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Cyclooxygenase inhibitors induce apoptosis in sinonasal cancer cells by increased expression of nonsteroidal anti-inflammatory drug-activated gene

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Nonsteroidal anti-inflammatory drug-activated gene-1 (NAG-1) has recently been shown to be induced by nonsteroidal anti-inflammatory drugs (NSAIDs) and to have proapoptotic and antitumorigenic activities. Although sulindac sulfide induced apoptosis in sinonasal cancer cells, the relationship between NAG-1 and NSAIDs has not been determined. In this study, we investigated the induction of apoptosis in sinonasal cancer cells treated by various NSAIDs and the role of NAG-1 expression in this induction. The effect of NSAIDs on normal human nasal epithelial (NHNE) cells was also examined to evaluate their safety on normal cells. Finally, the *in vivo* anti-tumorigenic activity of NSAIDs in mice was investigated. In AMC-HN5 human sinonasal carcinoma cells, indomethacin was the most potent NAG-1 inducer and caused NAG-1 expression in a time- and dose-dependent manner. The induction of NAG-1 expression preceded the induction of apoptosis. Conditioned medium from NAG-1-overexpressing *Drosophila* cells inhibited proliferation of sinonasal cancer cells and induced apoptosis. In addition, in NAG-1 small interfering RNA-transfected cells, apoptosis induced by indomethacin was suppressed. In contrast, NAG-1 expression and apoptosis were not induced by NSAIDs or conditioned medium in NHNE cells. Furthermore, indomethacin induced a dose-dependent *in vivo* increase in the expression of NAG-1 mRNA in the mice tumors and the volume of xenograft tumors of AMC-HN5 cells in indomethacin-treated nude mice was reduced compared to that in control mice. In conclusion, indomethacin exerts proapoptotic and antitumorigenic effects in sinonasal cancer cells through the induction of NAG-1 and can be considered a safe and effective chemopreventive agent against sinonasal cancer.

Key words: NSAIDs; NAG-1; sinonasal cancer; chemoprevention

Chemopreventive agents play an important role in interrupting the carcinogenic process and inhibiting the recurrence of precancerous lesions. At present, the pharmaceutical agents that have been most widely investigated as chemopreventive agents are the nonsteroidal antiinflammatory drugs (NSAIDs). NSAIDs have been clinically used as chemopreventive agents for familial adenomatous polyposis through the inhibition of cyclooxygenase-2 (COX-2).[1,2] However, the chemopreventive and antitumorigenic activities of NSAIDs were also observed in COX-2-deficient cells, indicating that NSAIDs also exert their anticancer effect through a mechanism other than COX-2 suppression. One such mechanism involves the induction of NSAID-activated gene-1 (NAG-1).[3]

NAG-1 was identified as a divergent member of the TGF-β superfamily and shares certain characteristic functions of the TGF-β superfamily cytokines.[3] NAG-1 exhibits proapoptotic and antitumorigenic activities through the induction of apoptosis in colorectal, lung, prostate, oral cavity and ovarian cancer cells.[4–8] NAG-1 is upregulated by some polyphenols, retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (AHPN), indole-3-carbinol, PPARγ ligands and conjugated linoleic acids in a COX-2- and p53-independent manner.[9–15] Furthermore, NAG-1 is highly expressed in the liver and intestine after oral administration of sulindac in mice and is inducible *in vivo* by NSAIDs.[5] Although sulindac sulfide induced apoptosis in sinonasal cancer cells, the relationship between sinonasal cancer and NAG-1 has not been revealed.[16] If NSAIDs induce NAG-1 expression and apoptosis in sinonasal cancer cells, these drugs may be useful chemopreventive agents against sinonasal cancer. It is essential to induce apoptosis selectively in sinonasal cancer cells without damaging NHNE cells, because if NAG-1 is also induced by NSAIDs in NHNE cells, it may cause apoptosis in normal cells. Therefore, when evaluating the efficacy of NSAIDs as chemopreventive agents, it is important to consider the induction of NAG-1 and apoptosis in both sinonasal cancer cells and NHNE cells.

Inverted papilloma is a benign sinonasal tumor originating mainly from the lateral nasal wall or the maxillary sinus that tends to recur and be associated with malignancy. Recently, studies have shown that a high risk of malignant transformation of inverted papilloma is significantly correlated with an increase in the epidermal growth factor receptor (EGFR) and Ki-67 index, HPV 6/11- and HPV 16/18-positivity, and a low apoptotic index.[17–19] Therefore, recurrence and malignant transformation can be suppressed by treatment with chemopreventive agents after radical resection of the inverted papilloma. However, it has not been established which chemopreventive agents are the safest and most effective against sinonasal cancer so it is clinically meaningful to investigate the efficacy of NSAIDs as chemopreventive agents.

The purpose of this study was to investigate whether NSAIDs induce apoptosis in sinonasal cancer cells and if so, whether induction of NAG-1 is directly linked to this apoptosis. In addition, the effects of NSAIDs on NHNE cells were examined to evaluate their safety on normal cells. Finally, the *in vivo* antitumorigenic activity of NSAIDs in mice was investigated to evaluate the use of NSAIDs as chemopreventive agents.

Material and methods

Cell lines and reagents

Passage-2 NHNE cells were cultured in Transwell-clear (Costa, Cambridge, MA) culture inserts (diameter 24.5 mm) with 0.45 mm pore size at a density of 10^5 cells/culture. The culture medium was a mixture of BEGM and DMEM (GibcoBRL, Rockvill, MD) in equal ratios with fetal bovine serum and supplements as previously published.[20] Cells were cultured in a submerged state for 9 days, then the media in the upper compartment was removed and

Abbreviations: COX, cyclooxygenase; NAG-1, nonsteroidal anti-inflammatory drug-activated gene-1; NCM, NAG-1 conditioned medium; NHNE cells, normal human nasal epitheial cells; NSAIDs, nonsteroidal anti-inflammatory drugs; siRNA, small interfering RNA; VCM, vehicle conditioned medium.

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nutrients were supplied from the membrane below the cells, creating an air-liquid interface (ALI) culture. For the study, cultured cells were used after 14 days in the ALI culture. The sinonasal cancer cell line used was the Asan Medical Center-Head and Neck Cancer 5 (AMC-HN5) cell line. These cells were maintained in DMEM medium supplemented with 10% fetal bovine serum, penicillin and streptomycin. Indomethacin and diclofenac were purchased from Sigma Chemical (St. Louis, MO), sulidac sulfide was purchased from Cayman Chemical (Ann Arbor, MI), and aceclofenac was provided by Daewoong Pharmaceutical. (Seoul, Korea). Rabbit anti-NAG-1 antibody was received as a generous gift from Dr. Thomas E. Eling (NIEHS, RTP, NC), and anti-α-tubulin antibody was purchased from Santa Cruz Biotechnology. (Santa Cruz, CA).

