

Review

NSAID Activated Gene (NAG-1), a Modulator of Tumorigenesis

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The NSAID activated gene (NAG-1), a member of the TGF- β superfamily, is involved in tumor progression and development. The over-expression of NAG-1 in cancer cells results in growth arrest and increase in apoptosis, suggesting that NAG-1 has anti-tumorigenic activity. This conclusion is further supported by results of experiments with transgenic mice that ubiquitously express human NAG-1. These transgenic mice are resistant to the development of intestinal tumors following treatment with azoxymethane or by introduction of a mutant *APC* gene. In contrast, other data suggest a pro-tumorigenic role for NAG-1, for example, high expression of NAG-1 is frequently observed in tumors. NAG-1 may be like other members of the TGF- β superfamily, acting as a tumor suppressor in the early stages, but acting pro-tumorigenic at the later stages of tumor progression. The expression of NAG-1 can be increased by treatment with drugs and chemicals documented to prevent tumor formation and development. Most notable is the increase in NAG-1 expression by the inhibitors of cyclooxygenases that prevent human colorectal cancer development. The regulation of NAG-1 is complex, but these agents act through either p53 or EGR-1 related pathways. In addition, an increase in NAG-1 is observed in inhibition of the AKT/GSK-3 β pathway, suggesting NAG-1 alters cell survival. Thus, NAG-1 expression is regulated by tumor suppressor pathways and appears to modulate tumor progression.

Key words: Anti-tumorigenic, Cancer, Cox inhibitor, *Min* mice, NAG-1, Tumor suppressor

Introduction

NAG-1 was identified in this laboratory as a divergent member of the TGF- β superfamily by PCR-based subtractive hybridization from an indomethacin-induced library obtained from human colorectal cells. NAG-1 was subsequently identified by other groups using a variety of different cloning strategies. These include Macrophage Inhibitory Cytokine-1 (MIC-1), Placental Transformation Growth Factor- β (PTGFB), Prostate Derived Factor (PDF), Growth Differentiation Factor 15 (GDF15), and Placental Bone morphogenetic protein (PLAB) (Baek and Eling, 2006a). Our initial focus was to study the expression of NAG-1 as a protein that possibly plays a role in the inhibition of tumor development by inhibitors of cyclooxygenase (COX). It is well established that the use of COX inhibitors prevents the development of colorectal cancer in humans, but the mechanisms are not fully elucidated. Considerable evidence supports the inhibition of prostaglandin formation by COX inhibitors as a critical component for the prevention of cancer. However, changes in gene expression induced by these drugs may also contribute to the attenuation of cancer. Results from these investigations on the role of NAG-1 in the prevention of cancer by COX inhibitors clearly indicated that NAG-1 may play an important role in the suppression of tumor progression. Studies with cells in cultures documented NAG-1 as an apoptosis inducer in cancer cells. Subsequent studies with a transgenic mouse, ubiquitously expressing NAG-1, provided additional support for suppression of tumor progression by this member of the TGF- β superfamily protein. However, the biological activity of this protein is very poorly characterized, and reports in the literature show high expression in tumors, a finding that initially would appear to be in disagreement with the proposed tumor suppressor activity of NAG-1. One explanation for this apparent discrepancy is that NAG-1 may act as a tumor suppressor at the early stages of tumor development but later as a pro-tumorigenic protein as the tumor progresses to a malignant tumor. This is not unexpected since NAG-1 is a member of the TGF- β superfamily, but an explanation for this change in biological activity is not clearly understood at the present time.

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NAG-1, a member of the TGF- β superfamily

The human NAG-1 has been mapped to 19p12.1-13.1 (Lawton *et al.*, 1997). The NAG-1 protein is encoded by two exons: the 309 bp Exon I contains a 71 bp 5' untranslated region (UTR) and a 238 bp coding region, and the 647 bp Exon II contains a 3' UTR. The gene contains a single 1820 bp intron (Lawton *et al.*, 1997). Human NAG-1 is most abundantly expressed in the placenta and is expressed at lower levels in the colon, kidney, and prostate (Paralkar *et al.*, 1998). The murine NAG-1 gene (also known as GDF-15), was also identified and characterized (Bottnar *et al.*, 1999). The murine NAG-1 gene is closely related to the human NAG-1, but the tissue distribution of mouse NAG-1 is different from human (Hsiao *et al.*, 2000). For example, the human NAG-1 gene is poorly or not at all expressed in the liver (Li *et al.*, 2000), whereas the mouse NAG-1 is highly expressed in the liver (Hsiao *et al.*, 2000). Sequence comparison between the human and mouse NAG-1 promoters in the ~700 bp region revealed only 39% homology (Baek *et al.*, 2001a). This suggests that human NAG-1 evolved from mouse NAG-1 to perform distinct functions in rodents and humans.

