

Suppression of tumor cell invasion by cyclooxygenase inhibitors is mediated by thrombospondin-1 via the early growth response gene *Egr-1*

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Abstract

Cyclooxygenase (COX) inhibitors have antitumorigenic activity and increase the expression of the early growth response gene *Egr-1*, a tumor suppressor gene and transcription factor. In this study, we have investigated the gene regulatory and anti-invasive activity of two traditional nonsteroidal anti-inflammatory drugs (NSAID), sulindac sulfide and indomethacin. These compounds inhibited tumor cell invasion and induced *Egr-1* expression in lung adenocarcinoma A549 cells. Overexpression of *Egr-1* reduced cellular invasion in the Matrigel system, whereas suppression of *Egr-1* by small interference RNA (siRNA) attenuated the inhibition of Matrigel invasion by these compounds, indicating that *Egr-1* is responsible for the decrease in invasion reported following treatment with NSAIDs. *Egr-1*-overexpressing cells were analyzed for genes involved in invasion and metastasis. Thrombospondin-1 (TSP-1) an antiangiogenic and anti-invasion protein was up-regulated by *Egr-1* overexpression, which was confirmed following treatment with sulindac sulfide. Furthermore, the induction of TSP-1 by sulindac sulfide was blocked by *Egr-1* siRNA. When TSP-1 was sequestered by the addition of anti-TSP-1 antibody, the inhibition of invasion by sulindac sulfide was attenuated, indicating that TSP-1 is involved in the inhibition of invasion by NSAIDs. We used the *Min* mouse model to determine if sulindac sulfide would increase *Egr-1* and TSP-1 *in vivo*, because this model is widely used to study

the effects of NSAIDs on tumor formation. Treatment of *Min* mice with concentrations of sulindac sulfide that inhibit tumor formation increased the expression of *Egr-1* and TSP-1 in colonic tissues and in the polyps of these mice. This is the first report suggesting that COX inhibitors suppress tumor cell invasion via TSP-1, which occurs downstream of *Egr-1*. [Mol Cancer Ther 2005;4(10):1551–8]

Introduction

Nonsteroidal anti-inflammatory drugs (NSAID) are used to relieve pain and inflammation but have also received considerable attention because of their protective effects against human cancer (1–4). NSAIDs are well known for their ability to inhibit the enzymatic activity of cyclooxygenase (COX), which metabolizes arachidonic acid into prostaglandin E₂. Prostaglandin E₂ is known to inhibit apoptosis, stimulate tumor growth, and enhance angiogenesis, tumor cell invasion, and metastasis in a variety of cancer models (5–7). NSAIDs enhance apoptosis and exert antimetastatic and antiangiogenic effects by inhibiting COX activity thereby inhibiting tumor growth (8–10). However, some lines of evidence suggested that NSAIDs modulate tumor growth by COX-independent signaling pathways (11, 12).

Recently, our laboratory reported that cyclooxygenase inhibitors induce the expression of the tumor suppressor gene, early growth response gene-1 (*Egr-1*; ref. 13). The protein product of *Egr-1* (also known as *knox24*, *zif268*, *NGFI-A*, and *Tis8*) is a Cys2-His2-typed zinc-finger transcription factor implicated in the regulation of a number of genes involved in inflammation, differentiation, growth, and development. *Egr-1* is a transcription factor responsible for the up-regulation of several genes related to the biological activity of COX inhibitors and other compounds. For example, *Egr-1* up-regulates NSAID activated gene-1 (*NAG-1*), also known as *PLAB*, *PTGFB*, *PDF*, *MIC-1*, and *HP00269*. *NAG-1* is an antitumorigenic protein (13–15) that stimulates apoptosis. Furthermore, *Egr-1* increases the expression of *ATF3* (16), which inhibits tumor growth in nude mouse models *in vivo* and tumor cell invasion *in vitro* (17). *Egr-1* is rapidly and transiently expressed in a number of cells after stimulation with a variety of agents, including cytokines and growth factors as well as diverse environmental toxicants (15, 18, 19). *Egr-1* has tumor-suppressive activity by modulating growth or transformation (20–22). *Egr-1* activates phosphatase and tensin (*PTEN*) homologue tumor suppressor gene during UV radiation (23) and is induced during the apoptotic process, where it mediates the activation of downstream gene regulators, such as *p53* (24–26) and *NAG-1* (14, 15). *Egr-1* also regulates the expression of diverse array of genes

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involved in tumor metastasis, such as thrombospondin-1 (TSP-1), plasminogen activator inhibitor-1 (PAI-1), and TGF- β 1 (23, 27, 28). TSP-1 is a potent inhibitor of neovascularization that limits tumor growth (29–31) and stimulates apoptosis and growth inhibition in neovascular endothelial cells (32, 33). The expression of TSP-1 is decreased in tumor cells that contain mutations in various oncogenes and tumor suppressor genes (34) and suppresses the growth of responsive tumor cells (35). High TSP-1 expression also is inversely correlated with invasiveness and lymph nodal metastasis (35, 36). Therefore, we tested the hypothesis that NSAIDs modulate the invasion of cancer cells *in vitro*, and that this occurs via the regulation of gene expression. Furthermore, we tested the hypothesis that TSP-1 is regulated by NSAIDs, and that this occurs via Egr-1.

