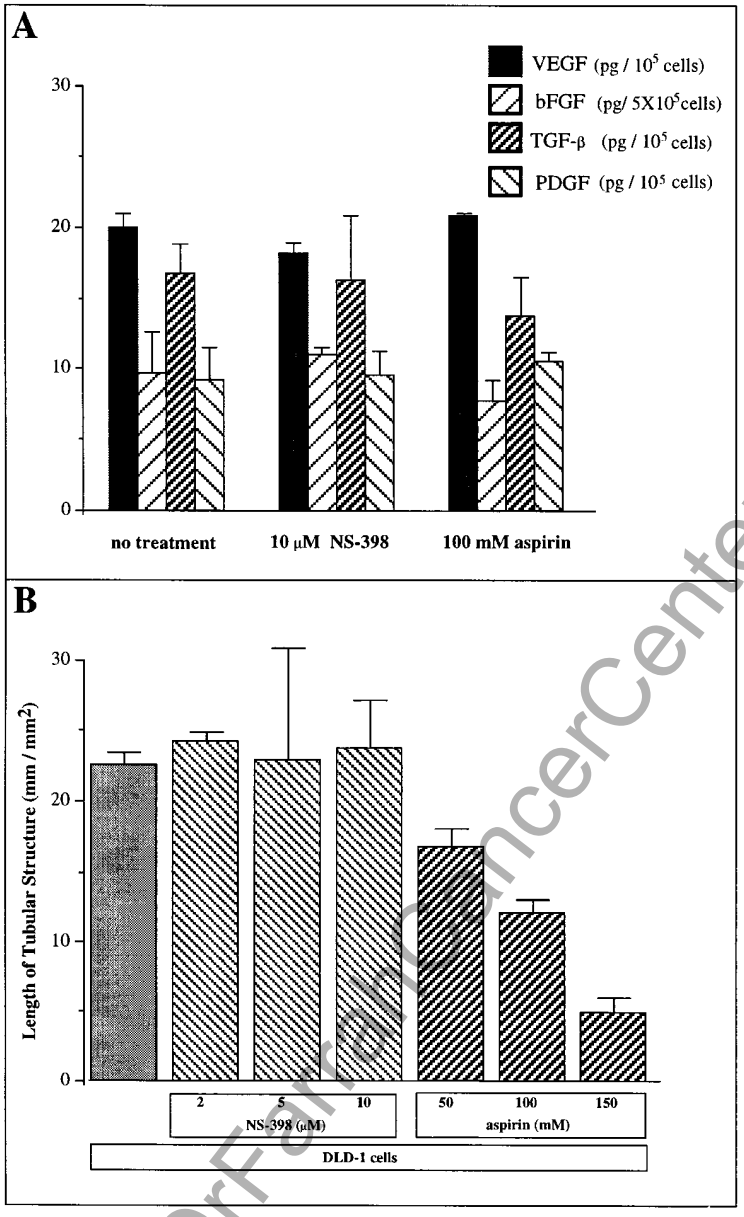

Figure 7. Production of Angiogenic Factors from DLD-1 Cells and Endothelial Tube Formation Induced by Coculture with DLD-1 Cells

(A) The effect of COX inhibitors on production of angiogenic factors by DLD-1 cells.

(B) The effect of COX inhibitors on induction of endothelial tube formation by coculture with DLD-1 cells. Quantitative analysis of angiogenesis in vitro by measuring the length of the tubular structures formed by HUVECs. The dose-dependent effect of NS-398 or aspirin treatment on endothelial tube formation was also assessed. The cell culture medium was changed daily with fresh addition of NS-398 or aspirin.

