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Enhancement of diallyl disulfide-induced apoptosis by inhibitors of MAPKs in human HepG2 hepatoma cells

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Abstract

We examined the effects of diallyl disulfide (DADS), an oil-soluble organosulfur compound found in garlic, on human HepG2 hepatoma cells to better understand its effect on apoptosis and apoptosis-related genes. Our study has demonstrated that DADS affects cell proliferation activity and viability and elicits typical apoptotic morphologic changes (chromatic condensation and nuclear fragmentation) in human HepG2 hepatoma cells. Also, treatment with DADS induces a temporary increase in phosphorylated p38 MAPK (phospho-p38) and phosphorylated p42/44 MAPK (phospho-p42/p44) in a time- and concentration-dependent manner. Inhibition of activated/phosphorylated mitogen-activated protein kinase (MAPK) with phospho-p38 or phospho-p42/44 specific inhibitors, SB203580 or U0126, induces apoptosis without DADS treatment, indicating that at least the endogenous activated forms of p38 MAPK and p42/p44 MAPK markedly exert cytoprotective roles from cell apoptosis in the HepG2 hepatoma cells. Combined treatment with these inhibitors followed by DADS further enhances the DADS-induced apoptosis. Taken together, these results show that both DADS and the specific inhibitors of MAPKs could induce apoptosis in HepG2 hepatoma cells and that the MAPKs inhibitors further enhance the apoptotic effect in DADS-treated HepG2 hepatoma cells.

Keywords: Diallyl disulfide; Phospho-p38; Phospho-p42/p44; Human HepG2 hepatoma cells; Apoptosis; MAPKs specific inhibitors

1. Introduction

Diallyl disulfide (DADS), an important component of garlic (*Allium sativum*) has been recently shown to inhibit the growth of human tumor cells from colon, lung, skin, and breast origins [1–4]. The anti-proliferative effect of DADS was attributed to suppression of the rate of cell division and induction of apoptosis in human tumor cells. Although the role(s) and/or mechanism(s) of DADS as an anticancer agent remain unclear, there is an increasing evidence for DADS-mediated modulation of signal transduction pathways [2,3,5]. Recent studies have demonstrated that DADS-induced apoptosis appears to occur via induction of p53 and activation of caspase-3 [2,3].

Apoptosis plays an essential role as a protective mechanism against neoplastic development by eliminating genetically damaged cells or excess cells that have been improperly produced. The maintenance of homeostasis in normal mammalian tissues by apoptosis reflects a critical balance between cell proliferation and cell loss. Inappropriate regulation of apoptosis is associated with a variety of diseases [6–8]. 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. On the other hand, since apoptosis represents an active, gene-directed mechanism, it should eventually be possible to control this process for therapeutic purposes.

In addition to the well-documented effects on apoptosis of some inducers such as Taxol and inducible nitric oxide synthase (iNOS) [9,10], a number of reports have described

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; DMEM, Dulbecco's modified Eagle medium; SB203580, 4-(4-fluoropheny)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)IH-imidazole; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(phenylthio) butadiene

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activation of components of the mitogen-activated protein kinase (MAPK) pathway in response to treatment with these inducers. MAPKs are serine–threonine protein kinases that are activated in response to a diverse range of stimuli including growth factors, hormones, neurotransmitters, and cellular stress. So far, the c-Jun NH2-terminal kinases (JNKs), p38 MAPK, AKT, and p42/44 MAPK have been identified as sub-families of MAPK [11–14]. The MAPK cascades are characterized with sequential signaling. The MAPK kinase kinases (MAPKKKs) phosphorylate and activate downstream MAPK kinases (MAPKKs) that ultimately activate MAPKs. Through these specific intracellular signaling cascades, a variety of extracellular stimuli are transduced through cells in a tightly regulated manner.

The p38 MAPK is more commonly activated in response to cytokines, stress and cellular damage, and the p44/42 MAPK is activated by growth stimuli and generally considered as a prosurvival mediator [15–17]. Several studies have described that cellular redox state, tyrosine kinases, and phosphatases are thought to be involved in the activation of stress responses, and represented the activation of various components of the MAPK family in different cell models after treatment with paclitaxel or TNF [18–23]. However, the role of the MAPKs in inducing tumor apoptosis by inducers including DADS is not entirely clear [23–28], although the activation of p38 MAPK is generally associated with induction of apoptosis, and p42/44 MAPK exerts cytoprotective effects [29].

In this report, we find that DADS and phosphorylated p38 MAPK (phospho-p38) or phosphorylated p42/44 MAPK (phospho-p42/44) specific inhibitors, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)IH-imidazole (SB203580) or 1,4-diamino-2,3-dicyano-1,4-bis(phenylthio) butadiene (U0126), significantly increase HepG2 hepatoma cell apoptosis. The activated MAPKs (p38 and p42/p44 MAPK) appear to play a cytoprotective role, and the MAPK specific inhibitors enhance apoptotic effects in HepG2 hepatoma cells with DADS treatment.

2. Materials and methods

2.1. Materials

Diallyl disulfide was purchased from Fluka Co. (80% purity). BSA, Dulbecco's modified Eagle medium (DMEM) medium, SB203580, and Hoechst 33258 were purchased from Sigma. Specific anti-phospho-and the cocktail of anti-total p38 and anti-total p42/44 MAPK antibodies, goat horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody were purchased from Santa Cruz Biotech. Phototope–HRP Western Detection Kit was purchased from New England Biolabs Inc. U0126 was a kind gift from Dr. J.M. Trzaskos (DuPont Merck Research Laboratory).

2.2. Cell culture

HepG2, a human hepatoma cell line, were cultured in DMEM with 10% heat-inactivated fetal bovine serum (FBS), benzylpenicillin (100 kU/L) and streptomycin (100 mg/L) at 37 °C in an incubator containing humidified air with 5% CO_2.

