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# Differential Regulation of Nonsteroidal Anti-Inflammatory Drug-Activated Gene in Normal Human Tracheobronchial Epithelial and Lung Carcinoma Cells by Retinoids

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## ABSTRACT

In this study, we analyze the effect of several retinoids on the expression of nonsteroidal anti-inflammatory drug-activated gene (*NAG-1*) in normal human tracheobronchial epithelial (HTBE) cells and several lung carcinoma cell lines. The retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (AHPN) greatly enhances the expression of *NAG-1* mRNA and protein in a time- and dose-dependent manner in human lung adenocarcinoma H460 cells and several other carcinoma cell lines. This induction was specific for AHPN because retinoic acid, a retinoic acid receptor-, and a retinoid X receptor pan-agonist were unable to induce *NAG-1*, suggesting that this induction is not mediated through activation of retinoid receptors. Although *NAG*-1 is a p53-responsive gene, AHPN-induced *NAG-1* expression does not require p53. The induction of *NAG-1* expression by AHPN is caused at least in part by an 8-fold increase in the stability of *NAG-1* mRNA. In contrast to carcinoma cells, *NAG-1* expression is effectively induced by retinoic acid and the RAR-selective pan-agonist in normal HTBE cells and accompanies the inhibition of squamous differentiation and the initiation of normal differentiation. In vivo, *NAG-1* expression was observed in the normal tracheobronchial epithelium, whereas no expression was found in either squamous metaplastic tracheal epithelium or in sections of human lung tumors. Our results suggest that the induction of *NAG-1* expression by retinoids in normal HTBE and lung carcinoma cells is regulated by distinct mechanisms and is associated with different biological processes. The linkage between AHPN treatment and *NAG-1* expression revealed in this study provides a new mechanism for the antitumorigenic activity of AHPN.

Retinoids play a critical role in prenatal and postnatal development of the lung and in the maintenance of normal homeostasis in the respiratory epithelium (Jetten et al., 1992; Massaro and Massaro, 1997). In the tracheobronchial epithelium retinoids are essential for the differentiation of tracheobronchial epithelial cells into mucous and ciliated cells (Floyd and Jetten, 1989; Jetten et al., 1992; Marvin et al., 1992; Reddy et al., 1995; Koo et al., 1999). Retinoid signaling pathways are also relevant to lung disease. Retinoic acid has been reported to reverse elastase-induced emphysema in rats (Massaro and Massaro, 1997) and retinoid signaling pathways are defective in human lung carcinoma cells (Adachi et al., 1998; Nervi et al., 1991; Virmani et al., 2000; Sun et al., 2002).

Many of the effects of retinoids are mediated by the retinoic acid receptors (RARs) and retinoid X receptors (RXRs). Recent studies have indicated that the regulation of differentiation in tracheobronchial epithelial cells by retinoids is mediated through these signaling pathways (Nervi et al., 1991; Koo et al., 1999). However, certain actions of retinoids, including the inhibition of cell proliferation and induction of apoptosis in many carcinoma cell lines by 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (AHPN, also named CD437), are independent of retinoid receptors (Shao et al., 1995; Adachi et al., 1998; Rishi et al., 1999; Sakaue et al., 1999; Sun et al., 1999, 2000, 2002; Zhao et al., 2001; Fontana and Rishi, 2002). The precise molecular mechanism(s) by which AHPN induces these changes has yet to be established.

In this study, we examine the effect of several retinoids on the expression of the nonsteroidal anti-inflammatory drug-activated gene-1 (*NAG-1*) (Baek et al., 2001a), a divergent member of the transforming growth factor-$\beta$ superfamily, in HTBE and lung carcinoma cells. NAG-1 is also referred to as

**ABBREVIATIONS:** RAR, retinoic acid receptor; RXR, retinoid X receptor; AHPN, 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene-carboxylic acid, NAG-1, nonsteroidal anti-inflammatory drug-activated gene 1; HTBE, human tracheobronchial epithelial; TTAB, 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-anthracenyl)-benzoic acid; GPDH, glyceraldehyde-3-phosphate dehydrogenase; ERK, extracellular signal-regulated kinase; UTR, untranslated region; TTP, tristetraproline; PD98059, 2′-amino-3′-methoxyflavone.

macrophage inhibitory cytokine-1 (Bootcov et al., 1997), placental transforming growth factor-$\beta$ (Lawton et al., 1997), placental bone morphogenetic protein (Hromas et al., 1997), prostate-derived factor (Paralkar et al., 1998), and growth/differentiation factor-15 (Bottner et al., 1999). NAG-1, like members of the transforming growth factor-$\beta$ family, contains a highly conserved, cysteine-rich domain of 80 residues. NAG-1 is synthesized as a 40-kDa propeptide that dimerizes in the endoplasmic reticulum. The inactive dimeric precursor is cleaved by a furin-like convertase, yielding an active 28-kDa homodimer that is secreted (Bauskin et al., 2000). Although the precise functions of NAG-1 have yet to be determined, several roles for NAG-1 are beginning to emerge. Recent studies suggested a role for NAG-1 in inflammatory responses, apoptosis, and tumorigenesis (Bootcov et al., 1997; Li et al., 2000; Baek et al., 2001b; Albertoni et al., 2002). Overexpression of *NAG-1* in colon carcinoma and glioblastoma cells has been shown to inhibit tumorigenicity in nude mice, indicating that NAG-1 exhibits antitumor activities.

In this study, we demonstrate that AHPN is a very effective inducer of *NAG-1* expression in a number of human carcinoma cell lines. This induction is independent of p53 and is caused at least in part by an increase in the stability of *NAG-1* mRNA. In most cell lines tested, retinoic acid and an RAR- and RXR-pan-agonist were unable to enhance *NAG-1* expression, suggesting that its induction by AHPN is mediated through an RAR/RXR-independent mechanism. In contrast to carcinoma cells, retinoic acid and the RAR pan-agonist were able to induce *NAG-1* expression in normal human tracheobronchial epithelial (HTBE) cells. This induction was associated with inhibition of squamous differentiation and induction of normal differentiation. Histochemical analysis demonstrated that *NAG-1* expression is associated with the normal tracheobronchial epithelium but is absent in squamous metaplasia. Our results suggest that the regulation of *NAG-1* expression by retinoids in normal HTBE and carcinoma cells is mediated by different mechanisms. The lack of *NAG-1* induction in tumor cells by retinoic acid and the RAR pan-agonist may be related to defects in the retinoid signaling pathway. Previous studies demonstrated that AHPN inhibits the tumorigenicity of lung carcinoma cells in mice (Lu et al., 1997) and that NAG-1 can inhibit tumor formation (Baek et al., 2001b; Albertoni et al., 2002). The induction of *NAG-1* by AHPN reported in this study provides a novel mechanism for the antitumor activity of AHPN.

