Non-steroidal anti-inflammatory drug activated gene (NAG-1) expression is closely related to death receptor-4 and -5 induction, which may explain sulindac sulfide induced gastric cancer cell apoptosis

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Non-steroidal anti-inflammatory drugs (NSAIDs) are powerful chemopreventive agents in various cancers. They act by inhibiting cyclooxygenase (COX) activity, or through other mechanisms. NSAID-activated gene (NAG-1) has antitumorigenic and pro-apoptotic activities, but the mechanisms of NAG-1-induced apoptosis are poorly understood. Here we examined whether NAG-1 expression is induced in gastric cancer cells treated with NSAIDs, and the effect of NAG-1 expression on cell death. NAG-1 cDNA was transfected into SNU601 cells, and the relation between the ectopic expression of NAG-1 and death receptor-4 (DR-4) and DR-5 levels was studied. We found that NAG-1 expression was strongly induced in SNU601 cells, which lack endogenous COX-2, by sulindac sulfide, and that this was closely related with increased apoptosis and decreased cell viability. Moreover, temporal expressions of DR-4 and DR-5 induced by sulindac sulfide were similar to that of NAG-1. Most SNU601 cells transfected with NAG-1 cDNA did not survive during expansion. Forced NAG-1 expression significantly induced apoptosis and DR-4 and DR-5 expression. We conclude that NAG-1 expression is closely related to DR-4 and DR-5 induction, which could provide a mechanistic basis for the apoptotic effect of COX inhibitors in gastric cancer cells.

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) may protect against cancers in the gastrointestinal tract. This effect is particularly well documented in the colon and rectum; for example, a 40–50% reduction in colon cancer incidence was reported among regular aspirin users (1). Recent studies have confirmed that regular NSAIDs usage was also associated with a reduced risk of stomach cancer (2,3). The molecular mechanisms underlying such chemopreventive effects of NSAIDs are less well understood and the subject of ongoing debate. One of the most widely accepted mechanisms for the anticancer effect of NSAIDs concerns reduced prostaglandin synthesis due to the inhibition of cyclooxygenase (COX) activity. The expression of COX-2 seems to increase angiogenesis in tumors (4), and COX inhibitors are known to attenuate angiogenesis (5). Moreover, forced COX-2 expression has been shown to increase cellular adhesion and to confer resistance to butyrate-induced apoptosis in rat intestinal epithelial cells, and this effect is reversed by COX inhibitors (6). NSAIDs also have growth inhibitory effects against colon cancer cell lines that do not express COX-1 or COX-2 (7–9), and against mouse embryo fibroblasts null for both COX-1 and COX-2 genes (10). Recently, the NSAID-activated gene, NAG-1, was identified in an indomethacin-induced gene library (7). NAG-1 has antitumorigenic and pro-apoptotic activities in vivo and in vitro assays, which are independent of COX inhibition (7,11). However, the mechanisms of NAG-1-induced apoptosis are poorly understood, as are the relations between NAG-1 expression and human stomach cancer cell apoptosis.

Apoptosis is controlled via two major pathways, one originates at the cell membrane, and another involves mitochondria (12–14). The apoptotic events comprising the mitochondrial pathway affect mitochondrial permeability and the release of cytochrome c from mitochondria into the cytosol. Cytosolic cytochrome c activates procaspase-9 by binding to Apaf1 in the presence of dATP, leading to caspase-9 activation and the subsequent activation of downstream effector caspases, including caspase-3, and apoptotic induction. A recent study showed that NSAIDs engage the mitochondrial pathway at the caspase-9 and Bax levels in human colon cancer cells (15). Moreover, COX-2 over-expression reduces apoptotic susceptibility by inhibiting the cytochrome c-dependent apoptotic pathway (16). The membrane death receptor (DR) pathway involves DRs like Fas, tumor necrosis factor-receptor 1 (TNF-R1), DR-3, DR-4 and DR-5, which are activated by their respective ligands and engage the intracellular apoptotic machinery (13,17). Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) is a newly identified member of the TNF ligand family and can induce caspase-dependent apoptosis in transduced cells by activating DR-4 and DR-5 (17,18). Huang et al. demonstrated that sulindac sulfide engages the membrane DR pathway involving DR-5 and proximal caspase-8 to induce apoptosis in human colon and prostatic cancer cells (19). Moreover, forced COX-2 expression inhibits DR-5 expression and confers resistance to TRAIL-induced apoptosis in human colon cancer cells (20).

