

**ERK IS INVOLVED IN THE DIFFERENTIATION INDUCED BY
DIALLYL DISULFIDE IN THE HUMAN GASTRIC CANCER CELL
LINE MGC803**

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Abstract: Diallyl disulfide (DADS) is a major constituent of garlic. Previously, we found that DADS both inhibited proliferation in human gastric cancer cells *in vitro* and *in vivo*, and induced G2/M arrest. In this study, we investigated whether this differentiation effect was induced by DADS in human gastric cancer MGC803 cells, and whether it was related to an alteration in ERK activity. The results showed that the growth of MGC803 cells was inhibited by DADS. Cells treated with DADS displayed a lower nucleocytoplasmic ratio and tended to form gland and intercellular conjunction structures. The ConA-mediated cell agglutination ratio and cells' ALP specific activity decreased. In MGC803 cells, dye transfer was limited to a few cells neighbouring the dye-injected cell and to a depth of 1-2 layers beneath the scrape site. However, after treatment with DADS, the LY (Lucifer Yellow) was transferred to several cells immediately neighbouring the microinjected cell and to a depth of 2-4 cell layers from the scrape site. This indicated that DADS induced differentiation in MGC803 cells. Western blot analysis revealed that although DADS did not influence the quantity of ERK1/2 protein expressed, it did decrease its phosphorylation in a concentration-dependent manner, compared with the controls. At 30 mg·L⁻¹, DADS inhibited the activation of ERK1/2 in 15-30 min. These results suggested that the DADS-induced differentiation of MGC803 cells involved an alteration of the ERK1/2 signaling pathway.

Key words: Diallyl disulfide, Stomach neoplasm, Differentiation, ERK

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Abbreviations used: ALP – alkaline phosphatase; ConA – concanavalin A; DADS – diallyl disulfide; DAS – diallyl sulfide; ERK – extracellular signal-regulated kinase; GJIC – gap junctional intercellular communication; LY – Lucifer Yellow; MAPK – mitogen-activated protein kinase; MTT – tiazolyl blue; PBS – phosphate buffered saline; RA – retinoic acid; SLDT – scrape-loading and dye transfer

INTRODUCTION

Gastric cancer is one of the leading causes of cancer death and the most common type of malignancy in China [1]. Despite recent advances in surgical and chemotherapeutic procedures, the 5-year survival rate for gastric cancer remains very low. Identifying alternative factors that may reduce the initiation and promotion of gastric cancer is therefore important for minimizing the incidence and severity of this disease.

A high intake of garlic is associated with a protective effect against various cancers such as stomach cancer in humans [2]. Several epidemiologic studies have suggested that garlic plays an important role in the reduction of deaths from cancer [3]. Garlic oil, or more specifically the diallyl disulfide (DADS) it contains, has recently become more appealing as an anti-carcinogenic agent, in part due to its ability to induce apoptosis *in vitro* [4] and inhibit the formation and growth of tumors *in vivo* [5, 6]. Generally, it is the lipid-soluble organic compounds in garlic that possess the most effective antiproliferative agents warranting further study as antitumorigenic agents [7]. Several studies have demonstrated the efficacy of garlic compounds as antitumorigenic agents. For example, DADS inhibits the *in vitro* growth of colon, lung, breast and gastric cancer cell lines, and leukemia cell lines [8-12], indicating that DADS may be a useful therapeutic tool in the prevention of environmentally induced cancers. We previously reported that DADS induced G2/M arrest and inhibited growth of MGC803 cells. Furthermore, it was found that the activation of the p38 MAP kinase pathway and decreased cdc25c protein expression are critical events in G2/M arrest by DADS [12, 13]. However, a systematic, detailed study of the differentiation effect of DADS on human gastric cancer cells has yet to be performed. Thus, the primary objective of our study was to examine the effect of DADS on the differentiation effect in human gastric cancer cell line MGC803.