2.3. Morphologic determination and quantification of apoptosis

After treatment with or without DADS (100 μmol/L) in phenol- and serum-free DMEM for 24 h, HepG2 hepatoma cells were observed by fluorescence microscopy after Hoechst 33258 staining. Apoptosis was routinely determined by counting the number of cells with condensed or fragmented chromatin as described previously [30]. Briefly, cells from different treatments were cytospun onto glass slides, followed by a mild hypotonic treatment (1:1, distilled water/growth medium ratio) for 10 min. The cells were pre-fixed for 5 min with a 50% fixative solution (3:1, methanol/acetic acid ratio), and then fixed with neat fixative solution for 10 min. The preparations were stained with Hoechst 33258 for 10 min, rinsed, and dried. The preparations were examined using fluorescence microscope. Condensed and fragmented nuclei, typical morphologic changes of apoptosis were easily distinguishable from intact nuclei and counted, and the percentages were subsequently calculated. Six randomly chosen fields of view were examined with a minimum number of 500 cells scored in each condition.

2.4. Determination of cell proliferation activity

HepG2 hepatoma cells were plated in 24-well plates and grown to 80% confluence in DMEM with 10% dialyzed and charcoal-stripped FBS. The cultures were then rinsed in phenol-free DMEM medium and incubated with respective test substances in phenol-free and serum-free DMEM for 12 h. [^{3}H] thymidine (1.35×10^4 Bq/L) was added. Cells were cultured for another 12 h. The supernatant was aspirated and then washed twice with PBS to remove excess [^{3}H] thymidine. Ice-cold 10% trichloroacetic acid (TCA) was applied for 30 min. The cells were then dissolved in 0.2 mol/L NaOH. Radioactivity (incorporation activity) was determined by scintillation counting.

2.5. Cell viability assay

To assess the cytotoxic effects of DADS in HepG2 hepatoma cells, we used a tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay [31]. In this assay, the MTT is used as a colorimetric substrate for measuring cell viability. When cells are injured, there is an alteration in the cellular redox activity such that cells are unable to reduce the dye.

For our MTT assay, the cells were plated in 24-well plates and grown to 80% confluence. The cultures were then rinsed in phenol-free DMEM medium and incubated with respective test substances in phenol-free and serum-free DMEM for 24 h. At the end of this time interval, 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 solubilization solution including 10% (v/v) Triton X-100 and 0.1 mol/L HCl in isopropanol equal to the volume of original culture medium. Colorimetric determination of MTT reduction was made at 570 nm.

2.6. Preparation of lysates

For MAPK kinases detection, HepG2 hepatoma cells cultured in six-well culture plates were grown to 80–90% confluence, then nutrient-starved for 24 h in serum-free DMEM, and then various concentrations of DADS were added for the indicated times. For MAPK inhibition studies, cells were pretreated for 10 min with SB203580 or U0126 before the addition of DADS. After being washed with ice-cold PBS three times, cells were lysed with 60 μL ice-cold lysis buffer containing (mmol/L) NaCl, 50; Na_3VO_4, 2; phenylmethylsulfonyl fluoride, 0.5; and HEPES, 10; at pH 7.4, along with 0.01% Triton X-100 and 10 mg leupeptin was added. The lysates were obtained by centrifugation at 18,000 × *g* at 4 °C for 15 min and the concentration of total cell protein was determined [32].

2.7. Western blot analyses

SDS sample buffer (one-forth volume) containing Tris–HCl (0.33 mol/L), SDS (10% (w/v)), glycerol (40% (v/v)), and bromophenol blue (0.4%) was added to the cell lysates. After being boiled for 5 min, 20 μg of extracted protein was electrophoresed 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, 50; pH 7.4; NaCl, 150 mmol/L containing 0.1% Tween-20). The blots were incubated with the primary antibodies against phospho-p38, phosphorylated p42/44 MAPK (phospho-p42/p44) or against total p38, total p42/p44 at 1:1000 dilution 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. To control equal protein concentrations in MAPK experiments, 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.

2.8. Statistics

Results are expressed as the mean ± S.E.M. with *N* being the sample size. Mean values were compared using a two-tailed *t*-test. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Induction of apoptosis by DADS on HepG2 hepatoma cells

To test the effect of DADS on the proliferation activity and cell viability of HepG2 hepatoma cells, the [^{3}H] thymidine incorporation assay and MTT conversion assay were used. As shown in Table 1, after treatment with DADS at 50, 100, and 150 μmol/L for 24 h, the [^{3}H] thymidine incorporation decreased by 4.4, 16.2, and 60.8%, respectively, compared to the control. This result suggests that DADS appears to be cytotoxic to HepG2 hepatoma cells in a dose-dependent manner. Exposure of HepG2 hepatoma cells to DADS at concentrations of 50, 100, and 150 μmol/L for 24 h decreased cell viability by 13.0, 23.7, and 36.5%, respectively, compared to the control.

To test whether DADS induce apoptosis in HepG2 hepatoma cells, DADS-treated HepG2 hepatoma cells were examined under fluorescence microscopy after staining with Hoechst 33258. After exposure to DADS (100 μmol/L) for 24 h, at least 23.6% of HepG2 hepatoma cells showed typical morphologic changes of apoptosis. The cell volume was reduced, the chromatin became

Table 1
Effects of DADS on incorporation activity and cell viability

DADS (mmol/L)	[^{3}H] thymidine incorporation		MTT absorbance	
	Bq/min	Normalized (%)	570 nm	Normalized (%)
0	23.62 ± 0.69	–	$0.523 \pm 8.66 \times 10^{-3}$	–
50	22.58 ± 0.70*	4.4	$0.455 \pm 8.66 \times 10^{-3}$**	13.0
100	19.80 ± 0.82**	16.2	$0.399 \pm 3.00 \times 10^{-3}$**	23.7
150	9.27 ± 0.30**	60.8	$0.332 \pm 4.58 \times 10^{-3}$**	36.5

HepG2 hepatoma cells were treated with DADS for 24 h, and their incorporation activity and cell viability were determined. Data are also normalized (%) with control (0). Data are shown as mean ± S.E.M., $N = 4$ experiments.

* $P < 0.05$.

