

# Cyclooxygenase Inhibitors Regulate the Expression of a TGF- $\beta$ Superfamily Member That Has Proapoptotic and Antitumorigenic Activities

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## ABSTRACT

The antitumorigenic activity of nonsteroidal anti-inflammatory drugs (NSAIDs), cyclooxygenase (COX) inhibitors, is well established, but responsible molecular mechanisms are not fully understood. NSAIDs stimulate apoptosis by COX dependent and independent mechanisms in colorectal cells in culture. Identification of genes regulated by COX inhibitors could lead to a better understanding of their proapoptotic and anti-neoplastic activities. Using subtractive hybridization, a cDNA which was designated as NSAID activated gene (NAG-1) was identified from NSAID-treated HCT-116, human colorectal cells. NAG-1 has an identical sequence with a novel member of the TGF- $\beta$  superfamily that has 5 different names. In the HCT-116 cells, NAG-1 expression is increased and apoptosis is induced

by treatment with some NSAIDs in a concentration and time-dependent manner. NAG-1 transfected cells exhibited increased basal apoptosis, increased response to NSAIDs and reduced soft agar cloning efficiency. Furthermore, transplantable tumors derived from NAG-1 transfected HCT-116 cells showed reduced tumorigenicity in athymic nude mice compared with vector-transfected HCT-116 cells. The increased NAG-1 expression by NSAIDs provides a suitable explanation for COX-independent apoptotic effects of NSAIDs in cultured cells. These data demonstrate that NAG-1 is an antitumorigenic and proapoptotic protein, and its regulation by COX inhibitors may provide new clues for explaining their proapoptotic and antitumorigenic activities.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are potent anti-inflammatory drugs and are also effective in reducing human and rodent colorectal cancer (Bohlbol et al., 1996; Taketo, 1998a,b). Epidemiological studies reveal a 40 to 50% reduction in mortality from colorectal cancer resulting from the use of NSAIDs (Thun et al., 1993). NSAIDs inhibit the two isoforms of prostaglandin H synthase [cyclooxygenase (COX)], COX-1 and COX-2, the enzymes responsible for the formation of prostaglandins from arachidonic acid. COX-1 is constitutively expressed, whereas mitogens, tumor promoters, and growth factors regulate COX-2 expression (Herschman, 1996). Some data link NSAID chemoprevention in colorectal cancer cells to COX inhibition (Watson, 1998). The expression of COX-2 seems to increase angiogenesis (Tsujii et al., 1998) in tumors, and COX inhibitors can attenuate angiogenesis (Jones et al., 1999). The over-expression of COX-2 in rat intestinal cells in culture attenuates butyrate-induced apoptosis (Tsujii and DuBois, 1995), a response re-

versed by incubation with the COX inhibitor, sulindac sulfide. Although there is data demonstrating that the antitumorigenic activity of NSAIDs is related to COX inhibition, other data suggest that NSAIDs have COX-independent effects.

Human colorectal cells in culture have served as useful models to examine the mechanisms by which COX expression contributes to cancer development and to assess how COX inhibitors reduce tumor development. COX inhibitors are reported to enhance apoptosis, particularly in cultured cells (Subbaramaiah et al., 1997), but many of these apoptotic responses required a higher concentration than necessary for COX inhibition (Hanif et al., 1996; Shiff et al., 1996; Piazza et al., 1997). Higher concentrations are also required to increase ceramide formation (Chan et al., 1998) and down-regulate the transcriptional activity of the peroxisome proliferator-activated receptor, PPAR $\delta$  (He et al., 1999). Thus, the proapoptotic activity of COX inhibitors in cultured cells may not only be dependent on inhibition of COX, but also independent of COX inhibition.

One mechanism that has not been explored is that NSAIDs

The INDO29 sequences are deposited to GenBank with accession number AF173860.

**ABBREVIATIONS:** NSAIDs, nonsteroidal antiinflammatory drugs; COX, cyclooxygenase; TGF- $\beta$ , transforming growth factor- $\beta$ ; NAG-1, NSAID-activated gene-1; DMSO, dimethyl sulfoxide; DFU, 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5H)-furanone; bp, base pair(s); PI, propidium iodide; INDO, indomethacin; FACS, fluorescence-activated cell sorting; PCR, polymerase chain reaction.

may stimulate apoptosis and other biological responses in cell culture by altering gene expression. To test this hypothesis, we looked for NSAID-inducible genes by suppression subtractive hybridization (Diatchenko et al., 1996) using the human colorectal adenocarcinoma cell line, HCT-116. Here, we report that cyclooxygenase inhibitors stimulate apoptosis and induce the expression of a novel member of the TGF- $\beta$  superfamily. We called this gene NSAID-activated gene (NAG-1), but it has a sequence identical to that of five recently reported genes (Bootcov et al., 1997; Lawton et al., 1997). In this report, we present evidence for the regulation of NAG-1 expression by COX inhibitors and demonstrate that NAG-1 has antitumorigenic and proapoptotic activity.

## Materials and Methods

**Cell Line and Reagents.** Cell lines in this study were purchased from ATCC (Manassas, VA). Human colorectal carcinoma cells, HCT-116, were maintained in McCoy's 5A medium. Media were supplemented with 10% fetal bovine serum and Gentamicin. Most NSAIDs in this study were purchased from Sigma (St. Louis, MO) and dissolved in DMSO, except sodium salicylate, which was dissolved in PBS. Sulindac sulfide and DFU (5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulfonyl)phenyl-2 (5*H*)-furanone) were from Merck, Celecoxib, and SC-58125 from Monsanto, NS-398 from Cayman. LM4101, LM4108, LM4115 were kindly provided by Dr. L. Marnett (Vanderbilt University, TN).