In this study, we examined whether NAG-1 expression is induced in gastric cancer cells by NSAIDs and the effect of NAG-1 expression on cell death. In addition, we studied whether the ectopic expression of NAG-1 alters DR-4 or DR-5 levels.

Abbreviations: COX, cyclooxygenase; DR, death receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MIC-1, macrophage inhibitory cytokine-1; NAG-1, NSAID activated gene; NSAIDs, non-steroidal anti-inflammatory drugs; PARP, poly ADP-ribose polymerase; PBS, phosphate-buffered saline; RT–PCR, reverse transcription-polymerase chain reaction; TBS, Tris-buffered saline; TGF-β, transforming growth factor-β; TRAIL, tumor necrosis factor-related apoptosis inducing ligand.

Materials and methods

Cell culture, reagents and drug treatment

Five well-defined gastric cancer cell lines (SNU601, 620, 668, 719 and MKN28) were obtained form the Korean Cell Line Bank (Seoul, Korea) and...
grown in RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Life Technologies) and gentamycin (10 μg/ml) in a 5% CO₂ humified atmosphere. NSAIDs in this study were purchased from Sigma (St Louis, MO) and dissolved in dimethyl sulfoxide. TRAIL was obtained from Calbiochem (San Diego, CA) and dissolved in phosphate-buffered saline (PBS).

**Transfection of NAG-1 cDNA**

SNU601 cells (2 x 10⁴ in 2 ml of RPMI 1640 without gentamycin) were plated in 6-well plates. Twenty-four hours later the cells were transfected with vector alone or with 4 μg of pcDNA3.1-NAG-1 plasmid containing a full-length NAG-1 cDNA (a gift of Dr Baek, University of Tennessee, Knoxville, TN), using Lipofectamine 2000 (Life Technologies) according to the manufacturer’s instructions. Briefly, 4 μg of DNA was mixed with 250 μl of RPMI 1640 without serum. Thereafter, the diluted DNA was combined with 10 μl of Lipofectamine 2000 diluted by 250 μl of RPMI 1640 without serum and incubated for 20 min at room temperature. This mixture was then added to cells that had attained 90% confluence in 6-well plates. After 48 h, the cells were harvested for western blot analysis, fluorescence-activated cell sorter (FACS) analysis and trypan blue dye exclusion assay. To ensure stable expression, the transfected cells were grown in RPMI 1640 supplemented with 300 μg/ml of gentamicin (Life Technologies) for 21 days.

**Measurement of apoptosis by FACS analysis**

Cells (3 x 10⁴ in 2 ml of RPMI 1640) were plated in 6-well plates, incubated for 24 h, and treated with the various agents. Thereafter, the cells were harvested, washed with PBS, and stained using a Cycle test plus DNA reagent kit (Becton Dickinson Immunocytometry Systems, San Jose, CA), according to manufacturer’s instructions. Briefly, 250 μl of solution A was added to the cells and incubated for 10 min at room temperature, and then 200 μl of solution B was added and incubated for 10 min at room temperature. Thereafter, 200 μl of cold solution C was added and cells were kept in the dark room for 10 min on ice. The cells (2 x 10⁴) were then examined by flow cytometry using a Becton Dickinson FACSort equipped with CellQuest software by gating on an area-versus-width dot plot to exclude cell debris and cell aggregates. Apoptosis was measured by determining the level of subdiploid DNA in cells.

**Trypan blue dye exclusion assay**

To determine cell viabilities, 4 x 10⁴ cells were seeded in triplicate in 24-well plates in 0.5 ml of medium for 24 h, and treated with the various agents. Thereafter, 10 μl of 0.4% trypan blue was added to cell suspensions and cells were observed under an inverted microscope.