** $P < 0.01$ vs. control.

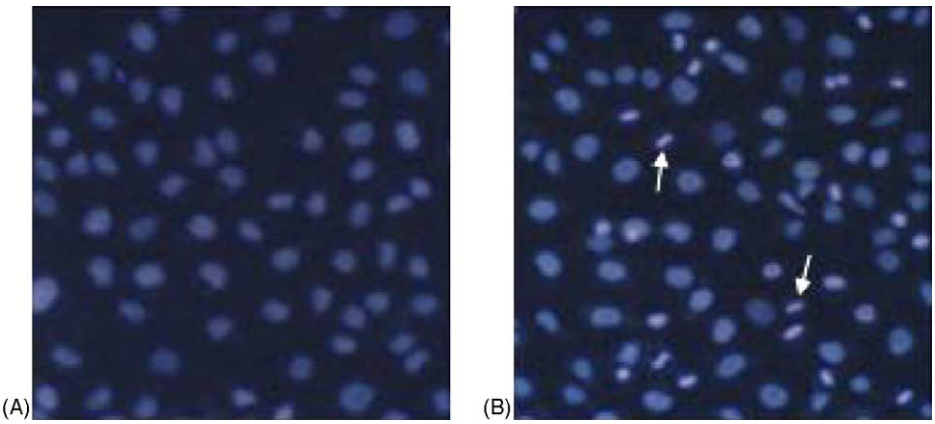

Fig. 1. HepG2 hepatoma cells were treated with or without 100 μmol/L DADS for 24 h, and then stained with Hoechst 33258 (1 μM phosphate-buffered saline) and photographed under fluorescence microscopy. (A) Untreated HepG2 hepatoma cells. (B) DADS-treated HepG2 hepatoma cells. The nuclei of dead cells appear to shrink shown with the symbols (↓), when compared to the normal nuclei. Six randomly chosen fields of view were observed with a minimum number of 500 cells scored in each condition. Condensed and fragmented nuclei appeared in DADS-treated cells (magnification, 660×).

condensed, and nuclei became fragmented (Fig. 1). Similar to DADS-induced cytotoxicity, DADS-induced apoptosis was observed in HepG2 hepatoma cells in a concentration-dependent manner. After DADS treatment of 50 or 150 μmol/L for 24 h, the number of apoptotic cells was increased to 14.2 or 37.5% when compared to the untreated controls (1.3%) (Table 2).

3.2. Transient increase in activation of endogenous MAPKs during the processing of DADS-induced HepG2 hepatoma cell apoptosis

To directly address the involvement of MAPKs in the processing of DADS-induced apoptosis, both phospho-p38 and phospho-p42/p44 were determined in DADS-treated HepG2 hepatoma cells using specific anti-phospho-p38 and anti-phospho-p42/p44 antibodies. As shown in Fig. 2, DADS caused a time-dependent activation of p38 MAPK and p42/p44 MAPK. DADS (100 μmol/L) stimulated the activation of p38 MAPK and p42/p44 MAPK in a similar time course with a maximal induction at about 15 and 20 min after stimulation, respectively. Levels returned nearly to baseline at 30 and 60 min. Treatment with DADS at concentrations of 50–150 μmol/L for 10 min stimulated an increase in phospho-p38 and phospho-p42/44 in a concentration-dependent manner (Fig. 3). These results suggest that some members of MAPKs play a role in the processing of DADS-induced apoptosis.

Table 2
Effects of DADS on HepG2 hepatoma cell apoptosis

DADS (μmol/L)	Proportion of apoptotic cells (% of total HepG2 cells)
0	1.30 ± 0.34
50	14.24 ± 0.41*
100	23.58 ± 1.31*
150	37.47 ± 3.78*

HepG2 hepatoma cells were treated with or without DADS for 24 h, and the numbers of apoptotic cells were determined by direct apoptotic cell counting after cell staining. The percentage of apoptotic cells was calculated with total HepG2 hepatoma cells. Data are shown as mean ± S.E.M., $N = 4$ experiments.

* $P < 0.01$ vs. control.

3.3. Protection of HepG2 hepatoma cell apoptosis by phosphorylated MAPKs

Generally, activation of p38 MAPK is associated with induction of apoptosis, whereas p42/44 MAPK exerts cytoprotective effects [29]. To test whether both phosphorylated MAPKs in HepG2 hepatoma cells have similar functions as reported before, the cell proliferation activity and apoptotic assays were performed after exposure of cells to active MAPK specific inhibitors. Pre-incubation with the phospho-p38 specific inhibitor, SB203580 (10 μmol/L), or the phospho-p42/p44 specific inhibitor, U0126 (5 μmol/L), for 10 min, markedly inhibited proliferation of HepG2 hepatoma cells. The [^{3}H] thymidine incorporation decreased by 16% (U0126) and 27% (SB203580), respectively, compared to the untreated control (0.006% Tween80) (Fig. 4A). Similar results were also observed using the MTT assay (Fig. 4B). A greater level of cell apoptosis was also found with both MAPK inhibitors. U0126 induced an 11-fold and SB203580 induced a 15-fold increase in apoptosis when compared to the control (Fig. 4C), indicating that endogenous activated MAPKs, both phospho-p42/p44 and phospho-p38, play a cytoprotective function in HepG2 hepatoma cell.

Result of Western blot analysis further confirmed that MAPK inhibitors affect the activation of endogenous MAPKs in HepG2 hepatoma cells. Treatment with SB203580 or U0126 blocked phosphorylated forms of p38 MAPK (Fig. 5A) and p42/p44 MAPK (Fig. 5B). Western blot bands for phospho-p38 and phospho-p42/p44 could not be detected using phospho-p38 or phospho-p42/p44 specific antibodies (lane 2, Fig. 5A and B).

