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Induction of Apoptosis and Transient Increase of Phosphorylated MAPKs by Diallyl Disulfide Treatment in Human Nasopharyngeal Carcinoma CNE2 Cells

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This study was undertaken to elucidate the effect of diallyl disulfide (DADS), an oil-soluble organosulfur compound found in garlic, in suppressing human nasopharyngeal carcinoma cells. A potent increase (of at least 9-fold) in apoptotic cells has accompanied 1) a decrease in cell viability, 2) a increase of the fraction of S-phase cells by up to 63.8%, and 3) a transient increase of the phospho-p38 and phospho-p42/44 (phosphorylated p38 MAPK and phosphorylated p42/44 MAPK) in a time- and concentration-dependent manner. These results indicate that DADS can induce apoptosis in human nasopharyngeal carcinoma cells via, at least partly, S-phase block of the cell cycle, related to a rise in MAPK phosphorylation.

Key words: Diallyl disulfide (DADS), Anticancer effect, Apoptosis; Cell cycle, Phosphorylated MAPKs, Human nasopharyngeal carcinoma CNE2 cells

INTRODUCTION

Diallyl disulfide (DADS) from garlic (*Allium sativum*) has been shown to have an antiproliferative effect on human tumor cells including those of colon, lung, skin, breast and liver origins (Hong *et al.*, 2000; Kwon *et al.*, 2002; Nakagawa *et al.*, 2001; Sundaram and Milner, 1996; Wen *et al.*, 2004). Although the role and mechanism of DADS as an anti-cancer agent have not been completely elucidated, DADS has been demonstrated to induce cell-cycle arrest (G2/M block) and apoptosis in some cultured cell lines, such as leukemia HL-60 (Kwon *et al.*, 2002), neuroblastoma SH-SY (Filomeni *et al.*, 2003), human colon tumor HCT-15 (Knowles *et al.*, 1998, 2003), and human colon adenocarcinoma HT-29 cell lines (Robert *et al.*, 2001).

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Cell-cycle arrest and apoptosis play essential roles as protective mechanisms against neoplastic development by eliminating genetically-damaged cells or improperly-produced excess cells. The maintenance of homeostasis in normal mammalian tissues by apoptosis reflects a critical balance between cell proliferation and cell loss, and inappropriate regulation of apoptosis is associated with a variety of diseases (Kolb, 2000; Meinhardt *et al.*, 1999; Thornberry and Lazebnik, 1998). In particular, the failure of dividing cells to initiate apoptosis in response to DNA damage has been implicated in the development and progression of cancer (Meinhardt *et al.*, 1999).

A number of reports have described activation of the components of the MAPK pathway during apoptosis induced by Taxol, inducible nitric oxide synthase (iNOS) or DADS (Iwai-Kanai *et al.*, 2002; McDaid and Horwitz, 2001; Wen *et al.*, 2004). The MAPK pathway is central to the control of growth, differentiation, and survival (Pouyssegur and Lenormand, 2003.). It is activated in response to a diverse range of stimuli including growth factors, hormones, neurotransmitters, and cell stressors. So far, c-Jun NH_2-terminal kinases (JNKs), p38-MAPK,

AKT, and p42/44-MAPK have all been identified as MAPK subfamilies (Berra *et al.*, 1998; Bonni *et al.*, 1999; Dent *et al.*, 1998; Widmann *et al.*, 1999). P38-MAPK is more commonly activated in response to cytokines, stress, and cellular damage; while p42/44-MAPK is activated by growth stimuli. Both are generally considered to be pro-survival mediators (Flamigni *et al.*, 2001; Mendelson *et al.*, 1996; Niisato *et al.*, 1999). Several studies have described cellular redox state, tyrosine kinases, and phosphatases to be involved in the activation of stress responses, and these represent the activation by phosphorylation of various components of the MAPK family in different cell models after treatment with paclitaxel or TGF-1 (Lieu *et al.*, 1998; Shtil *et al.*, 1999; Stone and Chambers, 2000; Wang *et al.*, 1999; Yujiri *et al.*, 1999; Vinals and Pouyssegur, 2001). Our previous reports show that both p38-MAPK and p42/44-MAPK produce cytoprotective effects in human hepatoma cells (Wen *et al.*, 2004). However, the role of MAPKs in inducing tumor apoptosis is not entirely clear (Amato *et al.*, 1998; Huang *et al.*, 1999; Lee *et al.*, 1998; Wen *et al.*, 2004; Wang *et al.*, 1998; Yujiri *et al.*, 1998).