cells, increased *COX-1* gene expression was observed during late fetal and early newborn life, a period that may be critical to successful cardiopulmonary transition and function in the newborn (Brannon et al., 1994). Recently, Narko et al. reported that COX-1-overexpressing endothelial cells undergo malignant transformation whereas COX-2-overexpressing endothelial cells were difficult to isolate (Narko et al., 1997). These results also suggest a role of COX-1 in the regulation of endothelial cell biology. Since aspirin (a COX inhibitor) is the most widely studied NSAID for cancer prevention, we sought to determine potential mechanisms for its chemoprotective effects. Here, we demonstrate that COX-1 expression and activity in HUVECs regulates tube formation induced by HCT-116 cells. Treatment of HUVECs with aspirin blocks angiogenesis stimulated by coculture with the COX-negative HCT-116 or DLD-1 cells. Therefore, prostaglandins produced by COX-1 in endothelial cells could be important in regulating genes required for endothelial tube formation. If fact, we did observe that aspirin treatment or inhibition of COX-1 synthesis (by antisense oligonucleotide treatment) markedly inhibited expression of Ets-1, a transcription factor shown to be involved in angiogenesis (Iwasaka et al., 1996).

NSAIDs Inhibit Growth of Colon Cancer Xenografts

Based on the results obtained in the in vitro angiogenesis studies, we predicted that parental Caco-2 cells would grow poorly as xenografts in nude mice, while COX-2-overexpressing cells would grow well. In vivo experiments supported this hypothesis. Moreover, both indomethacin and NS-398 inhibited tumor growth effectively. Additional support for our hypothesis comes from the finding that a selective COX-2 inhibitor does not affect HCT-116 xenograft growth, but a nonselective

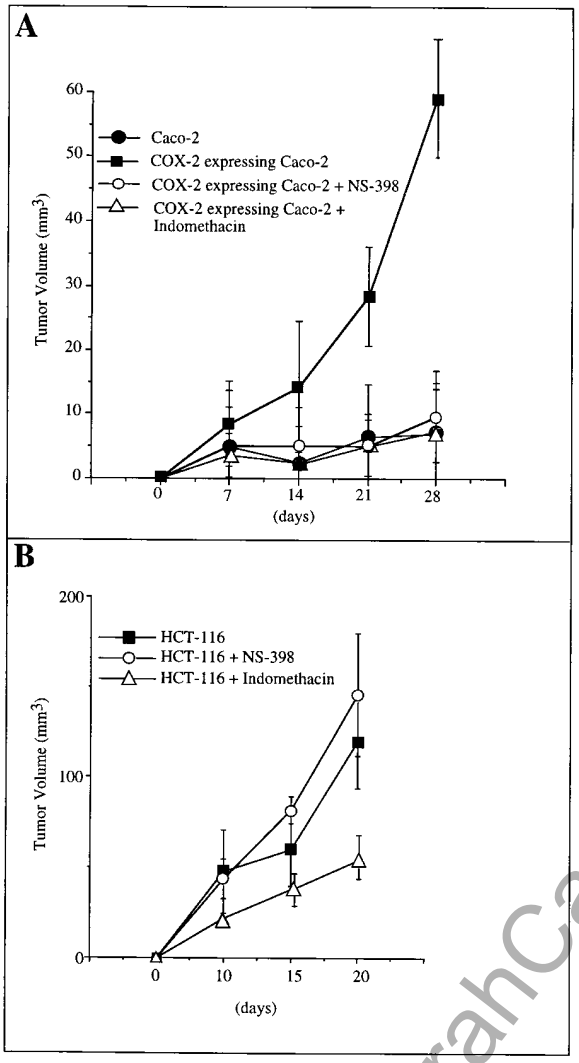

Figure 8. Effect of Cyclooxygenase Inhibitors on Growth of Xenografted Cells in Nude Mice

(A) 1×10^6 Caco-2 or COX-2-expressing Caco-2 cells were suspended in 0.2 ml of DMEM medium and injected into the dorsal subcutaneous tissue of athymic nude mice. Mice were given indomethacin (3 mg/kg), NS-398 (100 mg/kg), or saline daily by gavage tube, as noted in the figure. The treatment was continued for 28 days. Tumor volumes were determined by external measurement according to published methods (Wang et al., 1995). Volume was determined according to the equation $[V = L \times W^2] \times 0.5$, where V = volume, L = length and W = width. Values expressed are the means ± SE of 12 xenografts.

