Diallyl Disulfide (DADS) Induces the Antitumorigenic NSAID-Activated Gene (NAG-1) by a p53-Dependent Mechanism in Human Colorectal HCT 116 Cells

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ABSTRACT Garlic is appealing as an anti-carcinogenic agent due to its ability to induce apoptosis in vitro and inhibit the formation and growth of tumors in animals in vivo. Diallyl disulfide (DADS) is a constituent of garlic that suppresses neoplastic cell growth and induces apoptosis. We examined the effects of DADS on various cancer cell lines to better understand its effect on apoptosis and apoptosis-related genes. The nonsteroidal anti-inflammatory drug (NSAID)-activated gene (NAG-1) has proapoptotic and antitumorigenic activities and is upregulated by anticancer agents such as NSAIDs. In this study, human colorectal HCT-116 (wild-type p53), HCT-15 (p53 mutant) and human prostate PC-3 (p53 mutant) cells were exposed to DADS. DADS inhibited cell proliferation in all cell lines albeit to a lesser extent in HCT-15 and PC-3 cells at 11.5 and 23 μmol/L. In HCT-116 cells, DADS induced p53 and NAG-1 in a dose-dependent manner and the induction of p53 preceded that of NAG-1. In HCT-116 cells, NAG-1 protein expression was increased 2.4-fold ± 0.6 at 4.6 μmol/L and 6.1-fold ± 1.7 at 23 μmol/L DADS, whereas p53 was induced 1.5-fold ± 0.1 and 2.3-fold ± 0.4. DADS did not induce NAG-1 or p53 in p53 mutant cell lines; however, NAG-1 expression was induced by sulindac sulfide. HCT-116 cells treated with 4.6 and 23 μmol/L DADS resulted in a 1.9- and 2.9-fold increase in apoptosis, respectively. In contrast, 23 μmol/L DADS induced apoptosis only 1.8-fold in HCT-15 cells and not at all in PC-3 cells. Thus, DADS-induced apoptosis and NAG-1 protein expression appear to occur via p53.


KEY WORDS: • apoptosis • colorectal cancer • diallyl disulfide (DADS) • NAG-1 • p53
is forms of prostaglandin H synthase, cyclooxygenase-1 and -2. Our laboratory first identified NAG-1 as a NSAIId-acti-
vated gene using subtractive hybridization of indomethacin-
treated colorectal HCT-116 cells. This effort was undertaken to
better understand how NSAIId attenuates tumor growth (18).
Tumors derived from HCT-116 cells transfected to over-
express NAG-1 were reduced in number and in size as deter-
mined in athymic nude mice. These cells showed increased
basal apoptosis in vitro indicating that NAG-1 is both pro-
apoptotic and antitumorigenic (18). NAG-1 is identical to
and also known as macrophage inhibitory cytokine 1 (19),
placental bone morphogenetic protein (20) and placental
transforming growth factor-β (PTGF-β) (21). PTGF-β/NAG-1 is a secretory protein that can act in both an autocrine
and paracrine fashion (21).

**Materials and methods**

**Supplies and chemicals.** Dimethyl sulfoxide (DMSO), DADS, propidium iodide (PI), PBS and all other chemicals were purchased from Sigma Chemical (St. Louis, MO) unless otherwise noted. Sulfinilic acid (SS) was from Merck (Whitehouse Station, NJ). DADS was dissolved to 4.6 mmol/L (200–100X in DMSO). S-Alllylcysteine (SAC) and 3-allylmercaptoctethylamine (SAMC) were in the form of aged garlic extract (AGE) from Wakunaga Consumer Products (Mission Viejo, CA). SAC and SAMC are water-soluble allergen di-
terives. SAC is a major constituent of AGE, which can also be used to
form SAMC when reacted with the amino acid cysteine. SAMC was
freshly prepared in PBS at 4°C by combining stock solutions of SAC and
cysteine followed by shaking for several hours, then mild soni-
cation in a cold-room at 4°C. Stock solutions were then sterile
filtered through 0.2-μm Millipore filter cartridges (Bedford, MA).