In this report, we find that DADS induces cell cycle arrest and transient phosphorylation of p38-MAPK and p42/p44–MAPK, while inducing apoptosis in human nasopharyngeal carcinoma CNE2 cells.

MATERIALS AND METHODS

Materials

Diallyl disulfide (80% purity) was purchased from Fluka Co. BSA and Hoechst 33258 were purchased from Sigma Chemical Co. RPMI1640 medium was purchased from Gibco. Specific anti-phospho- and the cocktail of anti-total-p38 and anti-total-p42/44 antibodies, and goat horseradish peroxidase (HRP)-conjugated secondary antibody were purchased from Santa Cruz Biotech. The Phototope-HRP Western Detection Kit was purchased from New England Biolabs.

Cell culture

CNE2, a human nasopharyngeal carcinoma cell line, was cultured in RPMI1640 with 10% heat–inactivated fetal bovine serum (FBS), benzylpenicillin (100 kU/L), and streptomycin (100 mg/L) at 37°C in humidified air with 5% CO_2.

Cell viability assay

To assess the cytotoxic effects of DADS in CNE2 cells, we used a tetrazolium salt, MTT reduction assay (Hansen *et al.*, 1989). In this assay, MTT is used as a colorimetric substrate for measuring cell viability. When cells are injured there is an alteration in cellular redox activity such that cells are unable to reduce the dye. Cells were plated in 24-well plates and grown to 80% confluence. The cultures were then rinsed in phenol-free RPMI1640 media and incubated with specific test substances in phenol- and serum-free RPMI1640 for 24 h. At the end of this time, MTT was added to a final concentration of 0.5 g/L. After 1 h incubation, cultures were removed from the incubator and the formazan crystals were solubilized by adding 10% (v/v) Triton X-100 and HCl 0.1 μmol/L in isopropanol equal to the volume of original culture media. Colorimetric determination of the reduced MTT was made at 570 nm.

Cell cycle analysis

CNE2 cells were grown to 60% confluence and the culture media was changed to a complete medium with final concentrations of 0 μmol/L, 50 μmol/L, 100 μmol/L, and 150 μmol/L DADS, and then continued for 24 h. Afterwards, cells were harvested and fixed in 70% ethanol at 4°C for 24 h. Immediately before analysis the fixed cells were treated with RNase (100 mg/L) at 37°C for 30 min, and then propidium iodide (10 mg/L) added. After incubating at 4°C for 30 min in darkness, samples (3×10^4 cells for each assay) were analyzed for DNA content using an Epics ALTRA (Beckman-Coulter) flow cytometer and proprietary software.

Apoptosis analysis

Two methods were used to detect apoptosis induced by DADS. First, after treatment with or without DADS in phenol- and serum-free RPMI1640 for 24 h, morphological changes of CNE2 cells were observed by fluorescence microscopy after Hoechst 33258 staining. Apoptosis was routinely determined by counting the numbers of cells with condensed or fragmented chromatin as described previously (Bates *et al.*, 1994). Six randomly chosen fields of view were examined with a minimum number of 500 cells scored for each condition. Secondly, a flow cytometric assay by Epics ALTRA (Beckman-Coulter) was used to analyze the fraction of apoptotic cells as described above.

Preparation of lysates

For MAPK detection, CNE2 cells cultured in 6-well culture plates were grown to 80-90% confluence, then nutrient-starved for 24 h in serum-free RPMI1640. Various concentrations of DADS were added for the indicated times. After three washes with ice-cold PBS, cells were lysed with 60 L of ice-cold lysis buffer containing (in μmol/L) NaCl 50, Na_3VO_4 2, $Na_4P_2O_7$ 10, NaF 100, phenylmethylsulfonyl fluoride 1, DTT 2, and HEPES 50 at pH 7.5, along with 0.01% Triton X–100, leupeptin 20 μmol/L, and aprotinin 1×10^5 U/L. The lysates were obtained by centrifugation at 13,000×*g* at 4°C for 10 min and the concentration of total cell protein was determined by spectrophotometry

(Bradford, 1976).