Western blot analysis

Cells were scraped into 1 m radioimmunoprecipitation assay buffer (1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) and total protein concentration was measured by bicinchronic acid protein assay using bovine serum albumin. The cell lysates were centrifuged and the supernatants were subjected to Western blot analysis. Thirty micrograms of protein were loaded in each lane separated by 15% SDS-PAGE and transferred onto a nitrocellulose membrane. Blots were blocked with 10% nonfat dry milk in Tris-buffered saline with 0.05% Tween-20 (TBST) at 4°C for 12 hr and probed with anti-NAG-1 antibody (1:5,000) for 4 hr at room temperature. After washing, the blots were treated with horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ) diluted 1:5,000 in TBST for 1 hr, and washed several times. Bands were detected by enhanced chemiluminescence and autoradiography. The blots were stripped and reprobed with α-tubulin antibody (Santa Cruz Biotechnology) to normalize for loading.

Flow cytometry

Cells were plated at 4×10^5 cells/well in six-well plates, incubated for 16 hr, and treated with NSAIDs in the absence of serum. The Annexin V method was used to examine apoptosis of AMC-HN5 cells. Cells were harvested, washed with PBS, stained with propidium iodide (PI) and conjugated with Annexin V-FITC using the TACS Annexin V-FITC kit (Trevigen, Gaithersburg, MD) according to the manufacturer's protocol. Cells were examined by flow cytometry (FACSort, Becton-Dickson, San Diego, CA). Apoptotic populations were determined as Annexin V-positive/PI-positive and Annexin V-positive/PI-negative cell populations from the total gated cells. Experiments were performed at least three 3 times, and statistical significance was accepted for p-values <0.05 through the repeated measures ANOVA test and multiple comparisons analysis.

Generation of NAG-1 conditioned medium

NAG-1 conditioned medium (NCM) was prepared using *Drosophila* cells by a previously described method.[6] Briefly, *Drosophila* Schneider cell line (ATCC CRL-1963) was grown at 23°C in Schneider medium (Sigma). The open reading frame (ORF) corresponding to full-length NAG-1 from pcDNA3.1(+)-NAG-1 plasmid was subcloned into pMT/V5 vector (pMT/V5-His-NAG-1) under control of the metallothionein promoter (Invitrogen, Carlsbad, CA). Cell lines expressing hexahistidine-tagged NAG-1 were generated by transfection with pMT/V5-His-NAG-1 together with pCoHYGRO containing the *Escherichia coli* hygromycin B-phosphotransferase gene under control of the Drosophila copia promoter (Invitrogen). Transfection was performed according to standard $CaPO_4$ protocol and transfected cells were selected with hygromycin (300 μg/ml) for 6 weeks. Expression was induced by addition of $CuSO_4$ to the culture medium at a final concentration of 500 μM for 48 hr before use. The $CuSO_4$ induced culture supernatants were dialyzed against PBS and the supernatants were used as NCM. Vector conditioned medium (VCM) was prepared similarly but without the addition of $CuSO_4$. Although we used dialysis to remove $CuSO_4$, any remaining $CuSO_4$ could have affected the results. Thus, pMT/V5 and pCoHYGRO vectors were transfected into the Schneider cell line by the same method as used for NAG-1 overexpressing cells. These cells were treated with $CuSO_4$ for 48 hr, the culture supernatants were dialyzed, and the dialyzed supernatants were retained as vector medium. The media was concentrated using a Centriplus centrifugal filter device YM-50 (Millipore, Billelica, MA) at 3,000*g* according to the manufacturer's protocol. Expression of NAG-1 in NCM, VCM and vector medium was measured by Western blot analysis using anti-NAG-1 antibody.

NAG-1 small interfering RNA

The NAG-1 siRNA duplex (sense CUCAGUUGUCCUGCCC UGUdTdT and antisense ACAGGGCAGGACAACUGAGdTdT) and *ACCU Target* negative control siRNA duplex from Bioneer. (Daedeok, Daejeon, Korea) were used. Cells at 50–60% confluence were transfected using Lipofectamin (Gibco-BRL) with NAG-1 siRNA duplex (1 μg/well) or with negative control siRNA duplex (1 μg/well) according to the manufacturer's instructions. After incubation for 24 hr, the medium was removed and the cells were washed with PBS and treated with NSAIDs or vehicle for 24 hr. Cells were harvested and analyzed for NAG-1 expression and apoptosis. Experiments were repeated more than 3 times, and the statistical significance was accepted for p-values <0.05 through the repeated measures ANOVA test and multiple comparisons analysis.

Cell proliferation assay

Cells were plated in 96-well plates at a density of 2,000 cells/well. After incubation for 16 hr, the cells were washed with PBS and incubated for 48 hr in serum-free medium according to the experimental conditions. Cell proliferation was measured using a Cell Titer 96 AQ_{ueous} One Solution Proliferation Assay Kit (Promega, Madison, WI) according to the manufacturer's instructions. Two milliliters of tetrazolium products were mixed with 100 μl phenazine ethosulfate, and 20 μl of reagent was added to each well of the 96-well plates. Cells were incubated for 1 hr at 37°C under 5% CO_2 environment and optical density (O.D.) at 490 nm was measured using a spectrophotometer. The mean percentage of living cells was calculated as follows: [1-(mean O.D. of experimental group/mean O.D. of control)] × 100. The statistical significance was accepted for p-values <0.05 through the repeated measures ANOVA test and multiple comparisons analysis.

Tumor growth in nude mice

Twenty male nude mice (*BALB/c-nu*) were purchased at 5 weeks of age and were maintained in pathogen-free conditions until the experiments were performed at 8 weeks of age. A total of 10^7 AMC-HN5 cells in 0.1 ml of PBS were subcutaneously injected behind the anterior forelimb bilaterally in each mouse. As an experimental group, indomethacin (2 mg/kg/day) was administered to 10 mice in their drinking water. To put indomethacin in the drinking water, 10 mg of indomethacin was dissolved in 1 ml absolute ethanol and distilled water was added to 200 ml final volume. Indomethacin was omitted for the control group. Growth curves for grafts were determined by externally measuring tumors in 2D beginning when the tumor was more than 3 mm in diameter. Tumor volume was determined by the equation $V = [(L + W) 0.5] \times L \times W \times 0.5236$. Values are given as the mean ± SD of 20 xenografts per group. Statistical significance was accepted for p-values <0.05 through the paired t test.

