Chapter-II

Role of P53 And P21/Waf1 in Diallyl Sulfide Mediated Tumor Suppression in Mouse Skin
Introduction

Tumorigenesis is a multistep evolutionary process in which clones of normal cells incur changes, which provide a growth advantage and may predispose the cells in these clones to further growth promoting changes (Nowell, 1989). Underlying many of these growth-promoting changes are mutations in the growth regulatory genes of the cell, the ‘oncogenes’ and ‘tumor suppressor genes’ (Fearson & Vogelstein, 1990). These genetic alterations occur very frequently and therefore, could be utilized as diagnostic tools or as intermediate end points for chemoprevention studies (Conti & Gimenez-Conti, 1996).

The existence of tumor suppressor genes had been predicted in 1971 from the finding that the development of retinoblastoma involves two genetic alterations, presumably inactivation of both copies of cellular gene (Knudson, 1971). The development of second primary tumors in patients who had been successfully treated for retinoblastoma suggested that changes in the rb gene might be involved in the development of many human tumors, and several such tumor suppressor genes are cloned (Stanbridge, 1990). These genes normally function to inhibit or "put the breaks on" on the cell growth and thus controlling the cell cycle. The family of tumor suppressor genes encompasses several members namely p53, rb, WT1, BRCA1, NF1 etc. which have been found to be involved in controlling proliferation and differentiation in cells (Cowell, 1990; Levine, 1990; Yuspa et al, 1994). In particular, their function is associated with control of abnormal growth in carcinogenesis and thus named as ‘tumor suppressor genes’.

p53 is a gene that has received wide attention as a tumor suppressor gene (Cowell, 1990). The p53 gene was first identified in 1979 as a host cell protein, binding to the DNA virus SV40 (Lane & Crawford, 1979) and later on described as a tumor suppressor (Levine, 1990). In humans, the p53 gene contains 11 exons and is located on chromosome 17p13.1 while the mouse gene, which also contains 11 exons, is located on chromosome 11 (Soussi & May, 1996). In both human and mouse, the coded protein is of 53kD, the human protein containing 393 amino acids and mouse protein 390 amino acids. The p53 protein (Fig.17) consists of an acidic N-terminus with a transactivation domain, a hydrophobic central DNA-binding core and a basic C-terminus with regulatory and oligomerisation domains (Hainaut &
Figure 17: Structural features of the p53 tumor suppressor gene. The transcription activation site (TAS), heat shock protein binding site (HSP), SV40 large T-antigen binding sites (SV40), adenovirus E1b and papilloma virus E6 binding sites, cellular Mdm2 binding site, nuclear localization signal (NLS), oligmerization domain (OLIGO) and phosphorylation sites (cdc2 and CDK). The five evolutionarily conserved domains are labeled HCD I - V and the hot spot regions are HSR A - D.
The DNA binding domain of the p53 protein is composed of two beta sheets and a zinc atom, which stabilizes the structure (Cho et al, 1994).

p53 mutations are likely to be a primary tumor-initiating event in those humans and animals which inherit p53 mutations (Harvey et al, 1993; Malkin, 1994). In mouse skin model of cancer development, mutations of p53 and loss of heterozygosity (LOH) on chromosome 11 were found in about 25% of carcinomas, and in low frequencies in benign papillomas (Burns et al, 1991). Mutations detected in the p53 gene were found to be dependent upon the treatment regimen used (Brown et al, 1995). Tumors induced by initiation with DMBA and TPA promotion exhibited typical loss of function mutations in p53, such as frame shift mutations, deletions and point mutations introducing stop codons, in addition the wild type allele was lost (Ruggeri et al, 1991). In contrast, carcinomas induced by repeated carcinogen treatment using DMBA or the methylating agent MNNG showed carcinogen specific alterations in p53 (Ruggeri et al, 1991). The p53 mutations detected in carcinomas, induced by DMBA or MNNG treatment, mostly G to A transition was detected. Loss of remaining wild type allele was not observed in these tumors. Similarly, no p53 abnormalities were detected in early stage papillomas cell lines but 50% of late stage tumors cell lines had protein abnormalities (Ruggeri et al, 1991). The literature survey shows that the p53 mutations, play an important role in skin tumor development in the progression rather than initiation or promotion stage (Conti et al, 1991; Ruggeri et al, 1991).