NAG-1 protein has broad activity in inflammation and cancer as indicated by the diversity of nomenclature. Its biological activity is not well characterized nor is the molecular mechanism responsible for these functions, but NAG-1 may share some of the common functions of TGF- β superfamily cytokines. For instance, TGF- β 1 induces apoptosis and cell growth arrest in epithelial cells as reported for NAG-1, and TGF- β 1 knockout mice die of widespread inflammation. Indeed, NAG-1 appears to have some anti-inflammatory activity since its expression reduces TNF- α secretion in macrophages (Bootcov *et al.*, 1997). NAG-1 expression inhibits proliferation of primitive hemopoietic progenitors (Detmer *et al.*, 1999) and several epithelial cancer cell lines (Tan *et al.*, 2000; Baek *et al.*, 2001b; Baek *et al.*, 2002a; Baek *et al.*, 2002b; Baek *et al.*, 2005), reflecting the activity of a multi-functional cytokine. Ectopic expression of NAG-1 causes cell growth arrest of cancer cells as assessed in soft agar and cloning efficiency assays (Baek *et al.*, 2001b; Baek *et al.*, 2002a), and the expression of NAG-1 by a retrovirus system inhibits cell growth in MCF-7 human breast cancer cells (Li *et al.*, 2000). Further, overexpression of human NAG-1 in human colon and glioblastoma cells inhibits tumor formation in the nude mouse model (Baek *et al.*, 2001b; Albertoni *et al.*, 2002). NAG-1 has clearly documented anti-tumor activity in these mouse models of carcinogenesis.

The NAG-1 protein possesses an organization similar to other proteins in the TGF- β superfamily. NAG-1 is first formed as a pro-protein with the pro-domain consisting of 167 amino acids containing an N-linked glycosylation site at amino acid position 70 (Bauskin *et al.*, 2000). Proteolytic cleavage of the pro-protein at the amino acid target sequence RXXR results in the release of a 112 amino acid C-terminal mature protein, which is secreted. This mature region shares very little in common with other TGF- β superfamily proteins. Recently, it

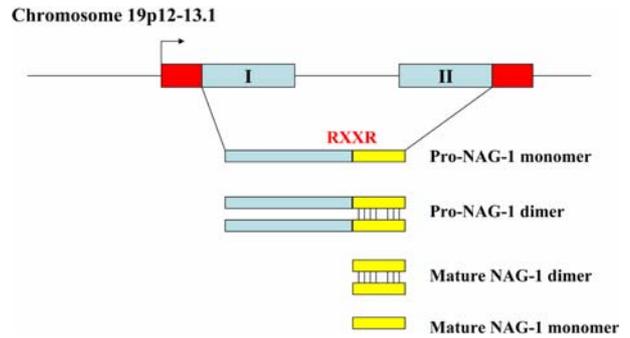


Fig. 1. NAG-1 can exist in several forms. NAG-1 is synthesized from two exons and cleavages at RXXR site to form C-terminal region. At the processing of mature NAG-1 protein, several forms could be produced from the cells.

has been reported that the pro-domain of NAG-1 selectively binds to an extracellular matrix (Bauskin *et al.*, 2005). Therefore, both the mature domain and pro-domain of NAG-1 are likely to play central roles in modulating the biological activity of NAG-1. These observations are strong evidence that NAG-1 represents the first member of a novel subfamily within the TGF- β superfamily of growth factors. Thus, NAG-1 exists in the intracellular compartment as predominantly the pro-form is cleaved and then secreted. Present in the plasma, under various pathological and physiological conditions is the secreted form of NAG-1. In acute injury, inflammation, and cancer, NAG-1 expression is highly increased, as detected by plasma levels of secreted NAG-1. Evidence indicates that NAG-1 exists primarily as a dimer formed by a disulfide bond, which raises the question as to which of the several forms of NAG-1 shown in Fig. 1 is the biologically active protein. One consideration is that the expression of the different forms can vary with the tumor and the extent of tumor progression, and may be different in comparison to normal tissue. Furthermore, the biological activity of the forms of NAG-1 has not been characterized and may not be the same.