The mitogen-activated protein (MAP) kinases, which comprise the extracellular signal-regulated kinase (ERK1/2,p44/p42), c-Jun N-terminal kinases (JNK), p38 and ERK5, have been shown to regulate a wide variety of cellular events, such as cell proliferation, differentiation and development [14]. MAPKs/(ERKs) have been shown to be pivotal elements in the processes that govern the fate of cells upon the reception of external stimuli, either promoting proliferation or switching on the genetic guidelines that will lead to terminal differentiation [15]. Much attention has been focused on the ERK pathway as a possible target for newly designed anti-neoplastic drugs [16]. Therefore, it is essential to establish the role that the ERK pathway plays in the process that leads to differentiation within a specific tissue or cell type. In this study, ERK activity is observed in the DADS-induced differentiation effect in human gastric cancer cell line MGC803.

MATERIALS AND METHODS

Reagents

The gastric cancer cell line MGC803 was provided by the Cancer Research Institute of Xiangya Medical School of Central South University. DADS (d420 = 1.0, Mr146.28, hallmark 80%, containing 10-20% DAS, dissolved in Tween-80) was purchased from Fluka Inc. MTT and trypsin were obtained from Amresco. Both ConA and Lucifer Yellow dye were purchased from Sigma Co. Ltd. An alkaline phosphatase specific activity detection kit was purchased from Nanjing Jiancheng Biotechnology Co. Ltd. MAPK monoclonal antibodies (anti-ERK), the HRP-conjugated anti-rabbit secondary antibody, a Phototope-HRP Western Detection kit and U0126 were purchased from New England Biolabs Inc.

Cell culture

MGC803 cells were obtained from the Institute of Oncology, Medical School of Central South University. Cells were maintained in RPMI 1640 medium supplemented with $100\text{ml}\cdot\text{L}^{-1}$ fetal bovine serum (FBS) at 37°C in a humidified 5% CO_2 incubator.

Cell viability assay

Cells were seeded out in 96-well plates and grown to 80% confluence in RPMI 1640 with $100\text{ml}\cdot\text{L}^{-1}$ FBS. The cultures were then rinsed and incubated with their respective test substances for 24 h. Cell viability was measured via the MTT assay. MTT was dissolved in 1.0 ml PBS. An amount of this solution equal to $100\text{ml}\cdot\text{L}^{-1}$ of the culture medium volume was added to the cell cultures. After 6 h incubation, the cultures were removed from the incubator and the formazan crystals solubilized by adding dimethyl sulfoxide. Metabolic activity was quantified by measuring light absorbance at 570 nm.

Transmission electron microscopy observation

The MGC803 cells and the cells treated with $30\text{ mg}\cdot\text{L}^{-1}$ DADS were rinsed twice with D-Hank's solution, shaved into a centrifuge tube with a plastic scraper, and centrifuged at 2000 rpm for 15 min. The supernatant was removed. The precipitate was prefixed in 2.5% glutaraldehyde for 2 h, postfixed in 1% osmium tetroxide for 2 h, dehydrated in ethanol series, embedded in epoxy resin 618, stained with lead citrate and uranyl acetate, and observed under a JEM-100CXII transmission electroscop.

ConA-mediated cell agglutination

Cells were incubated and digested with 0.2% trypsin. Then 0.01 M PH 7.4 PBS was used to form a single cell suspension of $1\times 10^5/\text{ml}$. The ConA stock solution was diluted to 25, 50, 100 and 200 $\mu\text{g}/\text{ml}$ ConA/PBS solution. 0.1 ml of the ConA solution was added to 0.1 ml of the appropriately modified cell suspension in 24-hole plates. The suspension was incubated with shaking on a Dubnoff

shaker. Then, the cells were placed at a standstill for observation of the agglutination phenomenon. A “+” means a result of more than 20 cell agglutination units per plate. The numbers of agglutinating cells with 100 µg/ml ConA solution was used to calculate the cell agglutination ratio by the following formula:

Cell agglutination ratio (%) = [(number of single cells without ConA - number of single cells with ConA) / number of single cells without ConA] × 100%

Duplicate samples were removed, each counted in triplicate and the results averaged.

