Modulation of p53 in 7,12-dimethylbenz[a]anthracene–induced skin tumors by diallyl sulfide in Swiss albino mice

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Abstract

Allium vegetables have been shown to have beneficial health effects against several chronic diseases including cancer. Diallyl sulfide (DAS), an organosulfur compound present in garlic, is well known for its chemopreventive properties in several tumor models. The pharmacologic role of DAS in prevention and treatment of cancer is well documented in the literature, but its molecular mechanism of action is not yet well defined. In the present study, modulation in p53 expression by topical application of DAS was recorded in 7,12-dimethylbenz[a]anthracene (DMBA)–induced skin tumors in Swiss albino mice. Western blot analysis and immunohistochemical protein detection, combined with multivariable flow cytometry, show that DAS application induces the expression of the wild-type (wt) p53 and down-regulates the expression of mutant (mut) p53. Immunoblotting analysis of tumors showed significant increase in levels of wtp53 by DAS application, whereas for mutp53 the DMBA-induced levels of protein were found to reduce to near normal levels with DAS application. The quantitative analysis of immunostained skin/tumor sections using image analysis and quantitative stereology showed 66.6% and 54.2% increases in wtp53 levels and 53.4% and 44.3% decreases in mutp53 levels in animals where DAS was applied 1 hour prior to or 1 hour after DMBA application, respectively. Flow cytometric analysis further confirmed modulation of wtp53 and mutp53 protein in DAS-supplemented tumors. The increase in the expression of wt tumor suppressor gene protein p53 was accompanied by elevation of the levels of cyclin-dependent kinase inhibitor p21/waf1. The percentage increase in the levels of p21/waf1 was found to be 72.9% and 61.3%, respectively, in DAS-supplemented groups before and after administration. These results thus show that DAS is a potential chemopreventive agent capable of modulating and regulating the tumor suppressor p53 along with its downstream effective molecule, p21/waf1. Thus, DAS can be a potential chemopreventive agent against skin tumor development.

Introduction

Garlic (Allium sativum) is known for its therapeutic properties since beginning of the recorded history and is probably the most widely studied medicinal plant (1, 2). Apart from its beneficial health effects, garlic has been reported to possess tumor inhibitory properties against various types of cancers (3, 4). The potential health benefits associated with garlic have been attributed to the presence of its organosulfur compounds. Diallyl sulfide (DAS), an organosulfide present in garlic, has received considerable attention as a potential chemopreventive agent. The anticarcinogenic properties of DAS have been shown in rodents with a variety of chemical carcinogens (5–8). Moreover, DAS exhibits a protective effect against cancer in a variety of target organs such as skin, lung, esophagus, colon, and liver (5–12), suggesting that the antitumorigenic properties of DAS are not limited to a single tissue or a carcinogen. The chemopreventive activity of DAS has been attributed to its ability to modulate phase I and II detoxifying enzymes, scavenging of free radicals, and its antimutagenic potential (13–16). The literature search revealed that studies on the molecular mechanism of tumor suppression by Allium derivatives are very few. However, some studies have shown that alliin, DAS, diallyl disulfide, and diallyl trisulfide possess inhibitory effects on the proliferation of tumor cells associated with induction of apoptosis and cell cycle regulation (17, 18). DAS has been reported by our group to suppress the growth of 7,12-dimethylbenz[a]anthracene (DMBA)–induced mouse skin tumors through the induction of apoptosis (8). Recently, we have shown that DAS can reverse the Vinca alkaloid–induced expression of P-glycoprotein both in vitro and in vivo (19). DAS, diallyl disulfide, and garlic extract were shown to be effective in reducing the expression of antiapoptotic gene bcl-2 and the induction of proapoptotic gene bax and the tumor suppressor gene p53 in non–small cell lung cancer cell lines (18). Diallyl disulfide–induced apoptosis has been shown to occur via p53-dependent mechanisms in human colorectal HCT 116 cells (20). Diallyl disulfide has also been shown to inhibit the growth of H-ras oncogene transformed tumors in nude mice by inhibiting membrane association of tumoral p21 ras (21).
Thus, the pharmacologic roles of allyl sulfides in prevention and treatment of cancer have been well documented in literature, but the mechanism of action of allyl sulfide compounds, particularly DAS, is not well defined. Therefore, to provide insight into the possible mechanism of tumor suppression by DAS, we investigated the effect of DAS on tumor suppressor gene p53 along with its downstream effective molecule p21/waf1 in DMBA-induced mouse skin tumors.