NAG-1 Expression in Cancer. Reports of NAG-1 expression in normal and transformed tissue have been inconsistent. We have shown by immunohistochemistry that expression of NAG-1 on the surface epithelium of human intestinal villi overlaps with regions undergoing apoptosis, and is down-regulated in colon tumors compared to adjacent normal tissue (Kim *et al.*, 2002). In addition, NAG-1 expression was observed in the normal tracheobronchial epithelia, whereas no expression was found in either squamous metaplastic tracheal epithelium or in sections of human lung tumors (Newman *et al.*, 2003). In contrast, evidence has also been presented to support a role for NAG-1 in the progression of tumors within the digestive tract. NAG-1 serum levels were elevated in patients with colorectal cancer compared to normal controls (Brown *et al.*, 2003). Furthermore, Lee *et al.* found that poorly differentiated

cells in the submucosa of the invasive areas of gastric cancers contained elevated levels of NAG-1 transcripts compared to normal cells (Lee *et al.*, 2003). This dichotomy was seen in prostate cancer as well. Microarray studies by Welsh *et al.* comparing matched samples from normal and primary prostate tumors also found increased NAG-1 expression in 21 out of 24 cancers (Welsh *et al.*, 2001). Karan *et al.* show higher NAG-1 expression in prostate cancer sections by immunohistochemistry (Karan *et al.*, 2003). However, inconsistent with previous reports, Thomas *et al.* found NAG-1 expression to be down-regulated in 8/10 primary prostate cancers (Thomas *et al.*, 2001). This apparent dichotomy in NAG-1 expression in normal cells and those in tumors of the intestinal tract and prostate has not been adequately explained and raises the possibility that NAG-1 plays distinctly different roles at different stages of tumor progression. The expression pattern of the different forms of NAG-1 may also change with tumor progression, but adequate methods to measure the distinct protein forms are not available. These observations also provide evidence for a dual role for NAG-1 in cancer progression: suppression of tumorigenesis in normal tissue at the early stages of cancer development, and promotion of tumor invasiveness and survival at more advanced stages of disease. In addition, identification of the signaling pathway of NAG-1 through the NAG-1 receptor may provide an answer for this dichotomy. How these changes in NAG-1 expression and function occur, and the precise role that this protein plays at these different stages of tumor progression, remains to be determined. Despite conflicting data on the expression in tumors, NAG-1 has been clearly documented to suppress tumor growth in mouse models of cancer.

NAG-1 transgenic mice. The mouse models for intestinal tumorigenesis have been very useful in investigating the inhibition of tumor formation by NSAIDs and other anti-cancer agents (Fearon and Vogelstein, 1990; Vogelstein *et al.*, 1988). Molecular studies of familial adenomatous polyposis (FAP) led to the discovery of the tumor suppressor gene adenomatous polyposis coli (*APC*) (Kinzler *et al.*, 1991; Kinzler and Vogelstein, 1996). Mutations in *APC* appear to be responsible not only for FAP but also for many sporadic cancers of the colorectal axis, stomach, and esophagus. The heterozygous C57BL/6J-*Apc*^{Min/+} mice develop numerous intestinal polyps with spontaneous loss of the wild type *APC* allele, and can serve as a murine model of human FAP. In addition to mutant mouse models, chemical carcinogens have been used to induce tumor formation.