**Isolation of an Indomethacin (INDO)-Induced Gene from HCT-116 Cells.** Messenger RNAs were isolated from INDO-treated (100  $\mu$ M) or vehicle-treated (0.2% DMSO) HCT-116 cells using a poly (A) spin mRNA isolation kit (New England Biolabs, MA). The cDNA Subtraction kit was used to make the INDO (+) and INDO (-) subtractive libraries according to the manufacturer's protocol (CLONTECH, Palo Alto, CA). A clone containing 159 bp was isolated from the INDO-induced library and designated INDO29. This sequence was identical to sequences reported by five different groups (Fig. 1A). The full-length cDNA containing the entire coding region was isolated by reverse transcriptase-PCR using two primers from PTGFB sequence (GenBank accession no. AF008393); sense strand, 5'-ACCTGCACAGCCATGCCCGGGCA-3' and anti-sense strand, 5'-CAGTGAAGGACCAGGACTGCTC-3'.

**Measurement of DNA Content and Apoptosis by FACS Analysis.** The DNA content for NSAIDs and vehicle treated HCT-116 cells was determined by FACS. Cells were plated at  $4 \times 10^5$  cells/well in six-well plates, incubated for 16 h, and then treated with NSAIDs in the presence of serum. After treatment, the cells were harvested, washed with PBS, fixed by the slow addition of cold 70% ethanol to a total of 1 ml, and stored at 4°C overnight. The fixed cells were pelleted, washed once with PBS, and stained in 1 ml of 20  $\mu$ g/ml propidium iodide (PI) and 1 mg/ml RNase in PBS for 20 min. Cells (7500) were examined by flow cytometry using Becton Dickinson FACSsort equipped with CellQuest software by gating on an area-versus-width dot plot to exclude cell debris and cell aggregates. Apoptosis was measured by the level of subdiploid DNA contained in cells after treatment with NSAIDs using CellQuest software. As a second method of detecting apoptosis, TACS Annexin V-FITC kit (Trevigen, Inc., Gaithersburg, MD) was used according to the manufacturer's protocol. Annexin V-positive/PI-positive and Annexin V-positive/PI-negative cell populations were determined as apoptotic populations from the total gated cells.

**Northern and Western Blot Analyses.** When reaching 60 to 80% confluence in 10-cm plates, the cells were treated at the indicated concentrations and times with different NSAIDs in the absence of serum. Total RNAs were isolated using TRIzol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's protocol. For Northern blot analysis, 10  $\mu$ g of total RNA was denatured at

55°C for 15 min and separated in a 1.2% agarose gel containing 2.2 M formaldehyde, and transferred to Hybond-N membrane (Amersham Pharmacia Biotech, Piscataway, NJ). After fixing the membrane by UV, blots were prehybridized in hybridization solution (Rapid-hyb buffer; Amersham) for 1 h at 65°C, followed by hybridization with cDNA labeled with [ $\alpha$ - $^{32}$ P]dCTP by random primer extension (DECAprimeII kit; Ambion, Austin, TX). The probes used were either full-length NAG-1 or placental TGF- $\beta$  clone (generously provided by Dr. Bento-Soar, University of Iowa, Iowa City, IA). After 4 h incubation at 65°C, the blots were washed once with  $2 \times$  SSC/0.1% SDS at room temperature and twice with  $0.1 \times$  SSC/0.1% SDS at 65°C. Messenger RNA abundance was estimated by intensities of the hybridization bands of autoradiographs using Scion Image (Scion Corporation, Frederick, MD). Equivalent loading of RNA samples was confirmed by hybridizing the same blot with a  $^{32}$ P-labeled  $\beta$ -actin probe that recognizes RNA of approximately 2 kilobase pairs.

The level of NAG-1 was evaluated using Western blot analysis with anti-human-NAG-1 antibody. The antibody was generated in this laboratory in rabbit from a specific peptide of the NAG-1 C-terminus (KTDGTGVSLQTYDDLLA). The antibody recognized both the precursor and secreted forms of NAG-1. HCT-116 cells were grown to 60 to 80% confluence in 10-cm plates and treated with NSAIDs for 48 h in the absence of serum. The media were harvested and concentrated approximately 15-fold using Centrprep 10 concentrators (Amicon Inc., Beverly, MA). Proteins (30  $\mu$ g) were separated by 15% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The blots were blocked for 1 h with 5% skim milk in Tris-buffered saline/Tween 0.05%, and probed with anti-NAG-1 antibody (1:5,000 in 1% skim milk in Tris-buffered saline/Tween 0.05%) at 4°C overnight. After washing, the blots were treated with horseradish peroxidase-conjugated secondary antibody for 1 h and washed several times. The signal was detected by enhanced chemiluminescence (Amersham Pharmacia Biotech) and autoradiography.

**Soft Agar Cloning Assay.** Soft agar assays were performed to compare the clonogenic potential of HCT-116 cells in semisolid medium. The stably transfected HCT-116 cells were resuspended at 3000 cells in 2 ml of 0.4% agar in McCoy's 5A medium and plated on top of 1 ml of 0.8% agar in six-well plates. Plates were incubated for 2 to 3 weeks at 37°C. Cell colonies were visualized by staining with 0.5 ml of *p*-iodonitrotetrazolium violet (Sigma).