(B) 1×10^6 HCT-116 cells were suspended in 0.2 ml of DMEM medium and injected into the dorsal subcutaneous tissue of athymic nude mice. The mice were given indomethacin (3 mg/kg), NS-398 (100 mg/kg), or saline daily by gavage tube. The treatment was continued for 20 days. Tumor volumes were measured as described above.

inhibitor is effective, as shown in Figure 8B. We are currently evaluating the effects of treatment with cyclooxygenase inhibitors on angiogenesis in these in vivo models.

In conclusion, further investigation will be required to determine the precise mechanism(s) by which COX-2 induces cancer cells to produce angiogenic factors and how COX-1 enhances the angiogenic potential of endothelial cells. However, our results raise novel ideas about the way in which COX inhibitors carry out their antitumor effects and open up new opportunities for developing agents for cancer treatment and prevention in humans.

Experimental Procedures

Reagents and Cells

Neutralizing antibodies against VEGF, bFGF, TGF-β, and PDGF were purchased from RandD Systems (Minneapolis, MN cat. #AB-293-NA, AB-233-NA, AB-101-NA, and AB-20-NA, respectively). Neutralizing antibody against endothelin-1 was purchased from Peptide Institute (Osaka, Japan) (Masuda et al., 1992). For controls in the blocking experiments, goat, chicken, or rabbit IgG (Zymed Laboratories, San Francisco, CA) was added at an amount equivalent to the final concentration of IgG (25 μg/ml). The antisense oligonucleotide sequences for COX-1 and COX-2 were developed in consultation with AGCT (San Diego, CA). We then evaluated a number of candidate sequences to determine which were the best at inhibiting the expression of COX-1 or COX-2 protein without affecting the expression of the other COX isoform. Phosphorothioate COX-1 and COX-2 antisense oligonucleotides were selected for the studies presented here: COX-1, AGAACCGGAGCAAGA; and COX-2, GGAAACATCGA CAGT, respectively. Sense sequence for each oligonucleotide was used as controls. Caco-2, DLD-1, and HCT-116 cells were obtained from the American Type Culture Collection and grown as described previously (Sheng et al., 1997; Tsujii et al., 1997). HCA-7 cells were kindly provided by Susan Kirkland (Sheng et al., 1997). Human umbilical vein endothelial cells were obtained from Clonetech (Palo Alto, CA) and used between passages 2 and 4 for each experiment.

Transfection

A 2.1 kb fragment containing an open reading frame of rat *COX-2* was isolated and cloned into the eukaryotic expression vector pCB6, which contains the *CMV* promoter for controlling transcription of the cDNA insert and a neomycin resistance gene expression cassette for G418 selection. This expression vector was transfected into the Caco-2 cells, and three independent clones showing a 10- to 12-fold increase in COX-2 expression were isolated by culturing the cells in medium containing 1.5 mg/ml of G418. We characterized all three clones and found that they all exhibited the identical phenotypic and biochemical alterations. Therefore, the results of our studies reported here focused on one of these clones. The Caco-2 cells were transfected with vector plasmid alone and selected in an identical fashion. These transfectants were used as controls and designated as "mock" cells.

Cell Culture

To quantitate a direct paracrine relationship between tumor cells and endothelial cells using neutralizing antibodies and antisense oligonucleotides, an in vitro coculture system was employed to assay for endothelial tube formation induced by tumor cells.