The p53 protein normally controlling the integrity of the genome is, wild type p53 (wtp53) and acts as a safe guard against cancer. However, mutational events can lead to the alterations in the p53 genes thus resulting in expression of mutant p53 (mutp53). In the course of studying the mechanism(s) by which wtp53 functions as a tumor suppressor, several biochemical properties and activities of wild type and mutant p53 protein have been documented, confirming their role in maintaining the balance between normal to neoplastic cells (Zambetti & Levine, 1993). In general, wtp53 protein has a short half-life of almost 30 min (Gronostajski et al, 1984), inhibits the growth of tumor cells in culture (Chen et al, 1990; Mercer et al, 1991) and inhibits the transformation of primary fibroblasts by cooperating oncogenes (Elihayu et al, 1989; Finlay et al, 1989). wtp53 protein displays a non-specific and specific DNA binding activity, which is required for its function in transcriptional transactivation (El-Deiry et al, 1992; Funk et al, 1992). This protein can form complexes with SV 40 large T antigen (Lane & Crawford, 1979; Linzler & Levine, 1979) as well as with
Modulatory Effects of DAS on p53 & p21/waf1

several other DNA tumor virus oncogenic products (Sarnow et al., 1982; Werness et al., 1990). In contrast mutp53 has a comparatively long half life of several hours (Hinds et al., 1990; Iggo et al., 1990), fails to suppress cell growth of tumor cells (Baker et al., 1990; Diller et al., 1990) and does not inhibit the transformation of primary fibroblasts by cooperating oncogenes (Elihayu et al., 1989; Finlay et al., 1989). The hot spot mutp53 proteins do not complex with SV 40 large T antigen (Chen et al., 1990).

The primary functions of the wtp53 are inhibition of abnormal growth of cells (Sionov & Haupt, 1999), DNA repair (Albrechtsen et al., 1999), cell cycle control (Stewart & Pietenpol, 2001), inhibition of angiogenesis (Vogelstein et al., 2000) and triggering of programmed cell death (Heinrichs & Deppert, 2003). Because these processes ensure genomic integrity or destroy the damaged cell, p53 gene has been called the “guardian of the genome” (Lane, 1992). p53 is a sequence specific nuclear transcription factor, that affects its target genes either by transcriptional activation or by modulating other protein activities by direct binding (El-Deiry, 1998; Murphy et al., 1999; Guimaraes & Hainaut, 2002). The p53-induced activation of target genes may result in the induction of growth arrest or apoptosis (Heinrichs & Deppert, 2003) (Fig. 18). Several biochemical activities have also been ascribed to p53 that may mediate the biological effects of the protein (Albrechtsen et al., 1999; Vousden & Lu, 2002).

One of transcriptionally upregulated gene by p53 is the waf1. The protein product of waf1 is the p21 protein, the first cyclin dependent kinase (CDK) identified (El-Deiry et al., 1993; Harper et al., 1993; Noda et al., 1994). The p21 protein is suggested to be the mediator of tumor suppressing activity of p53 through inhibition of CDK (Deng et al., 1995; Del Sal et al., 1996). The p21 is also known to be the regulator of CDK activity and to be induced in relation to cellular senescence (Harper et al., 1993; Noda et al., 1994). p21 expression is shown to increased in different cell types in response to a wide variety of stressful stimuli, including DNA damaging drugs or ionizing radiation, agents affecting DNA replication or mitosis, TGFβ, differentiating agent or oncogenic ras (El-Diery et al., 1993, 1994; Datto et al., 1995; Ahmad et al., 1998). The response to some of these factors (e.g. DNA damage or mutant ras) is mediated primarily through transcriptional activation of the p21/waf1 gene by p53 (El-Diery et al., 1993, 1994). Other p21 inducers such as TGF-β induces p21/waf1 in a p53 independent manner (Datto et al., 1995). The activation of p53 causes induction of p21/waf1, which in turn inhibits CDK cyclin activity and arrests
Figure 18: Schematic representation of the pathways following DNA damage which leads to p53 accumulation and subsequent changes in gene expression and protein–protein interactions.
the cell cycle at G1 or G2 cell cycle checkpoints (Colman et al, 2000; Taylor & Stark, 2001; Winters, 2002) This arrest gives time for DNA repair before replication or mitosis and thus links \( p21 \) directly to the tumor suppressor functions of \( p53 \).

Thus, therapeutic strategies targeting the oncogenic consequences of \( p21/waf1 \) expression may provide a new approach to chemoprevention and treatment of cancer. Being central to the pathways regulating cellular responses to DNA damaging agents, modulation of \( p53 \) and its effector molecules such as \( p21/waf1 \) levels could serve as an effective way of intervention against cancer (Conti & Gimenez-Conti, 1996; Mukhtar & Ahmad, 1999; Sherr & Roberts, 1999). Many chemopreventive natural food components and commonly used chemotherapeutic agents are known to exert their anticancer properties through the regulation of \( p53 \) dependent pathways (Lotem & Sachs, 1993; Schwartz et al, 1993; Dong, 2003). The antitumor activity of resveratrol was shown to occur through extracellular signal regulated protein kinases and p38 kinase mediated \( p53 \) activation and induction of apoptosis (She et al, 2001; Dong, 2003). Inhibition of BaP induced lung tumorigenesis in A/J mice by dietary N-acetylcysteine conjugates of benzyl and phenethyl isothiocyanates during the post-initiation phase is associated with activation of mitogen activated protein kinases and \( p53 \) activity and induction of apoptosis (Yang et al, 2002). Similarly, \( p53 \) has been implicated in the anticancer mechanism of vitamin E (Schwartz et al, 1993), retinoids (Shin et al, 1997), capsaicin (Kim et al, 1997) and n-butyrate (Mahyar-Roemer & Roemer, 2001).