**Tumor Growth in Nude Mice.** Thirty male nude mice (athymic NCr-nu) were purchased from NCI/Taconic at 5 weeks of age and were maintained in pathogen-free conditions. A total of  $3 \times 10^6$  cells in 0.1 ml of PBS were subcutaneously injected behind the anterior forelimb bilaterally in each mouse. Growth curves for xenografts were determined by externally measuring tumors in two dimensions. Tumor measurement began when the size was more than 3 mm in diameter (around 4 days after injection). Tumor volume was determined by the equation  $V = [(L + W) \cdot 0.5] \times L \times W \times 0.5236$ . Values are the mean  $\pm$  S.E. of 18 xenografts per group.

## Results

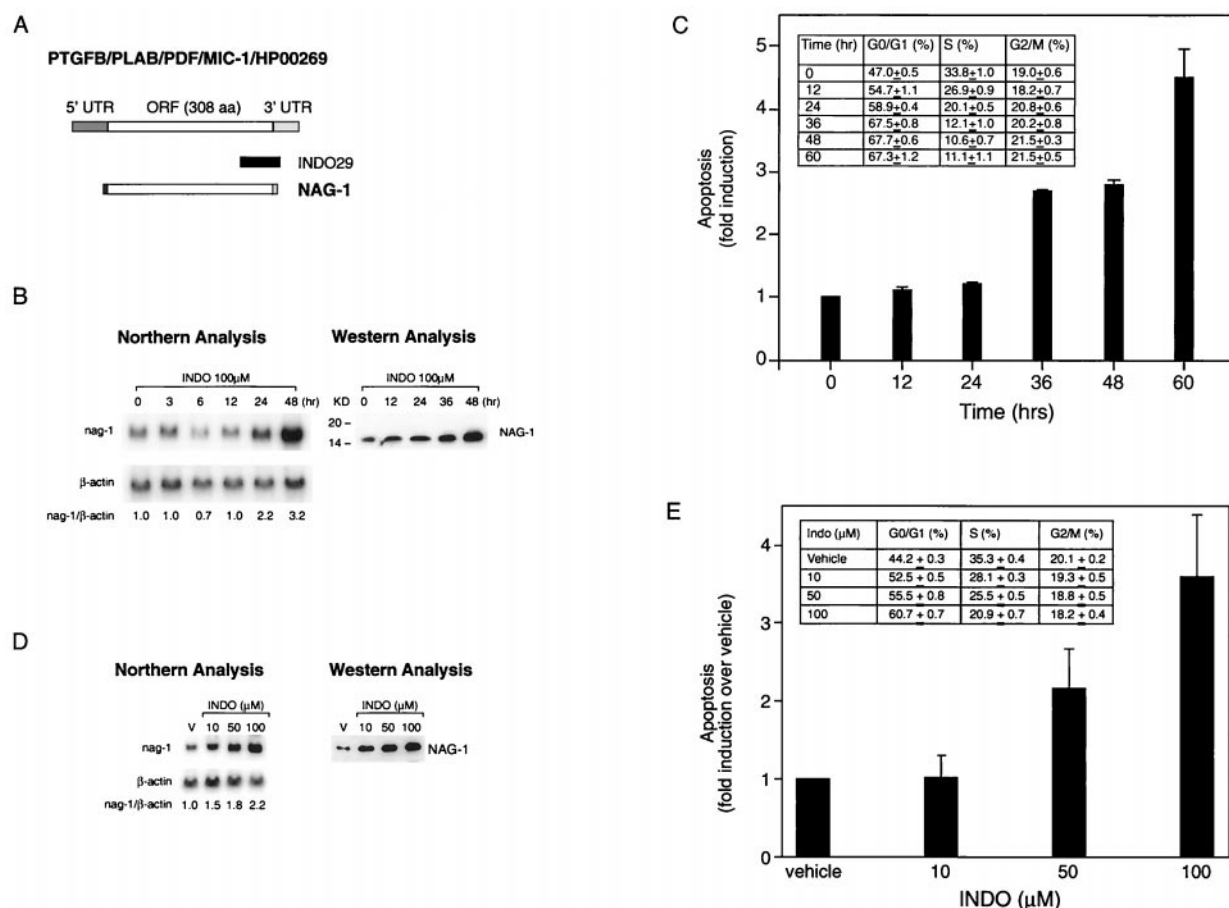
For these experiments, we chose the COX-deficient HCT-116 cells (Sheng et al., 1997) and confirmed the lack of COX expression and activity by Western and high-performance liquid chromatography analyses, respectively (Hsi et al., 2000). Despite the lack of COX expression, HCT-116 cells undergo apoptosis during treatment with INDO, making this cell line a useful tool to study COX-independent NSAID-induced gene expression.

**Identification of NSAIDs-Activated Gene.** The suppression subtractive hybridization method described by Diatchenko (1996) was used to determine whether NSAIDs could stimulate gene expression. A clone, designated INDO29, was isolated from the INDO-induced library. Char-

acterization of this 159-bp fragment by sequence analysis indicated that INDO29 is identical to the 3' region of a novel TGF- $\beta$  superfamily gene reported recently by five different groups (Fig. 1A) (Bootcov et al., 1997; Hromas et al., 1997; Lawton et al., 1997; Yokoyama-Kobayashi et al., 1997; Paralkar et al., 1998). Although these genes are named differently, sequence analyses revealed that the five genes are almost identical, and belong to a new, uncharacterized TGF- $\beta$  superfamily. Specific PCR primers were used to generate a full-length clone from HCT-116 cells with sequence identity to the genes shown in Fig. 1A. The full-length coding region was obtained, was sequenced completely, and was compared with the previously known five genes. We found that 1 bp in the coding region is different among the six genes, including our PCR product. Thus, the full-length NAG-1 is essentially identical to the genes reported by five

other groups. Based on sequence homology, NAG-1 is a divergent member of the TGF- $\beta$  family genes, because the seven-cysteine domain of NAG-1 shows 15 to 29% identity to the other TGF- $\beta$  superfamily members (data not shown). Because this branch of the TGF- $\beta$  superfamily has five different names, in this report, we designated this gene the NSAID-activated gene, NAG-1.