To assay the percentage of proliferating cells, a polycarbonate membrane (0.4 micrometer pore size) was coated with type I collagen and placed between the upper and lower well plates. Tumor cells were grown in the outer chamber, and at confluence, the medium was changed to serum-free RPMI. Separately, HUVECs suspended in serum-free RPMI containing 1% BSA were plated on the inner chambers and allowed to adhere for 24 hr. Then, the medium was changed to serum-free RPMI, and the inner chamber was transferred into the outer chamber. Proliferation of HUVECs was evaluated after 24 hr coculture with mock or COX-2-overexpressing Caco-2 cells.

To assay for migration of endothelial cells, polycarbonate filters (8 μm pore size) coated with type I collagen were used. Tumor cells were grown in the outer chamber, and at confluence, the inner chamber was transferred to the outer chamber, and HUVECs were suspended in serum-free RPMI and seeded in the inner chamber. Cell movement was evaluated toward a gradient of conditioned medium of mock or COX-2-overexpressing Caco-2 cells. The cells

were incubated at 37° for 4 hr. Following the incubation period, nonmigrated cells on the upper surface of the filter were removed, and the cells that had migrated onto the filter were counted manually by examination under the microscope.

For the assay of in vitro angiogenesis, tube formation of HUVECs in purified collagen was analyzed employing a coculture method. Tumor cells were grown in the outer chamber, and at confluence, the medium was changed with serum-free medium. Separately, for the preparation of collagen gel, 8 vol of Vitrogen 100 type I collagen (3.1 mg/ml) was mixed with 1 vol of 10 × RPMI and 1 vol of sodium bicarbonate (23.5 mg/ml). The mixture was quickly added to the inner chamber of collagen transwell inserts (12 mm diameter, 0.4 μm pores; Costar, Cambridge, MA) and allowed to gel for 30 min at 37°C. After solidification, the wells were washed twice with RPMI and then incubated for 2 hr with RPMI containing 10% FCS. The resultant gel was covered with culture medium containing sufficient HUVECs to form a confluent monolayer and incubated for 24 hr. At confluence, the medium was changed to 0.5% serum-containing medium, and the inner chamber was transferred into the outer chamber. After incubation for 4 days in the presence of cocultured colon cancer cells, tube-like structures that formed in the gel were measured by total length per field (X 200). Values were expressed as means and standard deviations of eight fields. To investigate the effect of COX-1 or COX-2 in endothelial cell tube formation, COX-1 or COX-2 antisense oligonucleotides were used. Before coculture was started, a monolayer of confluent HUVECs on collagen gel was treated with 0.5 μM for each antisense oligonucleotide and 10 μg/ml lipofectin. Four hours later, the medium was changed, and coculture was started.

These experiments were repeated with both human aortic endothelial cells (HA-4001) and human newborn skin microvascular endothelial cells (HE-4201) obtained from Kurabo Biomed. (Tokyo, Japan). Using these two endothelial cell lines, we obtained similar results for migration and tube formation as we have reported for the HUVECs in Figures 1–4.

Analysis of Expression of VEGF, bFGF, bFGF-Binding Protein, TGF-β, PDGF, iNOS, and Endothelin-1

The expression of angiogenic factors, *VEGF*, *bFGF*, and *TGF*-β were evaluated using Northern blot analysis and ELISA. COX-2-overexpressing Caco-2 cells and mock transfectants were cultured in the medium containing 10% FCS. At confluence, the medium was changed to serum-free medium, and after 24 hr culture, the medium was collected for ELISA, and total RNA was prepared from the cells. The *VPF/VEGF* cDNA fragment, *bFGF* cDNA fragment, *bFGF*-binding protein (a fragment comprising nucleotides 58–805 of human *bFGF*-binding protein; GenBank/EMBL accession number X04677), *TGF*-β cDNA fragment, *PDGF-B* cDNA fragment, *endothelin-1* cDNA fragment, and *iNOS* cDNA fragment were used as probes and labeled as previously described (Tsujii and DuBois, 1995). The concentration of VEGF, bFGF, TGF-β, PDGF, and endothelin-1 in the culture medium were measured using anti-human VEGF (Immuno-Biological Laboratories, code #17171, Gunma, Japan), anti-human fibroblast growth factor basic (RandD systems, cat #DFB00), human transforming growth factor-β1 (RandD systems, cat #DB100), and anti-human PDGF (RandD systems, cat #AB-20-NA), respectively. The concentration of nitric oxide was determined by NO-ANALYZER (FES-480; S. TEC, Osaka, Japan).