Studies on the molecular mechanism of tumor suppression by \textit{allium} derivatives are very few. In the only study reported for implicating \( p53 \) and \( p21/waf1 \), it was shown that DAS, DADS and garlic extract are effective in reducing the expression of anti-apoptotic genes (such as \( bcl-2 \)) and the induction of pro-apoptotic genes (such as \( bax \)) and the tumor suppressor gene \( p53 \) in non-small cell lung cancer cell lines (Hong et al, 2000). In the present set of investigations, we studied the role of tumor suppressor protein \( p53 \) and its effector molecule \( p21/waf1 \) in DAS mediated growth inhibition of DMBA induced mouse skin tumors.

**Material and Methods**

**Materials:** DMBA and DAS were purchased from Sigma (St Louis, USA). Anti-p53 antibody specific for wild type protein (clone PAb 1620, Ab-5) and polyclonal \( p21/
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waf1 (Ab-5) were procured from Oncogene research products (Cambridge, MA, USA). Mammalian mutp53, (Clone PAb 240) was obtained from Boehringer Mannheim (Germany). The horseradish conjugate isotypes were obtained from Banglore Genei (India) and the FITC conjugates were procured from Becton Dickinson (USA). The nitrocellulose membranes were obtained from Sartorious, (Germany). The rest of the chemicals were of analytical grade of purity and were procured locally.

Animal Bioassay: The mouse skin tumors were developed in Swiss albino mice using DMBA as a carcinogen. The tumors were developed on the dorsal skin of mice as described in detail in Material and Methods, Chapter 1 of this dissertation. The tumors so obtained were kept frozen (-80°C) till further experimentation.

Preparation of Nuclear Fractions Containing p53/p21: The skin/tumor tissue was removed and subcutaneous fat was scrapped off on ice. The nuclear fraction was prepared as per the method of Serpi et al (1999). Briefly, skin/ tumor tissue was chopped finely with the help of sterilized scissors and surgical blades, and 10% (w/v) homogenate was prepared by using Ultra-Turrax, homogenizer (Germany) in lysis buffer (containing 20% glycerol, 20mM HEPES, 10mM NaCl, 1.5 mM MgCl₂, 0.1% Nonidet P40, 0.2mM EDTA, 1mM DTT, 1μg/ml Pepstatin A, 1μg/ml Aprotinin and 100 μg/ml PMSF) on ice, and then centrifuged in a refrigerated centrifuge (M. B. Cooling, India) for 5 min at 2000 rpm. To the pellets, ~1ml of nuclear lysis buffer (500mM instead of 10mM NaCl) was added, mixed vigorously with the help of micropipette and incubated for 30 min in an ice bath. The samples were then centrifuged at 15000 rpm for 15 min at 4°C and the supernatant (nuclear extract) was collected and freeze in aliquots at -80°C until further used.

Protein Estimation: The protein estimation was performed routinely by the Folin-Phenol method of Lowry et al (1951). Peptide bonds form a complex with alkaline copper sulfate reagent, which gives a blue color with Folin's reagent. Briefly, 0.1 ml of the homogenate (10% w/v) was diluted to 1ml with distilled water. Then 5.0 ml of alkaline copper reagent (containing sodium carbonate (2%), CuSO₄ (1%) and sodium potassium tartarate (2%) in 1N NaOH) was added. Following 10 min after addition of alkaline copper sulfate reagent to allow complex formation, 0.5 ml of Folin's reagent (1:1 diluted with distilled water) was added. After 30 min the blue color developed, was read at 660nm. For standard bovine serum albumin (0.1 mg/ml) was used.
Western blotting: Western blotting was carried out as described by Towbin et al (1979). The proteins were separated electrophoretically (Laemmli, 1970) in a 10% polyacrylamide gel at constant voltage (80V) and transferred to nitrocellulose membrane for 2hrs. at constant voltage (300 V). Specific molecular weight standards (Sigma, USA) were also run in one lane in each gel. To avoid unspecific binding the membrane was incubated overnight at 4°C with TBS (1.29 gms Tris, 9.0 gms NaCl; in one liter distilled water, pH 7.4) containing 5% non-fat dry milk powder. The complete transfer of the proteins to the membrane was confirmed by staining the polyacrylamide gels with 0.1% coomassie brilliant blue dye (in propanol:acetic acid: distilled water, 20:12.5:67.5 ml v/v). The membrane was then incubated for 2 hrs at 4°C with appropriate antibodies i.e. anti-p53 wild type protein (0.25 μg/ml) (Clone PAb 1620), mutant anti-p53 protein (0.25 μg/ml) (Clone PAb 240) and polyclonal anti-

\[ p21/waf1 \]

(0.25 μg/ml) at dilutions recommended by the suppliers in 1% milk-TBS-T (0.05% Tween 20 in TBS). The blots were then washed five times for 10 min each in TBS-T. The blots were then conjugated with horse-radish peroxidase labeled anti-goat secondary antibody at 4°C (dilution 1:5000) for 6 hrs. After incubation, the blots were again washed five times for 10 min each with TBS-T and finally with TBS for 10 min. The complex formed by the protein and the antibody was detected using chromagen 3,3'-diaminobenzidine tetrahydrochloride (DAB) in 0.05M Tris, pH7.3 containing 30% (v/v) hydrogen peroxide in dark for 3-5 min. After staining blots were washed with distilled water, dried and recorded.