In vivo analysis of NAG-1 expression by RT-PCR

Samples of the mouse tumors were obtained by subcutaneous fine needle aspiration from the injected sites in 6 hr and by subcutaneous excision from the injected sites in 24 and 48 hr after indomethacin (2 mg/kg/day) treatment. Total cellular RNA was iso-

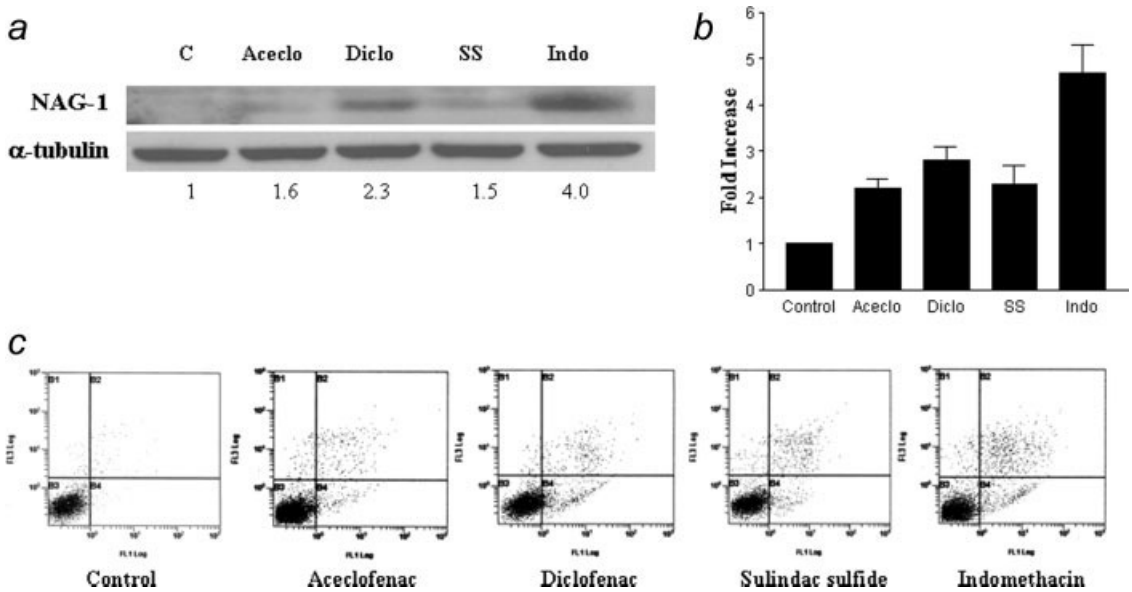

FIGURE 1 – Induction of NAG-1 expression and apoptosis in AMC-HN5 cells by NSAIDs. AMC-HN5 cells were treated with 100 μM aceclofenac (Aceclo), diclofenac (Diclo), sulindac sulfide (SS) or indomethacin (Indo) for 48 hr. Expression of NAG-1 was measured by Western blot analysis and normalized to the level of α-tubulin expression. Apoptosis was analyzed by flow cytometry using Annexin V-FITC and propidium iodide (PI), and represented by the fold increase in the percentage of apoptotic cells compared to the control. (*a*) NAG-1 expression is induced by NSAIDs in the following order: indomethacin (4.0 ± 0.5-fold increase), diclofenac (2.3 ± 0.3-fold increase), aceclofenac (1.6 ± 0.2-fold increase) and sulindac sulfide (1.5 ± 0.3-fold increase, compared to the control). (*b*) As to flow cytometry, apoptosis is induced by NSAIDs in the following order: indomethacin (4.7 ± 0.6-fold increase), diclofenac (2.8 ± 0.3-fold increase), sulindac sulfide (2.3 ± 0.4-fold increase) and aceclofenac (2.2 ± 0.2-fold increase, compared to the control). (*c*) Dot plots of DMSO- and NSAID-treated cells. The *x*-axis represents Annexin V-FITC and the *y*-axis represents PI. The results shown are representative of 3 independent experiments.

lated using the Tri-Reagent kit (Molecular Research Center, Cincinnati, OH) and was reverse transcribed into cDNA using random hexanucleotide primers and Moloney murine leukemia virus reverse transcriptase (Gibco-BRL). NAG-1 cDNA was amplified by PCR using a Perkin-Elmer Cetus DNA Thermal Cycler (Perkin-Elmer, Norwalk, CT). The oligonucleotide primers for PCR were designed based on GenBank™ sequence of human NAG-1 (GenBank™ accession number AF008303, 5′ primer ACCTGCACCTGCGTATCTCT; 3′ primer CGGACGAAGATTCTGCCAG). The following PCR conditions comprised of 35 cycles: denaturation at 95°C for 30 s, annealing at 60°C for 30 s and polymerization at 72°C for 30 s. The predicted size of the NAG-1 PCR product was 228 bp. The oligonucleotide primer of β_2-microglobulin (β_2M; used as a control gene for the RT-PCR) was based on the GenBank™ human sequence (GenBank™ accession number XM007650, 5′ primer CTCGCGCTACTCTC TTTCTGG; 3′ primer GCTTACATGTCTCGATCCCACTTAA). The PCR parameters used involved 23 cycles as follows: denaturation at 95°C for 30 s, annealing at 60°C for 60 s and polymerization at 72°C for 60 s. The PCR products obtained were analyzed by 1% agarose gel electrophoresis and visualized with ethidium bromide staining under a transilluminator. The signal intensity of the positive band was measured using the Scion Image (Scion, Frederick, MD) and the expression was normalized to the level of β_2M expression. Experiments were repeated more than 3 times, and the statistical significance was accepted for *p*-values <0.05 through the repeated measures ANOVA test and multiple comparisons analysis.

Results

Induction of NAG-1 and apoptosis in AMC-HN5 cells by various NSAIDs

AMC-HN5 cells were treated with 4 different NSAIDs at a concentration of 100 μM for 48 hr and the induction of NAG-1 and apoptosis was examined using Western blot analysis and flow cytometry. As shown in Figure 1, indomethacin was the most potent inducer of both NAG-1 expression ($p < 0.05$) and apoptosis ($p < 0.05$), and was therefore selected for further research.

Dose- and time-dependent induction of NAG-1 and apoptosis by indomethacin in AMC-HN5 cells

AMC-HN5 cells were treated with 0, 1, 10 or 100 μM indomethacin for 48 hr or with 100 μM indomethacin for 0, 6, 12, 24 or 48 hr. NAG-1 expression was observed after treatment with 10 μM indomethacin and increased in a dose-dependent manner ($p < 0.05$) (Fig. 2*a*). In apoptosis by indomethacin, a significant increase in apoptosis was noted from a dose of 10 μM indomethacin, ($p < 0.05$) (Figs. 2*b* and 2*c*). As for NAG-1 induction by incubation time, NAG-1 expression was observed from 12 hr and increased in a time-dependent manner ($p < 0.05$) (Fig. 3*a*). As for the apoptosis by incubation time, apoptosis was detected after 24 hr and increased with the duration of incubation ($p < 0.05$) (Figs. 3*b* and 3*c*). Thus, 10 μM of indomethacin and 24 hr incubation time were selected for further experiments.

Inhibition of cell proliferation and induction of apoptosis by recombinant NAG-1 in AMC-HN5 cells

NCM, VCM and vector medium were generated as described in Material and methods section. To confirm NAG-1 protein expression in the conditioned medium, we performed Western blot analysis. NAG-1 protein was detected in NCM but as expected, the NAG-1 band was not detected in VCM and the vector medium (Fig. 4*a*).