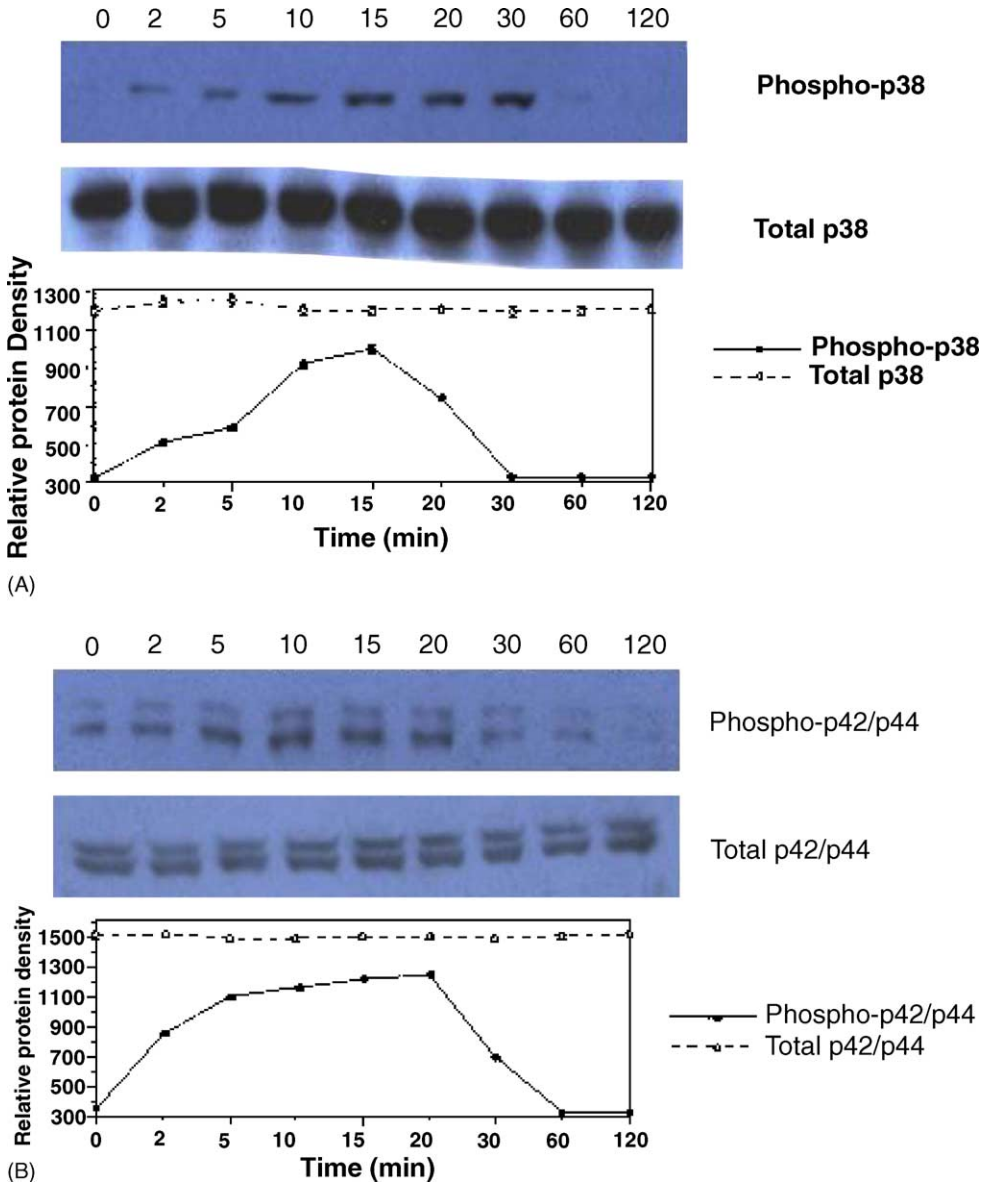

Fig. 2. Time course of DADS-induced activation of p38 MAPK and p42/p44 MAPK in DADS-induced apoptosis in HepG2 hepatoma cells. Cells were treated with 100 μmol/L DADS for 0–120 min, and Western blot analysis was performed. Extracts (20 μg of protein) from HepG2 hepatoma cells were resolved by SDS-PAGE and probed with anti-phospho-p38 antibody (top of A), or with anti-phospho-p42/p44 antibody (top of B). These experiments were repeated thrice with similar results, and typical data are presented. In parallel experiments, the amount of total p38 MAPK or p42/p44 MAPK was determined in the same cell extracts with anti-total p38 MAPK antibody (middle of A) or anti-total p42/p44 MAPK antibody (middle of B), that recognized p38 MAPK or p42/p44 MAPK independent of their phosphorylation state. Bands of MAPK were quantitatively determined by QuantiScan Version 2.1 Biosoft (bottom of A and B).

3.4. Enhancement of DADS-induced apoptosis by inhibition of MAPKs

To test whether MAPKs inhibitors could block DADS-induced activation of MAPKs in HepG2 hepatoma cells and subsequently enhance DADS-induced apoptosis, we first investigated the inhibition effect of these inhibitors by Western blot analysis. When SB203580 or U0126 were added to HepG2 hepatoma cells for 10 min before DADS-treatment, DADS-induced activations of p38 MAPK and p42/p44 MAPK were markedly decreased (lane 2 of Fig. 6), when compared to DADS-treatment alone (lane 1 of Fig. 6) or no treatment (lane 1 of Fig. 5). To examine further into whether this inhibition could achieve a cooperative apoptotic effect with DADS-induced apoptosis, a combined treatment of these inhibitor followed by DADS was performed. This combined treatment increased the proportion of apoptotic HepG2 hepatoma cells by 20% (DADS plus U0126 when compared with DADS- or inhibitor-treatment alone $P < 0.05$) and 40% (DADS plus SB203580, $P < 0.01$), respectively (Fig. 7A). Decreases of 45.9% (for DADS plus U0126) and 59.0% (for DADS plus SB203580) of proliferation activity were observed by preincubation of HepG2 hepatoma cells with MAPK