## Materials and Methods

**Materials.** The retinoid AHPN was obtained from Dr. U. Reichert (CIRD Galderma, Valbonne, France). The RAR-selective agonist [SRI-6751-84/TTAB, 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-anthracenyl)-benzoic acid], RXR-selective agonist [SR11217, 4-[1-(5,6,7,8-tetrahydro-5,5,8,8,-tetramethyl-2-naphthalenyl)-2-methyl-propenyl]-benzoic acid], and anti-AP1-selective retinoid [SR11302, (*E*)-3-methyl-9-(2,6,6-trimethylcyclohexenyl)-7-(4-methyl-phenyl)-2,4,6,8-nonatetraenoic acid] (Fanjul et al., 1994) were provided by Dr. M. Dawson (SRI, Menlo Park, CA). *All-trans* retinoic acid was obtained from F. Hoffman-La Roche (Nutley, NJ). Retinoids were dissolved in dimethyl sulfoxide. Control cells received dimethyl sulfoxide only. Human NAG-1 (MIC-1) protein was kindly provided by Dr. S. N. Breit (Centre for Immunology, St. Vincent's Hospital, Sydney, Australia).

**Cell Culture.** Normal HTBE cells were obtained from Cambrex Bio Science Walkersville (San Diego, CA). Cells were grown onto 24-mm permeable Transwell membranes (Costar, Cambridge, MA) in a 1:1 mixture of Dulbecco's modified Eagle's medium and bronchial epithelial cell growth medium (Cambrex Bio Science Walkersville) as described previously (Koo et al., 1999). Human carcinoma cell lines were obtained from Dr. A. Gazdar (University of Texas Southwestern Medical Center, Austin, TX) or from American Type Culture Collection (Manassas, VA). All cell lines were mycoplasm-free. Carcinoma cell lines were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin, and streptomycin. Cells were grown in the absence or presence of retinoids as indicated.

**RNA Isolation and Northern Analysis.** RNA from cultured cells was isolated using Tri-Reagent (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's protocol. Total RNA (30 $\mu$g) was electrophoresed through a formaldehyde 1.2% agarose gel as described previously (Sakaue et al., 1999), blotted to a Nytran Plus membrane (Schleicher & Schuell, Keene, NH), and UV cross-linked. Membranes were hybridized with radiolabeled probes for *NAG-1*, transglutaminase type I, and chicken *glyceraldehyde-3-phosphate dehydrogenase* gene (*GPDH*) as reported previously (Sakaue et al., 1999). A 602-base pair *Eco*RI/*Not*I cDNA fragment encoding human *NAG-1* was excised from IMAGE clone 1713523 (Research Genetics, Huntsville, AL) and used as a probe for *NAG-1*. Hybridizations were performed for 1 to 2 h at 68°C using QuikHyb reagent (Stratagene, La Jolla, CA), blots were washed twice with 2× standard saline citrate, 0.05% SDS for 15 min at room temperature, and in the final wash with 0.5× standard saline citrate, 0.1% SDS for 30 min at 65°C. Autoradiography was carried out with Hyperfilm-MP (Amersham Biosciences, Piscataway, NJ) at −70°C using double intensifying screens. MUC2 mRNA was analyzed by reverse transcription-polymerase chain reaction as described previously (Koo et al., 1999).

**Western Blot Analysis.** Cells were treated with retinoids in serum-free medium. Cells were collected in sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 10 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, aprotinin, and leupeptin) and phosphatase inhibitor mixture I and II (Sigma-Aldrich). Medium was collected separately, concentrated with Centricon Y-10 microconcentrators (Millipore Corporation, Bedford, MA), and mixed with 2× sample buffer. Proteins were examined by Western blot analyses using a specific antiserum against human NAG-1 (Baek et al., 2001b). Peroxidase-conjugated anti-rabbit IgG (AP156P GtXRbt) (1:20,000 dilution with 5% milk), purchased from Chemicon International (Temecula, CA), was used as secondary antibody. Antibodies were diluted in phosphate-buffered saline containing 1 or 5% milk powder and 0.05% Tween 20. Detection was carried out using Super Signal chemiluminescent substrate; luminol and peroxide purchased from Pierce Chemical (Rockford, IL).

**Immunohistochemical Staining.** Tissues were fixed in 4% formalin at 4°C for 16 h and then embedded in paraffin. Paraffin sections (5 $\mu$m) were deparaffinized in xylene and rehydrated through a graded series of ethanol solutions. The sections were incubated with blocking solution (5% milk powder, 1% bovine serum albumin in phosphate-buffered saline) for 60 min at room temperature, followed by a 60-min incubation with a 1000-fold dilution of anti-NAG-1 antiserum or preimmune serum in blocking solution. Subsequently, tissue sections were incubated for 60 min with biotinylated goat-anti-rabbit IgG (Jackson Laboratories, Bar Harbor, Maine) and then with streptavidin-horseradish peroxidase (Jackson Laboratories). Immunoreactivity was visualized using diaminobenzidine. The sections were counterstained with 1% methyl green.

## Results

**Induction of NAG-1 Expression by AHPN in Lung Adenocarcinoma H460 Cells.** Microarray analysis has identified a number of genes that are differentially regulated in lung carcinoma H460 cells during treatment with AHPN

(Sakaue et al., 1999, 2001). In this study, we characterize the expression of one of these genes, *NAG-1*. As shown in Fig. 1A, AHPN treatment of H460 cells enhanced NAG-1 mRNA expression about 16-fold. In contrast, the RAR pan-agonist TTAB, the RXR agonist SR11217, or SR11302, a retinoid with reported selective anti-activator protein 1 activity (Fanjul et al., 1994), was unable to increase NAG-1 mRNA expression in these cells. Retinoic acid also did not induce *NAG-1* in H460 cells (data not shown). These results demonstrate that induction of *NAG-1* is restricted to AHPN and suggest that regulation of *NAG-1* expression in H460 by AHPN is independent of the activation of RAR or RXR receptor signaling pathways.