**Cell line and reagents.** Cell lines were purchased from ATCC (Rockville, MD). Human colorectal cancer cell lines HCT-8, HCT-116, SW480 are maintained in McCoy's 5A medium. Media were supplemented with
10% fetal bovine serum (FBS) and 10 mg/mL gentamicin. Human
HCT-15 and PC-3 cells were maintained in RPMI-1640 plus 10%
FBS, 1 mmol/L l-glutamine, 1 mmol/L sodium pyruvate and 10 mg/mL
genamicin. Cell culture reagents were from Life Technologies
(Rockville, MD). Vehicle treatments consisted of 0.5% DMSO. Cells
were maintained at 37°C/5% CO₂ and split twice weekly with 0.25%
trypsin.

**Cell culture experiments.** Cells were plated overnight in complete
media and subsequently treated in serum-free media for various
time points for protein isolation and fluorescence-activated cell sort-
ing (FACS) analysis as indicated in the figure legends. Cells were
grown to 60–70% confluency in 12-well plates and then treated with
vehicle (0.5% final concentration DMSO), DADS or SS in the
absence of serum.

**Cell proliferation assay.** Cell proliferation was measured using the
MTS colorimetric assay by Promega (Madison, WI), which esti-
mates the number of viable cells in proliferation. Briefly, 500 cells per
well were plated in 96-well tissue culture dishes overnight. Cells were
then treated with various concentrations of vehicle or DADS as
indicated in the figure legend in a final volume of 0.1 mL complete
media containing 10% FBS. Cell viability was measured daily at 490
nm in an ELISA plate reader after the addition of 0.02 mL MTS
“Aqueous One” solution per well and a 1 h incubation at 37°C/5% CO₂.
Each experiment was carried out in quintuplet and repeated
three times. Data shown are mean OD 490 ± SD from a represen-
tative experiment.

**Protein isolation.** Protein was isolated in 1X RIPA buffer freshly
made the day of the experiment with one Complete-Mini protease
inhibitor tablet from Roche Diagnostics (Indianapolis, IN) per 0.1 L
RIPA buffer. DNA was sheared using a 23-gauge needle; cell lyses
were stored at 4°C for 30 min following centrifugation at 12000 x g
at 4°C for 20 min to remove cellular debris.

**Western blotting.** Proteins (15–20 μg) were separated by SDS-
PAGE and transferred onto nitrocellulose membranes. The blots were
blocked overnight with 10% skim milk in Tris buffered saline (TBS)
containing 0.1% Tween-20, and probed with anti-NAG-1 antibody
(1:2000 in 1% skim milk in TBS) for 1 h at room temperature as previously described (18). The antibody recog-
nized both the precursor and secreted forms of NAG-1. The second-
ary antibody used was anti-rabbit horseshadish peroxidase (HRP)
(Santa Cruz) (Santa Cruz, CA). For p53 studies, the primary antibody
for p53 was from Santa Cruz and was diluted 1:1000. The actin
antibody used was a goat polyclonal immunoglobulin G diluted 1:4000
(Santa Cruz). After washing, the blots were treated with HRP-conjugated secondary antibody for 1 h and washed
several times. Cell signal was detected by enhanced chemiluminescence
(Amersham Pharmacia Biotech, Piscataway, NJ) followed by autoradi-
ography. Where necessary, blots were stripped of antibody before
use while sealed in a plastic bag containing a solution of 62.5
mmol/L Tris-HCl, 2% SDS and 100 mmol/L β-mercaptoethanol
for 30 min with constant agitation in a 50°C water bath.

**Measurement of DNA content and apoptosis.** The DNA con-
tent for vehicle, DADS and SS treated cells were determined by
FACS. Cells were plated at 2.5 × 10⁵ cells/mL in 12-well plates, incubated until they reached 50% confluen-
cy before use while sealed in a plastic bag containing a solution of 23
mmol/L DADS, 10 μmol/L SS or vehicle for 48 h or more. After
washing, the cells were resuspended in PBS, harvested, then fixed by the slow addition of cold 70%
ethanol while mixing to a total of 1 mL and stored at 4°C
overnight. The fixed cells were pelleted, washed twice with PBS and
stained in 1 mL of 20 mg/mL PI, 1 g/L RNase in PBS for 20 min. Cells
(n = 7500) were examined by flow cytometry using Becton Dickinson
FACSort equipped with CellQuest (San Jose, CA) software by gating on an
area vs. width dot plot to exclude cell debris and cell aggregates.
Apoptosis was measured by the level of subdiploid DNA contents
in cells using CellQuest software from the total gated cells. Measurements are fold-increase over matched vehicle (DMSO)-treated cells.