Alkaline phosphatase (ALP) assay

Cells were digested with 0.2% trypsin and single cell suspensions were formed. These were centrifuged at 1000 rpm for 10 min. PBS solution was added to cell cultures by resuspension, and these were centrifuged at 1000 rpm for 10 min twice. 0.25% sodium deoxycholic acid/PBS solution 0.5 ml was added to split the cells, and then that mixture was centrifuged at 1500 rpm for 15 min, and determined without a depositor. The MGC803 cell total protein and alkaline phosphatase assays were carried out as in the technical manuals. The degree of light absorption of each tube was analyzed by spectrophotometer. Alkaline phosphatase activity was calculated by the following formula:

Alkaline phosphatase activity (Jinshi unit/100 ml) = light absorption degree of the assay tube / light absorption degree of a standard tube × phenol content (0.005) × dilution power (100/0.05)

Cell-cell communication assay – the scrape-loading and dye transfer (SLDT) technique

The culture medium from confluent cells was removed and saved. The cells were rinsed three times with Hanks' balanced salt solution containing 1% bovine serum albumin, after which a 27-gauge needle was used to create multiple scrapes through the cell monolayer in the presence of Dulbecco's phosphate-buffered saline, containing 0.5% rhodamine-dextran and 0.5% Lucifer yellow. After 3 min incubation at room temperature, the culture was rinsed three times with Hanks' balanced salt solution containing 1% bovine serum albumin, and then incubated for an additional 8 min in the saved culture medium to allow the loaded dye to transfer to adjoining cells. The cells were then rinsed and fixed with 4% paraformaldehyde and viewed using a fluorescence microscope with a UV light source. Several studies have demonstrated that such dye transfer is related to the morphological presence of gap junctions, radioactive metabolite transfer, and electrical coupling between cells [17].

Cell extracts and Western blotting

Cells were plated in 12-well plates. Semiconfluent cells were washed twice with PBS and incubated in a lysis buffer composed of 0.1 mol/L NaCl, 0.01 mol/L Tris·Cl (pH 7.6), 0.001 mol/L EDTA, 1 µg/ml aprotinin and 100 µg/ml phenylmethylsulfonyl fluoride (PMSF). The amount of protein in the resulting

supernatant was determined using a standard Bradford test kit. ERK-containing lysates from MGC803 cells were subjected to electrophoresis on a 12% SDS acrylamide gel, transferred onto polyvinylidene difluoride membranes (PVDF) and probed with anti-ERK or anti-P-ERK as the primary antibody and anti-rabbit IgG conjugated peroxidase as the secondary antibody. Bands were detected using enhanced chemiluminescence (ECL New England Biolabs) and quantified with a Bio Image Intelligent Quantifier 1-D, Version 2.2.1 (Nihon-BioImage Ltd).

Data analysis

All the values in this study were expressed as the means \pm SD (standard deviation) from three experiments. We did not observe differences between the results obtained in the three iterations of each treatment. The data was analyzed using the Chi-square test, Dunnett test and ANOVA's least significant difference test. A P value of less than 0.05 was considered statistically significant.

RESULTS

Cell viability

DADS was found to inhibit the growth of MGC803 cells in a dose-dependent manner. Exposure of MGC803 cells to 20 mg·L⁻¹ DADS for 96 h only yielded a ratio of 11.3%, whereas exposure to 35 mg·L⁻¹ DADS increased the ratio up to 60.6% as assessed by the MTT test ($P < 0.05$) (Tab. 1).

Tab. 1. The inhibition effects on MGC803 cells exposed to various concentrations of DADS for 96 h.

n = 6	Control	DADS (mg·L ⁻¹)			
		20	25	30	35
Mean	0.71	0.63	0.46	0.36	0.28
SD	0.021	0.025	0.033	0.046	0.039
Inhibition rate (%)		11.3	35.2	50.7	60.6

The effects of DADS on the morphology of MGC803 cells

Transmission electron microscopy revealed that the nucleo-cytoplasmic ratio of MGC803 cells was relatively large. The nucleus was irregular, with many heterochromatins in the nucleus. Nuclear indentation was obvious. Few organelles were found in the cytoplasm. However, after treatment with 30 mg·L⁻¹ DADS, the ultrastructure of the MGC803 cells underwent a significant change. The number of microvilli on the cell surface decreased, and the cells tended to form gland and intercellular junction structures. The nucleo-cytoplasmic ratio lessened. The amount of heterochromatin in the nucleus decreased while the amount of euchromatin increased, and the volume of the nucleolus lessened.