To determine whether NAG-1 inhibits cell proliferation, 5, 10 or 20 μl of conditioned medium was mixed with 95, 90 or 80 μl of culture medium and cell proliferation was measured. Treatment with 5 μl NCM did not significantly suppress cell proliferation but both 10 and 20 μl of NCM inhibited cell proliferation (Table I). As for the induction of apoptosis by recombinant NAG-1, vector medium and VCM had no significant effect on apoptosis. However, treatment with NCM significantly increased apoptosis ($p < 0.05$)

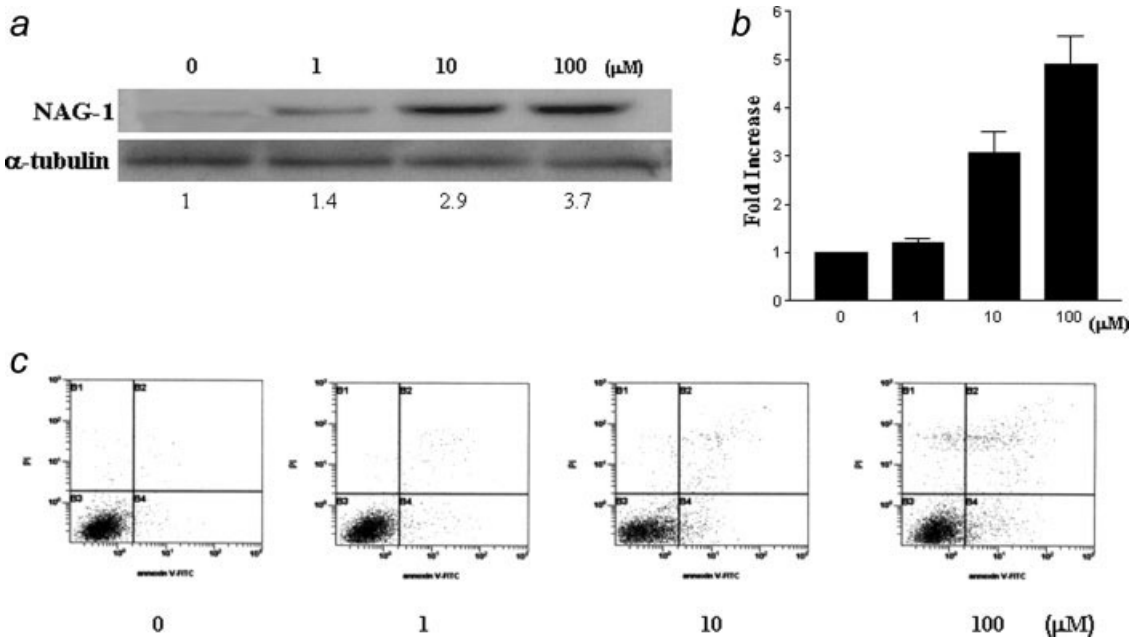

FIGURE 2 – Dose-dependent induction of NAG-1 expression and apoptosis in AMC-HN5 cells. AMC-HN5 cells were treated with various concentrations of indomethacin for 48 hr. Expression of NAG-1 was measured by Western blot analysis and normalized to the level of α-tubulin expression. Apoptosis was analyzed by flow cytometry using Annexin V-FITC and propidium iodide (PI), and represented by the fold increase in the percentage of apoptotic cells compared to the control. (*a*) The induction of NAG-1 expression by 1 μM, 10 μM and 100 μM indomethacin is 1.4 ± 0.1-fold, 2.9 ± 0.2- fold and 3.7 ± 0.4-fold compared to the control, demonstrating a dose-dependent increase in NAG-1 induction. (*b*) Bar graph showing fold increase of apoptosis. The increase in apoptosis induced by 1 μM, 10 μM and 100 μM indomethacin is 1.2 ± 0.1-fold, 3.1 ± 0.4-fold and 4.9 ± 0.4-fold compared to the control, showing a dose-dependent induction of apoptosis. (*c*) Dot plots of DMSO- and indomethacin-treated cells. The *x*-axis represents Annexin V-FITC and the *y*-axis represents PI. The results shown are representative of 3 independent experiments.

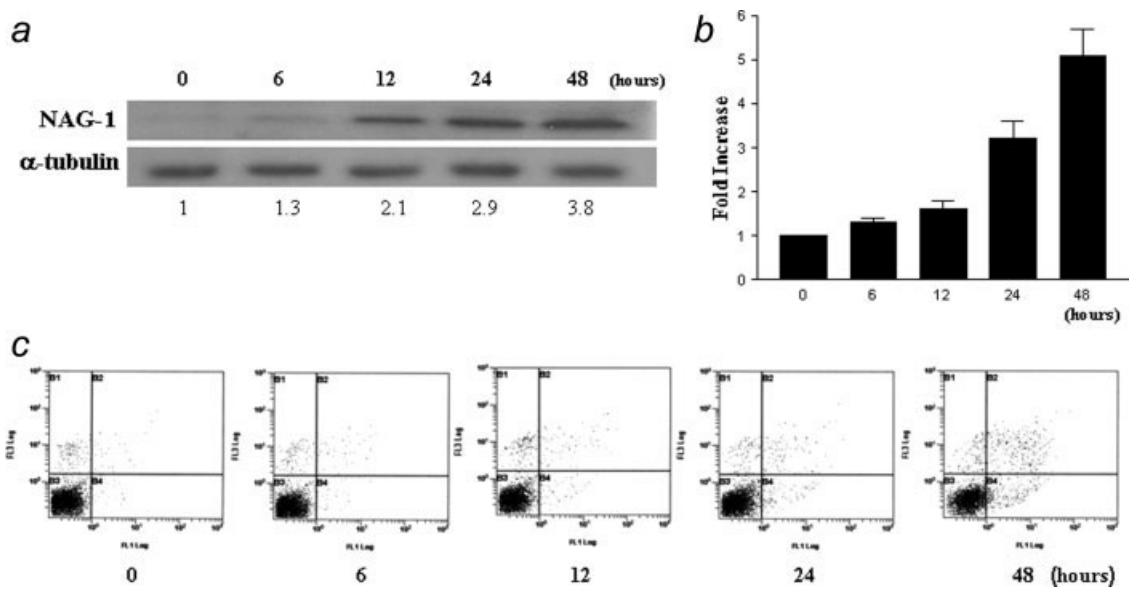

FIGURE 3 – Time-dependent induction of NAG-1 expression and apoptosis in AMC-HN5 cells. AMC-HN5 cells were treated with 100 μM indomethacin at the times indicated. Expression of NAG-1 was measured by Western blot analysis and normalized to the level of α-tubulin expression. Apoptosis was analyzed by flow cytometry using Annexin V-FITC and propidium iodide (PI), and represented by the fold increase in the percentage of apoptotic cells compared to the control. (*a*) The induction of NAG-1 expression by 100 μM indomethacin at 6, 12, 24 and 48 hr is 1.3 ± 0.2-fold, 2.1 ± 0.1-fold 2.9 ± 0.3-fold and 3.8 ± 0.5-fold compared to the control, showing a time-dependent increase in NAG-1 expression. (*b*) Bar graph showing fold increase in apoptosis. The induction of apoptosis at 6, 12, 24, and 48 hr is 1.3 ± 0.1-fold, 1.6 ± 0.2-fold, 3.2 ± 0.4-fold and 5.0 ± 0.5-fold compared to the control, showing a time-dependent increase in apoptosis. (*c*) Dot plots of indomethacin-treated cells. The *x*-axis represents Annexin V-FITC and the *y*-axis represents PI. The results shown are representative of 3 independent experiments.