We have generated Lox-P-NAG-1 transgenic mice that, when crossed with Protamine-Cre mice, over-express NAG-1 in all tissues (Baek *et al.*, 2006b). NAG-1-Protamine-Cre (NAG-Tg) mice were used to determine if the ubiquitous expression of human NAG-1 inhibited tumor formation. Mice were treated with azoxymethane (AOM), a colon carcinogen that is frequently used to study the pathogenesis of colorectal cancer. The NAG-1 mice showed smaller numbers of pre-

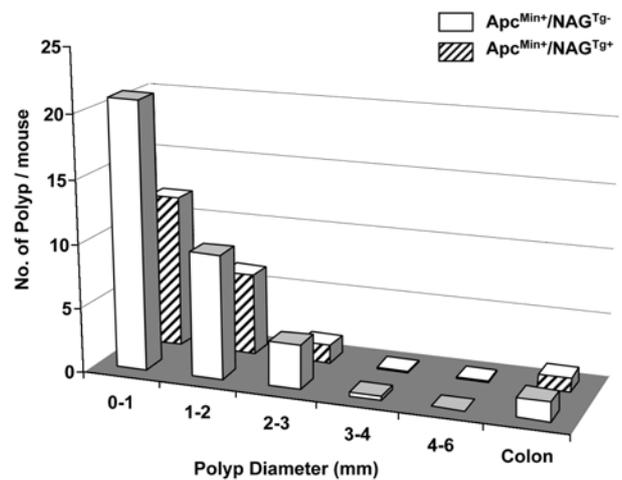


Fig. 2. Inhibition of Intestinal Polyps in *Min* mice by the Expression of Human NAG-1. *NAG*^{Tg+} mice carrying the *Apc*^{Min} mutation had a significant reduction in the number of adenomas and overall tumor load in comparison to non-transgenic littermates, but mean tumor size did not differ. The figure shows the comparison of the total numbers of polyps of different sizes between control littermates *Apc*^{Min+/+}/*NAG*^{Tg-} and *Apc*^{Min+/+}/*NAG*^{Tg+} mice (n=4 for *Apc*^{Min+/+}/*NAG*^{Tg-} and n=9 for *Apc*^{Min+/+}/*NAG*^{Tg+} mice). The data are represented as mean.

neoplastic lesions in their colon, compared to the wild type mice, suggesting that NAG-1 may suppress tumor formation. Mice carrying the *Apc*^{Min} mutation on a C57BL/6 background typically develop small adenomas or polyps, the vast majority of which occur in the small intestine. NAG-Tg mice were mated with *Min* mice, which spontaneously develop intestinal polyps. The *Apc*^{Min/+}/*NAG*^{+/-} mice showed an impressive 58% reduction in small intestine polyp numbers and 60% reduction of tumor load. The reduction in polyps was observed only in the smaller polyps and not the larger polyps (Fig. 2). Thus, expression of human NAG-1 in mice inhibits the development of chemically- and genetically-induced neoplasia in the intestinal tract. All these data strongly support the inhibitory role of NAG-1 in tumorigenesis and suggest it may function as a tumor suppressor gene.

NAG-1 and Prostate cancer

Prostate epithelia are composed of basal, luminal, and secretory epithelial cells that are in contact and interact with the prostate stromal compartment, which includes smooth muscle cells, fibroblasts, blood vessels, and nerves. NAG-1 mRNA is highly expressed in the human prostate epithelium (Paralkar *et al.*, 1998), suggesting its role in prostate homeostasis. There are several reports that NAG-1 inhibits cell growth and induces apoptosis. NAG-1 induced growth arrest in DU-145 human prostate carcinoma cells (Tan *et al.*, 2000). In another study, NAG-1 induced apoptosis involving

caspase-3 activation in DU-145 cells, but it did not affect proliferation (Liu *et al.*, 2003). Forced expression of NAG-1 inhibited the proliferation of PC-3 human prostate carcinoma cells as well as the growth of xenografted tumors (Lambert *et al.*, 2006). Inhibition of TPA-induced NAG-1 expression by NAG-1 short interfering RNA blocked TPA-induced apoptosis in LNCaP cells, suggesting induction of NAG-1 negatively affects LNCaP cell survival (Shim and Eling, 2005). While most *in vitro* studies suggest NAG-1 induces growth arrest or apoptosis, several studies showed that NAG-1 expression is up-regulated in human prostate cancer. Like TGF- β , genetic polymorphisms of NAG-1 have been described (Fairlie *et al.*, 2001). A GC polymorphism changes the basic amino acid histidine (H) to aspartic acid (D) at position 6 of the mature NAG-1 protein. Lindmark *et al.* has shown that the GC genotype, which results in aspartate at position 6 of mature NAG-1, may be associated with a lower risk of sporadic prostate cancer and of familial prostate cancer than the CC genotype (Histidine at position 6 of mature NAG-1 protein) (Lindmark *et al.*, 2004). However, Hayes *et al.* performed a similar study and found that the GC genotype is marginally associated with a lower risk of developing prostate cancer (Hayes *et al.*, 2006). The same study also found that GC genotype is associated with an increased risk of progression once the tumor is developed. Thus the role of NAG-1 in human prostate cancer is not clear at the present time. Additional experiments with mouse models of prostate cancer and NAG-1 transgenic mice may provide some new clues to elucidate the role of NAG-1 in prostate cancer.