**Induction of NAG-1 by AHPN in Other Carcinoma Cell Lines.** The induction of *NAG-1* by AHPN was examined in a number of other lung carcinoma cell lines. AHPN was able to induce *NAG-1* mRNA in adenocarcinoma A549 and H1355, and squamous carcinoma 226 cells (Fig. 1B). A small increase was observed in lung adenocarcinoma H441 and small cell carcinoma H69 cells, whereas levels did not change in adenocarcinoma Calu-6 cells. Analysis of *NAG-1* expression in several other carcinoma cell types showed induction of *NAG-1* mRNA in human mammary carcinoma MCF-7, T47D, BT549, ZR-75-1, and in prostate carcinoma LNCaP and PC3 cells. A small increase was observed in sarcoma HT1080-p53wt and HT1080-p53mt cells. The basal level of expression of *NAG-1* was significantly higher in HT1080-p53wt containing the wild-type p53 compared with HT1080-p53mt containing a mutated *p53* gene.

As demonstrated for H460 cells, the RAR pan-agonist TTAB was unable to induce *NAG-1* in lung carcinoma H441, A549, A1355, and Calu-6, and mammary carcinoma MDA-MB321, BT20, T-47D, and BT549 cells (Fig. 1C). However, TTAB was able to enhance *NAG-1* expression significantly in MCF-7 and caused a weak increase in ZR-75-1 cells. These results further support the conclusion that AHPN and TTAB regulate *NAG-1* expression by two different mechanisms.

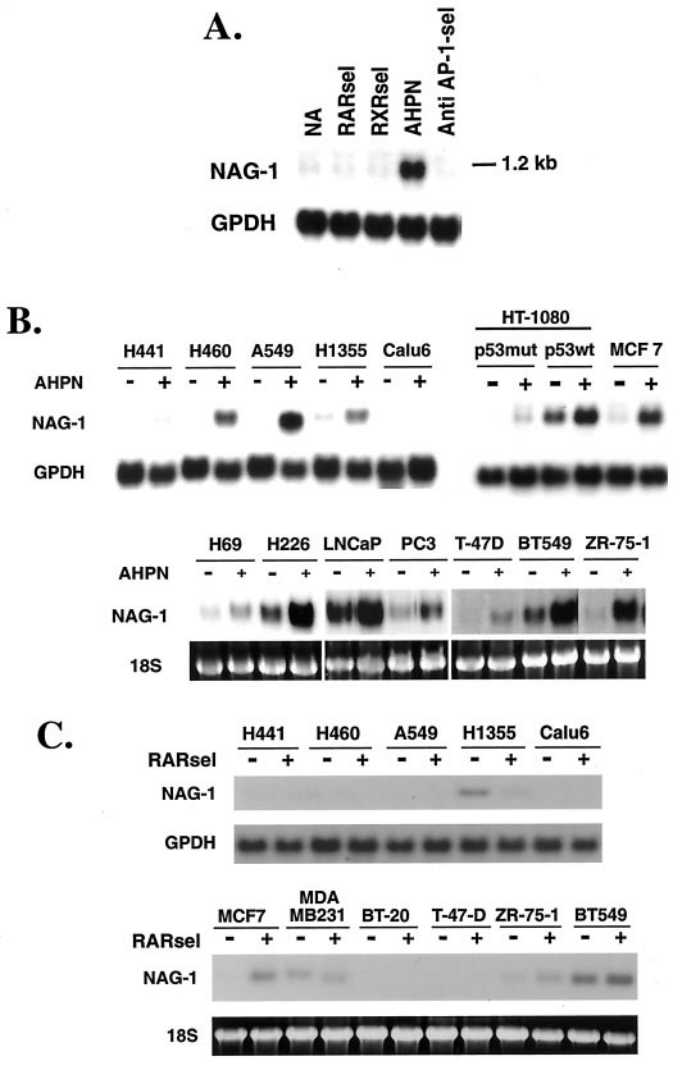


**Fig. 1.** AHPN is an effective inducer of *NAG-1* mRNA expression in several carcinoma cell lines. A, comparison of the effects of the RAR-selective pan-agonist TTAB (RARsel), the RXR-selective pan-agonist SR11217 (RXRsel), AHPN, and the reported anti-AP-1-selective retinoid SR11302 (anti-activator protein 1 sel) on *NAG-1* mRNA expression in human lung adenocarcinoma H460 cells. B and C, effects of AHPN (2.5 $\mu$M) and RARsel (1 $\mu$M) on *NAG-1* mRNA expression in several carcinoma cell lines. Carcinoma cells were treated with the indicated retinoid. After 16 h cells were collected and RNA isolated. Total RNA (20 $\mu$g) was examined by Northern blot analysis using radiolabeled probes for *NAG-1* and *GPDH*.

**Time- and Dose-Dependent Induction of NAG-1.** As shown in Fig. 2, AHPN induces *NAG-1* mRNA expression in adenocarcinoma H460 and A549 cells in a time- and dose-dependent manner. In both cell lines, an increase in *NAG-1* mRNA levels could be observed as early as 4 h after the addition of 2.5 $\mu$M AHPN. In H460, optimal induction of *NAG-1* mRNA was reached 16 h after addition of AHPN, whereas a concentration as low as 0.3 $\mu$M AHPN was able to induce *NAG-1* mRNA.