**Densitometry measurements.** Autoradiograms from Northern
and Western blots were scanned using a Umax Powerlook III scanner
(Fremont, CA) equipped with a transparency adapter and scanning
software. Bands were quantitated using Adobe Photoshop 5.0 (Adobe
Systems, San Jose, CA) software by gating on an
area vs. width dot plot to exclude cell debris and cell aggregates.
Values shown are fold-increase relative to vehicle-treated control as
determined in the figure legends. All experiments were repeated at least
three times using cells of different passages and freezer stocks.

**Statistical analyses.** Statistical analyses were performed on a
personal computer using SigmaStat (Jandel, San Rafael, CA). Values
represent mean ± SEM. Differences were determined using a
two-sided t-test with a 0.05 level of significance and by ANOVA with
Fisher’s Least Significant Difference method for multiple comparisons
where there were multiple treatment groups. DADS dose curve data
were normalized using a square-root transformation before ANOVA.

**RESULTS**

**Cell proliferation assay.** We first determined whether DADS inhibited cell proliferation in various human colorectal
(HCT-116 and HCT-15) and prostate (PC-3) cancer cell lines at various concentrations due to its ability to induce apoptosis in vitro (10,16). DADS inhibited growth in all cell lines tested, but the HCT-116 cells appeared to be the most sensitive (Table 1), warranting further study of these cells (Fig. 1A).

**Protein expression in p53 wild-type HCT-116 cells.** Because of the ability of DADS to inhibit cell proliferation, particularly in HCT-116 cells, we next determined whether NAG-1 protein expression was increased after treatment of HCT-116 cells (Fig. 1B). DADS induced NAG-1 protein expression 7.8-fold ± 1.6 in HCT-116 cells following a 23 μmol/L treatment for 48 h in serum-free media (Fig. 1C). We also tested the water-soluble garlic compounds SAC and SAMC for their ability to induce NAG-1 protein expression in HCT-116 cells because they inhibit cell growth in colorectal cancer cells (27). At 400 μmol/L, neither SAC nor SAMC induced NAG-1 protein expression; therefore, they were not considered further (data not shown). Subsequently, a dose-response curve was performed using DADS to confirm that the induction of NAG-1 was dose dependent. DADS treatment resulted in a dose-dependent induction of NAG-1 protein expression in HCT-116 cells (Fig. 2A). In HCT-116 cells, NAG-1 protein expression was increased 2.4-fold ± 0.6 at 4.6 μmol/L and 6.1-fold ± 1.7 at 23 μmol/L DADS, whereas p53 was induced 1.5-fold ± 0.1 and 2.3-fold ± 0.4, respectively (Fig. 2B). Values were adjusted using their corresponding actin levels.

To determine whether the increase in p53 by DADS in HCT-116 cells preceded that of NAG-1, a time course was performed. The expression of p53 after treatment with 23 μmol/L DADS occurred in a time-dependent manner with a peak protein expression occurring between 6 and 48 h (Fig. 3). SS did not induce p53 protein expression at 10 μmol/L.

The induction of NAG-1 also appeared in a time-dependent manner with the maximal increase occurring between 24 and 48 h (Fig. 3). SS is an NSAID known to induce NAG-1 expression and apoptosis in a p53-independent manner in HCT-116 cells; thus, it was used as a positive control for NAG-1 protein expression (18). SS (10 μmol/L) induced NAG-1 protein expression 11- and 15-fold at 24 and 48 h, respectively. Values are adjusted for time-matched, vehicle-treated controls. Thus, the induction of p53 protein expression by DADS precedes that of NAG-1, suggesting that NAG-1 protein expression may be p53 dependent.