Materials and Methods

Materials

DMBA, DAS, and β-actin were purchased from Sigma Chemical Co. (St. Louis, MO). Anti-p53 antibody specific for wild-type (wt) protein (clone PAB 1620, Ab-5) and polyclonal p21/waf1 (Ab-5) antibody were procured from Oncogene Research Products (Cambridge, MA). Mammalian mutant (mut) p53 (clone PAB 240) was obtained from Boehringer (Mannheim, Germany). The horseradish peroxidase–conjugated isotypes were obtained from Bangalore Genei (Bangalore, India) and the FITC conjugates were procured from Becton Dickinson (Franklin Lakes, NJ). The rest of the chemicals were of analytic grade of purity and were procured locally.

Animal Bioassay

Swiss albino mice (male, 10–12 g body weight) were obtained from the Industrial Toxicology Research Center (Lucknow, India) animal breeding colony. Animals were quarantined for 1 week on a 12/12 hour light/dark cycle and were fed solid pellet diet (Ashirwad, Chandigarh, India) and water ad libitum. The mouse skin tumors were obtained using DMBA as a complete carcinogen as described earlier (8). In brief, DMBA/DAS was applied topically on shaved dorsal skin in the interscapular region of 2 cm². The animals were divided into five groups comprising 25 animals each (Table 1).

After completion of the study period (28 weeks), all the animals were sacrificed 24 hours after the last treatment. Skin from the painted area (with or without tumors) was excised, cleaned, snap frozen in liquid nitrogen, and stored at −80°C until further use.

Preparation of Nuclear Fraction

The skin/tumor tissue was removed with sharp scalpel blades, and s.c. fat was scraped off, on ice. The samples were then homogenized in lysis buffer (containing 20% glycerol, 20 mmol/L HEPES, 10 mmol/L NaCl, 1.5 mmol/L MgCl₂, 0.1% NP40, 0.2 mmol/L EDTA, 1 mmol/L DTT, 1 μg/mL pepstatin A, 1 μg/mL aprotinin, 100 μg/mL phenylmethylsulfonyl fluoride) and processed according to the method of Serpi et al. (22). The homogenate was kept for 15 minutes in lysis buffer on ice and then centrifuged for 5 minutes at 2,000 rpm. To the pellets, nuclear lysis buffer (~1 mL), 500 mmol/L NaCl instead of 10 mmol/L NaCl) was added, mixed vigorously with the help of micropipette (or sonicated for 15 seconds, if needed), and incubated for 30 minutes in an ice bath. The samples were then centrifuged at 15,000 rpm for 15 minutes at 4°C and the nuclear extract was collected.

Preparation of Membrane Fraction

The membrane fractions containing ras protein was prepared according to the method of Limtrakul et al. (23). Briefly, skin/tumor tissues were washed with cold phosphate buffer saline, and 10% (w/v) homogenate was prepared in lysis buffer containing 20 mmol/L Tris-HCl (pH 7.5), 5 mmol/L MgCl₂, 1 mmol/L EDTA, 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, 20 μg/mL aprotinin, 10 μg/mL leupeptin] and centrifuged at 3,000 rpm for 5 minutes. The supernatant was taken out carefully and again centrifuged at 11,000 rpm for 30 minutes at 4°C. The supernatant was collected as cytosolic fraction, and the membrane pellet was resuspended in buffer containing 1% NP40, 0.5% sodium o xo cholate, 0.1% SDS, 0.6 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L sodium orthovanadate in PBS (pH 7.4), vortexed, and then centrifuged at 11,000 rpm for 30 minutes. The supernatant was used as membrane fraction.