**Western blot analysis**

Cells were washed with cold PBS and suspended in a lysis buffer (1 x PBS, 1% Triton X-100) supplemented with complete mini protease inhibitor mixture tablets (Boehringer Mannheim, Mannheim, Germany) on ice for 30 min. After removing cell debris by centrifugation, cell lysate protein concentrations were determined using BCA protein assay reagent (Pierce, Rockford, IL) with bovine serum albumin as a standard. Forty micrograms of protein were separated in 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The membranes were blocked with 5% skimmed milk in Tris-buffered saline (TBS) for 1 h at room temperature and probed with antibodies overnight at 4°C. The antibodies used were anti-NAG-1 (1.5 μg/ml; Upstate, Charlottesville, VA), anti-COX-2 (1:1000; Cayman Chemical, Ann Arbor, MI), anti-DR-5 (1: 500; ProSci, Poway, CA), anti-DR-4 (1: 200; Santa Cruz Biotechnology, Santa Cruz, CA), anti-caspase-3 (1:800; Upstate), anti-PARP (poly ADP-ribose polymerase) (1:800; Upstate) and anti-β-actin (1:2000; Santa Cruz Biotechnology). After washing with TBS-0.05% Tween 20, the blots were treated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:3000; Zymed Laboratories, San Francisco, CA) for 1 h at room temperature. Detection was performed by enhanced chemiluminescence (Pierce) and autoradiography.

**RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)**

Total RNA was extracted and purified from cultured cells using a GeneElute Mammalian Total RNA kit (Sigma) according to the manufacturer’s instructions. RNA was quantified by measuring absorbance at 260 nm. Two micrograms of total RNA from each sample was reverse transcribed into cDNA using Superscript II reverse transcriptase (Life Technologies) and random hexamer primers (Takara, Shiga, Japan). The PCR primers used were as follows (20): DR-4, 5'-GGA CAC GGC AGA GGC TGG GCTG ACC-3' (sense), 5'-GAGGGCAACCTTGTCAGCTGTATTACT-3' (antisense); DR-5, 5'-AAG ACC CTT GTG CTC GTT G-3' (sense), 5'-TCA CCC CAC TGG AAC CTT ATG ACC-3' (antisense); 5'-GACCGCGGAGACACTGGCATGCGC-3' (sense), 5'-AATGGTAAATGTCAAGCTTGTTACATT-3' (antisense). The PCR conditions used were:

- Initial denaturation at 94°C for 5 min, 30 cycles of amplification (94°C for 45 s, 60°C for 45 s, 72°C for 45 s), and an additional extension step of 72°C for 7 min. PCR products were electrophoresed in a 1.5% agarose gel containing ethidium bromide and then photographed under ultraviolet light.

**Results**

Of the five gastric cancer cell lines (SNU601, 620, 668, 719 and MKN28), two cell lines (MKN28 and SNU668) expressed COX-2, whereas the other cell lines (SNU601, 620 and 719) did not (Figure 1). The insignificant COX-2 expression in three cell lines (SNU601, 620 and 719) is explained by hypermethylation at the CpG island area of the COX-2 promoter (21). The COX-2 expression levels of MKN28 and SNU668 are in accord with previous findings (21,22). In order to study the COX-2-independent effect of NSAIDs, we chose the SNU601 cell line. Conventional NSAIDs that inhibit both COX-1 and COX-2, and a selective COX-2 inhibitor were used to determine whether NSAIDs increased NAG-1 expression. SNU601 cells were treated with various NSAIDs at the upper concentration limit used in HCT-116 cells by Baek et al. for 40 h (7). As shown in Figure 2, wild-type SNU601 did not express NAG-1, and sulindac sulfide was the most effective at increasing NAG-1. Indomethacin and aspirin also induced NAG-1 expression, whereas sulindac and NS-398 did not. We examined the relation between NAG-1 expression, and exposure time and sulindac sulfide dose. As shown in Figure 3A, the NAG-1 expression level increased with time, was highly expressed at 24 and 40 h and also showed dose dependence (P < 0.05). The number of round floating cells with multiple bodies connected with distorted cell membrane increased with

![Fig. 1. Western blot analysis of COX-2 expression in various gastric cancer cell lines. Forty micrograms of protein were separated by 12% SDS–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Immunoblots were probed with COX-2 polyclonal antibody. The bottom represents β-actin, which was used as a loading control.](image)

![Fig. 2. Western blot analysis of NAG-1 expression in various NSAID-treated SNU601 cells, wild SNU601 cells and SNU668 cells. SNU601 cells were grown for 40 h in the presence of NSAIDs at the upper limit of the concentration used by Baek et al. (7) (sulindac sulfide, 50 μM; aspirin, 10 μM; NS-398, 100 μM; sulindac, 40 μM; indomethacin, 100 μM). Forty micrograms of protein were separated by 12% SDS–PAGE and transferred to a nitrocellulose membrane. The bottom represents β-actin, which was used as a loading control.](image)
sulindac sulfide dose (Figure 3B), and cell viability after treatment of sulindac sulfide for 40 h was reduced with concentration, i.e. 98.3 ± 1.5, 87.7 ± 5.0 and 43.9 ± 5.5% at 12.5, 25 and 50 μM, respectively (P < 0.05) (Figure 3B). It is known that NSAIDs induce apoptosis in human gastric cancer cells (23,24), and therefore, flow cytometry was used to determine the subG1 population after treatment with sulindac sulfide. Apoptotic induction was found to be time and dose dependent.