Densitometric Analysis of Western blots: The intensity of the bands was quantitated using Easy Win 32 software on Gel Documentation System (Herolab, Gmbh, Germany). The intensity of each band was expressed in terms of pixel density for each band.

Immunohistochemistry: For immunohistochemistry, the frozen skin/tumor tissues were taken and sections of 10μm thickness were cut using cryostat microtome (Reichert-Jung, USA). The sections were fixed in neutral 10% formalin and the endogenous peroxidase activity was quenched with methanol: \( \text{H}_2\text{O}_2 \) solution (100 ml: 333 μl). After quenching the non-specific binding was minimized by incubation with normal goat serum (1:30) for 2 hrs. at 4°C. The slides were sequentially incubated with anti \( p53 \) antibody for wild type and mutant and \( p21/waf1 \) antibody in a moist chamber with for 72 hrs. After the incubation period, the sections were again
incubated for 2 hrs. with normal goat serum and then with HRP conjugated antigoat IgG for 6 hrs. at 4°C. The slides were washed with PBS between incubations and after a final rinse with PBS the slides were washed finally with acetate buffer (0.1M, pH 5.2). The color was developed using DAB in Tris buffer (0.05M, pH 7.3) containing H$_2$O$_2$.

**Image Analysis:** The immunostained slides were analyzed and scored, using Leica Image Analysis System, Germany (Fig. 19). The quantitative stereology was performed with Leica QWin 500 Image analysis software, for each slide in triplicates with at least six microscopic fields. The statistical analyses were done by one way ANOVA and P<0.05 was considered significant.

**Preparation of Single Cell Suspensions for Flow Cytometry:** The single cell suspensions of skin/tumor tissue from different treated and control groups for the flow cytometric analysis of p53/p21 protein were prepared as described in *Material and Methods, chapter-1*, using Medimachine (Becton Dickinson, U.S.A).

**Flow cytometric Analysis of p53 protein:** About 1 ml of cell suspension containing ~ 1x 10^6 cells (as counted on haemocytometer) were taken and fixed in chilled 70% ethanol for 30 min in ice bath. After fixation, cells were centrifuged (Tarson, India) at 2000 rpm at room temperature for 5 min. The cells were then permeabilized with 0.2% Triton X-100 for 20 min in ice bath and recentrifuged. The pellets were then resuspended in PBS and incubated with respective p53 antibody viz. anti-p53 antibody wild type protein (clone PAb 1620) and mutant anti p53 protein (clone PAb 240) (2|µg/ml in PBS containing 1% BSA) for 1 hr at room temperature. After incubation the cells were again pelleted, washed once with PBS and treated with FITC-conjugated isotype specific secondary antibody for detecting the respective protein levels. Cells were then analyzed on a flow cell cytometer (BD-LSR, Beckton Dickinson, USA) equipped with 488 nm Argon laser light source. Total 10,000 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.

**Flow cytometric Analysis of p21/waf1 protein:** The flow cytometric analysis of p21 protein was done as described above for p53 with the exception that the primary antibody used was polyclonal anti-p21/waf1 (2µg/ml in PBS containing 1% BSA).
**Figure 19**: Leica QWin Image Analysis System
**Statistical analysis:** Statistical analysis of the data was performed with student 't' test and ANOVA. P< 0.05 was considered significant.

**Results**

The results of the present investigations showed a modulatory effect of DAS on the expression of tumor suppressor gene p53 (both wild and mutant type) and its effector molecule p21/waf1 in mouse skin tumors. Thus the chemopreventive property of DAS seems to be through the p53 dependent mechanism. The results are summarized below:

**Effect of DAS on the expression of wtp53:**

The levels of wtp53 protein were over-expressed in tumors obtained by DMBA application (Gr. II) in comparison to normal mouse skin (Gr. I), where this protein was found in detectable limits (Fig. 20, lanes a & b). A comparatively high level of expression of this protein was observed in tumors of DAS supplemented groups (Gr. III & Gr. IV), indicating that wtp53 protein plays an important role in the inhibition of neoplastic changes. However, no significant change in the protein level was observed in tumors where DAS was given prior or after the carcinogen application (Fig. 20, lanes c & d). In Gr. V where only DAS was applied to mouse skin, levels of wtp53 were comparable to the normal levels (Fig. 20, lanes a & e).