Western Blotting

Western blotting was carried out as described by Towbin et al. (24) in the nuclear fractions of skin/tumor tissue. Protein concentration was estimated by the routine method of Lowry et al. (25) using bovine serum albumin as a standard. Proteins (30–50 μg) were resolved under non-denaturing conditions on PAGE for wtp53 and on 10% SDS-PAGE gels for mutp53 and electrophoblated onto nitrocellulose membranes. The blots were blocked overnight with 3% nonfat dry milk and probed with appropriate antibodies [i.e., anti-wtp53 (clone PAB 1620), anti-mutp53 (clone PAB 240), and polyclonal anti-p21/waf1 antibody] at dilutions recommended by the suppliers. Immunoblot were observed by horseradish peroxidase–conjugated anti-mouse IgG using chromagen 3,3′-diaminobenzidine tetrahydrochloride. To quantify equal loading,
identical gels were run simultaneously and probed with β-actin antibody. Data are presented as the relative density of protein bands normalized to β-actin. The intensity of the bands was quantitated using Easy Win 32 software on Gel Documentation System (Herolab, GmbH, Wiesloch, Germany).

Flow Cytometric Analysis
The single cell suspensions of skin/tumor tissue from different experimental and control groups were prepared using Medimachine (Becton Dickinson, San Jose, CA) as described by us earlier (8). For the flow cytometric analysis, cells (~1 x 10^6) in suspension were taken and fixed in chilled 70% ethanol. After fixation, cells were permeabilized with 0.2% Triton X-100 and centrifuged. The pellets were resuspended in PBS and incubated with respective antibodies for wtp53, mutp53, and p21/waf1 for 1 hour at room temperature. After incubation, the cells were treated with FITC-conjugated isotype-specific secondary antibody. Cells were then analyzed on a flow cell cytometer (BD-LSR, Becton Dickinson, San Jose, CA) equipped with 488 nm argon laser light source. Total 15,000 events were acquired for analysis using Cell Quest Software. Cells were properly gated and histogram plot of FITC fluorescence (x axis) versus counts (y axis) has been shown in logarithmic fluorescence intensity.

Immunohistochemistry
For immunohistochemistry, the frozen skin/tumor sections (10 μm) were cut using cryostat and were fixed in neutral 10% phosphate-buffered formaldehyde solution. The endogenous peroxidase activity was quenched with methanol and 30% hydrogen peroxide solution (9:1) and the nonspecific binding was blocked with 1% normal goat serum in TBS. The slides were sequentially incubated with the nonspecific binding was blocked with 1% normal goat serum in TBS. The endogenous peroxidase activity was quenched with neutral 10% phosphate-buffered formaldehyde solution. The sections were again incubated with normal goat serum and then with horseradish peroxidase–conjugated anti-goat IgG secondary antibody. The slides were washed with PBS between incubations. The color was developed using chromagen 3,3'–diaminobenzidine in Tris (0.05 mol/L, pH 7.3) buffer containing hydrogen peroxide.

Image Analysis
The immunostained slides were analyzed under microscope (Leica, Wetzlar, Germany) attached with CCD camera (JVC, Tokyo, Japan). The quantitative stereology was done with Leica QWin 500 Image analysis software for each slide in triplicates with at least six microscopic fields. The statistical analyses were done using one-way ANOVA. P < 0.05 was considered significant.

Results
No increased rate of mortality was observed among all the control and experimental groups during the experiment. The tumor incidence was observed only in the animals of groups 2 to 4; however, no tumor development was recorded in control groups, not exposed to carcinogen (i.e., DMBA; groups 1 and 5).