MGC803 cells showed some ultrastructural characteristics of their equivalent normal cells after being treated with DADS. (Fig. 1, 2).

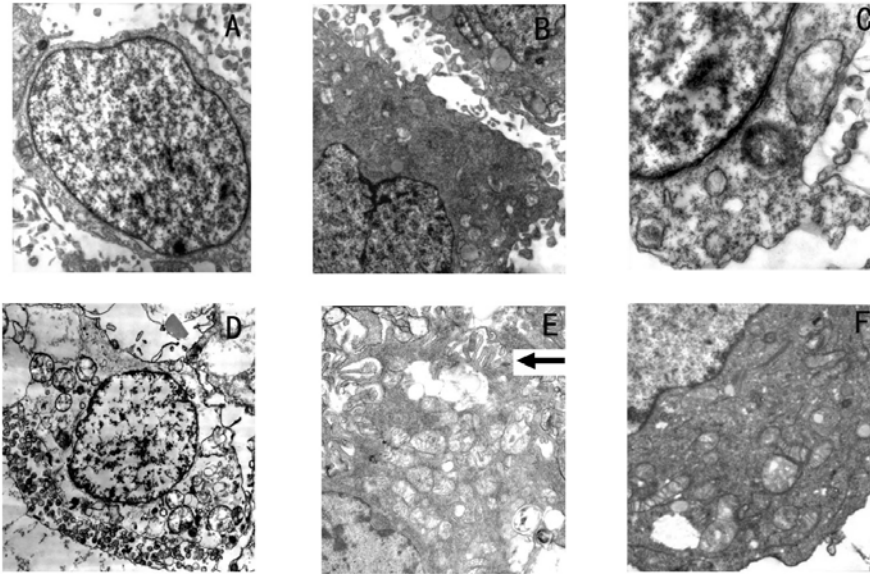


Fig. 1. The subcellular structure of untreated MGC803 cells (A-C), and treated with $30 \text{ mg}\cdot\text{L}^{-1}$ DADS (D-F). The nucleo-cytoplasmic ratio was large in the MGC803 cells (A) and nuclear indentation was obvious in untreated cells (B). The nucleo-cytoplasmic ratio was lessened by $30 \text{ mg}\cdot\text{L}^{-1}$ DADS (D) and an intercellular junctions developed (E) ($8,000\times$). Cytoplasmic organelles were few, and there was chondriosome swelling in untreated cells (C) ($20,000\times$), whereas organelles were abundant in the cytoplasm of cells treated with $30 \text{ mg}\cdot\text{L}^{-1}$ DADS (F) ($15,000\times$).

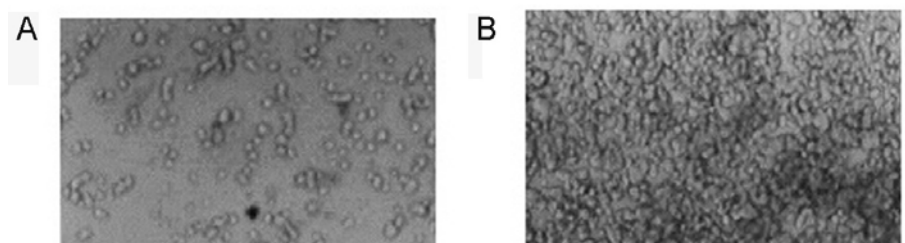


Fig. 2. The ConA-mediated cell agglutination ratio for MGC803 cells treated with $30 \text{ mg}\cdot\text{L}^{-1}$ DADS. A - untreated MGC803 cells, B - MGC803 cells treated with DADS ($20\times$).

Agglutination of MGC803 cells by ConA

ConA can cause cell agglutination if the cell has a corresponding membrane receptor. The ConA-mediated MGC803 cell agglutination ratio can thus indirectly indicate the number of microvilli on the cell surface. Tab. 2 showed that the ConA-mediated cell agglutination ratio lowered to 37.5% – 27% versus the control 79.1% when the MGC803 cells were treated with DADS at concentrations from 20 mg·L⁻¹ to 35 mg·L⁻¹ ($P < 0.05$); this effect occurred in a dose-dependent manner ($\chi^2 = 29.78$, $P < 0.05$, Tab. 2, Fig. 2).