Western blot analyses

SDS sample buffer containing Tris–HCl 0.33 μmol/L, SDS 8% (w/v), glycerol 40% (v/v), and bromophenol blue 0.4% was added to cell lysates. After boiling the extracted protein for 5 min, 20 g was resolved by SDS–PAGE. The protein was transferred to a nitrocellulose membrane which was then blocked at 25°C for 1 h with 5% BSA in TBST (Tris–HCl 20 μmol/L, pH 7.5, NaCl 137 μmol/L containing 0.1% Tween-20). The blots were incubated with the primary antibodies against phospho-p38, phospho-p42/p44, total-p38, or total-p42/p44 at 1:1000 dilutions at 4°C overnight, followed by incubation at room temperature for 1 h with secondary antibody (horseradish peroxidase conjugated) at 1:2000 dilution. Immunoreactive signals were visualized by the Phototope Western Detection System (New England Biolabs). As a protein loading control, two gels for each group were loaded in parallel with the same protein samples and blotted for activated/phosphorylated MAPKs or total MAPKs. Bands of MAPK were quantitatively determined by QuantiScan version 2.1 (Biosoft).

Statistics

Results are expressed as means±SEM with N being the sample size. Statistical analysis was performed on SPSS statistical package v7.1 (SPSS Inc). Values of $P<0.05$ were considered statistically significant, using a 2-tailed t-Test.

RESULTS

Induction of apoptosis by DADS on human nasopharyngeal carcinoma CNE2 cells

To test the effect of DADS on the viability of CNE2 cells, the MTT conversion assay was used. Exposure of CNE2 cells to DADS at concentrations of 50 μmol/L, 100 μmol/L, and 150 μmol/L for 24 h decreased cell viability by 10.1%, 13.9% and 22.3%, respectively, compared with untreated cells (Table I). This result suggests that DADS is cytotoxic to CNE2 cells in a concentration-dependent manner.

Table I. Effect of DADS on the growth of CNE2 cells

DADS (μmol/L)	MTT absorbance (570 nm)	Normalized cell viability (%)
0	$0.755\pm5.77\times10^{-3}$	–
50	$0.679\pm6.81\times10^{-3}$	10.01
100	$0.650\pm1.00\times10^{-3}$	13.91
150	$0.587\pm5.76\times10^{-3}$	22.25

Human CNE2 cells were treated with DADS for 24 h, and their cell viability was determined. Cell viability was normalized to control (0 μmol/L DADS). Data are shown as means±SEM, N=3 experiments. P<0.01 vs control.

To test whether DADS induces apoptosis in CNE2 cells, DADS-treated CNE2 cells were examined by fluorescence microscopy after staining with Hoechst 33258. After exposure to DADS (100 μmol/L) for 24 h, CNE2 cells showed typical morphologic changes of apoptosis: cell volume was reduced, chromatin became condensed, and nuclei became fragmented (Fig. 1B), compared with untreated cells (Fig. 1A). Similar to DADS-induced cytotoxicity, DADS also induces apoptosis of CNE2 cells in a concentration-dependent manner. Treatment with 150 μmol/L DADS for 24 h increased the number of apoptotic cells (measured by cell counting) to 24.4% compared with 2.7% in the untreated control, 12.8% when treated with 50 μmol/L, and 16.3% when treated with 100 μmol/L (Fig. 2). Similar results on apoptosis were also observed by DNA

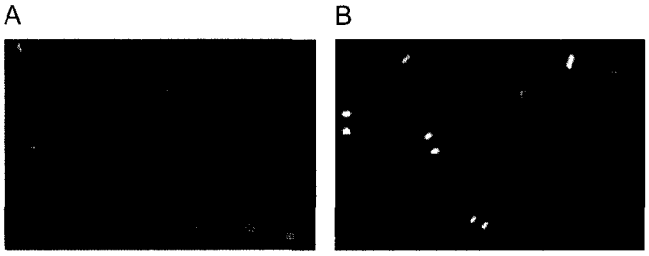

Fig. 1. CNE2 cells treated with or without 100 μmol/L DADS for 24 h. After staining with Hoechst 33258 (1 M phosphate-buffered saline), photographs were taken under fluorescence microscopy. Compared with normal nuclei (A), the nuclei of dead cells appear shrunken (B). (Magnification X660).