Regulation of NAG-1 expression

NAG-1 is up-regulated in human colorectal cancer cells by several NSAIDs (Baek *et al.*, 2002b), as well as by other anti-tumorigenic compounds, including resveratrol (Baek *et al.*, 2002a), genistein (Wilson *et al.*, 2003), diallyl disulfide (Bottone *et al.*, 2002), indole-3-carbinol (Lee *et al.*, 2005), PPAR γ ligands (Baek *et al.*, 2004; Yamaguchi *et al.*, 2006), 5F-203 (Martinez *et al.*, 2006), conjugated linoleic acids (Lee *et al.*, 2006), and retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (AHPN) (Newman *et al.*, 2003). Shown in Table 1 is a listing of most of the compounds we have tested for the induction NAG-1 expression with the human colorectal cancer line HCT-116. A very diverse number of chemicals with a wide range of chemical structures induce the expression of NAG-1, suggesting multiple mechanisms are responsible for the increase in expression. For the COX inhibitors, the structural characteristic for the inhibition of COX activity appears to be different from the structural features that regulate the induction of NAG-1 (Baek *et al.*, 2002b). The most potent COX inhibitor for the induction of NAG-1 expression is sulindac sulfide, with ample induction observed at 5 μ M, the physiological concentration achieved *in vivo*. In addition to colorectal cancer, NAG-1 induction by

Table 1. Drugs, Chemicals and NAG-1 Expression

	Concentration (μ M)	NAG-1 expression
Cyclooxygenase inhibitors		
Aspirin	1,000-10,000	↑
Sulindac	10-40	+/-
Sulindac Sulfone	100-400	+/-
Indomethacin	10-100	↑
Sulindac Sulfide	1-50	↑↑
Piroxicam	200-1000	↑
Diclofenac	50-200	↑
Acetaminophen	10-100	+/-
Ibuprofen	100-1,000	↑
Sodium Salicylate	1,000-5,000	↑
SC-58125	10-100	↑
Celecoxib ¹	0.01-0.1	toxic
Dietary agents		
Resveratrol	10-100	↑
Capsaicin	10-100	↑
EGCG	10-100	↑
ECG	50	↑
Indol Indol-3-carbinol	50	↑
DIM	1-10	↑
Genistein	10-100	↑
DADS	50-100	↑
Lycopene	10-100	+/-
Sulforaphane	10-100	+/-
Tran-10,cis-12 conjugated linoleic acid	25	↑
PPAR agonist		
Troglitazone	5	↑↑
15-Deoxy- $\Delta^{12,14}$ -prostaglandin J ₂	1-10	↑
MCC-555	5	↑
AHPN	1-10	↑
Anti-cancer drugs		
5F-203 ²	0.01-5	↑↑

1. Treatment of mice increased NAG-1 expression in intestinal tissue.
2. Treatment of mice increased NAG-1 expression in breast tumors.

NSAIDs was seen in lung, oral, prostate, bone, ovarian, and breast cancer cells (Baek *et al.*, 2002a; Kim *et al.*, 2003; Kim *et al.*, 2004; Shim and Eling, 2005). Of all the drugs and chemicals so far examined the most potent is the experimental anti-cancer drug 5F-203. Nanomolar concentrations of this drug produce high expression in human colorectal and breast cells in culture (Martinez *et al.*, 2006). Treatment of mice with the pro-drug Proress, converted to 5F-203 *in vivo*, induced the expression of NAG-1 in MCF-7-derived mammary tumors in an orthotropic model for breast cancer. The level of induced NAG-1 expression inversely correlated with tumor size. Thus, the anti-tumorigenic regulation of NAG-1 has been observed under *in vitro* and *in vivo* conditions.