These qualitative observations were confirmed by quantitating the blots by densitometry (Fig. 20). The normal wtp53 level recorded by densitometry was 845 pixels. An increase of 132% in the pixel density of wtp53 was observed in tumor tissue of DMBA exposed animals (Gr. II). However, a further significant increase (P< 0.05) in the wt p53 protein was recorded in DAS supplemented and DMBA induced tumors. In the tumors where DAS was given prior to DMBA (Gr. III), the expression of wtp53 was 224% more over control group. Similarly, in tumors of Gr. IV (DMBA+DAS) the wtp53 expression was 205% over normal tissue. When the expression of wtp53 was calculated over DMBA induced tumors, this increase was 40% and 31% indicating that wtp53 expression checks the cells to go into neoplastic state.

The quantitative stereology of immuno-stained skin/tumor sections for wtp53, further lended support to our observation that DAS supplementation increases the
Figure 20: Showing western blot and its densitometric analysis of wt*53 expression in mouse skin/tumors in different groups. (lane a: Untreated, b: ACE+ DMBA, c: DMBA+ DAS, d: DAS+ DMBA e: ACE+ DAS).

* Values are significantly different over untreated control, P<0.01
# Values are significantly different over positive control, P<0.05
wtp53 expression, thus attempting to control the cell to acquire the malignant phenotype. The photomicrographs showed a varied degree of wtp53 expression among the different treatment regimen (Fig. 21). In the normal mouse skin 2.68% of the total area showed presence of wtp53. The area showing staining of wtp53 was significantly increased (P<0.05) to 8.03% in DMBA induced tumors (Table 12). In DAS supplemented groups the levels of wtp53 were further increased showing 17.56 and 13.69% of the area positive for wtp53 in Gr. III and Gr. IV respectively. In the DAS exposed skin 2.87% of the area showed the presence of wtp53. The percent increase in the area (mm²/cm²) in the levels of wtp53 was 66.55 and 54.21% respectively in DAS pre and post supplemented group over DMBA exposed group (Table 12).

The results of the flow cytometric analysis further confirmed the findings of western blotting and immunohistochemistry. The FITC fluorescence was recorded in the form of histograms for each group and overlay was prepared showing differential expression of wtp53 (Fig. 22). The mean fluorescence intensity (MFI) for normal mouse skin was 1.68, which increased to 5.31 for DMBA induced tumors (Gr. II). A further increase in the fluorescence was recorded in the DAS supplemented groups (Gr. III & IV) suggesting increase in the levels of wtp53 by DAS. The MFI was 15.41 for DAS+ DMBA and 10.21 for DMBA+ DAS group respectively. In groups where only DAS was given the FITC fluorescence was 1.92, which was close to the normal values. The comparable levels of wtp53 in the normal skin (Gr. I) and DAS exposed skin (Gr. V) shows the nontoxic nature of DAS.

Effect of DAS on the expression of mutp53:

The mouse skin from the animals of control group (Gr. I) showed presence of detectable level of mutp53, which was found to over-expressed in tumors obtained with DMBA application (Fig. 23, lanes a & b). The over expression of mutp53 observed in DMBA induced tumors was significantly low (P<0.05) in tumors were DAS was given along with the DMBA (Gr. III & IV) indicating down regulation of induced levels of mutp53 by DAS (Fig. 23, lanes c & d). However, no significant difference in expression levels of mutp53 was observed in tumors where DAS was given prior and after the DMBA exposure (Fig. 23, lanes c & d).
Table 12: Effect of DAS on the Expression Levels of Oncogenic Proteins in DMBA Induced Mouse Skin Tumors.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Positive Area Fraction (%)</th>
<th>Area (mm²/ cm²) (Mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>wtp53</td>
<td>mutp53</td>
</tr>
<tr>
<td>Gr. I</td>
<td>Untreated</td>
<td>2.68</td>
<td>2.08</td>
</tr>
<tr>
<td>Gr. II</td>
<td>ACE+ DMBA</td>
<td>8.03</td>
<td>14.75</td>
</tr>
<tr>
<td>Gr. III</td>
<td>DAS+ DMBA</td>
<td>17.56</td>
<td>10.23</td>
</tr>
<tr>
<td>Gr. IV</td>
<td>DMBA+ DAS</td>
<td>13.69</td>
<td>11.02</td>
</tr>
<tr>
<td>Gr. V</td>
<td>ACE+ DAS</td>
<td>2.87</td>
<td>2.56</td>
</tr>
</tbody>
</table>

Values in the parentheses are % change over Gr. II
* P<0.05 was significant over Gr. I
** P<0.05 was significant over Gr. II
Figure 21: Photomicrographs of mouse skin/tumors showing immunohistochemical reactivity with wt p53 in different groups (128X). (a) Untreated, (b) ACE+DMBA, (c) DMBA+ DAS, (d) DAS+DMBA, (e) ACE + DAS
Figure 22: An overlay of flow cytometric analysis of *wt* p53 protein expression in mouse skin/tumors. The horizontal axis represents FITC fluorescence versus counts on vertical axis. Gr. I: Untreated; Gr. II: ACE + DMBA; Gr. III: DAS + DMBA; Gr. IV: DMBA + DAS; Gr. V: ACE + DAS
Figure 23: Showing western blot and its densitometric analysis of \textit{mutp53} expression in mouse skin/tumors in different groups. (lane a: Untreated, b: ACE+ DMBA, c: DMBA+ DAS, d: DAS+ DMBA e: ACE+ DAS).