The levels of wtp53 protein were overexpressed in tumors obtained by DMBA application (group 2) in comparison with normal mouse skin (group 1), where wtp53 protein was found in detectable limits (Fig. 1A, lanes 1 and 2). A comparatively high level of expression of wtp53 protein was recorded in tumors of DAS-supplemented groups (78% in group 3 and 60% in group 4) over group 2 (Fig. 1A), indicating that wtp53 protein plays a determining role in the inhibition of DMBA-induced neoplastic changes. However, no significant change in the wtp53 level was observed in tumors where DAS was given prior or after the DMBA application (Fig. 1A, lanes 3 and 4). In group 5, where only DAS was applied to mouse skin, levels of wtp53 were comparable with the normal levels (Fig. 1A). The flow cytometric analysis of skin/tumors from various groups revealed shifting of peak toward higher fluorescence with a mean fluorescence intensity (MFI) value of 15.41 and 10.21 in DMBA-supplemented groups (groups 3 and 4, respectively) from 5.31 for group 2 (ACE + DMBA; Fig. 2A). The MFI of groups 1 and 5 was significantly different, as the values were found to be 1.68 and 1.92, respectively. Analysis of wtp53 levels among the various groups by Western blotting and flow cytometry further lend support to immunohistochemical observations obtained by quantitative stereology. In tumors induced by DMBA (group 2), a significant change in the expression of wtp53 protein was observed in comparison with controls (Fig. 3A), whereas in DAS-administered groups (groups 3 and 4) further elevation in the expression of wtp53 was recorded (Table 2). The quantitation of immunostained slides showed 66.6% and 54.2% increase in the levels of wtp53 before and after DAS administration over group 2 (Table 2), indicating that the modulatory effect of DAS on wtp53 expression in DMBA-induced mouse skin tumors.

The mouse skin from the animals of control group showed presence of detectable level of mutp53, which was found to be overexpressed in Western blots of tumors obtained with topical application of DMBA (Fig. 1B, lanes 1 and 2). The overexpression of mutp53 observed in DMBA-induced tumors was found to be significantly low (P < 0.05) in tumors where DAS was given along with the DMBA (36% in group 3 and 46% in group 4), showing down-regulation of induced levels of mutp53 by DAS (Fig. 1B). However, no significant difference in expression levels of mutp53 was observed in tumors where DAS was given prior and after the DMBA exposure (Fig. 1B, lanes 3 and 4). Similarly, an increase in the MFI was apparent from 1.06 for control to 16.41 for DMBA-induced tumor cells (Fig. 2B). This increase in the MFI by DMBA recorded an inhibitory trend in DAS-supplemented groups as observed to be 8.13 in DMBA + DAS–treated group and 3.76 in DAS + DMBA–treated group. A 53.4% and 44.3% decrease in mutp53 levels was evident in groups 3 and 4, respectively, over group 2 (Fig. 3B), as shown by image analysis of the immunohistochemically stained slides, suggesting down-regulation of mutp53 by DAS (Table 2).

The effect of DAS treatment on the expression of p21/waf1 was assessed, which is transcriptionally up-regulated...
by the induced levels of wtp53. Immunoblot analysis showed overexpression of p21/waf1 in DAS-supplemented groups (70% in group 3 and 59% in group 4) when compared with the tumor cells induced by DMBA application only (group 2; Fig. 4). Flow cytometric analysis of the cells from various groups further confirmed the observations, as shown by the shift in MFI from 6.34 for group 1 to 22.42 for group 2. This shift in MFI was further magnified to 44.24 and 53.35 for groups 2 and 3, respectively (Fig. 5).

Discussion

Many classes of cancer chemopreventive agents, including naturally occurring and synthetic compounds, are studied for their efficacy in vitro and in vivo (26). Among the most extensively investigated are organosulfur compounds, which occur naturally in Allium vegetables (4, 27). DAS, a volatile organosulfide from garlic, is a potent antioxidant with anti-inflammatory and cancer preventive properties (3, 13). In vitro as well as in vivo studies have suggested that DAS may impart chemopreventive effects against many kinds of cancers including skin cancer (5, 7, 9, 10). The pharmacologic role of allyl sulfides in prevention and

The quantitative stereology of immunostained tissue sections showed an increase of 72.9% and 61.2% in the levels of p21/waf1 in DAS-supplemented groups over group 2 (Fig. 6; Table 2).
treatment of cancer has received considerable attention in the recent past, but the mechanism of action of allyl sulfide compounds is poorly defined, especially its role in cancer-controlling genes.