![Figure 3](image-url)

**Fig. 3.** The effect of sulindac sulfide on the expressions of NAG-1, DR-4 and DR-5, and on cell viability and apoptosis in SNU601 cells. (A) Expressions of NAG-1, DR-4 and DR-5 in the presence of sulindac sulfide at the indicated times (50 μM) and doses (0–50 μM). The bottom represents β-actin, which was used as a loading control. *Significantly different from 0, 6, 12 and 55 h (P < 0.05). "Significantly different from 0 and 12.5 μM (P < 0.05). (B) The morphology of cells treated with sulindac sulfide (50 μM) for 40 h. Cell viability was analyzed by trypan blue dye exclusion assay. *Significantly different from 0 and 12.5 μM (P < 0.05). **Significantly different from 0, 12.5 and 25 μM (P < 0.05). (C and D) The cells were treated with sulindac sulfide at the indicated doses for 40 h (C) and times (D), stained with propidium iodide, and analyzed by flow cytometry. Apoptosis is represented by fold increase in the subG1 population over 0 μM (C) and 0 h (D) treatment. *Significantly different from 0 μM (P < 0.05). **Significantly different from 0, 12.5 and 25 μM (P < 0.05). **Significantly different from 0 h (P < 0.05). ***Significantly different from the other groups (P < 0.05). (E) mRNA expression of DR-4 and DR-5 by RT-PCR in the cells treated with sulindac sulfide at indicated doses for 40 h. Mean values are shown for triplicate experiments; bars represent standard deviation.
dependent ($P < 0.05$) (Figure 3C and D), and to be slightly lower at 55 h than 40 h ($P < 0.05$), which corresponded with a mild decrease in NAG-1 expression at 55 h. A recent study showed that sulindac sulfide treatment increases DR-5 expression in human colon cancer cells (19), and thus we performed western blot and RT–PCR for DR-4 and DR-5 in sulindac sulfide treated cells. As shown in Figure 3A and E, the expression of DR-4 and DR-5 was negligible in wild SNU601 cells and was dependent on sulindac sulfide exposure time, showing the highest expression at 40 h and a marked decrease at 55 h ($P < 0.05$). Moreover, DR-4 and DR-5 expression levels were higher at 25 and 50 μM than that at 12.5 μM ($P < 0.05$). These expression patterns closely followed that of NAG-1.

SNU601 gastric cancer cells were transfected with human NAG-1 cDNA, and then the cells were harvested after 48 h. Strong NAG-1 expression in the transfected cells was confirmed versus the wild-type cells (Figure 4A). Most of the transfected cells lost adhesion and floated in culture, and exhibited distorted and shrunken cell membranes and nuclear fragmentation (Figure 4B). Thus, the viability of transfected cells was markedly decreased to 59.3 ± 1.6 versus 94.2 ± 1.3% for the wild-type cells ($P < 0.05$) (Figure 4B). Despite repeated attempts, we failed to obtain stable NAG-1 expressing cell lines, because the cells did not survive during expansion (Figure 4B). Moreover, the subG$_1$ population was markedly elevated in cells transfected with NAG-1 cDNA (Figure 4C). As shown in Figure 4A, forced NAG-1 expression induced DR-4 and DR-5. Caspase-3 exists in an inactive form as a pro-caspase, and its activation involves the cleavage of the pro-form into smaller active subunits, and activated caspase-3 specifically cleaves PARP into an 85-kDa apoptotic fragment. Forced NAG-1 expression reduced the level of procaspase-3 and induced smaller active fragments of caspase-3 (17 and 12 kDa) and an 85-kDa band of PARP at the expense of the 111-kDa band (Figure 4A).

**Discussion**

In this study, we report that NAG-1 expression is induced by sulindac sulfide in a gastric cancer cell line lacking...
endogenous COX-2, and that this induction is closely related with increased apoptosis and reduced cell viability. We also demonstrate for the first time that forced NAG-1 expression significantly increases DR-4 and DR-5 expression.