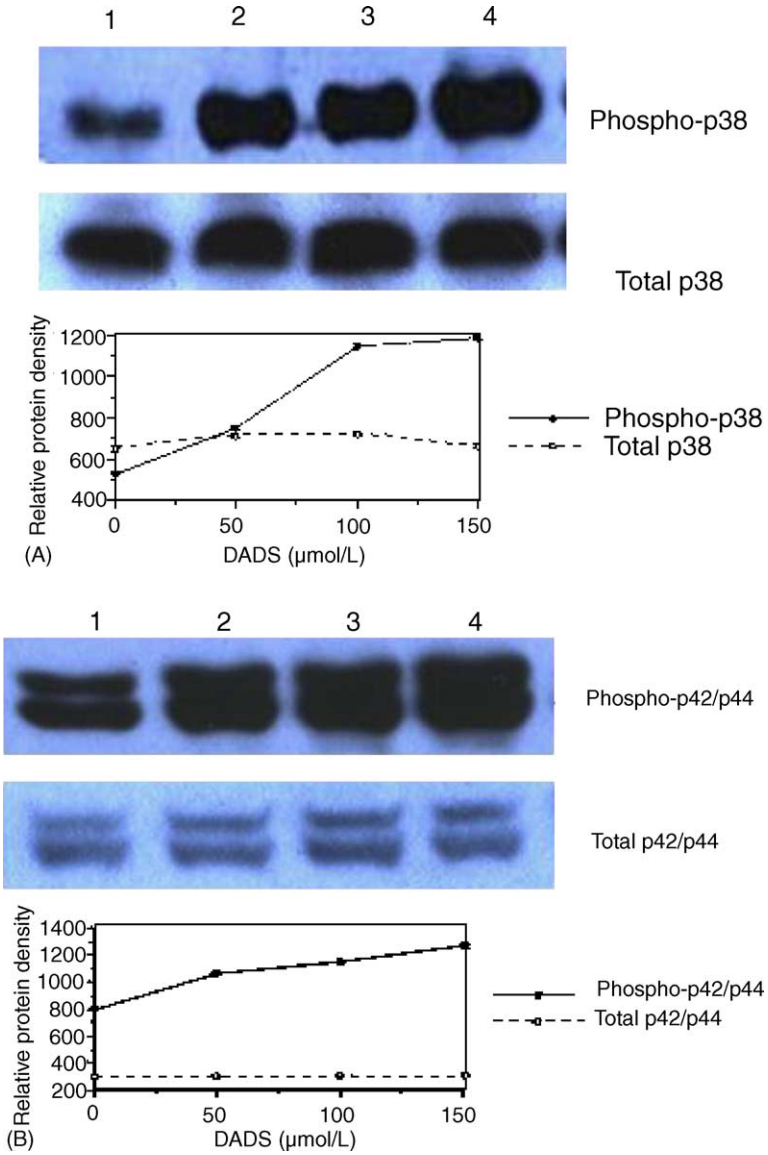

Fig. 3. Concentration-dependent activation of both p38 MAPK and p42/p44 MAPK by DADS treatment for 10 min in HepG2 hepatoma cells. Cells were treated with increasing concentrations (0–150 μmol/L) of DADS and Western blot assays were performed by applying 20 μg of the extracts and probing with anti-phospho-p38 (top of A) or anti-total p38 antibody (middle of A), and with anti-phospho-p42/p44 (top of B) or anti-total p42/p44 antibody (middle of B). Lane 1: control (untreated), lane 2: DADS (treated with 50 μmol/L), lane 3: DADS (100 μmol/L), and lane 4: DADS (150 μmol/L). Results are representative of three replicates. Bands of MAPK were quantitatively determined by QuantiScan Version 2.1 Biosoft (bottom of A and B).

inhibitors, when compared with DADS- or inhibitor-treatment alone (Fig. 7B). Decreased cell viability was also found by the MTT assay (Fig. 7C).

4. Discussion

It has been well documented that DADS inhibits growth of human MCF-7, T47D, and both ER-positive and ER-negative breast cancer cells in vitro [4,33], and induces apoptosis as determined from morphological changes and DNA fragmentation in HCT-15 human colon tumor cells [1]. In the present study, we found that DADS inhibits the growth of human HepG2 hepatoma cells as shown by decreases in both [^{3}H] thymidine uptake and cell viability (Table 1). The growth inhibitory properties of DADS are likely attributed to its induction of apoptotic cell death, at least partially, as indicated in Fig. 1 and Table 2.

Our present study shows that DADS may be an effective inducer of apoptosis in HepG2 hepatoma cells. A number of pathways are known to lead to apoptosis. The Bcl-2 family and caspase-3 are important regulators of apoptosis [34,35]. The Bcl-2 proteins were associated with up-regulation of Bax and down-regulation of Bcl-X_L. It has been shown that DADS-induced apoptosis is mediated via activation of caspase-3 [4]. In DADS-treated HL-60 cells, caspase-3 activation is evidenced by increase in protease activity and proteolytic cleavage activity of the proenzyme [36]. Excessive intracellular calcium is known to lead to apoptosis in several in vitro models [1]. In HCT-15 cells,

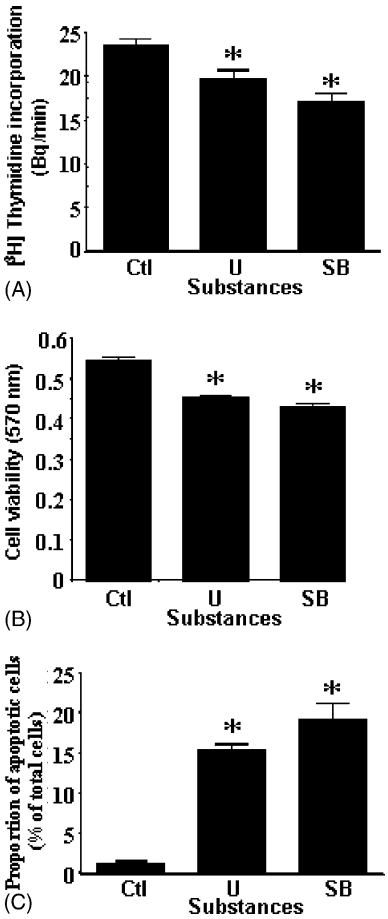

Fig. 4. Effect of MAPK inhibitors on incorporation activity (A), cell viability (B), and cell apoptosis (C). HepG2 hepatoma cells were treated with SB203580 (SB, 10 μmol/L) and U0126 (U, 5 μmol/L) for 10 min. The proliferation activity (Bq/min) and cell viability (570 nm) were determined, and cell survival was calculated as the ratio of the apoptotic cells to the total cell number. Data are shown as mean ± S.E.M., $N = 4$ experiments. $^{*}P < 0.01$ vs. Ctl (untreated).