Immunoblotting

Immunoblot analysis of cell protein lysates was performed as described (DuBois et al., 1996). In brief, after 48 hr of coculture, HUVECs were harvested from the collagen gels by treatment with collagenase, and the cells were lysed in RIPA buffer (1× PBS/1% Nonidet P-40/0.5% sodium deoxycholate/0.1% SDS at 10 mg/ml), then clarified cell lysates (50 μg) were denatured and fractionated by 12.5% SDS/PAGE. Following electrophoresis, the proteins were transferred to nitrocellulose, and the filters were probed with anti-human COX-1 (N-20, cat #sc-1753), COX-2 (N-20, cat #1746), and Ets-1 (N-276, cat #sc-111) antibodies (Santa Cruz Biotechnology), and the X-ray film was developed by the ECL chemiluminescence system (Amersham).

Tumor Growth in Nude Mice

Cells were suspended in 0.2 ml of DMEM medium and were injected into the dorsal subcutaneous tissue of athymic nude mice. Tumor volume was determined by external measurement according to published methods (Wang et al., 1995). Volume was determined according to the equation ($V = [L \times W^2] \times 0.5$, where V = volume, L = length, and W = width.

Acknowledgments

We thank Toshifumi Ito for critical input. We also thank Brigid Hogan for helpful comments and for a critical scientific review and Larry Marnett, Rebecca Shattuck-Brandt, Rajnish Gupta, and Chris Williams for helpful suggestions. The authors would like to acknowledge support from the A. B. Hancock, Jr. Memorial Laboratory, Lucille P. Markey Charitable Trust, the United States Public Health Services Grants DK-47297 (R. N. D.) and NIEHS-00267 and the Veterans Administration Merit Grant (R. N. D.). R. N. D. is the Mina Cobb Wallace Professor of Gastroenterology and Cancer Prevention.

Received April 15, 1998; revised May 14, 1998.

References

Abe, T., Okamura, K., Ono, M., Kohno, K., Mori, T., Hori, S., and Kuwano, M. (1993). Induction of vascular endothelial tubular morphogenesis by human glioma cells. A model system for tumor angiogenesis. J. Clin. Invest. *92*, 54–61.

Brannon, T.S., North, A.J., Wells, L.B., and Shaul, P.W. (1994). Prostacyclin synthesis in ovine pulmonary artery is developmentally regulated by changes in cyclooxygenase-1 gene expression. J. Clin. Invest. *93*, 2230–2235.

Breyer, M.D., Jacobson, H.R., and Breyer, R.M. (1996). Functional and molecular aspects of renal prostaglandin receptors. J. Am. Soc. Nephrol. *7*, 8–17.

Coffey, R.J., Hawkey, C.J., Damstrup, L., Graves-Deal, R., Daniel, V.C., Dempsey, P.J., DuBois, R.N., Jetton, T., and Morrow, J. (1997). EGF receptor activation induces nuclear targeting of COX-2, basolateral release of prostaglandins and mitogenesis in polarizing colon cancer cells. Proc. Natl. Acad. Sci. USA *94*, 657–662.

DuBois, R.N., Shao, J., Sheng, H., Tsujii, M., and Beauchamp, R.D. (1996). G1 delay in intestinal epithelial cells overexpressing prostaglandin endoperoxide synthase-2. Cancer Res. *56*, 733–737.

DuBois, R.N., Gupta, R., Brockman, J.A., Reddy, B.S., Karakow, S.L., and Lazar, M.A. (1998). The nuclear eicosanoid receptor, PPARγ, is aberrantly expressed in colonic cancers. Carcinogenesis *19*, 49–53.