**Induction of NAG-1 Protein by AHPN.** NAG-1 has been reported to be synthesized as a homodimeric precursor of about 80 kDa that is processed by proteolysis and secreted as a homodimer consisting of two ~14-kDa peptides. Western blot analysis of total cellular protein from AHPN-treated H460 cells demonstrated that the level of ~40-kDa precursor

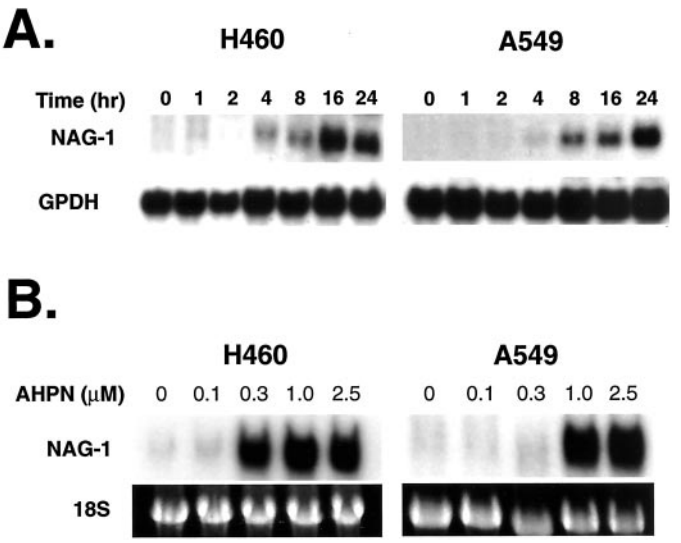


**Fig. 2.** A and B, time course and concentration dependence of the induction of *NAG-1* mRNA in lung adenocarcinoma H460 and A549 cells by AHPN. Cells were treated with 2.5 $\mu$M AHPN at the times indicated or for 24 h at the concentrations indicated. Total RNA (30 $\mu$g) was isolated and examined by Northern blot analysis using radiolabeled probes for *NAG-1* and *GPDH*.

protein increased in a time-dependent manner (Fig. 3A). A 24-fold induction in NAG-1 protein was observed 16 h after the addition of AHPN. Analysis of NAG-1 released into the medium showed a similar accumulation of the 14-kDa processed NAG-1 protein. This induction of NAG-1 protein was only observed with AHPN and not with the RAR- or RXR pan-agonists (Fig. 3B).

Because both AHPN and NAG-1 have been reported to promote apoptosis in certain cell lines, cells were treated with NAG-1 protein to determine whether the induction of apoptosis was caused by the synthesis and release of NAG-1 protein. However, treatment of H460 and A549 cells with NAG-1 did not affect proliferation or induce apoptosis and did not synergize with or inhibit AHPN-induced apoptosis (data not shown).

**Regulation of NAG-1 at the Level of RNA Stability.** AHPN has been reported to regulate gene expression by transcriptional and post-transcriptional mechanisms (Rishi et al., 1999; Sun et al., 2000; Sakaue et al., 2001). The 3.5-kilobase 5′-promoter flanking region of the *NAG-1* gene has been reported to contain several DNA elements involved in its regulation (Baek et al., 2001a; Wong et al., 2002). Using a reporter under the control of this regulatory region, we examined the effect of AHPN on transcriptional activation. AHPN had little effect on this transcriptional activation (data not shown), suggesting that it does not control the transcription of this gene through elements contained in this 3.5-kilobase region.

To determine whether *NAG-1* was regulated by AHPN at a post-transcriptional level, we examined its effect on *NAG-1* mRNA stability. H460 cells were treated with AHPN or vehicle for 8 h; transcription was then inhibited by the addition of actinomycin D and at different time intervals the level of *NAG-1* RNA determined by Northern blot analysis. Figure 4, A and B, demonstrates that the half-life of *NAG-1* mRNA in control H460 cells was 27 min compared with 3.5 h in AHPN-treated cells. These results suggest that this increase in stability of *NAG-1* mRNA is a major factor in the induction of *NAG-1* expression by AHPN.

The induction of *NAG-1* mRNA was found to be sensitive to cycloheximide because no increase in *NAG-1* mRNA was observed after AHPN treatment in the presence of cycloheximide (Fig. 4C), suggesting that the effect of AHPN on *NAG-1* is dependent on de novo protein synthesis. Cycloheximide may block the synthesis of a protein that stabilizes *NAG-1* mRNA.

The induction of several genes, including *Egr-1* and *Nur77*,

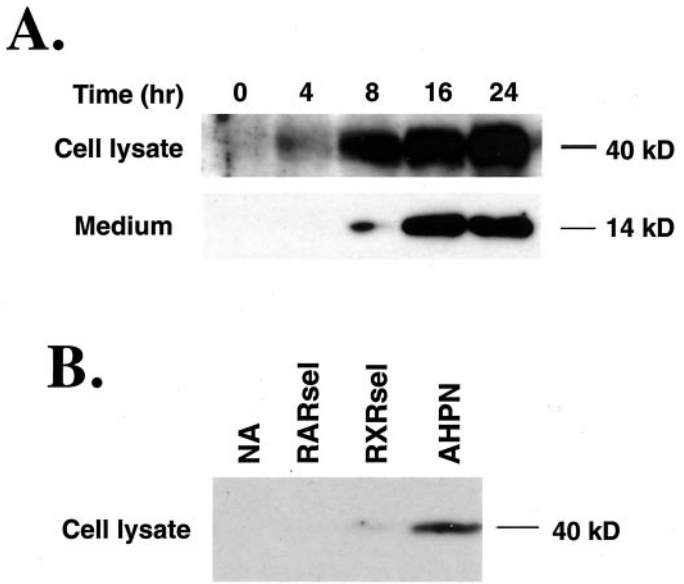


**Fig. 3.** AHPN causes an increase in cellular NAG-1 precursor protein and active NAG-1 in the medium. A, H460 cells were treated with AHPN and at the indicated times cellular proteins and proteins secreted into the medium were examined by Western blot analysis using a NAG-1-specific antibody. In cellular extracts the antibody recognized the ~40-kDa NAG-1 precursor and in medium the processed ~14-kDa NAG-1 protein. B, cellular protein from H460 cells treated with the RAR- and RXR-pan-agonist, and AHPN (1 $\mu$M for 24 h) was examined by Western analysis for the presence of NAG-1.