DADS was found to cause a sustained and dose-dependent increase in the intracellular calcium. On the other hand, a number of reports have described activation of components of the MAPK pathway in response to Taxol and iNOS-induced apoptosis [9,10]. Our present data demonstrate that DADS can activate both p38 and p42/p44 MAPKs in a time- and concentration-dependent manner during the processing of DADS-induced apoptosis in HepG2 hepatoma cells suggesting a plausible involvement of MAPKs in the regulation of apoptosis in these cells.

Our study has focused on the roles of MAPKs in the processing of DADS-induced apoptosis. Activation of p38 MAPK is generally associated with induction of apoptosis, whereas phospho-p42/44 exerts cytoprotective effects [29]. We found that the reduction of these activated/phosphorylated MAPKs by phospho-p38 or phospho-p42/44 specific inhibitors (SB203580 or U0126), markedly increases apoptosis (Fig. 4C) and enhances DADS-induced apoptosis (Fig. 7A) in HepG2 hepatoma cells. These data suggest that at least the basal levels of endogenous phospho-p38

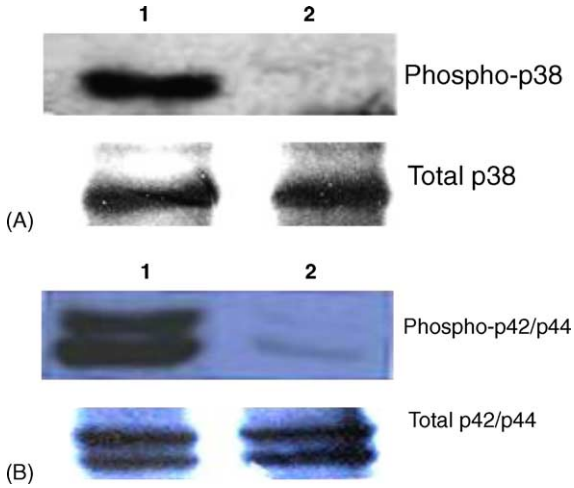

Fig. 5. Effect of MAPK inhibitors on the constitutive level of endogenous phospho-p38 and phospho-p42/p44 in HepG2 hepatoma cells. Cells were treated with or without MAPK inhibitors, SB203580 and U0126, for 10 min. Western blot analysis was performed with each 20 μg extract and with the indicated antibodies. (A) Lane 1: control (untreated), lane 2: SB203580 (treated with 10 μmol/L); (B) lane 1: control, 2: U0126 (with 5 μmol/L). Total p38 MAPK or p42/p44 MAPK was determined in the same conditions. This experiment was repeated thrice with similar results.

and phospho-p42/44 appear to play a role in maintaining the survival of the HepG2 hepatoma cells. In other words, our results indicate that DADS exerts a dual role: (1) a cytotoxic role leading to apoptosis and (2) a temporary protective role via members of the MAPK family. Our findings suggest a critical role for phospho-p38 and

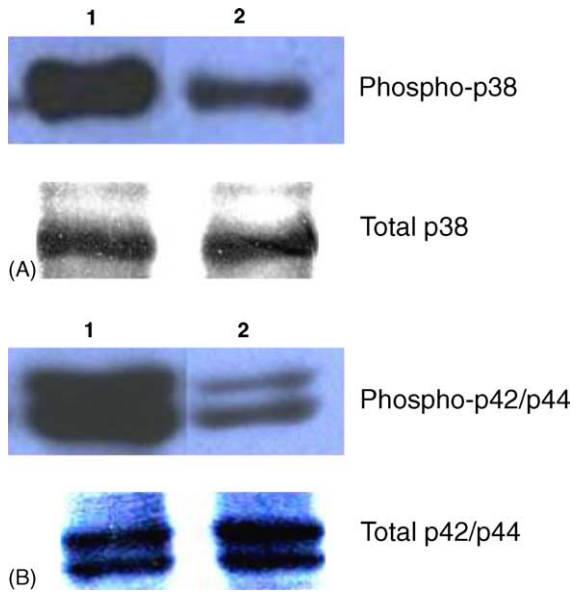

Fig. 6. Effect of MAPK inhibitors on DADS-induced activation of MAPKs in HepG2 hepatoma cells. Cells were treated with DADS (100 μmol/L) for 10 min, or preincubated with inhibitors, SB203580 (10 μmol/L) or U0126 (5 μmol/L), for 10 min before adding DADS reagent. The phospho-p38 and total p38 (A), or phospho-p42/p44 and total p42/p44 (B) were detected with anti-phospho-p38 and anti-total p38 antibodies, or with anti-phospho-p42/p44 and anti-total p42/p44 antibodies. (A) Lane 1: DADS-treatment, lane 2: DADS plus SB203580; (B) lane 1: DADS-treatment, 2: DADS plus U0126.