In this study, the inhibition of invasion was mediated by an increase in the expression of Egr-1, which subsequently increased the expression of TSP-1. The increase in expression of TSP-1 by NSAIDs was blocked in the presence of Egr-1 small interference RNA (siRNA). Furthermore, the inhibition of invasion was blocked by anti-TSP-1 antibody, indicating that TSP-1 is directly involved in NSAIDs anti-invasive activity. Therefore, the diverse biological activity of COX inhibitors in preventing cancer may be mediated in part by the up-regulation of the tumor suppressor Egr-1 and subsequently its downstream target, TSP-1.

Materials and Methods

Cell Culture Conditions and Reagents

Non-small cell lung cancer A549 cells were purchased from the American Type Culture Collection (Rockville, MD) and maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Hyclone, South Logan, UT), 50 units/mL penicillin (Sigma Chemical Co., St. Louis, MO), and 50 μ g/mL streptomycin (Sigma) in a 5% CO₂ humidified incubator at 37°C. HCT 116 colorectal cancer cells (American Type Culture Collection) were maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum, 50 units/mL penicillin (Sigma), and 50 μ g/mL streptomycin (Sigma). Cell number and viability were assessed by trypan blue (Sigma) dye exclusion using a hemacytometer. During incubation with chemicals, cells were cultured in serum-free DMEM. NSAIDs were purchased from Calbiochem (EMD Biosciences, Inc., La Jolla, CA).

Western Immunoblot Analysis

Levels of protein expression were compared using Western immunoblot analysis using goat polyclonal anti-human actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal anti-human TSP-1 antibody (Ab-11, NeoMarkers, Fremont, CA), and polyclonal anti-human Egr-1 antibody (Santa Cruz Biotechnology). Cell lysates were prepared in radioimmunoprecipitation assay buffer containing 1 \times PBS, 1% NP40, 0.5% sodium

deoxycholate, 0.1% SDS, 100 μ g/mL phenylmethylsulfonyl fluoride, 1 mmol/l sodium orthovanadate, and protease inhibitor cocktail (Sigma). After a brief sonication of samples at 4°C, lysates containing proteins were quantified using bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Fifty micrograms of protein were separated by NuPAGE Novex Bis-Tris gel electrophoresis (Invitrogen). Proteins were transferred onto a nitrocellulose membrane (Invitrogen), and the blots were blocked for 1 hour with 5% skim milk in TBS plus 0.05% Tween and probed with each antibody for 2 hours at room temperature or overnight at 4°C. After washing thrice with TBS plus 0.05% Tween, blots were incubated with horseradish-conjugated secondary antibody for 1 hour and washed with TBS plus 0.05% Tween thrice. Protein was detected by Supersignal WestPico Chemiluminescent substrate (Pierce).

Traditional Reverse Transcription-PCR

RNA was extracted with RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA (100 ng) from each sample was transcribed to cDNA by Superscript II RNase H- Reverse-transcriptase (Invitrogen). The amplification was done with Takara ExTaq DNA Polymerase (Chemicon, Temecula, CA) in GeneAmp PCR system 9700 (Applied Biosystems, Foster city, CA) using the following variables: denaturation at 94°C for 2 minutes and 25 cycles of reactions of denaturation at 98°C for 10 seconds, annealing at 59°C for 30 seconds, and elongation at 72°C for 45 seconds. An aliquot of each PCR product was subjected to 1.2% (w/v) agarose gel electrophoresis and visualized by staining with ethidium bromide. The 5' forward and 3' reverse complement PCR primers for amplification of each gene were as follows: human Egr-1 (5'-CAGTGGCC-TAGTGAGCATGA-3' and 5'-CCGCAAGTGGATCTTGG-TAT-3'), human TSP-1 (5'-AGAATGCTGTCTCGCTGTT-3' and 5'-TTTCTTGCAGGCTTTGGTCT-3'), and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH: 5'-TCAACGGATTGGTCGTATT-3' and 5'-CTGTGGTCAT-GAGTCTTCC-3').

Transfection Experiments and Generation of Stable Cell Lines

The full-length Egr-1 cDNA inserted into the pcDNA3.1/neo expression vector (pEgr-1) was described previously (15). Cells were transfected with plasmid DNA using FuGENE6 transfection reagent (Roche, Indianapolis, IN) according to the manufacturer's protocol. Transfection efficiencies were optimized to achieve 50% to 60% efficiency, which was confirmed with pMX-enhanced GFP vector. To create a pEgr-1-expressing stable cell line, A549 cells were transfected with pEgr-1 or pcDNA3.1-neo using FuGENE6 reagent as above. After 48 hours, the cells were subjected to selection for stable integrants by exposure to 1,000 μ g/mL G418 sulfate (Geneticin) purchased from Invitrogen in complete medium containing 10% fetal bovine serum. Selection was continued until monolayer colonies formed. The transfectants were maintained in complete medium supplemented with 500 μ g/mL G418 sulfate.