**Indomethacin Induces NAG-1 Expression and Apoptosis.** To confirm the increased expression of NAG-1 by INDO, Northern and Western blot analyses were performed on INDO-treated HCT-116 cells. The NAG-1 mRNA expression increased with time (Fig. 1B) with a marked increase in expression observed at 24 and 48 h. NAG-1 protein levels were also increased by INDO treatment dependent on the duration of exposure. The increases in NAG-1 protein were



**Fig. 1.** Identification and expression of NAG-1. A, schematic diagram for reported genes named PLAB, PTGFB, PDF, MIC-1, and HP00269. The bar indicates the coding region of cDNA with amino acids reported previously. Each clone is represented as follows: placenta bone morphogenic protein (PLAB; GenBank accession number U88323) (Hromas et al., 1997); placenta TGF- $\beta$  (PTGFB; GenBank accession number AF008303) (Lawton et al., 1997); prostate derived factor (PDF; GenBank accession number AF003934) (Paralkar et al., 1998); macrophage inhibitory cytokine-1 (MIC-1; GenBank accession number AF019770) (Bootcov et al., 1997); novel TGF- $\beta$  super family, HP00269 (GenBank accession number AB000584) (Yokoyama-Kobayashi et al., 1997). The black bar labeled INDO29 indicates 159-bp fragment first identified by subtractive hybridization from HCT-116 cells, whereas NAG-1 indicates the PCR-generated full-length cDNA. B, HCT-116 cells were treated with 100  $\mu$ M INDO in the absence of serum for various times. Left, Northern blotting was performed using the NAG-1 probe and reprobbed with  $\beta$ -actin probe as indicated under *Materials and Methods*. Levels of the 1.3-kb NAG-1 transcript were normalized to the levels of  $\beta$ -actin transcripts and are represented relative to 0 h treatment. Right, for the Western analysis, HCT-116 cells were treated with 100  $\mu$ M INDO for various times in the absence of serum. The media were harvested and concentrated, and 30  $\mu$ g of total protein was subjected to 15% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and probed with anti-NAG-1 antibody. C, apoptosis and cell cycle kinetics of INDO-treated HCT-116 cells at different time points. HCT-116 cells were treated with INDO, stained with PI, and analyzed by flow cytometry. Apoptosis is represented by the fold increase in the subG1 population over 0 h treatment. D, concentration-dependent expression of NAG-1. HCT-116 cells were grown in varying concentrations of INDO for 24 h or 48 h, and Northern (left) and Western analyses (right) were performed as described above. V indicates 0.2% DMSO. E, apoptosis and cell cycle kinetics of INDO-treated HCT-116 cells at different concentrations. Apoptosis was analyzed by FACS using HCT-116 cells treated with different concentrations of INDO for 48 h as described above.

observed at 36 and 48 h and occurred at later time points than the increases in mRNA.

It is known that NSAIDs induce apoptosis in cultured colorectal cells, so flow cytometric analysis of the distribution of cells at various stages of the cell cycle was performed. A prolonged G<sub>1</sub> phase and shortened S phase were observed that were dependent on the duration of INDO treatment (Fig. 1C). The changes in the cell cycle are consistent with the previous report that NSAIDs affect cell cycle progression in colon cancer cells (Shiff et al., 1996). However, because G<sub>1</sub> arrest occurs at an earlier time point than NAG-1 protein expression, the cell cycle arrest and NAG-1 induction may be independent events. Apoptotic cells were identified as the subG<sub>1</sub> population. The induction of apoptosis by INDO was time-dependent, with nearly 3- and 4.5-fold increases observed at 36 h and 60 h, respectively (Fig. 1C). These data suggest a correlation between INDO-induced apoptosis and INDO-induced NAG-1 protein expression, because similar time courses were observed.

The induction of NAG-1 mRNA and protein by INDO were also concentration-dependent (Fig. 1D). HCT-116 cells were treated with concentrations of INDO ranging from 0 to 100  $\mu$ M. Increases in both NAG-1 mRNA and protein were observed at INDO concentrations as low as 10  $\mu$ M, but were maximum at 100  $\mu$ M. Similarly, the induction of apoptosis was dependent on the concentration of INDO, with a significant increase at 50  $\mu$ M and a maximum at 100  $\mu$ M (Fig. 1E).

A correlation was observed between the increases in NAG-1 expression and the induction of apoptosis, but these results did not address whether NAG-1 expression may be a consequence of apoptosis. HCT-116 cells were treated with sodium butyrate, a known apoptotic inducer. With this treatment, apoptosis and/or G<sub>1</sub> cell cycle arrest was observed, but NAG-1 expression was not enhanced (data not shown). Thus, the increased expression of NAG-1 is not the result of apoptosis, cell cycle arrest, or cytotoxic effects, but is specific for treatment with NSAIDs.

NSAIDs can also induce apoptosis in breast (Han et al., 1998), lung (Castonguay et al., 1998), leukemia (Finstad et al., 1998), and prostate (Palayoor et al., 1998) cell lines. Thus, A549 lung epithelial cells, MCF-7 mammary cells, PC-3 prostate cancer cells, and U937 leukemia cell lines were incubated with INDO, and Northern blot analysis was performed. NAG-1 gene expression and apoptosis was stimulated by INDO treatment in the cell lines tested (data not shown). These data show that the ability of NSAIDs to increase the expression of NAG-1 is not restricted to colorectal carcinoma cells.