Folkman, J. (1990). What is the evidence that tumors are angiogenesis dependent? J. Natl. Cancer Inst. *82*, 4–6.

Forman, B.M., Chen, J., and Evans, R.M. (1996). The peroxisome proliferator-activated receptors: ligands and activators. Ann. N. Y. Acad. Sci. *804*, 266–275.

Giardiello, F.M., Offerhaus, G.J.A., and DuBois, R.N. (1995). The role of nonsteroidal antiinflammatory drugs in colorectal cancer prevention. Eur. J. Cancer *31A*, 1071–1076.

Hoff, T., DeWitt, D., Kaever, V., Resch, K., and Goppelt-Struebe, M. (1993). Differentiation-associated expression of prostaglandin G/H synthase in monocytic cells. FEBS Lett. *320*, 38–42.

Iwasaka, C., Tanaka, K., Abe, M., and Sato, Y. (1996). Ets-1 regulates angiogenesis by inducing the expression of urokinase-type plasminogen activator and matrix metalloproteinase-1 and the migration of vascular endothelial cells. J. Cell. Phys. *169*, 522–531.

Kawamori, T., Rao, C.V., Seibert, K., and Reddy, B.S. (1998). Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, against colon carcinogenesis. Cancer Res. *58*, 409–412.

Koolwijk, P., van Erck, M.G., de Vree, W.J., Vermeer, M.A., Weich, H.A., Hanemaaijer, R., and van Hinsbergh, V.W. (1996). Cooperative effect of TNFα, bFGF, and VEGF on the formation of tubular structures of human microvascular endothelial cells in a fibrin matrix. Role of urokinase activity. J. Cell Biol. *132*, 1177–1188.

Masuda, E., Kawano, S., Nagano, K., Tsuji, S., Ishigami, Y., Tsujii,

M., Hayashi, N., Fusamoto, H., and Kamada, T. (1992). Effect of intravascular ethanol on modulation of gastric mucosal integrity: possible role of endothelin-1. Am. J. Physiol. *262*, G785–G790.

Montesano, R., Orci, L., and Vassalli, P. (1983). *In vitro* rapid organization of endothelial cells into capillary-like networks is promoted by collagen matrices. J. Cell Biol. *97*, 1648–1652.

Murakami, M., Matsumoto, R., Urade, Y., Austen, K.F., and Arm, J.P. (1995). c-kit ligand mediates increased expression of cytosolic phospholipase A2, prostaglandin endoperoxide synthase-1, and hematopoietic prostaglandin D2 synthase and increased IgE-dependent prostaglandin D2 generation in immature mouse mast cells. J. Biol. Chem. *270*, 3239–3246.

Narko, K., Ristimaki, A., MacPhee, M., Smith, E., Haudenschild, C.C., and Hla, T. (1997). Tumorigenic transformation of immortalized ECV endothelial cells by cyclooxygenase-1 overexpression. J. Biol. Chem. *272*, 21455–21460.

Oshima, M., Dinchuk, J.E., Kargman, S.L., Oshima, H., Hancock, B., Kwong, E., Trzaskos, J.M., Evans, J.F., and Taketo, M.M. (1996). Suppression of intestinal polyposis in $APC^{\Delta 716}$ knockout mice by inhibition of prostaglandin endoperoxide synthase-2 (COX-2). Cell *87*, 803–809.

Papapetropoulos, A., Desai, K.M., Rudic, R.D., Mayer, B., Zhang, R., Ruiz-Torres, M.P., Garcia-Cardena, G., Madri, J.A., and Sessa, W.C. (1997). Nitric oxide synthase inhibitors attenuate transforming-growth-factor-β1-stimulated capillary organization in vitro. Am. J. Pathol. *150*, 1835–1844.