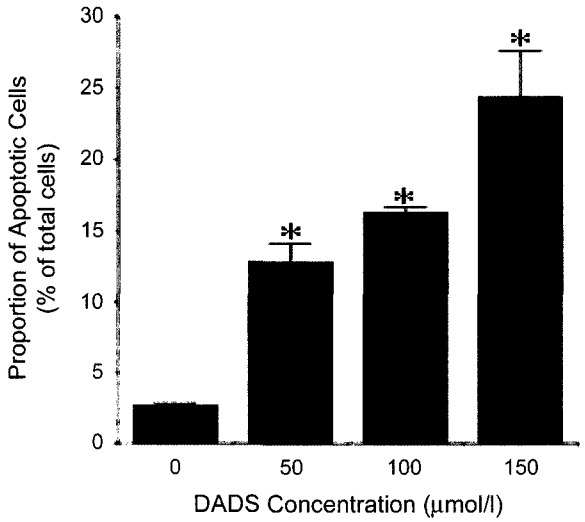

Fig. 2. Effect of DADS on cell apoptosis. CNE2 cells were treated with 0–150 μmol/L DADS for 24 h. After staining with Hoechst 33258, six randomly chosen fields of view were observed with a minimum number of 500 cells scored in each condition. Apoptotic cells (condensed with fragmented nuclei) were calculated as a ratio to the total number of cells counted. *$P < 0.01$ *vs* control (untreated).

flow cytometric analysis. The percentages of apoptotic cells when treated with 0 μmol/L, 50 μmol/L, 100 μmol/L and 150 μmol/L DADS were 1.8%, 9.5%, 15.1%, and 24.4%, respectively (Table II).

Induction of cell cycle arrest by DADS on human nasopharyngeal carcinoma CNE2 cells

The cell cycle effects that follow DADS treatment in CNE2 cells were analyzed by performing cytofluorometric analyses. We detected cell cycle distribution with concentrations of DADS treatment of 0 μmol/L, 50 μmol/L, 100 μmol/L and 150 μmol/L. As displayed in Table II, the percentages of cells in G1-phase were 45.0%, 27.2%, 37.1%, and 26.1%; and the percentages in S-phase were 45.0%, 60.8%, 53.0%, and 63.8%, respectively. Percentages did not differ during the G2/M-phase. These results indicate that DADS induces cell cycle arrest in S-phase in CNE2 cells in a concentration-independent manner.

Table II. Effect of DADS on the cell cycle distribution in CNE2 cells

DADS (μmol/L)	G1 (%)	S (%)	G2/M (%)	Apoptosis (%)
0	45.0±2.1	45.0±1.8	9.9±0.7	1.8±0.1
50	27.2±1.0	60.8±2.7	12.0±1.4	9.5±0.5
100	37.1±1.2	53.0±2.5	9.9±0.5	15.1±0.8
150	26.1±0.9	63.8±3.1	10.2±1.2	24.4±1.1

Human CNE2 cells were treated with DADS for 24 h, and cell cycle distribution was analyzed by flow cytometry. Data are shown as means ±SEM, N=3 experiments. P<0.01 vs control.