COX inhibition is not required for the chemopreventive effect of NSAID, a well-known observation in colon carcinogenesis (25). Sulindac sulfone, an oxidant product of sulindac, and which does not inhibit COX, inhibited proliferation and induced apoptosis in HT-29 colon cancer cells (26,27). Compared with controls, sulindac sulfone reduced the number of colonic neoplasms in azoxymethane-treated rats (28). Sulindac sulfide inhibited proliferation, altered the cell cycle phase distribution, reduced the expression of proliferation markers and induced apoptosis in the HCT-15 colon cancer cell line, which does not express COX (29,30). A recent study reported that NSAIDs also showed anti-neoplastic properties in a gastric cancer cell line by inhibiting mitogen activated protein kinase (23). NAG-1 is a newly identified member of the transforming growth factor-β (TGF-β) superfamily, but only shares a sequence identity of 25% with other family members (7). It has also been reported as a placental bone morphogenic protein (31), placental TGF-β (32), prostate-derived factor (33) and macrophage inhibitory cytokine-1 (MIC-1) (34). Members of the TGF-β family are multifunctional growth factors, and the nature of their effects depends on the cellular context (35). Baek et al. demonstrated that NAG-1 has antitumorigenic and pro-apoptotic activities in HCT-116 colon cancer cells devoid of COX expression (7). In the present study, sulindac sulfide induced the expression of NAG-1 in SNU601 cells devoid of COX-2 expression, and this was found to be closely related to decreased cell viability and increased apoptosis. Moreover, forced expression of NAG-1 remarkably reproduced this finding. Therefore, increased NAG-1 expression by sulindac sulfide may be an acceptable explanation for the COX-2-independent chemopreventive effect of NSAIDs in gastric cancer cells. However, the receptor or transduction pathway engaged by NAG-1 is unknown, and further study is needed.

As shown in recent and the present studies (19,20), sulindac sulfide induces DR-4 and DR-5 expression in gastric cancer cells. Temporal sequence of induction of NAG-1, DR-4, DR-5 and apoptosis exists. Furthermore, forced NAG-1 expression strongly induced DR-4 and DR-5. The data suggest that the induction of DR-4 and DR-5 by sulindac sulfide are closely related to NAG-1 expression and apoptosis. Moreover, DRs can be activated in ligand-dependent or -independent manners (13). Ligand-independent activation occurs via agonist antibody or increased DRs. Over-expression of exogenous or endogenous DR-5 has been shown to induce apoptosis (36–38). Thus, it is possible that DR-4 and DR-5 over-expression coupled with NAG-1 induction cause apoptosis even in the case of no TRAIL treatment. However, the possibility that increased DR-4 and DR-5 may sensitize SNU-601 cells to pre-existing TRAIL should be considered. Caspase-3 is the one of the most distal caspases activated by mitochondrial and membrane DR pathways (12–14). We identified the reduced expression of procaspase-3 and the increased expression of smaller active fragments of caspase-3. In order to investigate the general status of the genes related to apoptosis induced by NAG-1, we intend to perform a cDNA array analysis on gastric cancer cells after co-transfection with COX-2 and NAG-1, and to compare the results with those of cells transfected with NAG-1 or COX-2 alone.

Lee et al. demonstrated that the stable transfection of MIC-1 cDNA into SNU216 gastric cancer cells significantly increases invasiveness by activating the urokinase-type plasminogen activator (uPA) system (39). In addition, they reported that recombinant MIC-1 induced gastric cancer cell invasion and the activation of the uPA system in a dose-dependent manner. Moreover, recent findings that MIC-1 is highly and specifically expressed in prostate and colorectal cancer tissues suggest the possible role of MIC-1 in tumor development and progression (40–42). Therefore, it is likely that NAG-1, like TGF-β (43), functions both positively and negatively in tumorigenesis, and their modes of action depend on their molecular and cellular contexts.

In conclusion, this study shows that NAG-1 expression induced by sulindac sulfide is closely related to the inductions of DR-4 and DR-5. We believe that this could provide a mechanistic basis for the apoptotic effects of COX inhibitors in gastric cancer cells. Moreover, the identification of NAG-1 as an antitumorigenic gene regulated by NSAIDs may result in the development of new drugs for the treatment of human gastric cancer.

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References