Tab. 2. The ConA-mediated cell agglutination ratio for MGC803 cells treated with DADS at various concentrations.

	MGC803 cells	DADS (mg·L ⁻¹)			
		20	25	30	35
Single cell number with ConA	28	150	68	59	46
Single cell number without ConA	134	240	105	85	63
Cell agglutination ratio (%)	79.1	37.5	35.2	30.6	27.0

Alkaline phosphatase assay

The alkaline phosphatase (ALP) specific activity of MGC803 cells was 0.120 U/g (Units per gram of protein). After treatment with DADS from 20 to 35 mg·L⁻¹, the ALP specific activity decreased from 0.102 to 0.040 U/g, and the decreasing ratio was from 15.7% to 66.1% ($F = 207.6$, $P < 0.05$), which indicated that DADS inhibited the ALP specific activity of MGC803 cells in a dose-dependent manner.

Tab. 3. The ALP specific activity of MGC803 cells treated with DADS.

	MGC803 cells	DADS (mg·L ⁻¹)			
		20	25	30	35
ALP specific activity (U/g)	0.120	0.102	0.066	0.059	0.040
Decreasing ratio (%)		15.7	15.5	51.2	66.1

Scrape-loading and dye transfer technique (SLDT)

To verify the existence of GJIC in the endometrial carcinoma cells, scrape-loading/dye transfer was performed. In the MGC803 cells, dye transfer was limited to a few cells neighbouring the dye-injected cell; similarly, dye transfer was restricted to a depth of 1-2 layers beyond/beneath the scrape site (Fig. 3A). However, after treatment with DADS, LY was transferred to several cells immediately neighbouring the microinjected cell and to a depth of 2-4 cell layers from the scrape site (Fig. 3B).

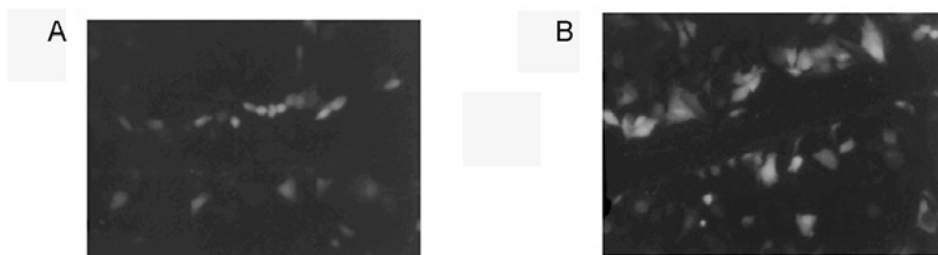


Fig. 3. MGC803 cells were exposed to $30 \text{ mg}\cdot\text{L}^{-1}$ DADS for 1 day. The cells were then assayed for intercellular communication using Lucifer yellow dye transfer after scrape-loading. In untreated MGC803 cells, the dye spread was one cell layer thick (A). After treatment with $30 \text{ mg}\cdot\text{L}^{-1}$ DADS, the dye reached 2 or 3 cells from the scrape line (B).

Expression of phospho-ERK1/2

$30 \text{ mg}\cdot\text{L}^{-1}$ DADS inhibited the activation of ERK1/2, with a maximal inhibition after 15 min incubation, and then a return to near baseline levels by 2 h after treatment. At concentrations of 20, 25, 30 and $35 \text{ mg}\cdot\text{L}^{-1}$, treatment with DADS for 15 min inhibited the expression of phospho-ERK1/2 in a dose-dependent