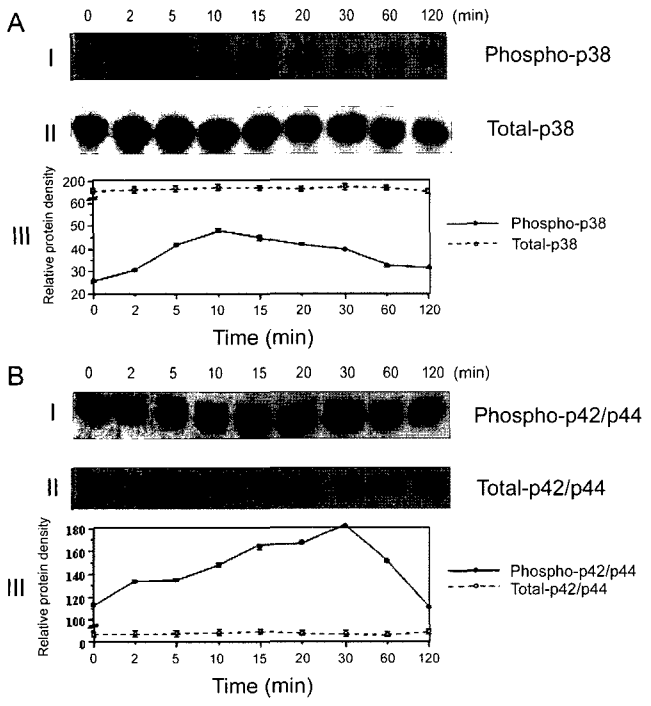

Fig. 3. Time-course of DADS-induced activation of p38-MAPK and p42/p44-MAPK in CNE2 cells. Cells were treated with 100 μmol/L DADS for 0-120 min, and Western blot analysis was performed. Extracts (20 g of protein) from CNE2 cells were resolved by SDS-PAGE and probed with anti-phospho-p38 antibody (A-I), or with anti-phospho–p42/p44 antibody (B-I). In parallel experiments, the amount of total-p38 or p42/p44 was determined in the same cell extracts with anti-total-p38 antibody (A-II) or anti-total-p42/p44 antibody (B-II). Representative graphs of Western blot densities shown in A-III and B-III were quantitatively determined by QuantiScan version 2.1 biosoft. Results were confirmed in triplicate.

Transient increase in activation of endogenous MAPKs during DADS-induced human nasopharyngeal carcinoma CNE2 cell apoptosis

To directly address the involvement of MAPKs in DADS-induced apoptosis, both phospho-p38 and phospho-p42/p44 proteins were assayed in DADS-treated CNE2 cells using anti-phospho-p38 and anti-phospho-p42/p44 antibodies. As shown in Fig. 3, DADS caused a time-dependent activation of p38-MAPK and p42/p44-MAPK. DADS (100 μmol/L) stimulated the activation of p38-MAPK and

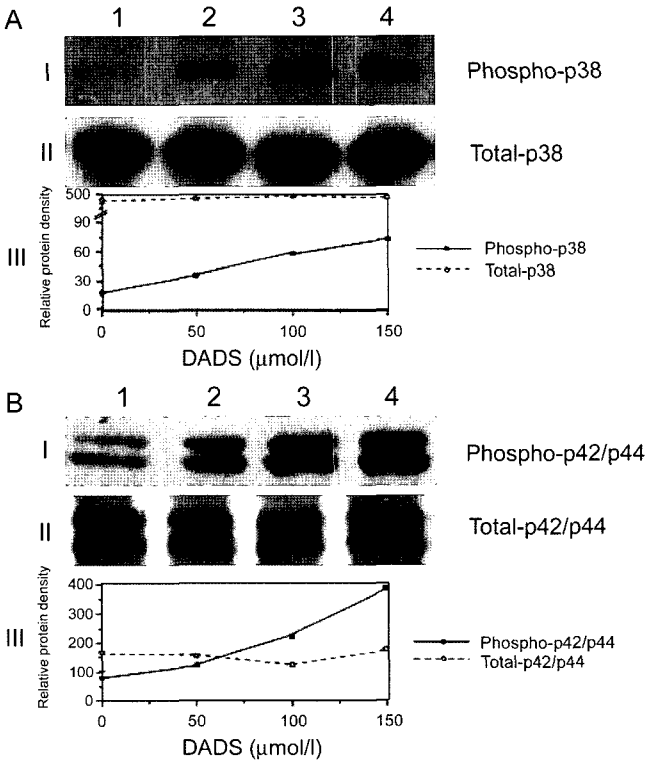

Fig. 4. Concentration-dependent activation of both p38-MAPK and p42/p44-MAPK by DADS treatment for 10 min in CNE2 cells. Cells were treated with increasing concentrations (0-150 μmol/L) of DADS and Western blot assays were performed by applying 20 g of the extracts and probing with anti-phospho-p38 or anti-total-p38 antibodies (A-I, A-II), and with anti-phospho-p42/p44 or anti-total-p42/p44 antibodies (B-I, B-II). Lane 1: control (untreated), 2: 50 μmol/L DADS, 3: 100 μmol/L DADS, 4: 150 μmol/L DADS. Representative graphs of Western blot densities shown in A-III and B-III were quantitatively determined by QuantiScan version 2.1 biosoft. Results were confirmed in triplicate.