Treatment with siRNA

Cells were transfected with Egr-1 siRNA (Dharmacon, Lafayette, CO), targeting the sequence AAGTTACTACC-TCTTATCCAT. As a control, cells were treated with an equal amount of nonspecific control RNA (Dharmacon). Transfection of synthetic RNA was done with LipofectAMINE 2000 according to the manufacturer's protocol (Invitrogen). Following transfection with siRNA, cells were incubated for 48 hours followed by the conditions indicated.

Matrigel Invasion Assay

The 8- μ m pore size upper chambers were coated with growth factor-reduced Matrigel (2.5 mg/mL; BD Biosciences Discovery Labware, Bedford, MA), and dry coatings were reconstituted in DMEM for 1 to 2 hours before cell passage. Cells were seeded onto the precoated upper transwells at 3×10^4 per 500 μ L serum-free medium. Outer medium was filled with 10% serum containing media. Cells were incubated for 48 hours, and then Matrigel were swabbed with a Q-tip. Cells were stained with Diff-Quik stain set according to the manufacturer's protocol (Dade Behring, Inc., Newark, DE) counted under phase-contrast microscope for quantitative analysis. The cells invading through the Matrigel were counted under microscope in five predetermined fields at $\times 100$ original magnification.

Min Mice Experiments

All animal procedures were approved by the University of Tennessee Animal Care and Use Committee and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Tissue samples were obtained during the course of a previously reported study and details of the experimental protocol are presented therein (37). Briefly, after acclimation to AIN93-G diets (Dyets, Inc., Bethlehem, PA) for a period of 1 week, *Min* mice ($n = 4$ each) were fed diet containing 320 ppm sulindac, the prodrug for sulindac sulfide that is equivalent to 30 mg/kg/d, or control diets for 40 hours then sacrificed at 85 days old as previously reported (37). Mice were euthanized by CO₂ inhalation, blood was drawn, and the intestines removed. Intestines were flushed with cold PBS (to remove fecal material) followed by a flush with RNAlater (at room temperature). Intestines were opened longitudinally, submerged in RNAlater, and labeled sequentially with the animal number. Tissues were stored at -20°C until RNA was isolated using Qiagen's Midi RNA isolation kit. Cells were homogenized by centrifugation through a QIAshredder spin column (Qiagen). Real-time reverse transcription-PCR (RT-PCR) was done as previously reported by this laboratory (38). Values are fold mRNA expression (\pm SE) according real-time RT-PCR and expressed relative to time-matched, control-fed mice and adjusted for mouse GAPDH. Results are from mRNA analysis of the colons ($n = 4$) and tumors ($n = 2$) of mice repeated twice and subsequently pooled (pseudoreplicated) to allow for SE calculations.

Real-time RT-PCR mRNA Analysis

Real-time RT-PCR was done as previously described by this laboratory (37, 39). The following mouse primers were used for real-time RT-PCR from mRNA isolated from *Min*

mouse tissues: mouse GAPDH, forward AATGTGTC-CGTCGTGGATCT and reverse GCTTACCACCTTCTT-GATGT; mouse Egr-1, forward CCTTTCTGACATCGC-TCTGAA and reverse CGAGTCGTTGGCTGGGATA; mouse TSP-1, forward TCGGCCTTAACGAATGAGAA and reverse AGCGGGCACCTTCCTAGTG.

Statistical Analyses

Data were analyzed using SigmaStat for windows (Jandel Scientific, San Rafael, CA). For comparison of two groups of data, Student's *t* test was done. For multiple comparisons, data were subjected to ANOVA and pairwise comparisons made by the Student-Newman-Keuls method. Data not meeting normality assumption were subjected to Kruskal-Wallis ANOVA on ranks and then pairwise comparisons made by the Student-Newman-Keuls method.

Results

Induction of Egr-1 mRNA and Protein Expression by COX Inhibitors in A549 Cells

A549 cells were selected to investigate the effect of NSAIDs on the invasion of tumors cells, because they serve as a well-established model for the study of invasion of cancer cells and express the COX enzymes (40, 41). We first needed to determine if COX inhibitors would increase the expression of Egr-1 in A549 cells as observed in other cancer cell lines (13). A549 cells were incubated with two concentrations of NSAIDs, and Egr-1 protein expression was measured (Fig. 1A). Among the tested NSAIDs, sulindac sulfide was a most potent inducing agent of Egr-1 followed by indomethacin, and the specific COX-1 inhibitor, SC-560. Physiologic concentrations of sulindac sulfide are in the 10 to 20 μ mol/L range based upon reported plasma levels in human subjects (42). Thus, in subsequent studies, sulindac sulfide was used in this concentration range, whereas indomethacin was used for comparison and to further validate our findings. SC-560 was not selected because its biological activity is not well established. Egr-1 protein induction by sulindac sulfide was maximal around 3 to 4 hours, and Egr-1 mRNA was detected as early as 1 hour after treatment with these compounds (Fig. 1B). These results confirm the increase in Egr-1 expression by COX inhibitors in A549 cells; therefore, they were further studied.