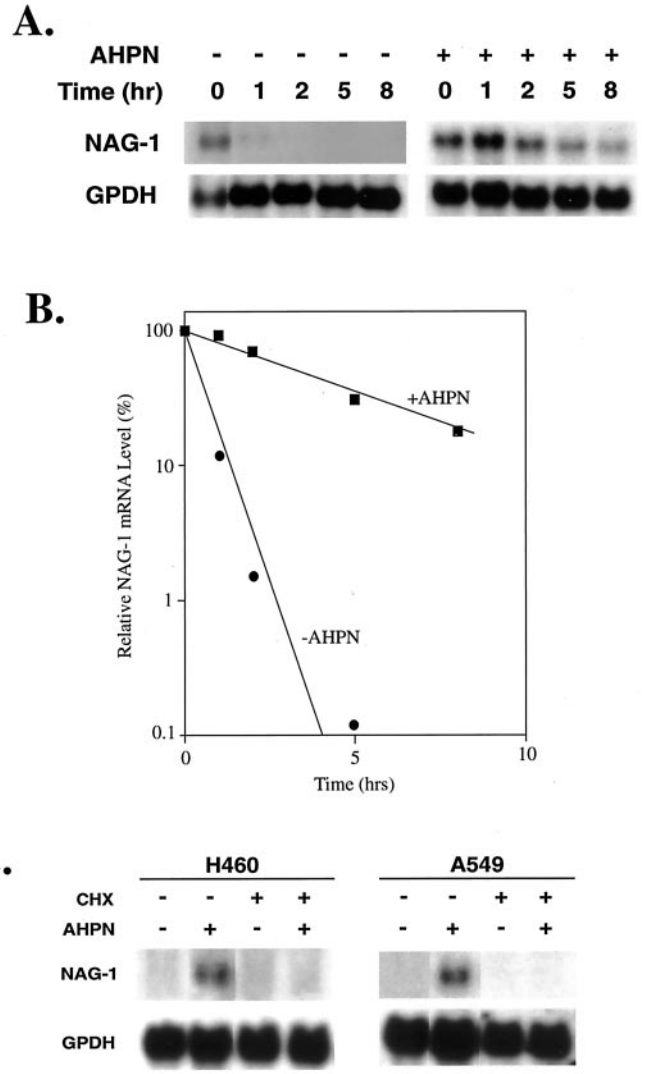


**Fig. 4.** Effect of AHPN on the stability of *NAG-1* mRNA. A, H460 cells were treated with AHPN for 8 h and subsequently with actinomycin D (5 $\mu$g/ml). At the indicated times, RNA was isolated and examined by Northern blot analysis with a radiolabeled probe for *NAG-1* and *GPDH*. Poly(A)$^+$ RNA was used in the case of untreated cells (●) and total RNA for AHPN-treated cells (■). B, hybridization signals were quantitated with a PhosphorImage analyzer using ImageQuant software as described under *Materials and Methods*. The relative level of *NAG-1* RNA (relative to the level of *GPDH*) was calculated, and the results plotted as the percentage of the RNA level present at time 0 of actinomycin D addition. C, cycloheximide inhibits the induction of *NAG-1* mRNA by AHPN. Cycloheximide (5 $\mu$g/ml; CHX) or vehicle was added 30 min before H460 or A549 cells were treated with AHPN (2.5 $\mu$M). After 8 h, RNA was isolated and examined by Northern blot analysis for *NAG-1* mRNA expression.

by AHPN is blocked by the mitogen-activated protein kinase/extracellular signal-regulated kinase 1 (MEK1) inhibitor PD98059 (Sakaue et al., 2001); however, inhibition of the MEK1/ERK1/2 signaling pathway had little effect on AHPN-mediated induction of *NAG-1* (data not shown), suggesting that this induction is regulated by an ERK1/2-independent mechanism.

**Induction of NAG-1 Expression by Retinoids in Normal HTBE Cells.** Next, the effect of several retinoids on *NAG-1* expression was examined in normal HTBE cells. In contrast to human lung carcinoma H460 cells, the RAR pan-agonist TTAB, retinoic acid, and AHPN all induced *NAG-1* mRNA expression in normal HTBE cells (Fig. 5A). TTAB was more effective than RA and AHPN in inducing *NAG-1*, whereas the RXR-selective retinoid SR11217 had little effect on *NAG-1* expression. In contrast to H460 cells, AHPN did not induce apoptosis in HTBE (data not shown) in agreement with previous studies (Sun et al., 2002). Clearly, the specificity by which retinoids induce *NAG-1* expression in normal HTBE cells differed substantially from that of H460 cells, suggesting that in these two cell types *NAG-1* expression is regulated by two different mechanisms. The induction of *NAG-1* mRNA by TTAB occurred in a dose-dependent manner (Fig. 5B). TTAB was able to induce *NAG-1* at concentrations as low as 1 nM. The enhancement in *NAG-1* mRNA was accompanied by an increase in *NAG-1* proteins levels (Fig. 5C).

**NAG-1 Expression Is Associated with Normal Differentiation of HTBE Cells.** Previous studies have shown that retinoids are essential for the normal differentiation of HTBE cells. In the absence of retinoids, HTBE cells in vivo as well in vitro undergo squamous differentiation, whereas in the presence of retinoids cells differentiate into mucosecretory and ciliated cells. (Koo et al., 1999). As shown in Fig. 6A, HTBE cells grown to confluence in the absence of retinoic acid expressed little *NAG-1* mRNA. Subsequent treatment with retinoic acid caused a rapid increase in the level of *NAG-1* mRNA expression. An increase in *NAG-1* mRNA could be observed as early as 12 h after the addition of