p42/p44-MAPK in a similar time-course with maximal induction at 10 to 30 min after stimulation. Treatment with DADS at concentrations of 0-150 μmol/L for 10 min stimulated an increase in phospho-p38 and phospho-p42/44 in a concentration-dependent manner (Fig. 4). These results suggest that activation of MAPKs plays a role in the mechanism of DADS-induced apoptosis.

DISCUSSION

In the present study, we have found that DADS inhibits the growth of CNE2 cells, as shown by decreases in cell viability (Table I). The growth inhibitory properties of DADS are likely to be attributable to its induction of apoptotic cell death as indicated in Fig. 1, Fig. 2, and Table II. It has been well documented that DADS inhibits growth of estrogen receptor (ER)-positive (KPL-1 and MCF-7) and -negative (MDA-MB-231 and MKL-F) human breast cancer cell lines *in vitro* (Nakagawa *et al.*, 2001). Furthermore, it induces apoptosis as determined by morphological changes and DNA fragmentation in human colon tumor and hepatoma cell lines (Sundaram and Milner, 1996; Wen *et al.*, 2004).

A number of pathways are known to lead to apoptosis. The Bcl-2 family and caspase-3 are important regulators of apoptosis (Farrow and Brown, 1996; Kroemer, 1997). The Bcl-2 proteins are associated with up-regulation of Bax and down-regulation of Bcl-X_L. Other studies have suggested that induction of apoptosis might be through activation of the mitochondrial pathway involved in Bcl-2 down-regulated cytochrome release into the cytosol, and activation of caspase-9 and caspase-3 (Filomeni *et al.*, 2003). It has also been shown that DADS-induced apoptosis is mediated by activation of caspase-3 (Nakagawa *et al.*, 2001). In DADS-treated HL-60 cells, caspase-3 activation is evidenced by an increase in protease activity and proteolytic cleavage activity of the proenzyme.

Excessive intracellular calcium is known to lead to apoptosis in several *in vitro* models [1]. In HCT–15 cells, DADS was found to cause a sustained and dose-dependent increase in intracellular calcium (Sundaram and Milner, 1996). We have already described that DADS can activate the components of the MAPK pathway and phosphorylated MAPK inhibitors (SB203580 and U0126) cause apoptosis in human HepG2 hepatoma cells (Wen *et al.*, 2004). Our present data demonstrate that DADS can transiently activate both p38-MAPK and p42/p44-MAPK in a time- and concentration-dependent manner during DADS-induced apoptosis in CNE2 cells (Figs. 3 and 4). This suggests that DADS-induced phospho-p38 and phospho-p42/44 appear to play a role in maintaining the regulation of apoptosis in CNE2 cells.

DADS has been demonstrated to induce cell cycle arrest in G2/M-phase in some cultured cell lines. The block at G2/M-phase might explain the increase in apoptosis through activation of the mitochondrial pathway (Filomeni *et al.*, 2003). In our present study, the effects of DADS on the cell cycle shows that DADS induces an increase in the number of CNE2 cells in S-phase reaching values of 63.8% (Table II). The percentage of the cell population showing apoptotic features was 24.4% (Table II), suggesting that the block in S-phase finally results in triggering of the apoptotic program.

In summary, our results indicate that DADS can induce apoptosis in human nasopharyngeal carcinoma CNE2 cells *via*, at least partly, S-phase block of cell cycle, related to a rise in MAPK phosphorylation.

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Abbreviations

DADS, Diallyl disulfide; MAPK, Mitogen-activated protein kinase; phospho-p38, phosphorylated p38 MAPK; phospho-p42/p44, phosphorylated p42/44 MAPK; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide.

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