Tumor Cell Invasion Is Inhibited by COX Inhibitors and Requires Egr-1

The invasion of A549 cells into the Matrigel matrix was inhibited by indomethacin as shown in Fig. 2A. Because Egr-1 is known to regulate the expression of genes involved in tumor metastasis (43, 44), we tested if the overexpression of Egr-1 altered the invasion of these cells. The empty vector (pCDNA3.1-neo) and this vector containing a sequence expressing Egr-1 (pEgr-1) was transfected into A549 cells and the stable cell lines were isolated and assessed. There was no significant difference in the cell proliferation between these two cell lines (data not shown).

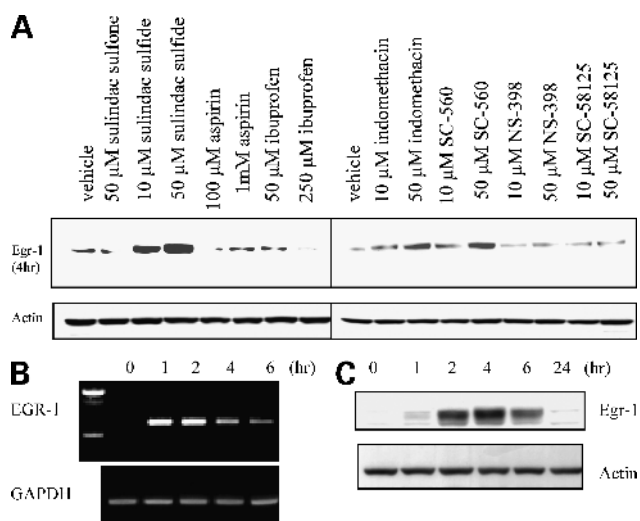


Figure 1. Effects of NSAIDs on Egr-1 protein and mRNA expression. **A**, a wide variety of NSAIDs were tested for their ability to regulate Egr-1 protein expression in A549 cells. The cells were incubated for 16 h in serum-free medium and treated with the compound indicated for 4 h. **B** and **C**, cells were treated for the times indicated in the presence of 25 μ M/L sulindac sulfide followed by analysis of Egr-1 mRNA (**B**) and protein (**C**) expression. Representative of three independent experiments.

However, the overexpression of Egr-1 attenuated the cellular invasion of A549 cells into the Matrigel matrix (Fig. 2A). Moreover, the expression of Egr-1 increased the inhibition of invasion by indomethacin treatment. These findings suggest that Egr-1 or its downstream targets mediate the inhibition of invasion by indomethacin and other COX inhibitors. Suppression of Egr-1 expression by siRNA enhanced the invasion of A549 cells compared with control RNA-transfected cells (Fig. 2B). Incubation with either indomethacin or sulindac sulfide inhibited the invasion of the cells. The suppression of Egr-1 expression by the siRNA attenuated the reduction in the invasion of NSAID-treated cells by restoring the level of invasion comparable to untreated cells. Subsequently, siRNA efficiency was confirmed by analyzing Egr-1 expression (Fig. 2C). These results indicated that the inhibition of tumor cell invasion by sulindac sulfide and indomethacin is, at least in part, mediated by the increased expression of Egr-1.

Regulation of TSP-1 by COX Inhibitors Occurs via Egr-1

Egr-1 is a transcription factor known to regulate the expression of a variety of genes, some of which have possible anti-invasion activities. One possible candidate gene proposed to mediate the invasion is *TSP-1*. *TSP-1* is reported to inhibit invasion and to inhibit angiogenesis. A549 cells stably transfected with pEgr-1 or vector DNA were treated with serum to simulate the invasion process. Egr-1 mRNA induction was maximal as early as 30 minutes after serum addition and then returned to basal level (Fig. 3A). *TSP-1* expression was enhanced by exogenous Egr-1 expression and was more prominently expressed

when the serum stimulus was provided to the cells. To determine if NSAID treatment would also induce *TSP-1* expression, cells were incubated with indomethacin or sulindac sulfide for 24 hours and whole cellular lysate was analyzed by Western blot analysis (Fig. 3B). Sulindac sulfide and indomethacin induced *TSP-1* in a concentration-dependent manner in A549 cells. Compared with Egr-1 induction (Fig. 1B), *TSP-1* protein expression was observed at 24 hours, whereas *TSP-1* mRNA was maximal 4 hours after sulindac sulfide treatment (Fig. 3C). These findings suggest that NSAID-induced Egr-1 expression results in the expression of *TSP-1* during tumor cell invasion and *TSP-1* acts as an anti-invasive mediator in A549 lung adenocarcinoma cells.