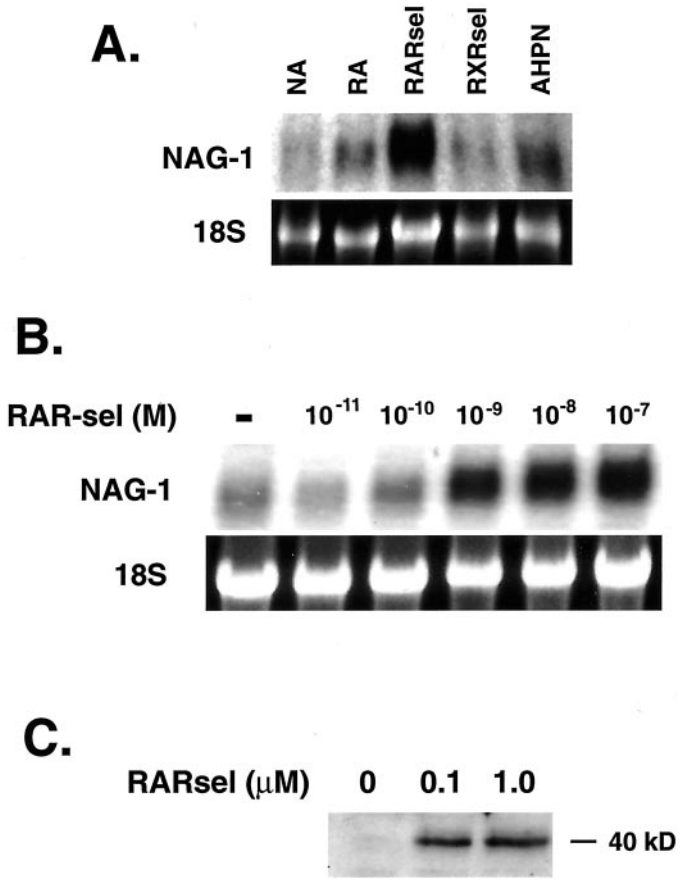


**Fig. 5.** A, induction of *NAG-1* mRNA expression by retinoid receptor-selective retinoids. Normal HTBE cells were cultured as described under *Materials and Methods*. At day 7, cells were treated with either retinoic acid, the RAR-selective pan-agonist TTAB (100 nM; RARsel), the RXR-selective pan-agonist SR11217 (1 $\mu$M; RXRsel), or AHPN (1 $\mu$M). RNA was examined by Northern blot analysis using $^{32}$P-radiolabeled probes for *NAG-1*. B, dose dependence of *NAG-1* mRNA induction by the RAR-pan-agonist TTAB in normal HTBE cells. HTBE cells were grown in Transwell dishes in the presence of the indicated concentration of TTAB. Total RNA was isolated and examined by Northern analysis using $^{32}$P-radiolabeled probes for *NAG-1* and *GPDH*. C, induction of NAG-1 protein by the RAR-pan-agonist TTAB in normal HTBE cells.

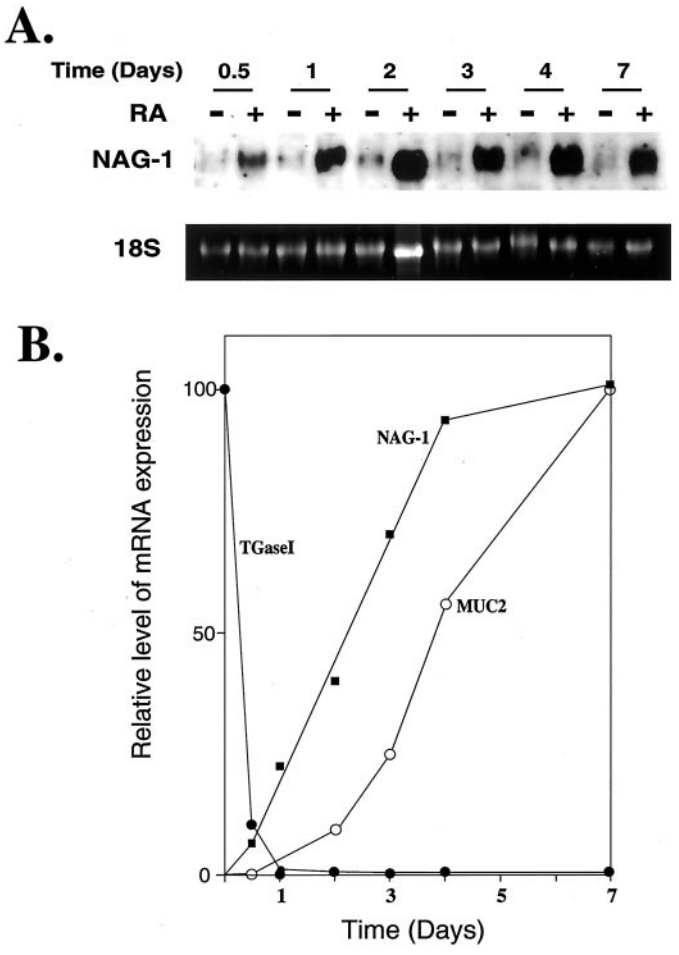


**Fig. 6.** A, induction of *NAG-1* mRNA expression in normal HTBE cells by retinoic acid. Cells were grown in Transwell dishes in the absence of retinoids for 7 days and then in the presence or absence of 1 $\mu$M RA. At different time intervals cells were collected and RNA isolated. Total RNA (10 $\mu$g) was examined by Northern blot analysis using $^{32}$P-radiolabeled probe for *NAG-1*. B, expression of *NAG-1* in relation to squamous and mucous cell differentiation. The relative level of *NAG-1*, *transglutaminase type I* (*TGase I*), and *MUC2* mRNA expression was calculated and plotted as function of time. *NAG-1* and *TGase I* mRNA expression were determined by Northern blot analysis, whereas *MUC2* mRNA expression was analyzed in the same samples by reverse transcription-polymerase chain reaction as described under *Materials and Methods*. The mRNA levels (relative to that of 18S rRNA) were determined by densitometry.

retinoic acid and accompanied the suppression of *transglutaminase I* mRNA expression, a squamous cell-specific marker (Fig. 6B). The induction of *NAG-1* mRNA preceded the increase in the expression of the mucin gene *MUC2*, which was first observed at 24 h of retinoic acid treatment in agreement with a previous report (Koo et al., 1999). These results suggest an association of *NAG-1* expression with the induction of the normal pathway of differentiation in HTBE cells.

To determine whether *NAG-1* had any effect on differentiation, HTBE cells were treated with NAG-1 in the presence or absence of retinoic acid. No significant differences were observed between treated and untreated cells with regard to the expression of *transglutaminase I* and *MUC-2* (data not shown).