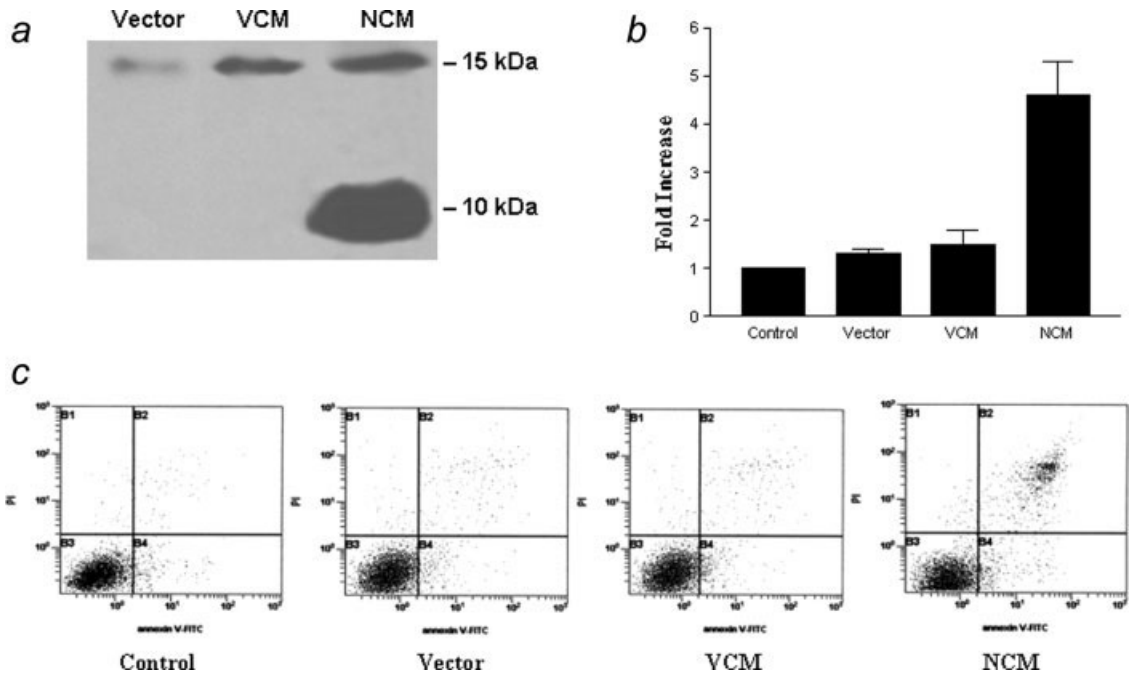

FIGURE 4 – Recombinant NAG-1 induces apoptosis in AMC-HN5 cells. (*a*) Western blot analysis of vector medium, VCM and NCM. Recombinant NAG-1 protein of approximately 10 kDa is detected in 5 μl of concentrated NCM but not in vector medium and VCM. (*b*) Bar graph showing the fold increase in apoptosis. AMC-HN5 cells were treated with DMSO (control), vector medium, VCM or NCM (1:10, conditioned medium:culture medium), and apoptosis was measured by flow cytometry. The apoptotic ratio of treatment with vector medium and VCM is 1.3 ± 0.1 and 1.5 ± 0.3 compared to DMSO-treated cells. In contrast, NCM induces a 4.6 ± 0.7-fold increase in apoptosis. (*c*) Dot plots of AMC-HN5 cells treated with DMSO, vector medium, VCM, and NCM. The results shown are representative of 3 independent experiments.

TABLE I – INHIBITION OF AMC-HN5 CELL PROLIFERATION BY VECTOR MEDIUM, VCM AND NCM

	Inhibition (%)
5 μl	
Vector medium	1.2 ± 0.9[a]
VCM	1.5 ± 1.0
NCM	32.5 ± 4.8
10 μl	
Vector medium	8.7 ± 2.8
VCM	10.2 ± 2.6
NCM	57.4 ± 6.1
20 μl	
Vector medium	21.2 ± 4.7
VCM	28.5 ± 7.2
NCM	73.6 ± 9.2

[a]Mean ± standard deviation.

(Figs. 4*b* and 4*c*). These data support the hypothesis that NAG-1 protein induces apoptosis in AMC-HN5 cells.

Suppression of NAG-1-induced apoptosis by NAG-1 siRNA in AMC-HN5 cells

Even though recombinant NAG-1 induced apoptosis in AMC-HN5 cells, it was necessary to clarify that indomethacin-induced apoptosis is mediated by NAG-1. Thus, we transfected AMC-HN5 cells with NAG-1 siRNA or negative control siRNA and incubated the cells for 24 hr with 10 μM indomethacin and measured apoptosis and NAG-1 expression. The induction of NAG-1 was observed in cells transfected with the negative control siRNA and treated with indomethacin compared to cells transfected with the negative control siRNA and untreated with indomethacin ($p < 0.05$). However, in cells transfected with NAG-1 siRNA, NAG-1 induction was not observed regardless of the indomethacin treatment (Fig. 5*a*). In the negative control siRNA-transfected cells, apoptosis was significantly induced with indomethacin treatment ($p < 0.05$). However, in NAG-1 siRNA-transfected cell, apoptosis was not induced regardless of indomethacin treatment (Figs. 5*b* and 5C). These data further confirm the hypothesis that NAG-1 protein induces apoptosis in AMC-HN5 cells.

Induction of NAG-1 and apoptosis by NSAIDs and recombinant NAG-1 in NHNE cells

To determine the effect of NSAIDs on normal cells, NHNE cells were treated with the 4 NSAIDs at a concentration of 100 μM for 48 hr and the induction of NAG-1 and apoptosis was examined using Western blot analysis and flow cytometry. Contradictory to the result in cancer cells, none of the NSAIDs induced NAG-1 expression (Fig. 6*a*) and apoptosis. (Figs. 6*b* and 6*c*).

NHNE cells were also treated with DMSO (control), vector medium, VCM or NCM at a ratio of 1:10 for conditioned medium:-culture medium for 24 hr to determine whether NAG-1 in conditioned medium could induce apoptosis in normal cells. In contrast to the effect of NCM on AMC-HN5 cells, none of the conditioned media induced apoptosis in NHNE cells (Fig. 7).

In vivo expression of NAG-1 and anti-tumorigenic activity of indomethacin

AMC-HN5 cells were subcutaneously inoculated into *BALB/c-nu* nude mice and the level of NAG-1 in the tumors and the anti-tumorigenic effect of indomethacin were measured. Samples of the tumors were removed and *in situ* NAG-1 expression was measured by RT-PCR. Indomethacin induced an apparent dose-dependent increase in the expression of NAG-1 mRNA in the tumors ($p < 0.05$) (Fig. 8*a*).