The tumor suppressor gene p53 is regarded as a key factor in maintaining the balance between cell growth and cell death (28, 29). The importance of p53 gene can be drawn from the fact that this gene is reported to be mutated in ~80% of the all human malignancies (30). Because of its role in regulation of cell cycle, alterations in p53 are critical events in carcinogenesis. The wt*p53* in response to toxic insults to DNA triggers a chain of cell cycle regulatory...

**Figure 3.** Modulatory effect of DAS application on p53 expression: *wt*p53* (A) and *mut*p53* (B) in different groups. Details are described in Materials and Methods. The DAS application leads to increased expression of *wt*p53 and down-regulates *mut*p53 expression in DMBA-induced tumors. The brown deposits of 3,3′-diaminobenzidine mark the reactivity with p53. a, untreated control; b, DMBA; c, DMBA + DAS; d, DAS + DMBA (×128).
events to check the proliferation of altered cells to repair or minimize the damage (28, 29). In tumors, loss of wtp53 function prevents the activation of this growth control pathway (31). This failure to induce transcriptionally active wtp53 plays a role in the unregulated growth of the tumors and also in the failure to respond to chemotherapeutic agents, which normally trigger wtp53, and regulates cell cycle arrest or cell death (32). Because the balance between wtp53 and mutp53 determines the fate of the cell, many chemopreventive agents are known to exert their anticancer effects by modulating their expression levels. The results of the present study showed up-regulation of wtp53, and suppressing the expression of mutp53 is in accordance with the previous reports where natural and dietary compounds have been shown to exert their preventive property through modulating the balance between wt and mutp53 protein expression. Vitamin E has been shown to inhibit DMBA-induced buccal pouch tumor development by stimulating the expression of wtp53 (33). Similarly, retinoids, including vitamin A and its synthetic analogues (i.e., all-trans and 9-cis retinoic acid), and 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid have been shown to induce the levels of wtp53 to check the growth of cancer cells (34, 35). The carotenoids and retinoids are also reported to prevent DMBA-induced in vitro malignant transformation of oral human keratinocytes by depressing mutp53 and enhancing tumor suppressor p53 (36). Supplementation of reduced glutathione has been shown to inhibit the experimental oral carcinogenesis by increasing the levels of wtp53 in DMBA-induced dysplasia and squamous cell carcinoma (37). Certain other chemopreventive agents such as indole-3-carbinol, tea polyphenol epigallocatechin gallate, and soy isoflavone genistein have been shown to inhibit benzo(a)pyrene-induced aberrant proliferation through the induction of p53-dependent apoptosis in human mammary epithelial cells (38). The up-regulation of wtp53 by chemopreventive agents is most likely responsible for the transcriptional induction of the p53-Mediated Skin Tumor Inhibition by DAS

Table 2. Effect of DAS on the expression levels of oncogenic proteins in DMBA-induced mouse skin tumors as quantitated using Leica QWin 500 Image analysis system

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Positive area fraction</th>
<th>Area (mm²/cm²), mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wtp53</td>
<td>mutp53</td>
<td>p21/waf1</td>
</tr>
<tr>
<td>1</td>
<td>Untreated</td>
<td>2.68</td>
<td>2.08</td>
</tr>
<tr>
<td>2</td>
<td>ACE + DMBA</td>
<td>8.03</td>
<td>14.75</td>
</tr>
<tr>
<td>3</td>
<td>DAS + DMBA</td>
<td>17.56</td>
<td>10.23</td>
</tr>
<tr>
<td>4</td>
<td>DMBA + DAS</td>
<td>13.69</td>
<td>11.02</td>
</tr>
<tr>
<td>5</td>
<td>ACE + DAS</td>
<td>2.87</td>
<td>2.56</td>
</tr>
</tbody>
</table>

NOTE: Values in parentheses are percentage change as calculated over group 2.
* indicates P < 0.05, significant over group 1.
** indicates P < 0.05, significant over group 2.