* Values are significantly different over untreated control, $P<0.01$
# Values are significantly different over positive control, $P<0.05$
The quantitation of mutp53 levels by densitometric analysis of blots confirmed the down-regulation of the protein by DAS. The intensity of band in normal skin was recorded to be 356 pixels. An increase of 318% in the level of mutp53 was observed in tumor tissue of DMBA exposed animals over normal. However, a significant decrease (P<0.05) in the levels of mutp53 was recorded in DAS administrated groups (Gr. III & Gr. IV). In DAS supplemented tumors the expression of the mutp53 was found to decrease by 44.9% in DAS pretreated, whereas in DAS post treated tumors the expression of the protein was decreased by 41.7% over DMBA induced tumors (Gr. II).

The immunohistochemical analysis of mutp53 in different groups showed varied degrees of expression (Fig. 24). In the control mouse skin 2.08% of total area showed presence of mutp53 whereas, in the DMBA induced tumors the mutp53 levels were significantly higher showing 14.75% area stained positive towards mutp53. A significant decrease (P<0.05) in percent area showing the presence of mutp53 was observed in tumors obtained from DAS supplemented groups (Gr. III & Gr. IV). In tumors where DAS was given before the DMBA exposure, 10.23% area was positive. Whereas, in group where DAS was given after the DMBA administration, 11.02% area showed presence of mutp53 (Table 12). No significant difference in the percentage area showing mutp53 levels was obtained in tumors where DAS was given prior to and after the DMBA exposure. The DAS exposed skin showed 2.56% area positive for mutp53, which was found to near to normal levels. The percent decrease in the area (mm²/cm²) showing staining for mutp53 was 53.41 and 44.26% in DAS supplemented groups (Table 12).

The observations of western blotting and immunohistochemistry that DAS inhibits the levels of mutp53 in DMBA induced tumors were further confirmed by flow cytometry (Fig. 25). The normal skin showed low expression levels of mutp53 with MFI of 1.06. In DMBA induced tumors a significant increase in the fluorescence was recorded with MFI 16.41 indicating over expression of mutp53. The FITC fluorescence was found to decrease in DAS supplemented groups with MFI 3.76 in Gr.III and 8.13 in Gr. IV, suggesting down regulation of mutp53 by DAS (Fig. 25). The DAS exposed skin showed levels of mutp53 close to normal levels with MFI 1.65.
Figure 24: Photomicrographs of mouse skin/tumors showing immunohistochemical reactivity with mutp53 in different groups (128X) (a) Untreated, (b) ACE+DMBA, (c) DMBA+ DAS, (d) DAS+ DMBA, (e) ACE + DAS
Figure 25: An overlay of flow cytometric analysis of $mutp53$ protein expression in mouse skin/tumors. The horizontal axis represents FITC fluorescence versus counts on vertical axis. Gr. I: Untreated; Gr. II: ACE+ DMBA; Gr. III: DAS+ DMBA; Gr. IV: DMBA+ DAS; Gr. V: ACE+ DAS.
Effect of DAS on the expression of p21/waf1:

The effect of DAS on the levels of p21/waf1 was also investigated, as it is known to be transcriptionally upregulated by the induced levels of wtp53. The upregulation of p21/waf1 is associated with the tumor suppressor function of the wtp53 and thus might be involved in the molecular mechanism of antitumorigenic properties of DAS. The western blot analysis showed detectable levels of p21/waf1 in normal mouse skin (Fig. 26, lane a). The DMBA induced tumor showed significantly elevated levels of p21/waf1 (Fig. 26, lane b). The DMBA induced levels of p21/waf1 were further found to increased in DAS supplemented groups (Gr. III & IV). However, in tumors where DAS was given prior to and after DMBA, no significant change in the levels of p21/waf1 was observed (Fig. 26, lanes c & d). In Gr. V, where only DAS was given, the levels of p21/waf1 were comparable to that of the normal levels (Fig. 26, lanes a & e).

These qualitative results were also quantitated by densitometric analysis of the blots. The normal p21/waf1 level recorded by densitometry was 478 pixels. An increase of 72% in the pixel density of p21/waf1 was observed in tumor tissue of DMBA exposed animals (Gr. II). A further significant increase in the levels of p21/waf1 was recorded in the tumor tissue of the animals where DAS was given along with the DMBA exposure. In the DAS pretreated tumors, the expression of p21/waf1 was 200% more over control group (Gr. I). Similarly in the DAS post treated group (Gr. IV), the p21/waf1 expression was 184% higher over normal skin (Gr. I). When the expression of p21/waf1 was calculated over positive control (Gr. II), the increase in the levels were 74 and 65% in DAS+DMBA and DMBA+DAS groups respectively. This suggests that DAS induces levels of p21/waf1.