TSP-1-Mediated Inhibition of Tumor Cell Invasion

To confirm the involvement of *TSP-1* in NSAID-mediated inhibition of tumor invasion, cells were allowed to invade into the Matrigel matrix in the presence or absence of *TSP-1* protein, anti-*TSP-1* antibody, and/or vehicle or sulindac sulfide. Incubation of cells with sulindac sulfide or *TSP-1* protein attenuated the invasion of the cells into the Matrigel matrix relative to the appropriate controls. The addition of anti-*TSP-1* antibody (α *TSP-1*) enhanced the basal invasiveness of A549 cells and reversed the inhibition of invasion by the addition of sulindac sulfide and/or *TSP-1* protein (Fig. 4). Thus, *TSP-1* directly inhibits invasion of the A549 cells and seems to mediate the suppression of invasiveness by sulindac sulfide.

NSAIDs Regulate the Expression of TSP-1 Downstream of Egr-1

Overexpression of Egr-1 enhanced *TSP-1* production; therefore, we hypothesized that inhibition of Egr-1 would attenuate *TSP-1* induction by NSAIDs. Sulindac sulfide treatment increased the expression of Egr-1 and *TSP-1* in cells transfected with control vector. In contrast, cells transfected with Egr-1 siRNA showed decreased expression of Egr-1 and mitigated the induction of *TSP-1* by sulindac sulfide (Fig. 5). These results support the conclusion that the induction of *TSP-1* by NSAIDs occurs in an Egr-1-dependent manner.

Induction of Egr-1 and TSP-1 by Sulindac Sulfide Occurs in Colorectal Cancer Cells and *Min* Mice Treated with the Prodrug Sulindac

A suitable model for studying the inhibition of pulmonary tumors by COX inhibitors is not available. Therefore, to determine if the treatment of mice with sulindac sulfide would result in the increased expression of Egr-1 and *TSP-1* *in vivo*, we used the *Min* mouse model, which is a well-established model for the inhibition of colorectal cancer, by COX inhibitors. First, we set out to confirm that alterations in Egr-1 and *TSP-1* expression were not cell line dependent. Therefore, human colorectal cancer HCT-116 cells, which are commonly studied to measure the effects of gene regulation by anticancer compounds, were used. Sulindac sulfide induced both Egr-1 and *TSP-1* in a concentration-dependent manner in HCT-116 cells (Fig. 6), suggesting that the induction of *TSP-1* was not restricted to human