**Stimulation of NAG-1 Expression by Other NSAIDs.** To determine whether other NSAIDs increased apoptosis and NAG-1 expression, conventional NSAIDs that inhibit both COX-1 and COX-2, as well as selective COX-2 inhibitors were examined. HCT-116 cells were treated with various NSAIDs at the concentrations shown in Table 1 for 24 h, and Northern analysis was performed using NAG-1 cDNA as a probe. Treatment with different NSAIDs increased NAG-1 expression in a concentration-dependent manner. The conventional NSAIDs increased NAG-1 gene expression by 2- to 5-fold, whereas acetaminophen did not induce NAG-1 expression at any concentration. Sulindac sulfide was the most effective at increasing NAG-1 mRNA. The prodrug sulindac and its metabolite sulindac sulfone, which are weak cyclooxygenase

inhibitors, did not induce NAG-1 expression. Interestingly, COX-2 specific inhibitors did not increase NAG-1 expression, with the exception of LM-4101 and SC-58125. The COX-2 inhibitor Celecoxib could not be fully tested because it was toxic to these cells after 24-h incubation. The drugs LM-4101, 4108, and 4115 are derivatives of indomethacin and are COX-2 specific inhibitors (Kalgutkar et al., 2000). In general, a correlation was observed between the ability of various NSAIDs to inhibit COX and to induce NAG-1, suggesting that specific structural characteristics are necessary for NAG-1 induction.

Because sulindac sulfide was the most potent inducer of NAG-1 mRNA, NAG-1 protein expression was evaluated by Western blot analysis (Fig. 2). At a concentration of 1  $\mu$ M sulindac sulfide, some increases in NAG-1 protein (1.5-fold) were observed, and much greater increases in NAG-1 protein were achieved at concentrations from 5 to 50  $\mu$ M. Thus, an excellent correlation exists between the sulindac sulfide concentrations required to enhance NAG-1 mRNA (Table 1) and protein (Fig. 2).

NSAID stimulation of apoptosis was measured at the concentration yielding the highest fold increase in NAG-1 mRNA. As reported in Table 1, every NSAID that increased NAG-1 expression also induced apoptosis, suggesting a correlation between apoptosis and NAG-1 expression ( $r^2 = 0.94$ ). The NSAID-induced apoptosis was confirmed by annexin V assay, which can detect early apoptotic and late apoptotic/necrotic cell populations (data not shown). These data confirmed the apoptosis data generated using the PI staining method. The association between NSAID-induced apoptosis and the increase in NAG-1 expression provides evidence that NSAID-induced apoptosis may be mediated, in part, by the NAG-1 expression.

**NAG-1 Expression Enhances INDO-Induced Apoptosis.** The correlations observed between the induction of NAG-1 and of apoptosis necessitated construction of NAG-1

TABLE 1

NSAID-induced apoptosis and NAG-1 expression

Each NSAID was tested using at least three different concentrations to measure an increase in NAG-1 mRNA. These data are representative of two independent experiments. The concentration yielding maximal increase in NAG-1 mRNA was used for apoptosis determination. The apoptosis data are reported as mean  $\pm$  S.D. by PI staining methods ( $n = 6$ ).

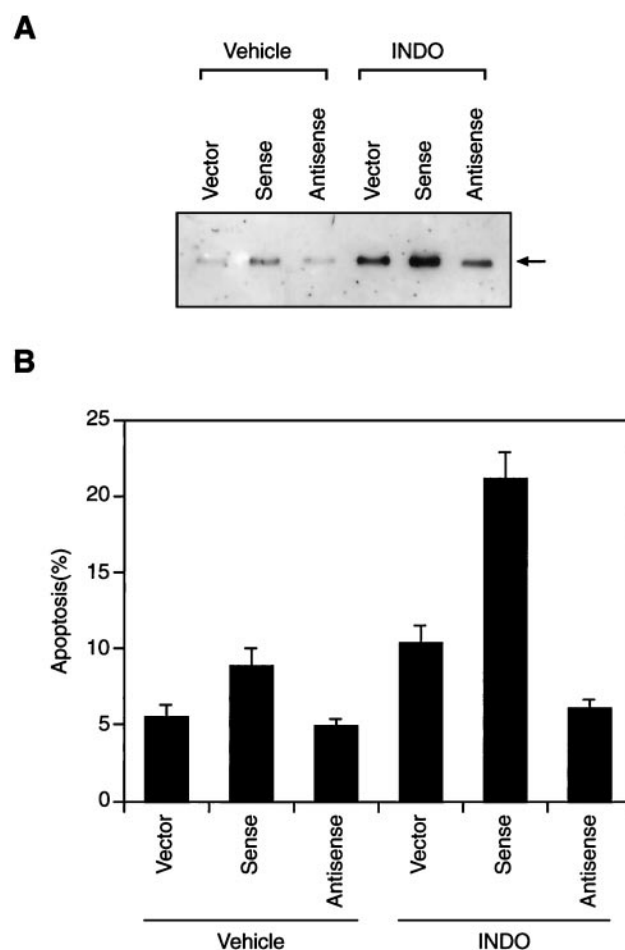
Conventional NSAIDs	Concentration $\mu$ M	NAG-1 mRNA induction Fold increase	Apoptosis Ratio Fold induction
Sulindac	10–40	1.5	1.1 $\pm$ 0.2
Sulindac sulfone	100–400	1.3	1.6 $\pm$ 0.1
Sulindac sulfide	1–50	4.6	4.3 $\pm$ 0.7
Indomethacin	10–100	2.2	2.9 $\pm$ 0.1
Piroxicam	200–500	1.9	1.5 $\pm$ 0.1
Diclofenac	50–200	3.7	4.0 $\pm$ 0.3
Aspirin	1,000–10,000	3.5	3.5 $\pm$ 0.5
Ibuprofen	100–500	2.0	1.9 $\pm$ 0.4
Sodium salicylate	1,000–5,000	3.0	1.8 $\pm$ 0.3
Acetaminophen	10–100	0.9	0.9 $\pm$ 0.2
COX-2 specific inhibitors			
NS-398	10–100	0.9	1.0 $\pm$ 0.1
DFU	10–100	1.0	0.5 $\pm$ 0.1
Celecoxib <sup>a</sup>	0.01–0.1	1.0	0.7 $\pm$ 0.1
SC-58125	10–100	5.0	3.2 $\pm$ 0.2
LM-4101	10–100	3.0	4.1 $\pm$ 0.1
LM-4108	10–100	1.0	1.7 $\pm$ 0.2
LM-4115	10–100	1.0	N.D.