**In Vivo Localization of NAG-1.** To determine the expression of *NAG-1* in the normal tracheobronchial epithelium, sections of normal human trachea and several tumor tissues from adeno-, small cell, large cell, and squamous cell carcinomas were stained with an anti-NAG-1 antiserum. As shown in Fig. 7, the columnar cells in normal tracheobronchial epithelium stained positively for NAG-1 protein, whereas no staining was observed in basal cells or in any of the tumor sections analyzed regardless of the histological subtype of the tumor (Fig. 7, A and B; data not shown). In the normal epithelium, staining was most pronounced in ciliated cells. A previous study (Sueoka et al., 2000) showed that when small sections of human trachea are cultured in the presence of retinoic acid the normal architecture of the tracheobronchial epithelium is maintained; however, when sections are cultured in the absence of retinoic acid the epithelium becomes squamous metaplastic. As shown in Fig. 7C, the epithelium of sections cultured in the presence of retinoic acid stained positively for NAG-1, whereas sections cultured in the absence of retinoic acid stained negatively for NAG-1 (Fig. 7D). These results demonstrate that NAG-1 expression is associated with the normal tracheobronchial epithelium and is not expressed in basal cells or in squamous differentiated cells. These observations are in agreement with the findings shown in Fig. 6.

## Discussion

In this study, we demonstrate that treatment of human lung carcinoma and normal HTBE cells with retinoids dramatically induces the expression of *NAG-1*. The induction of *NAG-1* in carcinoma and normal cells is dependent on the type of retinoid used and seems to be regulated by distinct mechanisms and associated with different biological processes.

Previous studies have shown that the synthetic retinoid AHPN inhibits cell proliferation and is an effective inducer of apoptosis in several human lung carcinoma and other carcinoma cell lines (Shao et al., 1995; Adachi et al., 1998; Rishi et al., 1999; Zhao et al., 2001; Sun et al., 2002). Although AHPN can bind selectively to the RARγ receptor and weakly activate transcription through this receptor, many of its responses associated with inhibition of cellular proliferation and induction of apoptosis have been found not to involve RAR or RXR nuclear receptor-mediated signaling pathways. In this study, we demonstrate that AHPN is an effective inducer of *NAG-1* expression in lung carcinoma H460 and A549 cells and several other carcinoma cell lines. The time course and concentration dependence of this induction are very similar to those reported for AHPN-induced apoptosis and induction of other genes, including *GADD45* and *MYD118* (Adachi et al., 1998; Sakaue et al., 1999). The induction of *NAG-1* is rather specific for AHPN because in most cell lines retinoic acid or the RAR- or RXR-pan-agonist were unable to induce *NAG-1* mRNA. These results suggest that AHPN regulates *NAG-1* expression by a mechanism that is

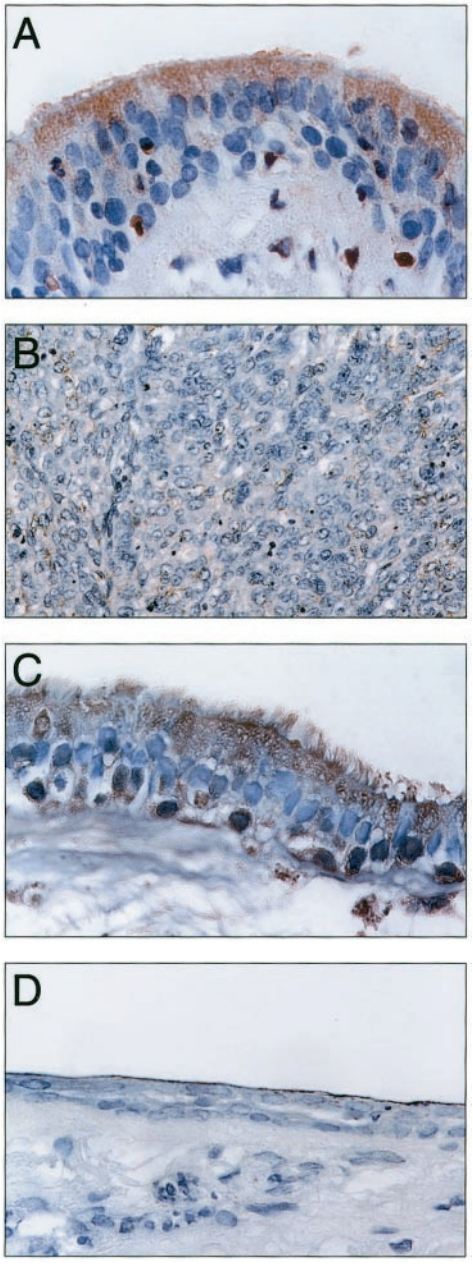


**Fig. 7.** Immunohistochemical localization of NAG-1 protein in normal human bronchus and human lung carcinoma. Sections of human bronchus and lung carcinoma were examined by immunohistochemical analysis as described under *Materials and Methods* using a specific anti-NAG-1 antiserum. Preimmune serum was used in controls and did not show any staining (data not shown). A, normal human bronchus. B, human lung adenocarcinoma. C, human bronchus cultured in the presence of retinoic acid; normal pseudostratified epithelium is maintained. D, human bronchus cultured in the absence of retinoic acid; epithelium becomes squamous metaplastic.

independent of retinoid receptors. Many retinoids, including the RXR pan-agonist SR11217 and SR11302, have been reported to exhibit anti-AP-1 activity (Fanjul et al., 1994). Because these retinoids do not affect *NAG-1* expression in H460 cells, the induction of *NAG-1* seems not to involve the anti-AP-1 activity of retinoids (Fig. 1A).

AHPN has been reported to regulate gene expression by transcriptional as well as post-transcriptional mechanisms (Rishi et al., 1999; Sakaue et al., 1999, 2001). In H460 cells, AHPN causes an 8-fold increase in the half-life of *NAG-1* mRNA, from 27 min in control cells to ~3.5 h in treated cells. These results suggest that the increase in *NAG-1* mRNA expression by AHPN may be largely regulated by a post-transcriptional mechanism and be caused by increased stability of *NAG-1* mRNA. Stability of mRNAs can be mediated by different mechanisms. The control of mRNA stability can involve adenylate/uridylate-rich instability elements in the 3′-UTR or specific RNA stemloop motifs (Chen and Shyu, 1995; Ross, 1996). Specific mRNA binding proteins interacting with such motifs may protect *NAG-1* RNA from degradation by endo- and exonucleases, thereby increasing RNA stability. Inversely, increased stability might be caused by a reduction in the level of proteins that destabilize *NAG-1* mRNA. Recently, tristetraproline (TTP) has been reported to interact with specific AU-rich motifs and target certain mRNAs such as *tumor necrosis factor-α* mRNA for enhanced degradation (Lai et al., 2000). Interestingly, the 3′-UTR of human *NAG-1* mRNA contains three AU-rich elements that may play a role in determining the stability of *NAG-1* mRNA (Yokoyama-Kobayashi et al., 1997). However, the induction of *NAG-1* seems to be unrelated to TTP because *TTP* expression was enhanced severalfold rather than decreased in AHPN-treated H460 cells (Sakaue et al., 2001). Therefore, the stabilization of *NAG-1* mRNA may involve interaction with other proteins. Our observation that cycloheximide suppresses the induction of *NAG-1* mRNA by AHPN would be in agreement with the hypothesis that it inhibits the synthesis of a protein stabilizing *NAG-1* RNA rather than of an RNA destabilizing protein. In addition to *NAG-1* mRNA, AHPN has been reported to enhance the stability of several other RNAs, including *GADD45* and *MYD118* mRNA (Rishi et al., 1999; Sakaue et al., 1999). In the case of *GADD45*, a 45-base pair region in the 5′-UTR was found to be involved in the increased stability caused by AHPN (Rishi et al., 1999).