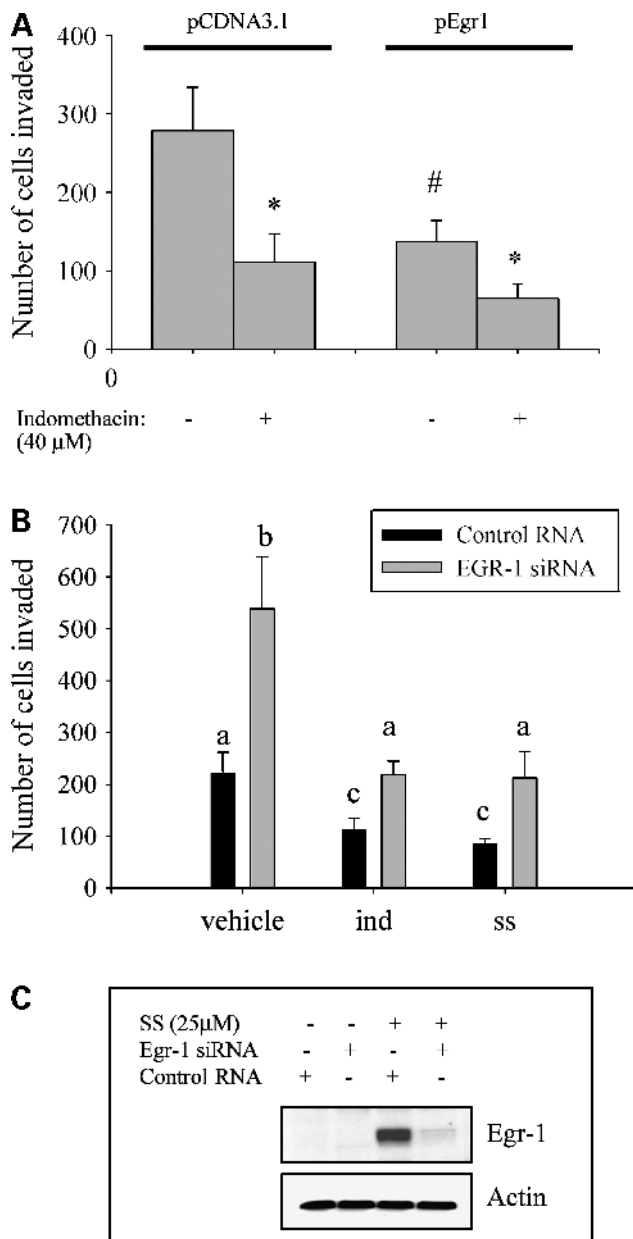


Figure 2. Effects of Egr-1 and sulindac sulfide on Matrigel invasion. **A**, vector or Egr-1-overexpressing A549 cells were subjected to Matrigel invasion assays. Suspended cells were loaded onto the Matrigel-coated transwell in the presence of vehicle or 40 μ mol/L indomethacin and incubated for 48 h followed by quantification of migrated cells. The cells invading through the Matrigel were counted under microscope in five predetermined fields at $\times 100$ original magnification. Each sample was assayed in triplicate. *, $P < 0.05$, significantly different from the corresponding vehicle-treated group; #, significant difference between the two vehicle-treated groups according to a Student's t test. **B**, Egr-1 siRNA or control RNA-transfected A549 cells were subjected to Matrigel invasion assay. Suspended transfected cells were loaded onto the Matrigel-coated transwell with or without 40 μ mol/L indomethacin (*ind*) or 10 μ mol/L sulindac sulfide (*ss*) and incubated for 48 h to quantify migrated cells. Groups with different letters are significantly different ($P < 0.05$) by the Student-Newman-Keuls method. **C**, A549 cells were transfected with Egr-1 siRNA and incubated for 48 h followed by treatment with 25 μ mol/L sulindac sulfide for 4 h. Cellular lysates were analyzed by Western blot analysis. Representative of three independent experiments.

pulmonary cells. Subsequently, real-time RT-PCR on mRNA from *Min* mice fed diets containing the prodrug for sulindac sulfide, sulindac, or control diets was done. After acclimation to normal AIN93-G diets for 1 week, *Min* mice were fed a diet of vehicle or 320 ppb or 30 mg/kg/d sulindac, the prodrug for sulindac sulfide, for 40 hours and mice were sacrificed as previously reported (37, 39). The level of Egr-1 and TSP-1 message was analyzed by real-time RT-PCR. Results are from mRNA analysis of the colons ($n = 4$) and tumors ($n = 2$) of mice repeated twice and pooled. Treatment with sulindac increased the expression of Egr-1 by 1.8 ± 0.1 -fold in the colon but increased the expression 14.5 ± 0.4 -fold in the polyps relative to mice fed the control diets. Treatment of the mice also increased the expression of TSP-1 by 2.0 ± 0.2 -fold in the colon and by 2.2 ± 0.03 -fold in the polyps of *Min* mice. Therefore,

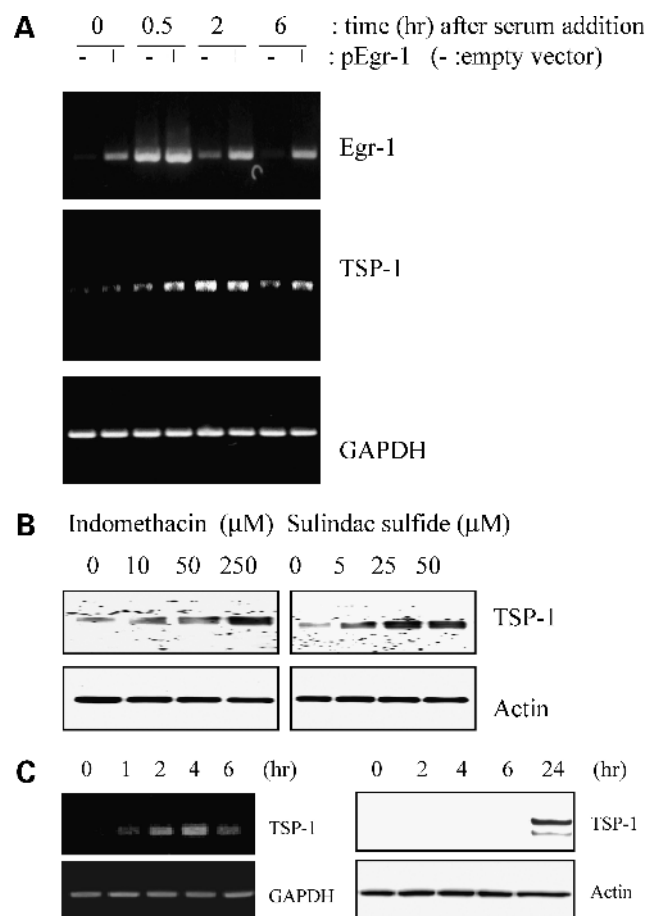


Figure 3. Effects of Egr-1 overexpression and treatment with sulindac sulfide on the expression of thrombospondin-1. **A**, Egr-1-overexpressing or vector-transfected A549 cells were incubated with serum-free medium for 24 h followed by incubation with serum containing complete medium for the time points indicated. Subsequently, total RNA was analyzed by RT-PCR for Egr-1, TSP-1, and GAPDH. **B**, A549 cells were cultured in the presence of indomethacin or sulindac sulfide for 24 h, and total cellular protein lysates were analyzed by Western blot analysis. **C**, A549 cells were treated with sulindac sulfide (25 μ mol/L) for the times indicated followed by analysis of mRNA (*left*) or total protein lysates (*right*). Representative of three independent experiments.