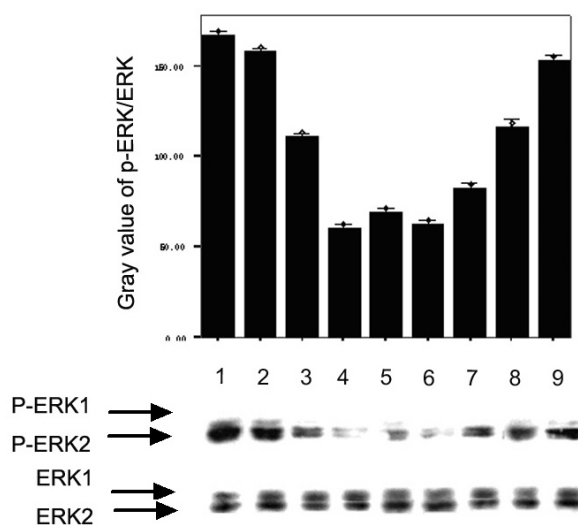


Fig. 4. DADS inhibits ERK1/2 phosphorylation. MGC803 cells were treated with $30 \text{ mg}\cdot\text{L}^{-1}$ DADS. Lysates were prepared at the times shown. ERK1/2 phosphorylation and expression were determined by Western blot with antiphospho-ERK and anti-ERK antibodies. Expression and phosphorylation of ERK1/2 were analyzed by Western blot. The Gray values for phospho-ERK1/2 and the total ERK1/2 were determined by thin-layer chromatography. $n = 3$ experiments, mean \pm SEM. Average of duplicates constitutes one determination.

manner (Figs 4, 5). In Fig. 6, U0126 is shown to weakly reinforce the inhibition of phosphorylation by DADS, causing an increase in total ERK1/2 (Fig. 6). In parallel experiments, the amount of total ERK1/2 was determined in the same cell extracts using an antibody that recognized all ERK1/2 independent of their phosphorylation state.

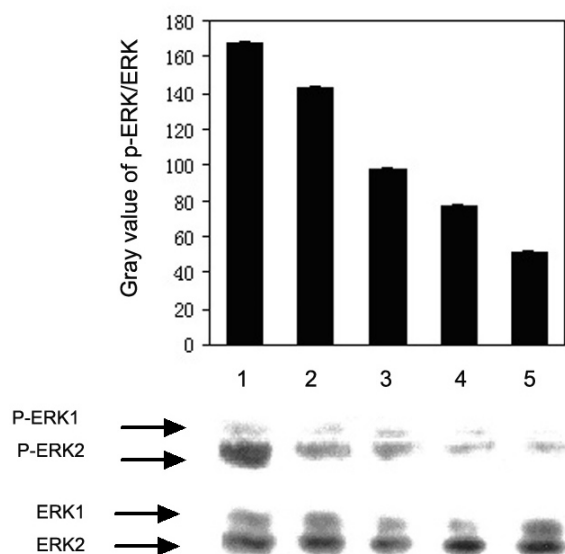


Fig. 5. MGC803 cells were treated with 20, 25, 30 or 35mg·L⁻¹ DADS, and lysates were prepared for Western blot after 15 min. Expression and phosphorylation of ERK1/2 were analyzed by Western blot. The Gray values for phospho-ERK1/2 and total ERK1/2 were determined by thin-layer chromatography. n = 3 experiments, mean ± SEM. Average of duplicates constitutes one determination.

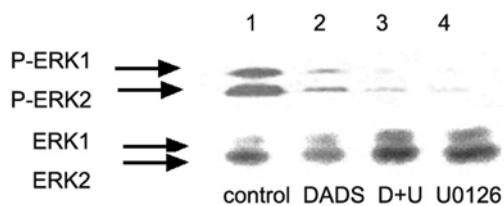


Fig. 6. The effect of U0126 (10 μmol·L⁻¹) on DADS-induced differentiation in MGC803 cells. MGC803 cells were treated with 30 mg·L⁻¹ DADS for 15 min (lane 2) or 10 μM U0126 (MEK inhibitor) for 1 h (lane 4). In lane 3, MGC803 cells were pretreated with U0126 for 1 h and then treated with 30 mg·L⁻¹ DADS for 15 min; then, lysates were prepared for Western blot.

DISCUSSION

In this study, apparent growth inhibition could be seen in the MGC803 cell line treated with DADS in a dose-dependent model. At the higher concentration of $35\text{mg}\cdot\text{L}^{-1}$, DADS displayed a time-dependent model unlike at $25\text{mg}\cdot\text{L}^{-1}$, and inhibited MGC803 cell growth steadily.