All mice survived throughout the experimental period. The weight of mice in the control group was 24.7 ± 1.7 g before the experiment and 27.2 ± 2.2 g on sacrifice, and the weight of mice in the indomethacin-treated group was 24.6 ± 1.5 g before treatment and 27.4 ± 1.4 g on sacrifice; thus, there was no significant difference between the 2 groups. The amount of drinking water

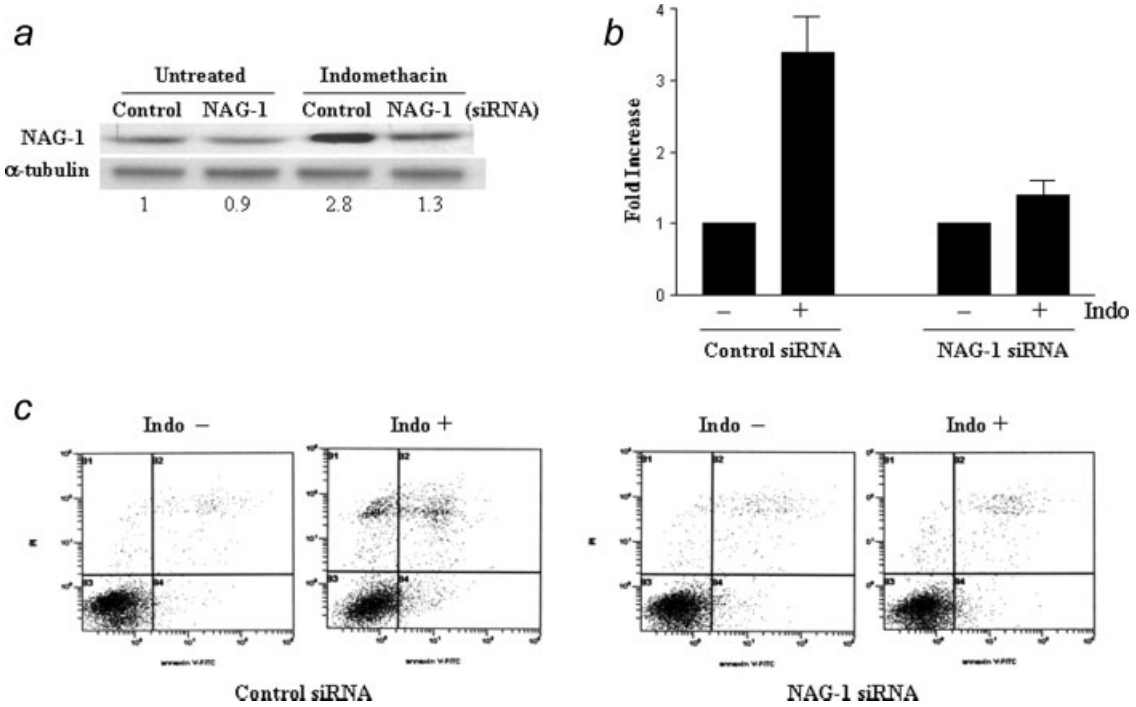

FIGURE 5 – Apoptosis is suppressed by NAG-1 siRNA in AMC-HN5 cells. AMC-HN5 cells were transfected with NAG-1 siRNA or negative control siRNA and incubated for 24 hr with 10 μM indomethacin and measured for apoptosis and NAG-1 expression. Expression of NAG-1 was measured by Western blot analysis and normalized to the level of α-tubulin expression. (*a*) The induction of NAG-1 in the negative control siRNA-transfected indomethacin-treated cells is 2.8 ± 0.4-fold compared to negative control siRNA-transfected indomethcin-untreated cells (Untreated Control). However, in the NAG-1 siRNA-transfected cells without indomethacin treatment or with indomethacin treatment, the induction of NAG-1 is 0.9 ± 0.1-fold and 1.3 ± 0.4-fold, respectively, compared to untreated control. (*b*) The apoptotic ratio of negative control siRNA-transfected indomethacin-treated cells is 3.4 ± 0.5 compared to the siRNA-transfected indomethcin-untreated cells. However, the apoptotic ratio of the NAG-1 siRNA-transfected indomethacin-treated cells is 1.4 ± 0.2 compared to the NAG-1 siRNA-transfected indomethacin-untreated cells. (*c*). Dot plots of AMC-HN5 cells treated with indomethacin in negative control siRNA- and NAG-1 siRNA-transfected cells. The results shown are representative of 3 independent experiments.

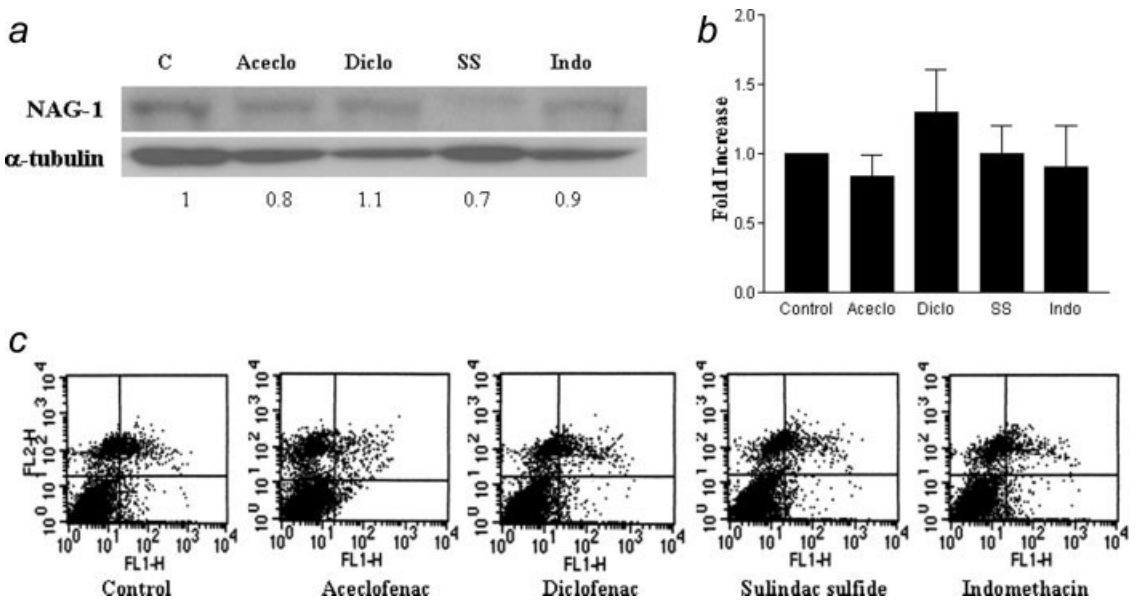

FIGURE 6 – NSAIDs do not induce NAG-1 expression and apoptosis in NHNE cells. NHNE cells were treated with 100 μM of aceclofenac (Aceclo), diclofenac (Diclo), sulindac sulfide (SS) or indomethacin (Indo) for 48 hr. Expression of NAG-1 was measured by Western blot analysis and normalized to the level of α-tubulin expression. Apoptosis was analyzed by flow cytometry using Annexin V-FITC and propidium iodide (PI), and represented by the fold increase in the percentage of apoptotic cells compared to the control. (*a*) The induction of NAG-1 by NSAIDs is as follows: a 1.1 ± 0.4-fold increase for diclofenac, 0.9 ± 0.3-fold increase for indomethacin, 0.8 ± 0.2-fold increase for aceclofenac, and 0.7 ± 0.2-fold increase for sulindac sulfide compared to control. (*b*) The induction of apoptosis by the NSAIDs is as follows: a 1.3 ± 0.3-fold increase for diclofenac, 1.0 ± 0.2-fold increase for sulindac sulfide, 0.9 ± 0.3-fold increase for indomethacin, and 0.8 ± 0.1-fold increase for aceclofenac compared to control. (*c*) Dot plots of DMSO- and NSAIDs-treated NHNE cells. The *x*-axis represents Annexin V-FITC and the *y*-axis represents PI. The results shown are representative of 3 independent experiments.