<sup>a</sup> This compound was toxic to the HCT-116 cells at micromolar concentrations. N.D., not determined.

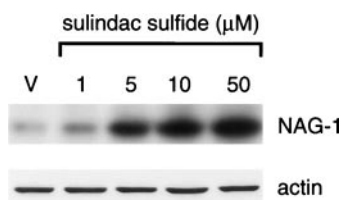
overexpressing cells to directly assess the biological activities of NAG-1. HCT-116 cells were stably transfected with an expression vector containing the full-length NAG-1 coding region in the sense and antisense orientations. Despite repeated attempts, individual clones with high NAG-1 expression could not be isolated, because the clones did not survive during expansion, possibly reflecting a high rate of apoptosis (data not shown). Thus, a pooled population of cells obtained after selection with G418 was used. These cells expressed NAG-1 protein at 2.0-fold greater than the vector-transfected cells. The anti-sense construct did not completely suppress basal NAG-1 expression, because slightly lower NAG-1 levels (0.7-fold) were observed compared with vector-transfected cells (Fig. 3A). The sense-HCT-116 cells exhibited a slower growth rate compared with vector-transfected cells or antisense-HCT-116 cells (data not shown). A higher percentage of the sense-HCT-116 cells underwent spontaneous apoptosis compared with the vector-transfected HCT-116 cells, in agreement with the higher level of NAG-1 expression (Fig. 3B). In contrast, the antisense-HCT-116 cells demonstrated slightly lower spontaneous apoptosis, concomitant with slightly lower basal expression of NAG-1 (Fig. 3B). These stably transfected cells were incubated with INDO for 48 h and percent apoptosis was determined by FACS analysis. INDO enhanced NAG-1 expression and the percentage of apoptotic cells by approximately 2-fold in the vector-HCT-116 cells, similar to results in Fig. 1C at 48 h in wild-type HCT-116 cells. The NAG-1 expression in the sense cells increased the apoptotic response to INDO, correlating with the increased NAG-1 expression. In contrast, INDO did not increase apoptosis or the expression of NAG-1 in antisense HCT-116 cells (compare vehicle treated vector and antisense to INDO treated vector and antisense in Fig. 3). These results support the conclusion that the INDO induced expression of NAG-1 is responsible, in part, for the INDO-induced apoptosis in HCT-116 cells.

**NAG-1 Has Antitumorigenic Activity.** The antitumorigenic activity of NAG-1, independent of NSAIDs treatment, was evaluated by determining whether NAG-1 expression would affect cell growth in vitro and in vivo. Cloning efficiency was examined by the soft agar cloning assay. The ability to form colonies in soft agar is reflective of tumorigenicity. NAG-1 overexpression resulted in a dramatic reduction (~50%) of the clonogenic capacity of the cells (Fig. 4A). The effect of NAG-1 on the growth of tumors was evaluated as xenografts in nude mice. NAG-1 transfected (sense and antisense) and vector-transfected HCT-116 cells were in-

jected subcutaneously into the flanks of athymic nude mice. In comparison with the vector-transfected HCT-116 cells, the antisense NAG-1 HCT-116 cells rapidly developed visible tumors and exhibited dramatic growth throughout the time course. The difference between the antisense and vector-transfected cells was statistically significant ( $P = 0.05$ ). In contrast, the sense NAG-1 HCT-116 cells grew at a slower rate and the resulting tumors were smaller than those from vector-transfected cells ( $P = 0.02$ ). The differences in growth of tumors derived from the antisense NAG-1 cells and tumors from sense NAG-1 cells were even more dramatic. After 25 days of growth, the tumors derived from the sense NAG-1 cells were approximately 35% the size of the antisense NAG-1 cells. This difference between the tumors from sense and antisense NAG-1 cells was highly statistically significant ( $P = 0.0002$ ). Even with the rather modest changes in the levels of NAG-1 in the transfected cells (Fig. 3A), profound effects were observed in the growth of tumors derived from these cells (Fig. 4B). Thus, data from in vitro and in vivo



**Fig. 3.** Altered apoptosis in sense- and anti-sense- NAG-1 cells. A, Western analysis of ectopic expression of NAG-1 in HCT-116 cells. The full-length NAG-1 (sense strand and antisense strand) was cloned into pCDNA3.1 expression vector and transfected into HCT-116 cells. Each cell was grown under G418 (500  $\mu$ g/ml) for 3 weeks and treated with either vehicle or INDO (100  $\mu$ M). After growing for 48 h, the media were harvested, concentrated, and subjected to Western analysis. Total protein (30  $\mu$ g) was loaded in each lane, and the arrow indicates the NAG-1 protein band. B, the stably transfected HCT-116 cells (vector, sense, or antisense NAG-1) were treated with vehicle or 100  $\mu$ M INDO for 48 h. The cells were harvested and apoptotic populations were examined using PI staining as described in Fig. 1C. The data represent mean  $\pm$  S.D.