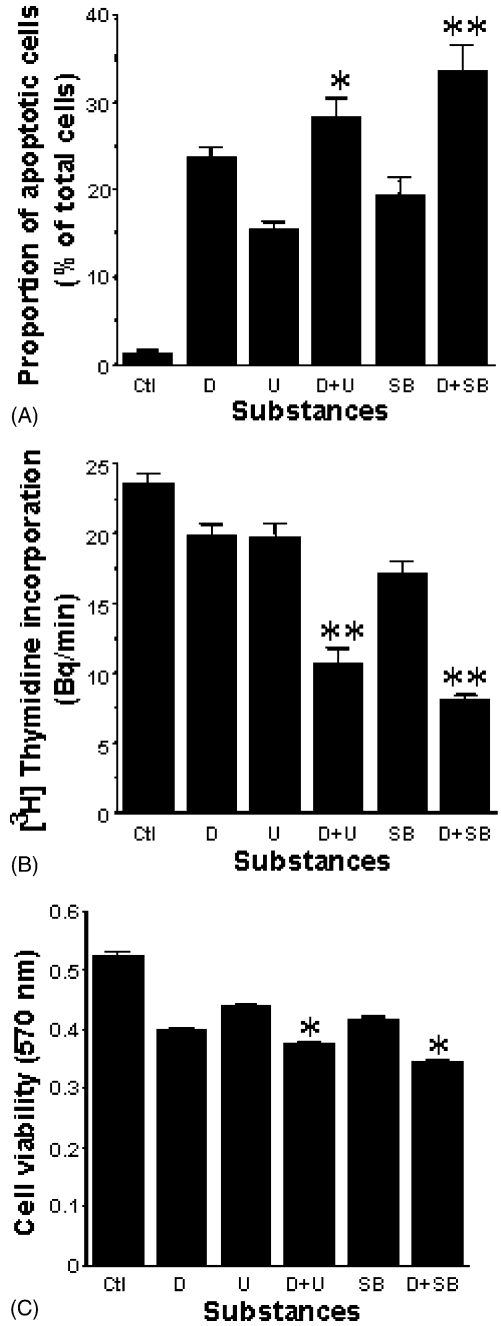

Fig. 7. Cooperation effects of SB203580 or U0126 on cell apoptosis (A), incorporation activity (Bq/min) (B), and cell viability (C) of DADS-treatment HepG2 hepatoma cells. Cells were pre-incubated with SB203580 (SB, 10 μmol/L) or U0126 (U, 5 μmol/L) for 10 min before adding DADS (D, 100 μmol/L) treatment for 24 h. Their proliferation activity, cell viability, and cell survival activity were determined. Data are shown as mean ± S.E.M., $N = 4$ experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. DADS for cell apoptosis (A), $^{**}P < 0.01$ vs. SB203580 or U0126 for incorporation activity (B), and $^{*}P < 0.05$ vs. DADS for cell viability (C). Ctl, untreated control.

phospho-p42/44 in spontaneous apoptosis and the processing of DADS-induced apoptosis of HepG2 hepatoma cells. However, our data do not rule out the possibility that other MAPKs may also play a role in these processes.

Even though our data support the idea that the effects of DADS on MAPKs inhibit rather than promote apoptosis, an alternative interpretation may exist for some of our discoveries. For example, a marked subsequent down-regulation of phospho-p38 and phospho-p42/44 by DADS treatment for more than 1 h was observed (Fig. 2). It is plausible that this down-regulation of phospho-p38 and/or phospho-p42/44 may constitute an apoptotic stimulus. Further studies are needed to elucidate the mechanism of the effect of DADS on apoptosis.

Our results presented in this study also establish a potential role for MAPK inhibitors in apoptosis of HepG2 hepatoma cells. First, inhibitor-treatment not only blocked the constitutive level of phospho-p38 and phospho-p42/44 (Fig. 5), but also blocked DADS-induced MAPK activation although it is not complete (Fig. 6). Second, a combination treatment with both DADS and MAPK inhibitor (SB203580 or U0126) decreased proliferation activity and viability of HepG2 hepatoma cells, and led to increase in apoptotic activity (Fig. 7). The combined effect is greater than that by DADS- or inhibitor-treatment alone, suggesting the possibility that DADS and MAPK inhibitors, especially the combination of both, may have chemocytotoxic value for human hepatoma.

In summary, our results show that DADS induces apoptosis and temporarily activates MAPKs in HepG2 hepatoma cells. Both phospho-p38 and phospho-p42/44 negatively regulate HepG2 hepatoma cell apoptosis. The MAPK inhibitors, SB203580 and U0126, not only induce apoptosis but also enhance an apoptotic effect in DADS-induced apoptosis of HepG2 hepatoma cells.

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References

[1] Sundaram SG, Milner JA. Diallyl disulfide induces apoptosis of human colon tumor cells. Carcinogenesis 1996;17:669–73.
[2] Hong YS, Ham YA, Choi JH, Kim J. Effects of allyl sulfur compounds and garlic extract on the expression of Bcl-2, Bax, and p53 in non small cell lung cancer cell lines. Exp Mol Med 2000;32:127–34.
[3] Kwon KB, Yoo SJ, Ryu DG, Yang JY, Rho HW, Kim JS. Induction of apoptosis by diallyl disulfide through activation of caspase-3 in human leukemia HL-60 cells. Biochem Pharmacol 2002;63:41–7.
[4] Nakagawa H, Tsuta K, Kiuchi K, Senzaki H, Tanaka K, Hioki K. Growth inhibitory effects of diallyl disulfide on human breast cancer cell lines. Carcinogenesis 2001;22:891–7.