*NAG-1* expression has been reported to be regulated by p53-dependent and -independent mechanisms. Recent studies have identified two p53 binding sites in the *NAG-1* promoter regulatory region and one p53-repressor element that suppresses p53-mediated transactivation (Wong et al., 2002). The induction of *NAG-1* in colon and mammary carcinoma cells by resveratrol (Baek et al., 2002) and irradiation (Li et al., 2000), respectively, is regulated through a p53-dependent mechanism. However, nonsteroidal anti-inflammatory agents have been shown to enhance *NAG-1* by a cyclooxygenase-1/2- and p53-independent mechanism (Baek et al., 2001a,b). The induction of *NAG-1* in human glioblastoma by anoxia occurs also independently of p53 (Albertoni et al., 2002). Although AHPN induces p53 in H460 and other carcinoma cell lines, the induction of *NAG-1* by AHPN does not require p53 induction because AHPN induces *NAG-1* in cell lines, including H1355 and BT549, which contain mutant *p53*. Previously, we have shown that several other genes, including *GADD45*, *MyD118*, and *Egr-1*, are induced by AHPN independently of the p53 status (Sakaue et al., 1999, 2001). However, we cannot rule out that in certain cell lines p53 may act synergistically with the p53-independent mechanism (Sun et al., 1999). The latter is supported by the higher levels of *NAG-1* expression observed in HT1080-p53wt compared with HT1080-p53mt cells (Fig. 1B).

In contrast to lung carcinoma H460 cells, both retinoic acid and the RAR pan-agonist are able to induce effectively (10–30-fold) the expression of *NAG-1* mRNA in normal HTBE cells. AHPN treatment also caused a small increase in *NAG-1* expression but in contrast to carcinoma cells AHPN does not induce apoptosis in normal HTBE cells in agreement with previous studies (Sun et al., 2002). HTBE cells grown in the absence of retinoids undergo squamous differentiation, as characterized by the expression of the squamous cell markers *transglutaminase type I* and *cornifin*, whereas treatment with retinoic acid, TTAB, or AHPN induces mucociliated cell differentiation as indicated by the enhanced expression of several genes, including *RARβ*, *CYP26*, *MUC2*, and *MUC5AC* (Koo et al., 1999; Kim et al., 2000). Our results show that the increase in *NAG-1* mRNA expression in HTBE accompanies the inhibition of the squamous phenotype and the induction of the mucociliated cell differentiation. In HTBE cells, *NAG-1* expression is therefore associated with the induction of normal differentiation. This conclusion is supported by immunohistochemical analysis of sections from normal and squamous metaplastic tracheobronchial epithelium. In the normal tracheobronchial epithelium NAG-1 seems to be most highly expressed in the ciliated cells. The precise role(s) of NAG-1 in the normal tracheobronchial epithelium is not yet fully understood. Clearly, in normal cells NAG-1 is not associated with apoptosis. Preliminary studies did not reveal any influence of NAG-1 on the expression of several differentiation markers in HTBE cells. The rapid induction seen in HTBE after the addition of retinoic acid occurs at a time when cell cultures undergo a lot of remodeling, suggesting that NAG-1 may play a role in this process. Another study has proposed a similar role for NAG-1 during regeneration in mouse liver (Hsiao et al., 2000). The mechanism by which NAG-1 transduces its signal is still poorly understood but probably involves interaction with cell surface receptors. Future identification of such receptors will help to determine which cells are targets for NAG-1 action.

Although the precise biological functions of NAG-1 are not yet well understood, roles in inflammation, embryonic development, and tumorigenesis have been suggested. *NAG-1* is highly expressed in activated macrophages and may play a role in late anti-inflammatory responses (Bootcov et al., 1997). Overexpression of *NAG-1* has been reported to inhibit cell proliferation and induce apoptosis in mammary carcinoma MDA-MB-468 and colon carcinoma HCT-116 cells (Li et al., 2000; Baek et al., 2001b), whereas other studies showed little effect of NAG-1 on proliferation of glioblastoma cells (Albertoni et al., 2002). Addition of human NAG-1 to human lung carcinoma H460 and A549 cells did not affect proliferation or apoptosis (data not shown), suggesting that treatment with NAG-1 alone may not be sufficient to induce apoptosis in these cells. AHPN increases the expression of many growth-suppressor and apoptosis-promoting genes (Li et al., 1996; Rishi et al., 1999; Sakaue et al., 1999, 2001; Sun et al., 2000). It seems likely that the growth inhibitory,

apoptosis-inducing, and antitumorigenic effects of AHPN depend on the synergistic/cooperative action of many proteins.

Several studies have recently demonstrated that expression of NAG-1 greatly inhibits the tumorigenic capacity of colon carcinoma and glioblastoma cells in mice (Baek et al., 2001b; Albertoni et al., 2002). It has been suggested that the antitumorigenic activity of NAG-1 may involve both autocrine and paracrine mechanisms. The latter may involve antiangiogenic effects of NAG-1. The antitumorigenic effects of NAG-1 are of particular interest to the reported inhibition of tumor growth by AHPN in mice (Lu et al., 1997). The linkage between NAG-1 induction and AHPN revealed in this study provides a new molecular mechanism that may contribute to the antitumorigenic activities of AHPN.

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