Figure 4. Western blot analysis of p21/waf1 in mouse skin tumors following DAS application. Skin/tumor lysates were prepared as described in Materials and Methods and proteins (30 – 50 μg) were employed for Western blots. An increased expression of p21/waf1 was recorded in the tumors obtained in DAS-supplemented groups (groups 3 and 4) over DMBA group (group 2). Lane 1, untreated; lane 2, DMBA; lane 3, DAS + DMBA; lane 4, DMBA + DAS; lane 5, DAS.

Figure 5. An overlay of flow cytometric analysis of p21/waf1 protein expression in mouse skin/tumor. Single cell suspensions were prepared as described in Materials and Methods. FITC fluorescence (x axis) versus counts (y axis). A total of 15,000 events were counted on argon 488 laser. Topical application of DAS resulted in increased expression of p21/waf1 as shown by higher MFI values over DMBA.
of p21/waf1 by directly interacting with its regulatory elements (32). Thus, the up-regulation of p21/waf1 by DAS as observed in the results of the present study in DMBA-induced tumors could be responsible for the growth inhibitory effects of DAS because of its role in cell cycle arrest. However, further insights into the effect of DAS on cyclin-dependent kinase complexes operated by cyclin-dependent kinase 2 and Cdc2 (cyclin-dependent kinase 1) and cell cycle checkpoints are required.

The wt p53 is reported to be up-regulated in the cells by its increased half-life through inhibition of its degradation time (39, 40) as well as modulation of its stability by post-translational events such as phosphorylation and acetylation (41, 42). Therefore, it is likely that up-regulation of wt p53 by DAS can occur via a similar mechanism of action. The up-regulation of wt p53 may also induce the expression of several p53-regulated downstream genes including DR5, bax, fas, and p21/waf1, which may cause growth arrest or apoptosis of the neoplastic cells (43, 44). Moreover, another plausible reason could be the prevention of mutation of p53 to oncogenic forms by promoting DNA repair. This may also be attributed to the antioxidant and antigenotoxic properties of the DAS (16, 45), as oxidants produced during tumorigenetic transformation promote genetic instability and mutational damage to the DNA. Thus, it could be suggested that DAS exerts its anticancer effect either by stimulating a cancer suppressor gene to prevent the action of carcinogenic influences or by preventing the mutation of other proto-oncogenes that may function together with mut p53.

The role of p53, in addition to the induction of p21/waf1, is also in the protection of the genome integrity, via physical interaction with DNA, as well as in regulation of cell propensity to apoptosis (29). Many chemopreventive agents are known to exert their anticancer effects through the induction of apoptosis via p53-dependent mechanisms (46–48). Studies on the Allium derivatives have shown that diallyl disulfide induces apoptosis and nonsteroidal anti-inflammatory drug–activated gene (NAG-1) protein expression via p53-dependent mechanisms in human colorectal HCT 116 cells (20). Similarly, Hong et al. (18) have shown that the mechanism of apoptosis induced by organoallyl sulfur compounds such as DAS, diallyl disulfide, or garlic extract is regulated through p53-dependent or p53-independent related bax/bcl2 dual pathway in non–small cell lung cancer cell lines. Earlier, we have shown that DAS can induce apoptosis in DMBA-induced tumor cells (8). The results of the present investigations showed a modulatory effect of DAS on the expression of cell cycle regulatory and signal transducing genes [i.e., p53 (both wt and mut) and p21/waf1] in mouse skin tumors. Therefore, a correlation between the tumor suppressor p53 and apoptosis in the anticancer mechanism of DAS could be established.

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p53-Mediated Skin Tumor Inhibition by DAS

References


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