There was a significant difference in the morphology and ultrastructure between the tumor cells and their relevant normal cells. Tumor cells usually display some malignant morphological and ultrastructural characteristics, such as a relatively large nucleocytoplasmic ratio, a large malformed nucleus with several nucleoli, underdeveloped organelles, and abundant microvilli. Therefore, it is important to observe and identify the changes in the ultrastructure in tumor cells when determining the effects of differentiation inducers. A series of studies on chemically induced differentiation of gastric carcinoma, leukemia, hepatoma and lung carcinoma cells have made it clear that the ultrastructure of carcinoma cells after induction underwent a restorative alteration similar to that of relevant normal cells [18]. MGC803 is a poorly-differentiated human gastric adenocarcinoma cell line with fast-proliferation and highly malignant characteristics. This study showed that MGC803 cells had the typical malignant phenotypical characteristics of ultrastructure in tumor cells. However, in MGC803 cells treated with $30\text{mg}\cdot\text{L}^{-1}$ DADS, they displayed the following changes: the number of microvilli on the cell surface decreased; cells tended to form glands and intercellular junction structures; cellular apparatus were abundant in the cytoplasm; the nucleocytoplasmic ratio decreased; the heterochromatin level in the nucleus decreased while the euchromatin level increased; the volume of the nucleolus decreased. All showed some ultrastructural characteristics of their equivalent normal cells after being treated with DADS.

The enzyme-digested solution from *Mensamaria intercedens* (Lampart) has the effect of inhibiting the cancerous phenotype including the changes of morphology, the percentage of ConA-mediated agglutination and so on, and can somewhat induce the differentiation of the MGC803 cell line [19]. Using a transmission electron microscope and ConA-mediated cell agglutination, we found that the number of microvilli on the cell surface decreased and the ConA-mediated cell agglutination ratio lowered when MGC803 cells were treated with DADS; this indicated that the MGC803 cells' malignant characteristics were declining, and that decreasing ratio was found to be dose-dependent. It was remarkable that the activity of alkaline phosphatase (ALP), which is not present in normal human gastric mucosa, was highly expressed in MGC803 cells which have a low level of differentiation. DMSO was able to induce differentiation of MGC 803 cells and change their malignant phenotypes, including a decline in ALP specific activity [20]. The treatment with isoverbascoside also significantly inhibited both ALP and LDH activities, suggesting that isoverbascoside induced MGC803 cells to revert against the malignant phenotypes [21]. Our results revealed that the treatment with $30\text{mg}\cdot\text{L}^{-1}$ DADS significantly inhibited ALP

activity, suggesting that DADS can impell MGC803 cell reversion against the malignant phenotypes. All of the above indicated that DADS induced differentiation in MGC803 cells. Recently, other studies showed that DADS inhibits proliferation and induces differentiation through increased histone acetylation on rat liver and hepatoma and colon tumors [22, 23]. The mechanism induced by DADS in MGC803 cells will be explored in our next study.

Gap junctional communication permits the direct exchange of small molecules and ions and has been implicated in tissue homeostasis/metabolite exchange. The lack of gap junctional intercellular communication (GJIC) plays important roles in the promotion and progression of carcinogenesis [24]. Intercellular communication is necessary in the control of cell growth and differentiation in multicellular organisms. Gap junction channels play an important role in intercellular communication by providing a direct pathway for the movement of molecular information, including ions, and polarized and non-polarized molecules up to a molecular mass of 1 kDa between adjacent cells [25, 26]. Compounds able to restore GJIC in junctionally deficient cells may be used for the development of new strategies in the prevention and/or treatment of several human malignancies [27]. The modulation of cell-cell channel communication by RA occurs differently in these two experimental models: RA is able to revert cell transformation in Hep G2 cells, while in fetal hepatocytes, it may induce the expression of a more differentiated phenotype [24]. In this study, treatment with DADS significantly increased the GJIC of MGC803 cells, as was determined using the SLDT assay. The effect on GJIC caused by DADS suggests that it might be beneficial in tumor therapy. Diallyl disulfide (DADS) was able to induce apoptosis in several tumor cell types such as human colon carcinoma, AGS gastric adenocarcinoma, lung carcinoma, bladder cancer and hepatoma cells [4, 28-31]. However, in our study, it is interesting that DADS induced not apoptosis but differentiation in the human gastric cancer cell line MGC803, which suggest that DADS can cause a different anti-cancer effect.