### TABLE 1

**The percentage of inhibition of cell proliferation by diallyl disulfide (DADS)**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>DADS μmol/L</th>
<th>% Inhibition</th>
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<tr>
<td>HCT-116</td>
<td>4.6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>11.5</td>
<td>73*</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>90*</td>
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<td>PC-3</td>
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<tr>
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<td>23</td>
<td>44*</td>
</tr>
<tr>
<td>HCT-15</td>
<td>4.6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>11.5</td>
<td>33*</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>52*</td>
</tr>
</tbody>
</table>

1 Values are means, n = 5.
2 Relative to vehicle-treated controls, * P < 0.01.
3 Cells were treated for 4 d in complete media containing various amounts of DADS and measured for growth inhibition as indicated in Materials and Methods.

**Protein expression in p53 mutant HCT-15 and PC-3 cells.** HCT-15 cells contain a mutation in the p53 gene rendering it nonfunctional (25,26). PC-3 cells are an immortalized human prostate adenocarcinoma cancer cell line that also contain a mutant p53 gene (28,29). NAG-1 was not induced in HCT-15 or PC-3 cell lines after treatment with DADS (Fig. 4A). In contrast, at 10 μmol/L SS, NAG-1 protein expression was induced 11.3-fold ± 0.94 (P < 0.001) in HCT-15 cells after a 48-h treatment and 12.5-fold ± 0.99 (P < 0.001) in PC-3 cells (Fig. 4B). Expression of p53 was not...
changed in these cell lines after treatment with DADS or SS (Fig. 4A).

**DADS-induced apoptosis.** To confirm that the inhibition of cell growth by DADS resulted from the induction of apoptosis and to support the hypothesis that this induction was due in part to NAG-1 protein expression, HCT-116 cells were treated with 4.6 and 23 μmol/L DADS or vehicle for 48 h in serum-free media and apoptosis measured by FACS analysis. Apoptotic cells were identified as the subG1 population of 7500 gated cells. Treatment of HCT-116 cells with 4.6 μmol/L DADS resulted in a 1.9-fold increase in apoptosis, whereas 23 μmol/L DADS resulted in a 2.9-fold increase in apoptosis over vehicle according to FACS analysis. Thus, the induction of apoptosis by DADS appears to be due to NAG-1 protein expression and occurs in a dose-dependent manner (Fig. 5).

In HCT-116 cells, 4.6 and 23 μmol/L DADS differed from the vehicle control (P < 0.03) as did 10 μmol/L SS (P < 0.02); 10 μmol/L SS was used as a positive control because it induces apoptosis in HCT-116 cells (18,30). To obtain additional evidence that DADS-induced apoptosis is mediated by p53-dependent NAG-1 induction, we treated p53 mutant PC-3 and HCT-15 cells with 23 μmol/L DADS or vehicle for 48 h in serum-free media and apoptosis was measured by FACS analysis.
DISCUSSION

NAG-1 is a p53 target gene that has proapoptotic and antitumorogenic activities and is upregulated by anticancer agents such as NSAIDs. NAG-1 expression is induced by multiple mechanisms and is regulated by both p53-dependent and -independent mechanisms. Here, we demonstrate that DADS induces both p53 and NAG-1 protein expression in a time- and dose-dependent manner in p53 wild-type HCT-116 cells. Induction of NAG-1 by DADS occurred only in p53 wild-type cells, suggesting a link between DADS-induced NAG-1 protein expression and p53. Treatment of p53 mutant HCT-15 and PC-3 cells with SS, a NSAID known to induce NAG-1 protein expression in a p53-independent manner, resulted in an 11- and 12-fold induction of NAG-1 protein expression, respectively (18). Furthermore, SS induced apoptosis in HCT-15 (31) and PC-3 cells, confirming the ability of the p53 mutant cell lines to undergo apoptosis mediated by DADS. Taken together, these data indicate that DADS induction of NAG-1 and apoptosis is in part p53 dependent. Conversely, no significant increase in apoptosis after treatment with DADS was observed in PC-3 cells and only a small and nonsignificant 1.8-fold increase was seen in HCT-15 cells, further suggesting that p53 is an essential link among DADS treatment, NAG-1 protein expression and apoptosis. However, induction of apoptosis by DADS in HCT-15 cells has been reported, but at 100–500 μmol/L DADS (7). Furthermore, DADS-induced inhibition of cell growth was far less dramatic in both HCT-15 and PC-3 cells as measured by cell proliferation (Table 1). This is further evidence that similar to apoptosis, the inhibition of cell growth by DADS occurs at least in part via a p53-dependent mechanism. This is consistent with previous reports that DADS reduces cell proliferation 90% in HCT-15 cells, albeit at much higher concentrations (7,32). Thus, the concentration of DADS appears to be an important factor in determining the mechanism involved in inhibition of cell growth and induction of apoptosis; however, at low concentrations, NAG-1 and p53 appear to play key roles in mediating this response.