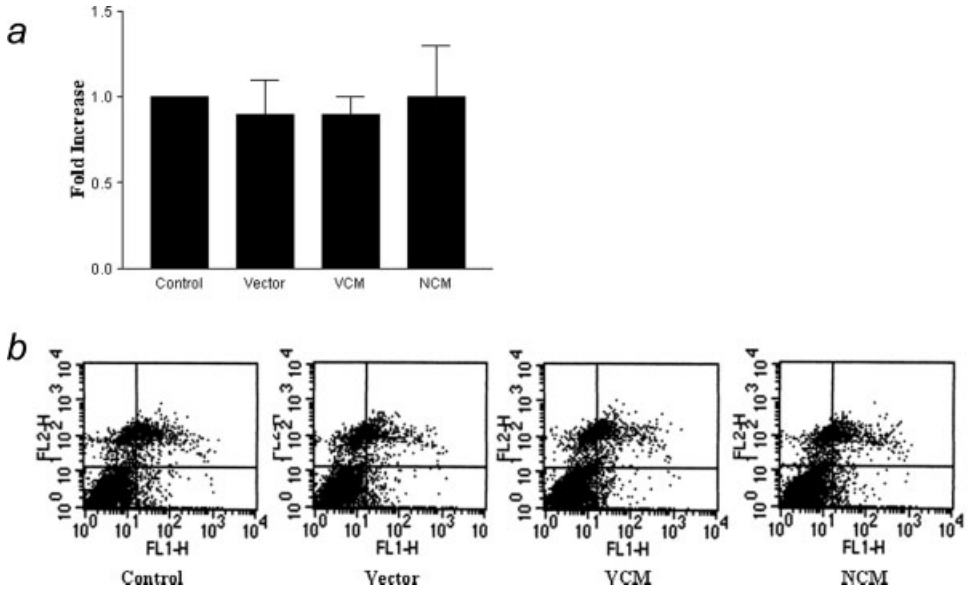

FIGURE 7 – Recombinant NAG-1 does not induce apoptosis in NHNE cells. (*a*) Bar graph showing fold increase of apoptosis. NHNE cells were treated with DMSO (control), vector medium, VCM and NCM (1:10, conditioned medium:culture medium), and apoptosis was measured by flow cytometry. Induction of apoptosis is not observed in cells treated with vector medium, VCM, or NCM. (*b*) Dot plots of DMSO-, vector medium-, VCM- and NCM-treated NHNE cells. The results shown are representative of 3 independent experiments.

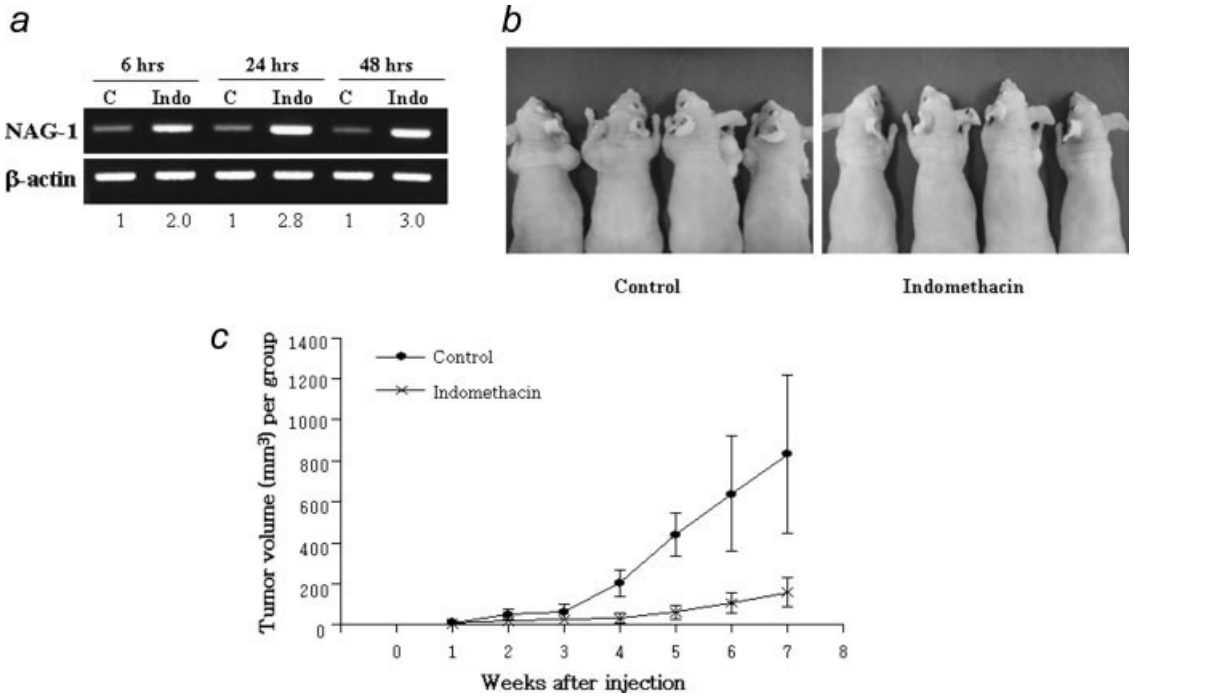

FIGURE 8 – *In situ* expression of NAG-1 and anti-tumorigenic activity of indomethacin in nude mice. AMC-HN5 cells (10^7 cells/100 μl) were inoculated subcutaneously in *BALB/c-nu* nude mice. In control group (Control), mice drank distilled water without indomethacin but in the indomethacin-treated group (Indomethacin), mice drank indomethacin-treated water at a dose of 2 mg/kg/day. Tumors were removed in 6, 24 and 48 hr after indomethacin treatment and RT-PCR was done as described in Material and methods section. Tumors were measured externally on the indicated days in 2D using calipers. Values are the mean ± standard deviation of 20 xenografts. (*a*) NAG-1 expression in implanted AMC-HN5 tumors. The induction of NAG-1 by indomethacin is as follows: a 2.0 ± 0.3-fold increase for 6 hr; 2.8 ± 0.6-fold increase for 24 hr and 3.0 ± 0.7-fold increase for 48 hr, compared to each control. (*b*) Photos showing tumors in the control group and indomethacin-treatd group. In indomethacin-treated group, the volume of tumors is smaller than the control group. (*c*) Graph of the size of tumors. Tumor volume measured weekly from the 1st to the 7th week is 11.5 ± 3.3 mm^3; 52.0 ± 23.2 mm^3; 66.5 ± 35.7 mm^3; 205.4 ± 63.1 mm^3; 440.3 ± 105.2 mm^3; 639.1 ± 280.7 mm^3; and 831.9 ± 384.5 mm^3 in the control group compared to 7.7 ± 4.1 mm^3; 19.5 ± 8.8 mm^3; 26.0 ± 12.1 mm^3; 33.6 ± 22.4 mm^3; 64.1 ± 32.7 mm^3; 108.1± 50.6 mm^3 and 160.7 ± 71.8 mm^3 in the indomethacin-treated group.

consumed was not significantly different between the groups: 4.2 ± 0.2 ml/day/mouse in the control group compared to 4.1 ± 0.1 ml/day/mouse in the indomethacin-treated group. With the indomethacin treatment, the volume of tumors was significantly reduced compared to that of the control ($p < 0.05$) (Figs. 8*b* and 8*c*). Thus, the data suggest that indomethacin increases the expression of NAG-1 in tumors and the induced NAG-1 suppresses the size of tumors.