The mitogen-activated protein kinase ERK pathway is crucial in the control of cell proliferation and differentiation [32]. Cytokine-induced myeloid differentiation is dependent on the activation of the MEK/ERK pathway [33]. Thyroid hormone-induced morphological differentiation and maturation of astrocytes involves an initial decline in the p-ERK level and then sustained activation of p-ERK levels [34]. G proteins and ERK activation play roles in the hemin-induced erythroid differentiation of K562 cells [35]. The activation of the ERK mitogen-activated protein kinase pathway stimulates neuroendocrine differentiation in prostate cancer cell lines [36]. Mitogen-activated protein kinase is required for the proliferation and cardiomyocyte differentiation of P19 embryonal carcinoma cells [37]. The activation of the ERK2-ERK1 signaling pathway is needed to elicit megakaryocytic differentiation of K562 leukemic cells [38]. The activation of the MEK/ERK signaling pathway is necessary for bryostatin-1-induced differentiation of the human acute lymphoblastic leukemia cell line Reh [39]. Oncostatin M-induced growth inhibition and morphological

changes in MDA-MB231 breast cancer cells are abolished by blocking the MEK/ERK signaling pathway [40]. The ERK pathway plays a major role in ovarian cancer pathogenesis, and down-regulation of this master signaling pathway is highly effective for the inhibition of ovarian tumor growth [41]. Diallyl disulfide induces ERK phosphorylation in human colon tumor cells. High levels of ERK phosphorylation may serve as a compensatory mechanism, mediating the block in G2/M progression by DADS [42]. All of the above show that ERK phosphorylation is activated in the growth inhibition and differentiation effects induced by anti-cancer agents. However, using human gastric cancer MGC803 cells, we observed five differentiation patterns in the protein phosphorylation of ERK1/2: 1) a progressive decrease for 30 min followed by an increase; 2) a time-dependent decrease; 3) no change over a 2-h incubation period; 4) concentration-dependent changes in the activation of ERK1/2 induced by DADS from 25 to 35 mg·L⁻¹; 5) U0126 weakly reinforcing the inhibition of phosphorylation by DADS and causing an increase in the total ERK 1/2 level. The increase may be related to a change in the expression of the ERK gene induced by U0126. It is necessary to further perform a systematic, detailed study of the effect of U0126 on human gastric cancer cells. DADS inhibits ERK1/2 in MGC803 cells, which may be correlated with protein tyrosine phosphatases, which are frequently up-regulated in association with decreased cell proliferation and terminal differentiation. A membrane-associated tyrosine phosphatase acts to decrease peak ERK activity; the negative effects of DADS may be in part mediated by protein tyrosine phosphatase inhibition of ERK and cell proliferation. These findings are supported by studies showing that sustained ERK1/2 activation is the result of MKP-1 degradation through the ubiquitin/proteasome pathway, and that overexpression of MKP-1 directly decreases ERK1/2 phosphorylation [41, 43]. Further studies to examine the interaction between these and other signaling pathways may yield important insights into the molecular basis for the regulation of cell growth and differentiation in gastric cancer cells. In our study, exposing MGC803 cells to DADS revealed that cell proliferation was inhibited in a concentration-dependent manner on day 4. However, DADS had an inhibitory effect on the phosphorylation of ERK within 2 h. The cell population doubling time is usually longer than 24 h, and the phosphorylation of the signaling molecule always initiates in the early phase and with a maximal induction after about 30 min stimulation. DADS inhibited the activation of ERK1/2 with a maximal induction after 15 to 30 min stimulation, then returned to near baseline levels 2 h after treatment. It was only considered by us that phosphorylation usually occurred within about 30 min. However, we have not considered its changes in transcription level after 4 h and *de novo* synthesis in the protein level after 24 h. Therefore, these contents will be involved in our next experiments.

Acknowledgements. This study was supported by the Key Project Foundation of the Science and Technology program of Hunan Province (04SK1004), the Key Project of the Scientific Research Foundation of the Hunan Province Education Department (04A047) and the Scientific Research Foundation of the Hunan Province Education Department (No. 04C538).

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