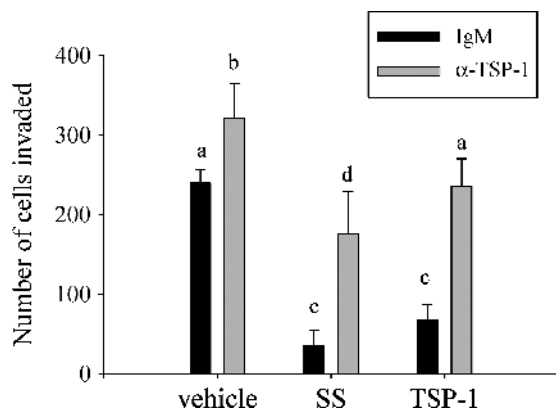


Figure 4. Suppression of TSP-1 enhances tumor cell invasion. A549 cells were subjected to Matrigel invasion in presence of vehicle, sulindac sulfide (10 μ mol/L, SS) or TSP-1 protein (5 μ mol/L) in the presence of immunoglobulin M (IgM, 10 μ g/mL, black columns) or anti-TSP-1 antibody (α -TSP-1, gray columns). The cells invading through the Matrigel were counted microscopically in five predetermined fields at $\times 100$ original magnification. Each sample was assayed in triplicate.

treatment of *Min* mice with doses of sulindac that inhibit polyp growth increases the *in situ* expression of both Egr-1 and TSP-1, indicating the potential biological significance of the gene regulatory activity of these compounds.

Discussion

NSAIDs have potent antiangiogenesis (45) and antimetastatic (7) activity both *in vitro* and *in vivo*. However, the molecular mechanisms for these activities are largely uncharacterized. Data from a number of experimental approaches illustrate the importance of both COX-1 and COX-2 in the promotion of cancer. In particular, the elevated levels of expression seen with COX-2, and its metabolite prostaglandin E_2 in tumors, interest in COX-2, has resulted in the onset of several clinical trials to determine the feasibility of using COX-2-specific inhibitors as chemopreventive drugs. However, the increase in cardiovascular events associated with COX-2 inhibitors may prevent the use of these drugs as long-term chemoprevention drugs. Therefore, it is important to identify the downstream targets and alternate mechanistic approaches to the development of chemopreventive drugs.

In an attempt to explain the biological activity of these compounds in the prevention of cancer, our laboratory has focused on the gene regulatory activity of COX inhibitors, the molecular pathways involved, and the biological significance of this regulation. Recently, we reported that certain COX inhibitors up-regulate the expression of the tumor suppressor gene *Egr-1* independent of their COX inhibitory activity. Egr-1 is a transcription factor that regulates the expression of various downstream genes, several of which could be responsible for the biological activity of COX inhibitors. For example, Egr-1 increases the expression of the antitumorigenic

gene, *NAG-1*, a protein that modulates apoptosis and may contribute to the enhanced apoptosis observed in the presence of COX inhibitors. This gene is an attractive alternate target for COX inhibitors, because Egr-1 regulates the expression of genes not only linked to apoptosis but also genes associated with angiogenesis and metastasis. Therefore, we next investigated the relationship between COX inhibitors and metastasis, a multistep process including angiogenesis, detachment, invasion, and extravasation. The focus of this study was the invasion of tumor cells and report here findings that support the hypothesis that COX inhibitors suppress tumor cell invasion by increasing the expression of TSP-1, a protein with documented anti-invasion activity. A number of COX inhibitors were examined and sulindac sulfide, which is a potent inhibitor of tumor formation in animal models and used in humans to treat persons with familial adenomatous polyposis (46), a genetic disorder resulting in abnormal colorectal polyp formation in humans, was found to be the most potent inducer of Egr-1 expression. Therefore, sulindac sulfide was selected for subsequent studies. The COX inhibitor-induced expression of TSP-1 was dependent on the expression of Egr-1. Overexpression of Egr-1 and incubation of A549 cells with indomethacin and sulindac sulfide at concentrations that increase Egr-1 expression suppressed invasion of the cells into the Matrigel matrix. Blockage of COX inhibitor-induced Egr-1 expression with Egr-1 siRNA attenuated the NSAID-inhibited invasion and the increase in the expression of TSP-1. The induction of Egr-1 preceded that of TSP-1, suggesting that NSAID-induced Egr-1 expression results in the expression of TSP-1. Furthermore, the sequestering of the TSP-1 protein by a TSP-1 antibody increased tumor cell invasion, indicating that the inhibition of invasion of these cells by NSAIDs and Egr-1 is mediated, in part, by TSP-1 downstream of the transcription factor Egr-1 in A549 cells. These findings