Discussion

In this study, we demonstrated that NSAIDs induce NAG-1 expression and apoptosis in sinonasal cancer cells and that indomethacin is the most potent NSAID of those tested. Although the induction of apoptosis by NSAIDs has been observed in several cancer cells,[4–8] the specific NSAID that causes maximal induction of NAG-1 expression depends on the type of cancer cell. For example, sulindac sulfide is the most potent NAG-1 inducer in colorectal cancer and diclofenac is the most potent in oral cavity cancer.[3,5] These differences are probably related to the complex regulation of NAG-1 expression, which involves both transcriptional and posttranscriptional mechanisms. Furthermore, its expression seems to involve *cis*- and *trans*-acting promoter elements, various transcription factors and several antitumorigenic substances. NAG-1 is also known to be a downstream target of the diverse p53, ERG-1 and AKT/GSK-3 tumor suppressing pathways.[9,10,12–15,21,22] Thus, the mechanism of NAG-1 regulation depends on cell type and the mode of induction, and further study on the induction of NAG-1 by indomethacin in sinonasal cancer cells is necessary.

Our results indicate that the induction of apoptosis by indomethacin in sinonasal cancer cells is associated with increased expression of NAG-1. Moreover, we show that indomethacin induces NAG-1 expression and apoptosis in a dose- and time-dependent manner and that the induction of NAG-1, which was observed after 12 hr, precedes the induction of apoptosis, which was observed after 24 hr. We postulated that indomethacin induces expression of NAG-1, resulting in apoptosis of AMC-HN5 cells. Although these observations strongly indicate a correlation between NAG-1 and apoptosis, they are insufficient to establish a causal relationship. Therefore, we investigated the ability of NAG-1 conditioned medium to induce apoptosis and investigated the effects of NAG-1 siRNA to inhibit apoptosis. Our results showed that apoptosis was significantly increased by NCM, whereas vector medium and VCM had no effect and apoptosis was significantly suppressed by NAG-1 siRNA. The data support the hypothesis that NAG-1 protein exhibits proapoptotic activity in sinonasal cancer cells.

As the AMC-HN5 cells used here are COX-2-expressing cells (data not shown), the induction of apoptosis by indomethacin could be caused by the inhibition of COX-2 or induction of NAG-1. We believe that the induction of apoptosis in AMC-HN5 cells by indomethacin is caused by NAG-1 for the following reasons: (*i*) Although indomethacin inhibits both COX-1 and -2, it selectively inhibits COX-1[23]; (*ii*) Apoptosis caused by the inhibition of COX-2 is induced at a concentration of 1 μM indomethacin and is induced most potently by diclofenac,[24] whereas our results show that apoptosis was not observed at 1 μM of indomethacin and that indomethacin was more potent than diclofenac; (*iii*) Induction of NAG-1 by NASIDs has been reported to be independent of inhibition of COX-1 and COX-2.[4] Thus, we assume that the induction of apoptosis by indomethacin is not associated with COX-2 inhibition.

Since nontoxicity toward normal cells is a prerequisite of chemopreventive agents, we examined the effect of indomethacin on NHNE cells. Apoptosis was not induced by indomethacin or NCM in NHNE cells under the same conditions in which apoptosis was induced in sinonasal cancer cells. Thus, indomethacin appears to be a safe agent with respect to normal cells. The reason for this dichotomy in the reaction to indomethacin is uncertain. The development of cancer involves changes related to specific genes, epigenetic events and signal transduction in the cells. Theses changes may be connected to the contradictory reaction to indomethacin. Another possible explanation is the homeostasis of normal cells. In human nasal epithelium and NHNE cells, abundant NAG-1 proteins exist.[20] In this abundant state of NAG-1, even indomethacin is applied to NHNE cells, NAG-1 cannot be induced due to homeostasis of cells. However, the mechanism of this contradictory reaction will be researched in a future study.

Indomethacin induced NAG-1 in tumors and also demonstrated *in vivo* antitumorigenic activity in this study. Indomethacin treatment caused a significant decrease in the volume of xenograft tumors of AMC-HN5 cells in indomethacin-treated nude mice compared to controls. Similar antitumorigenic activity of NSAIDs was reported in HCT-116 human colorectal cancer cell lines and MDA-435 breast cancer cell lines.[3,25] Induction of NAG-1 expression and antitumorigenic activity of NSAIDs has also been demonstrated in transgenic mice overexpressing human NAG-1.[26] However, there are also contradictory reports concerning the antitumorigenic activity of NAG-1 since expression of NAG-1 is elevated in the serum of colorectal cancer patients, submucosal tissue of advanced gastric cancer patients and prostate cancer patients.[27–29] It appears that NAG-1 exerts anti-cancer and antitumorigenic activity in the early stages of cancer, but increases tumor invasiveness and decreases survival rates in advanced stages.[21] At present, this contradictory action cannot be explained and further investigation into NAG-1 function is required.

Indomethacin is the NSAID used in the treatment of ankylosing spondylitis, shoulder bursitis, gout, osteoarthritis, tendonitis, and rheumatic arthritis. The maximal dose of oral and rectal administration is 200 mg/day in adults and 2 mg/kg/day in children; therefore, we selected a dose of 2 mg/kg/day for the mice experiments.[30,31] A dose of 2 mg/kg/day indomethacin can be converted into 2.8 μM peak plasma concentration, which is lower than the dose used in the *in vitro* study. Considering that 75 mg/day oral administration of indomethacin to a 70-kg individual is converted into 7.6 μM peak plasma concentration, which is higher than the concentration used in our experiment, indomethacin may be used as a chemopreventive agent in a high-risk group of inverted papilloma patients.[32] With respect to the general toxicity of indomethacin, many adverse reactions, such as gastrointestinal disturbance and neurologic symptoms, are possible.[33] Although mortality and serious complications were not observed in this study, it would be necessary to carefully monitor for possible complications in a future clinical study on high-risk inverted papilloma patients.

This study examined several NSAIDs known at the time to induce NAG-1, but does not include the experimental anti-cancer drug 5F-203 that has recently been shown to be the most potent inducer of NAG-1 in MCF-7 cells. Further studies on 5F-203 and clinical investigations on chemoprevention of high-risk cases of inverted papilloma will be required.

Conclusion

Indomethacin demonstrated proapoptotic and anti-tumorigenic effects in sinonasal cancer cells through the induction of NAG-1 and can be considered a safe and effective chemopreventive agent against sinonasal cancer.

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