**Fig. 2.** Western analysis of sulindac sulfide-treated HCT-116 cells. HCT-116 cells were grown for 48 h in the presence of sulindac sulfide at the indicated concentrations. The cell lysates were harvested in radioimmunoprecipitation assay buffer and Western analysis was performed as described in Fig. 1. NAG-1 protein was expressed as a precursor form (35 kDa) in the cell lysates. The membrane was stripped and reprobed for actin (Santa Cruz Biotechnology, Santa Cruz, CA) to evaluate equivalent loading.

studies support the hypothesis that NAG-1 has antitumor activity and that expression attenuates tumor development.

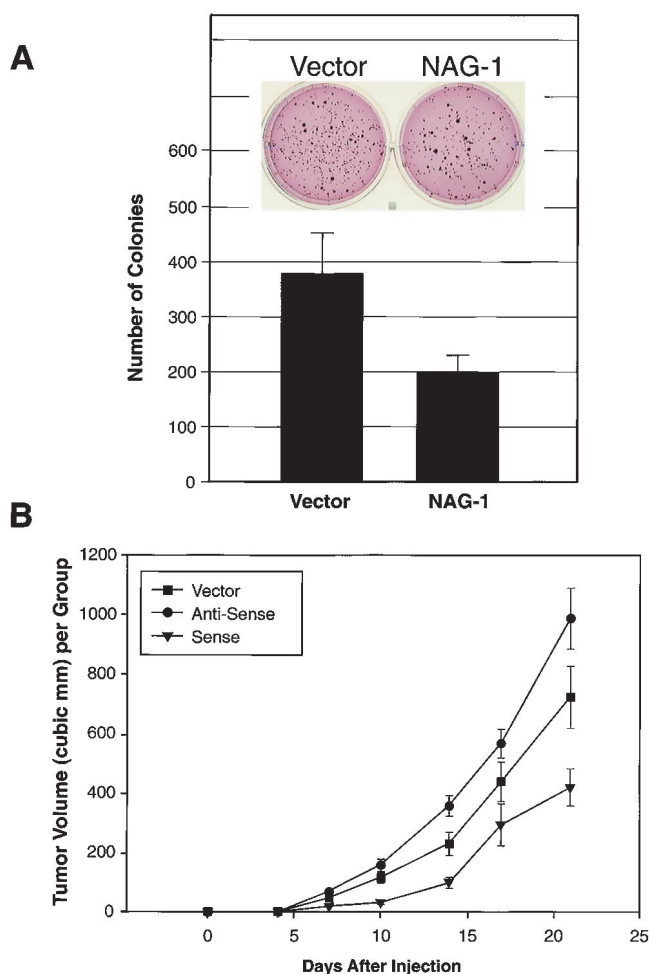
## Discussion

In this report, we present evidence that some NSAIDs increase the expression of an uncharacterized and divergent member of the TGF- $\beta$  superfamily that we called NAG-1 (NSAIDs-activated gene). This protein possesses proapoptotic and antitumorigenic activity. Incubation of the cells with COX inhibitors at concentrations higher than required to inhibit COX initiated apoptosis and increased expression of NAG-1, suggesting a link between apoptosis and NAG-1 expression. Further evidence for this association between NAG-1 expression and apoptosis was obtained using NAG-1

sense and antisense transfected HCT-116 cells. Overexpression of NAG-1 in sense-NAG-1 cells enhanced basal and INDO-stimulated apoptosis, whereas the antisense-NAG-1 exhibited an attenuated response to INDO. Sense-NAG-1 HCT-116 colorectal cells display less clonogenic growth in soft agar than control HCT-116 cells. The reduction of colony growth in soft agar suggests that NAG-1 expression resulted in apoptosis and/or cell growth arrest independent of NSAID treatment. Furthermore, the growth rate in nude mice of transplantable tumors derived from HCT-116 cells was attenuated by NAG-1 expression, providing evidence for the antitumorigenic activity of NAG-1. Our investigation was focused on colorectal cells because the antitumorigenic effect of NSAIDs is well documented for human colorectal cancer. However, the increased expression of NAG-1 in response to some NSAIDs was also observed in human breast, prostate, and leukemia cell lines, suggesting that the increased NAG-1 expression is not restricted to colorectal tissue. The results provide evidence supporting the following hypotheses: 1) NAG-1 has antitumorigenic and proapoptotic properties; 2) some NSAIDs regulate the expression of NAG-1; and 3) the proapoptotic effects reported for COX inhibitors in cell culture are mediated, in part, by NAG-1 expression.