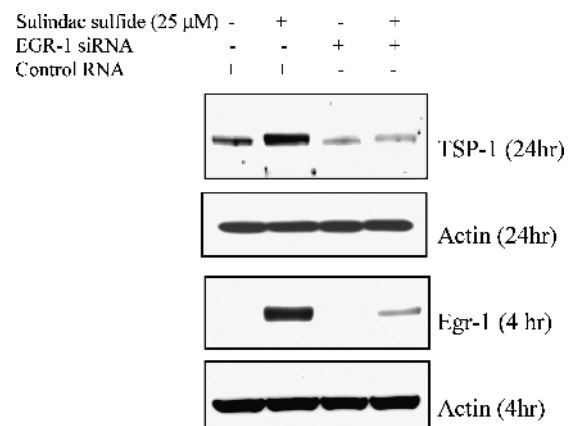


Figure 5. Egr-1 is involved in the induction of TSP-1 by sulindac sulfide. A549 cells were transfected with Egr-1 siRNA or control RNA followed by treatment with vehicle or sulindac sulfide for the times indicated. Whole cell lysates were used for Western blot analysis. Representative of three independent experiments.

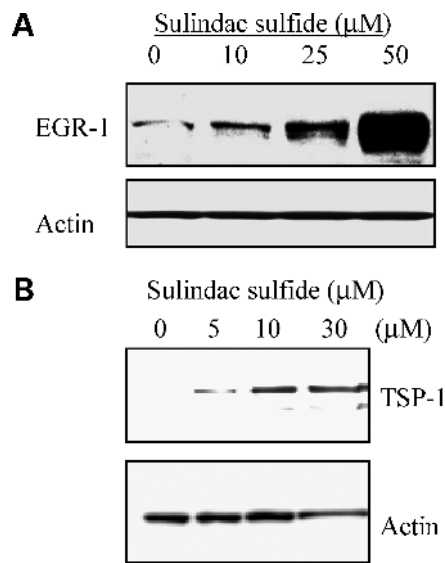


Figure 6. Regulation of Egr-1 and TSP-1 by sulindac sulfide is not cell line dependent. **A**, HCT-116 cells were incubated for 16 h in serum-free medium followed by treatment with each dose of sulindac sulfide for 4 h. **B**, HCT-116 cells were treated with each dose of sulindac sulfide in serum-free medium for 24 h. Whole cell lysates were used for Western blot analysis. Representative of three independent experiments.

support the hypothesis that COX inhibitors increase the expression of Egr-1 and subsequently mediate the expression of TSP-1 that inhibits the invasion of the tumors cells into the matrix.

Reduced expression of TSP-1 correlates with a poor prognosis in patients with non-small cell lung cancer (47). TSP-1 is an inhibitor of invasion but also an inhibitor of angiogenesis, and its suppression is crucial for the angiogenic switch in many tumor models (30, 48, 49). The cellular mechanism is not well known but TSP-1 can inhibit endothelial cell growth via the apoptotic pathway (50). TSP-1 inhibits the activity of matrix metalloproteinase-9, which causes release of vascular endothelial growth factor sequestered in the extracellular matrix, thereby increasing invasion potential (51). Moreover, TSP-1 can promote secretion of PAI-1, which was shown to inhibit the invasiveness of human lung carcinoma cells (44, 52). The promoter region of human *TSP-1* contains one Egr-1 binding site, and *TSP-1* transcription is modulated by Egr-1 (27, 28).

An important question addressed in this study was to determine if treating an experimental animal model with an anticancer dose of a COX inhibitor would increase the expression of *Egr-1* and *TSP-1*. Although our initial findings in this report show that NSAIDs inhibit invasion used lung adenocarcinoma cells, which are commonly used to study invasion, we selected the *Min* mouse model to determine if gene regulation could occur *in vivo*, because it is an accepted model used to show the inhibition of tumorigenesis by COX inhibitors. The *Min* mouse is an excellent model for human colorectal cancer, and treatment with sulindac sulfide inhibits polyp formation. Our

findings indicate that physiologically achievable concentrations of sulindac sulfide increased the expression of Egr-1 and TSP-1, suggesting that treatment with COX inhibitors occurs *in vivo*. However, further work is needed to determine if the gene regulatory activity of these compounds results in the inhibition of tumor formation, invasion, or polyp formation shown by these compounds in the literature.

In summary, the inhibition of tumor development by COX inhibitors seems to be a complex event involving several different mechanisms. Considerable evidence supports the notion that the inhibition on COX activity is as an important mechanism for the regulation of these pathways and that they are associated with the increase in apoptosis, inhibition of angiogenesis, and suppression of tumor cell invasion observed after treatment with COX inhibitors. In this report, we have presented data for an additional mechanism for this activity. Some COX inhibitors increase the expression of the tumor suppressor gene *Egr-1* both in cells in culture and as illustrated *in vivo* following treatment of *Min* mice with the COX inhibitor, sulindac sulfide. Egr-1 is regulated in a variety of cancer cell lines following treatment with NSAIDs. Subsequently, Egr-1 regulates the expression of several genes linked to the reported biological activity of COX inhibitors as illustrated by TSP-1, which inhibit tumor cell invasion and angiogenesis. Thus, the inhibition of tumors growth by COX inhibitors may occur by mechanisms dependent of inhibition of COX activity and by altering the expression of genes.

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