The results of western blotting were confirmed by immunohistochemical detection of the p21/WAF1 expression in different treatment regimen (Fig. 27). The image analysis of the immunostained section of normal skin showed 1.59% area positive for p21/waf1. In the DMBA induced tumor the total area showing presence of p21/waf1 increased significantly as compared to normal levels. In DMBA induced tumors 5.09% of the total area showed the presence of p21/waf1. In DAS supplemented groups, the total area showing p21/waf1 expression increased further to 7.09% and 9.12% in DMBA+DAS and DAS+DMBA groups respectively (Table 12). In DAS exposed skin 1.46% area showed the expression of p21/waf1, which was found to be similar to that of the normal levels suggesting nontoxic nature of DAS.
Figure 26: Showing western blot and its densitometric analysis of *p21/waf1* expression in mouse skin/tumors in different groups. (lane a: Untreated, b: ACE+ DMBA, c: DMBA+ DAS, d: DAS+ DMBA, e: ACE+ DAS)

* Values are significantly different over untreated control, *P*<0.01

# Values are significantly different over positive control, *P*<0.05
Figure 27: Photomicrographs of mouse skin/tumors showing immunohistochemical reactivity with p21/waf1 in different groups (128X). (a) Untreated, (b) ACE+DMBA, (c) DMBA+DAS, (d) DAS+DMBA, (e) ACE + DAS
The percent increase in the area (mm^2/cm^2) positive for p21/waf1 levels was 72.86 and 61.22% in groups where DAS was given before or after the DMBA exposure in comparison to group where DMBA was given alone (Table 12).

The flow cytometric analysis of p21/waf1 expression in different experimental and control groups provided further support to our observations. A significant increase in the fluorescence was observed in the DMBA induced tumors as compared to the normal levels indicating over expression of p21/waf1 in Gr. II. The shift in the fluorescence was from 6.34 for untreated to 22.42 for DMBA induced tumors (Fig. 28). This shift in the fluorescence was further enhanced in DAS treated tumors. In tumors where DAS was given before the DMBA exposure, MFI of 53.35 was recorded. Similarly, in tumors where DAS was given after DMBA exposure, MFI was 44.24. In group where animals were given DAS only, the fluorescence was recorded at 8.07 channel value indicating normal levels of p21/waf1 expression.

Discussion

The tumor suppressor gene p53 is regarded as a key factor in maintaining the balance between cell growth and cell death in the living system (Agarwal et al, 1998; Mowat, 1998). The importance of this gene can be drawn from the fact this gene is reported to be mutated in about 80% of the all human malignancies (Hollstein et al, 1991). Due to its importance in regulation of cell cycle, alterations in p53 are critical events in carcinogenesis. The wtp53 was found to be polymorphic with a single nucleotide polymorphism in the exon 4 in codon 72 (Matlashewski et al, 1987). The substitution of guanine to cytosine changes the resultant amino acid from arginine to proline, thus leading to the expression of mutp53. This altered protein is biologically and biochemically differs in nature (Thomas et al, 1999; Martin et al, 2000). The wtp53 in response to toxic insults to DNA triggers a chain of cell cycle regulatory events to check the proliferation of altered cells to repair or minimize the damage (Agarwal et al, 1998; Mowat, 1998). In tumors, loss of wtp53 function prevents the activation of this growth control pathway (Burns et al, 1991). This failure to induce transcriptionally active wtp53 play a role in the unregulated growth of the tumors and also in the failure to respond to chemotherapeutic agents, which normally trigger wtp53, regulate cell arrest or death (El-Diery et al, 1994).

Studies have shown the accumulation of both wt and mutp53 following exposure with known carcinogens such as DMBA and BaP (Schwartz & Shklar,
Figure 28: An overlay of flow cytometric analysis of p21/waf1 protein expression in mouse skin/tumors. The horizontal axis represents FITC fluorescence versus counts on vertical axis. Gr. I: Untreated; Gr. II: ACE+ DMBA; Gr. III: DAS+ DMBA; Gr. IV: DMBA+ DAS; Gr. V: ACE+ DAS
1996; Ember et al, 1998, Serpi et al, 1999). In consistent with these findings, in the present study, the DMBA exposure was found to induce the levels of \( wtp53 \) in mouse skin tumors (Fig. 20-22). DAS supplementation further increased the levels of \( wtp53 \) in DMBA induced mouse skin tumors. The increase in the \( wtp53 \) expression was consistent in all the three methods viz. western blotting, flow cytometry and immunohistochemistry employed for the detection of \( p53 \), thus increasing the credibility of the results. The immunohistochemical staining of the skin tumors revealed an increase of 66 and 54% in the levels of \( wtp53 \) following pre and post DAS supplementation (Table 12). The importance of immuno-localization further indicates that DAS increases the stability of the \( wtp53 \). The densitometry of western blots shows an increase in DAS supplemented tumor cells by 40 and 31% respectively (Fig. 20). This increase in immunoreactivity to the \( wtp53 \) has also been shown in various other studies for evaluating the chemopreventive potential of natural products (Schwartz et al, 1993; Schwartz & Shklar, 1996; Narayanan et al, 1998). These immunohistochemical observations and increased expression of \( wtp53 \) in western blots suggesting that DAS exerts its chemopreventive property by modulating \( wtp53 \) expression in mouse skin tumor cells (Fig. 20 & 21). In case of \( mutp53 \) an increased expression was recorded in the tumor cells from DMBA induced mouse skin tumors mainly because of the malignant characteristics of tumors (Fig. 23-25). The administration of DAS along with DMBA was able to check the levels of \( mutp53 \) expression as evident in all the three methods employed in the present investigation. The immunohistochemical localization of the \( mutp53 \) showed 53 and 44% decrease in the levels of the protein following DAS supplementation (Fig. 24). A shift in fluorescence towards the affinity to \( mutp53 \) was evident in DAS supplemented tumors (Fig. 25). Thus, it is likely that DAS suppresses the growth of DMBA induced tumors by increasing the levels of \( wtp53 \) and down regulating the levels of \( mutp53 \) as observed with other chemopreventive agents e.g. carotenoids, retinoids etc. (Schwartz, 1999).