The human NAG-1 promoter contains several *cis-acting*

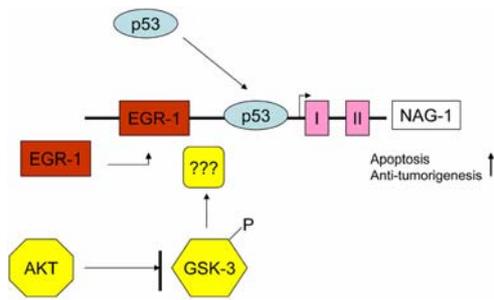


Fig. 3. Regulation of NAG-1 expression by tumor suppressors.

and *trans-acting* elements (Baek *et al.*, 2001a). The comparison of human and mouse promoter sequences revealed very little homology between the two species. Less homologous promoter sequences may provide an explanation for the differential transcription in different tissues. However, despite diversity in promoter sequences and expression, Sp1, AP-1, and Nkx-2 may play a common role to regulate NAG-1 in the two species (Baek *et al.*, 2001a). The Sp1 transcription factors regulate the basal transcription of NAG-1 through the GC box located within -133 bp of the NAG-1 promoter, whereas the three p53 sites play a pivotal role in dietary compound-induced NAG-1 expression (Li *et al.*, 2000; Baek *et al.*, 2002a). Furthermore, several COX inhibitors and troglitazone induce NAG-1 expression at the transcriptional level via EGR-1 transcription factors (Baek *et al.*, 2004; Baek *et al.*, 2005). Interestingly, we have found that the EGR-1 binding site in the NAG-1 promoter overlaps with an Sp1 site. Thus, the transcriptional activity of NAG-1 depends upon the balance of EGR-1 and Sp1 family members. The expression of Sp1 is not altered in the presence of sulindac sulfide, whereas EGR-1 expression is increased (Baek *et al.*, 2005). The expression of EGR-1 also increases NAG-1 transcription and will enhance sulindac sulfide induced NAG-1 expression. Post-transcriptional regulation was also involved in NAG-1 expression. MCC-555 (one of the PPAR γ ligands), the anti-cancer drug 5F-203 (Martinez *et al.*, 2006; Yamaguchi *et al.*, 2006) and the RXR ligand APHN (Newman *et al.*, 2003) increase NAG-1 RNA stability in cells, resulting in the up-regulation of NAG-1. Thus, NAG-1 is regulated by several transcriptional factors and different mechanisms, suggesting its diverse regulation by anti-tumorigenic compounds. In addition, from our studies on the regulation of NAG-1 expression by tumor prevention drugs it can be concluded that NAG-1 is an important downstream target of three tumor suppressor pathways (Fig. 3): p53 (Baek *et al.*, 2002a), EGR-1 (Baek *et al.*, 2005), and AKT/GSK-3 β (Yamaguchi *et al.*, 2004). These findings also suggest that NAG-1 maybe a key mediator for tumor suppression.

Summary

The evidence linking NAG-1 with cancer is compelling, but there are multiple unknown and conflicting findings. Much needs to be done to elucidate the role of NAG-1 in tumorigenesis. The biological activity of the protein is poorly understood and recombinant NAG-1 proteins are not readily available. Furthermore, the receptor and the downstream signaling pathways have not been delineated. The effects of NAG-1 can be contradictory and under different conditions, NAG-1 can exhibit either tumorigenic or anti-tumorigenic activity. This is likely dependent upon the tumor stage and the origin of the tumor as well as cell context. Clearly, the anti-tumorigenic activity is well established, but how the transition to a pro-tumorigenic protein occurs is unclear. Investigations on the regulation of NAG-1 expression have revealed complex mechanisms that can be modulated by a number of drugs and chemicals. Noteworthy is the finding that the tumor suppressor pathways p53 and EGR-1 up regulate NAG-1 expression. Likewise, inhibition of the AKT/GSK-3 β pathway increases NAG-1 expression. These findings link NAG-1 as a potentially important downstream target in tumor suppression.

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