Prescott, S.M., and White, R.L. (1996). Self Promotion? Intimate connections between APC and prostaglandin H synthase-2. Cell *87*, 783–786.

Sankar, S., Mahooti-Brooks, N., Bensen, L., McCarthy, T.L., Centrella, M., and Madri, J.A. (1996). Modulation of transforming growth factor β receptor levels on microvascular endothelial cells during in vitro angiogenesis. J. Clin. Invest. *97*, 1436–1446.

Seed, M.P., Brown, J.R., Freemantle, C.N., Papworth, J.L., Colville-Nash, P.R., Willis, D., Somerville, K.W., Asculai, S., and Willoughby, D.A. (1997). The inhibition of colon-26 adenocarcinoma development and angiogenesis by topical diclofenac in 2.5% hyaluronan. Cancer Res. *57*, 1625–1629.

Sheng, H., Shao, J., Kirkland, S.C., Isakson, P., Coffey, R., Morrow, J., Beauchamp, R.D., and DuBois, R.N. (1997). Inhibition of human colon cancer cell growth by selective inhibition of cyclooxygenase-2. J. Clin. Invest. *99*, 2254–2259.

Shiff, S.J., and Rigas, B. (1997). Nonsteroidal anti-inflammatory drugs and colorectal cancer: evolving concepts of their chemopreventive actions. Gastroenterology *113*, 1992–1993.

Skobe, M., Rockwell, P., Goldstein, N., Vosseler, S., and Fusenig, N.E. (1997). Halting angiogenesis suppresses carcinoma cell invasion. Nat. Med. *3*, 1222–1227.

Smalley, W., and DuBois, R.N. (1997). Colorectal cancer and non steroidal anti-inflammatory drugs. Adv. Pharmacol. *39*, 1–20.

Smith, C.J., Morrow, J.D., Roberts, L.J., and Marnett, L.J. (1993). Differentiation of monocytoid THP-1 cells with phorbol ester induces expression of prostaglandin endoperoxide synthase-1 (COX-1). Biochem. Biophys. Res. Comm. *192*, 787–793.

Tsujii, M., and DuBois, R.N. (1995). Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase-2. Cell *83*, 493–501.

Tsujii, M., Kuwano, S., and DuBois, R.N. (1997). Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. Proc. Natl. Acad. Sci. USA *94*, 3336–3340.

Ueda, N., Yamashita, R., Yamamoto, S., and Ishimura, K. (1997). Induction of cyclooxygenase-1 in a human megakaryoblastic cell line (CMK) differentiated by phorbol ester. Biochim. Biophys. Acta *1344*, 103–110.

Wang, J., Sun, L., Myeroff, L., Wang, X., Gentry, L.E., Yang, J., Liang, J., Zborowska, E., Markowitz, S., Willson, J.K., and Brattain, M.G. (1995). Demonstration that mutation of the type II transforming growth factor β receptor inactivates its tumor suppressor activity in replication error-positive colon carcinoma cells. J. Biol. Chem. *270*, 22044–22049.

Warren, R.S., Yuan, H., Matli, M.R., Gillett, N.A., and Ferrara, N. (1995). Regulation by vascular endothelial growth factor of human colon cancer tumorigenesis in a mouse model of experimental liver metastasis. J. Biol. Chem. *95*, 1789–1797.

Williams, C.S., Smalley, W., and DuBois, R.N. (1997). Aspirin use and potential mechanisms for colorectal cancer prevention. J. Clin. Invest. *100*, 1–5.

Yamazaki, K., Lehr, J.E., Rhim, J.S., and Pienta, K.J. (1996). Establishment of an immortalized Copenhagen rat bone marrow endothelial cell line. In Vivo *10*, 459–462.

Yang, E.Y., and Moses, H.L. (1990). Transforming growth factor β1-induced changes in cell migration, proliferation, and angiogenesis in the chicken chorioallantoic membrane. J. Cell Biol. *111*, 731–741.