Since the balance between the \( wt \) 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 showing upregulation of \( wtp53 \) and suppressing the expression of \( mutp53 \) are 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 \( wtp53 \) (Schwartz et al,
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Similarly retinoids, including vitamin A and its synthetic analogs viz. all trans and 9-cis retinoic acid, 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. (Narayanan et al, 1998; Sun et al, 2002). 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 (Schwartz, 1999). Supplementation of 10 mg/kg reduced glutathione by oral intubation in Syrian Golden hamsters, has been shown to inhibit the experimental oral carcinogenesis by increasing the levels of wtp53 in DMBA induced dysplasia and squamous cell carcinoma (Schwartz & Shklar, 1996). Certain other chemopreventive agents such as I3C, tea polyphenols EGCC and soy isoflavone genistein have been shown to inhibit BaP induced aberrant proliferation through the induction of p53 dependent apoptosis in human mammary epithelial cells (Katdare et al, 1998).

The upregulation of wtp53 by chemopreventive agents is most likely responsible for the transcriptional induction of p21/waf1 by directly interacting with its regulatory elements (El-Diery et al, 1994). Thus, the upregulation 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. The levels of p21/waf1 were increased upto the extent of 73 and 65% in DAS pre and post supplemented groups as observed in immunohistochemical staining (Fig. 27). The increase in the levels of p21/waf1 was also consistent in western blotting and flow cytometry (Fig. 26 & 28). However, further insights into the effect of DAS on CDK complexes operated by Cdk2 and Cdc2 (Cdk1) and cell cycle checkpoints are required.

The wtp53 is reported to be upregulated in the cells by its increased half-life through inhibition of its degradation time (Harris, 1996; Colman et al, 2000) as well as modulation of its stability by post-translational events such as phosphorylation and acetylation (Kapoor & Lozano, 1998; Oshiro et al, 2003). Therefore, it is likely that upregulation of wtp53 by DAS can occur via a similar mechanism of action. The upregulation of wtp53 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 (El-Deiry, 1998; Murphy et al, 1999; Guimaraes & Hainaut, 2002). 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 (Sai-
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Kato et al, 1995; Wu et al, 2001; Shukla et al, 2003) as oxidants produced during tumorigenic 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 protooncogenes that may function together with mutp53.

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 (Mowat, 1998; Heinrichs & Deppert, 2003). Many chemopreventive agents are known to exert their anticancer effects through the induction of apoptosis via p53 dependent mechanisms. Shin et al (1997) have shown the accumulation of p53 protein and retinoic acid receptor beta as a mechanism of chemopreventive efficacy of retinoids in clinical settings. Resveratrol, a natural chemopreventive phytoalexin of wine and grapes, is known to suppress cell transformation and induce apoptosis through a p53 dependent pathway (Huang et al, 1999; She et al, 2001). Capsaicin, a principal pungent ingredient from hot red and chili peppers has been shown to induce apoptotic death of cultured human gastric cancer cells mediated via over expression of the p53 tumor suppressor gene and/or c-myc proto-oncogene (Kim et al, 1997). Similarly DADS, has been shown to induce apoptosis and non-steroidal anti-inflammatory drug (NSAID) activated gene (NAG-1) protein expression, via p53 dependent mechanisms in human colorectal HCT 116 cells (Bottone et al, 2002). Similarly, Hong et al (2000) have shown that the mechanism of apoptosis induced by organoallyl sulfur compounds such as DAS, DADS or garlic extract was 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 (Arora & Shukla, 2003a). Thus on this basis and results of the present chapter of this dissertation a correlation between the tumor suppressor p53 and apoptosis in the anticancer mechanism of DAS could be established. The upregulated wtp53 by DAS induces levels of p21/waf1, which in turn may cause tumor cells to undergo apoptosis.

Thus, from the present study, it can be concluded that dietary agents like DAS can act to up-regulate wtp53 and p21/waf1 and down-regulate of mutp53 to provide protection in cancer development and retard malignancy.