The induction of NAG-1 by DADS is dependent on p53. The induction of p53 by DADS precedes that of NAG-1, and p53 and NAG-1 are not induced in p53 mutant cell lines. The induction of apoptosis by DADS is greater in p53 wild-type than p53 null cells (17). NAG-1 is a p53 target gene that controls cell growth (21), and NAG-1 is a transcriptionally regulated gene that is activated by various wild-type p53 inducible systems in p53 mutant H1299 human lung cancer cells (33). Also, NAG-1 is induced by etoposide, a known inducer of p53, in p53 wild-type but not p53 mutant cell lines (21). NAG-1 contains two p53 binding sites as well as several other transcription binding sites in its promoter, indicating that the induction of NAG-1 expression may occur via multiple mechanisms (24). Additionally, the p53-dependent transactivation of NAG-1 is blocked by a dominant negative p53 mutant and other p53 mutants (21). NAG-1 is a known inducer of growth arrest and apoptosis and appears to be an important component in p53-dependent apoptosis (23). Furthermore, HCT-116 cells transfected to overexpress NAG-1 result in decreased tumorigenesis in athymic nude mice, reduced growth in soft-agar and undergo apoptosis (18).

Is the use of garlic (Allium sativum) and its constituents feasible as an anticarcinogenic agent rivaling that of NSAIDs? Dietary intake of garlic is associated with a reduction in a variety of cancers (4). Garlic has been shown to have antimicrobial, antithrombotic, antitumorigenic, antiarthritic and other useful properties (34). Garlic oil’s antitumorigeninicity in gastric cell lines indicates that garlic oil or its constituents may prevent colorectal cancer (15). Generally it is the lipid-soluble organic compounds from garlic such as DADS that possess the most effective antiproliferative effects (10). SAC and SAMC did not induce NAG-1 in HCT-116 cells at concentrations as high as 400 μmol/L. Interestingly, in p53 wild-type HCT-116 and p53 mutant HT-29 and SW-480 cells, SAMC induced apoptosis at 200 μmol/L (27). DADS was as effective as the anticancer compound 5-fluorouracil at reducing the growth of tumors in nude mice at equivalent doses (8). DADS also inhibited the toxicity of benzo(a)pyrene carcinogenicity in mice (12). Therefore, garlic oil, and DADS in particular, is toxic to cancer cells in vitro, resulting in apoptosis and cell death. Suggesting that it may have therapeutic value (7,10, 16). Thus, DADS may be effective in the prevention of some cancers. The intake of dietary compounds such as garlic, fruits, vegetables and soybeans is inversely associated with colorectal polyp formation (3). Thus cancer prevention may
be achievable through diets rich in antitumorogenic compounds such as garlic. The same has been said about the regular intake of NSAIDs. NAG-1 is an important link bet-
 tween reduced tumor growth in mice treated with or fed diets containing NSAIDs. Many food items have antitumorogenic properties. Therefore, a diet rich in foods containing sub-
 stances with antitumorogenic properties may provide some protection against cancer development and therefore should be further investigated. Interestingly, many dietary compounds such as genistein (35), selenium (36), resveratrol (37) and DADS (15) as illustrated here act through a p53-dependent mechanism. However, the p53 gene is frequently mutated in a
 variety of cancers, resulting in a loss in p53’s tumor suppressor function and thereby diminishing the ability of such compo-
nents to prevent cancers from developing through p53-de-
pendent mechanisms. Conversely, SS exerts its proapoptotic effect in a p53-independent manner (18,38,39). Therefore, it may be especially beneficial to further investigate dietary compo-
nents that act through p53-independent mechanisms similar to that of SS because of its ability to act through both p53-de-
pendent and -independent mechanisms. Meanwhile, dietary compo-
nents that act in a p53-dependent manner may be better suited for the prevention of cancer before mutations arise.

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