[5] Bottone Jr FG, Baek SJ, Nixon JB, Eling TE. Diallyl disulfide (DADS) induces the antitumorigenic NSAID-activated gene (NAG-1) by a p53-dependent mechanism in human colorectal HCT 116 cells. J Nutr 2002;132:773–8.
[6] Thornberry NA, Lazebnik Y. Caspases: enemies within. Science 1998; 281:1312–6.
[7] Meinhardt G, Wendtner CM, Hallek M. Molecular pathogenesis of chronic lymphocytic leukemia: factors and signaling pathways regulating cell growth and survival. J Mol Med 1999;77:282–93.
[8] Kolb JP. Mechanisms involved in the pro- and anti-apoptotic role of NO in human leukemia. Leukemia 2000;14:1685–94.
[9] McDaid HM, Horwitz SB. Selective potentiation of paclitaxel (taxol)-induced cell death by mitogen-activated protein kinase kinase inhibition in human cancer cell lines. Mol Pharmacol 2001;60:290–301.
[10] Iwai-Kanai E, Hasegawa K, Fujita M, Araki M, Yanazume T, Adachi S, et al. Basic fibroblast growth factor protects cardiac myocytes from iNOS-mediated apoptosis. J Cell Physiol 2002;190:54–62.
[11] Widmann C, Gibson S, Jarpe MB, Johnson GL. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol Rev 1999;79:143–80.
[12] Dent P, Jarvis WD, Birrer MJ, Fisher PB, Schmidt-Ullrich RK, Grant S. The roles of signaling by the p42/p44 mitogen-activated protein (MAP) kinase pathway a potential route to radio- and chemo-sensitization of tumor cells resulting in the induction of apoptosis and loss of clonogenicity. Leukemia 1998;12:1843–50.
[13] Bonni A, Brunet A, West AE, Datta SR, Takasu MA, Greenberg ME. Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. Science 1999;286:1358–62.
[14] Berra E, Diaz-Meco MT, Moscat J. The activation of p38 and apoptosis by the inhibition of Erk is antagonized by the phosphoinositide 3-kinase/Akt pathway. J Biol Chem 1998;273:10792–7.
[15] Mendelson KG, Contois LR, Tevosian SG, Davis RJ, Paulson KE. Independent regulation of JNK/p38 mitogen-activated protein kinases by metabolic oxidative stress in the liver. Proc Natl Acad Sci USA 1996;93:12908–13.
[16] Niisato N, Post M, van Driessche W, Marunaka Y. Cell swelling activates stress-activated protein kinases, p38 MAP kinase and JNK, in renal epithelial A6 cells. Biochem Biophys Res Commun 1999;266: 547–50.
[17] Flamigni F, Facchini A, Stanic I, Tantini B, Bonavita F, Stefanelli C. Control of survival of proliferating L1210 cells by soluble guanylate cyclase and p44/42 mitogen-activated protein kinase modulators. Biochem Pharmacol 2001;62:319–28.
[18] Lieu CH, Liu CC, Yu TH, Chen KD, Chang YN, Lai YK. Role of mitogen-activated protein kinase in Taxol-induced apoptosis in human leukemic U937 cells. Cell Growth Differ 1998;9:767–76.
[19] Shtil AA, Mandlekar S, Yu R, Walter RJ, Hagen K, Tan TH, et al. Differential regulation of mitogen-activated protein kinases by microtubule-binding agents in human breast cancer cells. Oncogene 1999;18:377–84.
[20] Wang TH, Popp DM, Wang HS, Saitoh M, Mural JG, Henley DC, et al. Microtubule dysfunction induced by paclitaxel initiates apoptosis through both c-Jun N-terminal kinase (JNK)-dependent and -independent pathways in ovarian cancer cells. J Biol Chem 1999;274:8208–16.
[21] Yujiri T, Fanger GR, Garrington TP, Schlesinger TK, Gibson S, Johnson GL. MEK kinase 1 (MEKK1) transduces c-Jun NH2-terminal kinase activation in response to changes in the microtubule cytoskeleton. J Biol Chem 1999;274:12605–10.
[22] Stone AA, Chambers TC. Microtubule inhibitors elicit differential effects on MAP kinase (JNK, ERK and p38) signaling pathways in human KB-3 carcinoma cells. Exp Cell Res 2000;254:110–9.
[23] Liu WL, Guo X, Guo ZG. Estrogen prevents bovine aortic endothelial cells from TNF-alpha-induced apoptosis via opposing effects on p38 and p44/42 CCDPK. Acta Pharmacol Sin 2002;23:213–8.
[24] Lee LF, Li G, Templeton DJ, Ting JP. Paclitaxel (Taxol)-induced gene expression and cell death are both mediated by the activation of c-Jun NH2-terminal kinase (JNK/SAPK). J Biol Chem 1998;273:28253–60.
[25] Wang TH, Wang HS, Ichijo H, Giannakakou P, Foster JS, Fojo T, et al. Microtubule-interfering agents activate c-Jun N-terminal kinase/stress-activated protein kinase through both Ras and apoptosis signal-regulating kinase pathways. J Biol Chem 1998;273:4928–36.
[26] Yujiri T, Sather S, Fanger GR, Johnson GL. Role of MEKK1 in cell survival and activation of JNK and ERK pathways defined by targeted gene disruption. Science 1998;282:1911–4.
[27] Amato SF, Swart JM, Berg M, Wanebo HJ, Mehta SR, Chiles TC. Transient stimulation of the c-Jun-NH2-terminal kinase/activator protein 1 pathway and inhibition of extracellular signal-regulated kinase are early effects in paclitaxel-mediated apoptosis in human B lymphoblasts. Cancer Res 1998;58:241–7.
[28] Huang Y, Sheikh MS, Fornace Jr AJ, Holbrook NJ. Serine protease inhibitor TPCK prevents Taxol-induced cell death and blocks c-Raf-1 and Bcl-2 phosphorylation in human breast carcinoma cells. Oncogene 1999;18:3431–9.
[29] Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 1995;270:1326–31.
[30] Bates RC, Buret A, van Helden DF, Horton MA, Burns GF. Apoptosis induced by inhibition of intercellular contact. J Cell Biol 1994;125:403–15.
[31] Hansen MB, Nielsen SE, Berg K. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J Immunol Methods 1989;119:203–10.
[32] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 1976;72:248–54.
[33] Lea MA, Ayyala US. Differentiating and growth inhibitory effects of diallyl disulfide on cancer cells. Int J Oncol 1997;11:181–6.
[34] Farrow SN, Brown R. New members of the Bcl-2 family and their protein partners. Curr Opin Genet Dev 1996;6:45–9.
[35] Kroemer G. The proto-oncogene Bcl-2 and its role in regulating apoptosis. Nat Med 1997;3:614–20.
[36] Kwon KB, Yoo SJ, Ryu DG, Yang JY, Rho HW, Kim JS, et al. Induction of apoptosis by diallyl disulfide through activation of caspase-3 in human leukemia HL-60 cells. Biochem Pharmacol 2002;63:41–7.