During preparation of this article, two reports were published that presented evidence for the proapoptotic activity of NAG-1. In addition, they report that NAG-1 (called PTGF- $\beta$  in these studies) is regulated by p53 tumor suppressor gene (Li et al., 2000; Tan et al., 2000). NAG-1 was identified by DNA chip technology as a target for p53 and two putative p53 binding sites were identified in the promoter of this gene. Evidence was presented suggesting that PTGF- $\beta$  (NAG-1) could mediate the p53-dependent growth suppression. Adenovirus-mediated PTGF- $\beta$  (NAG-1) expression in MCF-7, breast cancer cells resulted in growth arrest and the induction of apoptosis (Li et al., 2000), a finding that is in agreement with the results reported here for colorectal cells. Because HCT-116 cells express wild-type p53, one question is if the NSAID-induced NAG-1 expression is mediated by the p53 sites in the promoter. However, NSAID-induced NAG-1 expression was observed in p53-null, U937, and PC-3 cells (Herrmann et al., 1998; Akashi et al., 1999). The biochemical pathway for NSAID-induced apoptosis seems to not require p53 induction (Piazza et al., 1997). Thus, NSAID and p53 regulation of NAG-1 expression may occur via independent mechanisms.

Although most of these studies were done in HCT-116 cells that are devoid of COX activity, interestingly, the NSAIDs that stimulate the expression of NAG-1 are also characterized as potent inhibitors of COX enzymes. For example, the COX inhibitor, sulindac sulfide, up-regulates NAG-1 expression, but the prodrug sulindac and the inactive metabolite sulindac sulfone do not stimulate expression of this protein. Furthermore, most COX-2 specific inhibitors were not effective at increasing NAG-1 expression in HCT-116 cells. However, one COX-2 specific inhibitor, SC-58125, was one of the most effective stimulators of NAG-1 expression. The potency for NAG-1 induction is sulindac sulfide > diclofenac > indomethacin > ibuprofen  $\approx$  sodium salicylate  $\approx$  aspirin  $\approx$  piroxicam. The order potency for enhanced NAG-1 expression is poorly correlated with the rank order of the inhibition of COX. This suggests that the structural characteristics responsible for induction of NAG-1 are similar to, but distinct



**Fig. 4.** Antitumorigenic activity of NAG-1. **A**, soft agar assay using NAG-1 overexpressing cells. Cells were grown in 0.4% soft agar for 2 weeks, and stained with *p*-iodonitrotetrazolium violet solution. The results are representative of three different experiments. The data represent mean  $\pm$  S.D. **B**, exponentially growing vector and NAG-1-transfected HCT-116 cells ( $3 \times 10^6$  cells) were inoculated subcutaneously in athymic nude mice. Tumors were measured externally on the indicated days in two dimensions using calipers. Values are the mean  $\pm$  S.E. of  $n = 18$  xenografts. The statistical difference between the tumor volumes at the final data point was determined by paired *t* test (antisense versus sense,  $P = 0.0002$ ; vector versus sense,  $P = 0.02$ ; vector versus antisense,  $P = 0.05$ ). Vector represents vector-transfected HCT-116 cells, whereas sense represents NAG-1-transfected and antisense represents antisense NAG-1-transfected HCT-116 cells. The data are representative of two independent experiments.

from the structural requirements responsible for inhibition of COX. Interestingly, the most potent NAG-1 inducer is sulindac sulfide, which has potent antitumorigenic activity and is fairly effective in the treatment of familial adenoma polyposis patients. The concentration of sulindac sulfide required to enhance NAG-1 expression (5–10  $\mu$ M) was similar to the peak plasma concentration observed in human patients (Kwan and Duggan, 1977). The plasma concentration of INDO in patients is also approximately 5  $\mu$ M. This concentration is lower than the concentrations required increase NAG-1 expression in cultured cells. Thus, additional evidence is required to determine whether NAG-1 expression is increased in patients receiving usual doses of NSAIDs and if NAG-1 expression plays an important role in reduction of colorectal cancer in humans and in experimental animals.

NAG-1 is a newly identified member of the TGF- $\beta$  superfamily, but only shares 25% sequence identity with other family members. However, it does contain the characteristic consensus RXXRA/S cleavage signal for processing the immature pro-form to the active secreted protein. Members of the TGF- $\beta$  family exert a wide range of activities regulating cell growth, differentiation, matrix formation, and apoptosis. The biological activity is not fully characterized. NAG-1 seems to induce cartilage and bone formation (Lawton et al., 1997; Paralkar et al., 1998) and may suppress inflammation by inhibiting macrophage activation (Bootcov et al., 1997). TGF- $\beta$  is recognized as an important negative regulator of growth of colonic epithelial cells. Multiple lines of evidence suggest that the TGF- $\beta$  pathway is a potent tumor suppressor of human colorectal cancer. Ectopic expression of NAG-1 in HCT-116 cells showed reduction in the growth rate of transplantable tumors in nude mice, suggesting that NAG-1, like other TGF- $\beta$  proteins, has antitumorigenic activity. Thus, NAG-1 inhibits cell growth and suppresses inflammation. Its regulation by COX inhibitors reveals a potentially important mechanism to influence these biological processes. The regulation of NAG-1 is not clearly understood and extensive promoter analysis is required to delineate the mechanisms by which NSAIDs induce NAG-1 expression. The structural and chemical characteristics of NSAIDs responsible for COX inhibition may be similar to, but distinct from the structural characteristics responsible for the up-regulation of this member of the TGF- $\beta$  superfamily. Once better understood it should be possible to develop new antitumorigenic and anti-inflammatory drugs that are potent stimulators of NAG-1 expression but are not COX inhibitors and, thus, devoid of the undesirable side effects of NSAIDs.

In summary, evidence is presented that NAG-1, a novel member of the TGF- $\beta$  superfamily, has proapoptotic and antitumorigenic activities. NAG-1 expression is regulated by some NSAIDs and therefore, the proapoptotic activity of NSAIDs observed in cell culture systems seems to be linked to the expression of NAG-1. The identification of NAG-1 as an antitumorigenic gene regulated by NSAIDs may result in the development of new drugs used in the treatment of human cancers.

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