

Diallyl Disulfide Induced Signal Transducer and Activator of Transcription 1 Expression in Human Colon Cancer Colo 205 Cells using Differential Display RT-PCR

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Abstract. Diallyl disulfide is one of the components of garlic and has been demonstrated to induce apoptosis in many cancer cell lines, though it is not reported to be associated with signal transducer and activator of transcription 1 (STAT1) expression. Moreover the role of STAT1 does not directly affect apoptosis in cancer cells after exposure to chemotherapy agents, though some reports showed that STAT1 is associated with apoptosis. In this study, differential display RT-PCR was used to examine the effects of diallyl disulfide (DADS) on human colon cancer cells (colo 205). The results demonstrated that DADS induced the expression of STAT1 which was also confirmed using Western blotting. STAT1 decoy oligonucleotides were also used to block STAT1 mRNA and led to a decrease in the levels of STAT1 and to subsequent decrease in the percentage of apoptosis induced by DADS in examined colo 205 cells.

Epidemiological and laboratory investigations demonstrated that garlic (*Allium sativum*) has anticancer properties and the consumption of garlic is associated with the reduction of cancer related deaths (1). Garlic-derived organosulfur compounds such as diallyl disulfide (DADS) have been shown to exhibit a wide range of antitumor activation against human cancer cells such as colon, lung, skin, breast,

leukemia and neuroblastoma (2-6). Recent studies have shown that DADS and the specific inhibitors of MAPK induced apoptosis in HepG2 hepatoma cells and the MAPK inhibitors further enhanced the apoptotic effect in DADS-treated HepG2 hepatoma cells (7). In F344 rats, it was reported that DADS acts as an inhibiting potential in colon and renal carcinogenesis induced by N-diethylnitrosamine (8). Our previous studies had demonstrated that DADS induced apoptosis in human colon and bladder cancer cells through the caspase-3 activation pathway (9). It is reported that STAT1 acts as a pro-apoptotic factor in many cell systems such as cardiac myocytes (10-12) and lymphocytes (13). However, there are no reports that address DADS affecting the expression of STAT1 in human colon cancer cells. Therefore, in the present studies, we investigated the role of DADS STAT1 expression in human colon cancer colo 205 cells.

Materials and Methods

Chemicals and reagents. Diallyl disulfide (DADS) was purchased from Fluka Chemika Co. (Bucha, Switzerland). Epigallocatechin-3-gallate (EGCG), propidium iodide (PI), ribonuclease-A, trypan blue, Tris-HCl and Triton X-100 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), potassium phosphates and TE buffer were purchased from Merck Co. (Darmstadt, Germany). RPMI-1640, fetal bovine serum (FBS), and glutamine, penicillin-streptomycin and trypsin-EDTA were obtained from Gibco BRL (Grand Island, NY, USA).

Human colon cancer cell line. Human colon cancer colo 205 cell line was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). The cells were immediately placed into 75-cm² tissue culture flasks and grown at 37°C under a humidified

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5% CO₂ and 95% air at one atmosphere in RPMI 1640 medium supplemented with 10% FBS, 1% glutamine and 1% penicillin-streptomycin (10 ng/ml penicillin and 10 ng/ml streptomycin).

Flow cytometry analysis of apoptosis from colo 205 cells treated with DADS. Approximately 5x10⁵ cells/well of colo 205 cells in 12-well plate with 50 μM DADS were incubated in an incubator for 24 and 48, and the cells harvested by centrifugation. The cells were fixed gently (drop by drop) by adding 70% ethanol (in PBS) in ice overnight and underwent re-suspension in PBS containing 40 μg/mL PI and 0.1 mg/mL RNase (Sigma, St. Louis, MO, USA) and 0.1% Triton X-100 in the dark. After half an hour at 37°C, the cells were analyzed with a flow-cytometer (Becton-Dickinson, San Jose, CA, USA) equipped with an argon laser at 488 nm wave length. The cell cycle distribution was determined (14, 15).

RNA isolation, mRNA differential display and gene identification. Total RNA of colo 205 cells with or without DADS cotreatment was isolated using TRIzol reagent according to the manufacturer's protocol (Life Technologies, Inc., Grand Island, NY, USA). For mRNA differential display, the protocol was as described elsewhere (14). Arbitrary primer H-AP76 and reverse primer H-T11A were used in this experiment as in previous studies (16).

Effect of DADS on the production of STAT1 and apoptosis from colo 205 cells. approximately 5x10⁵ cells/well of colo 205 cells in 12-well plate with concentrations (0, 0.5, 5, 10, 25 and 50 μM) of DADS or treated with 50 μM DADS were incubated for different time and the cells harvested by centrifugation. The isolated cells were divided into two parts, part one assayed for apoptosis as described above. The other was treated with primary anti-STAT1 for 2.5 h then washed with PBS. The cells then were stained with secondary antibody with fluorescence for 30 min. The percentage staining for STAT1 was assayed as described elsewhere (16).

Effect of DADS on the production of STAT1 and apoptosis from colo 205 cells after pretreatment with STAT1 decoy oligonucleotides. Approximately 5x10⁵ cells/well of colo 205 cells in 12-well plate with 50 μM DADS or pretreated with STAT1 decoy oligonucleotides (CATGTTATGCATATTCCTGTAAGTG) were incubated for 3 h followed by 50 μM DADS treatment for 24 h, before the cells were harvested by centrifugation. The isolated cells were divided into two parts, one part was assayed for apoptosis by flow cytometric methods as described above, the other was treated with primary anti-STAT1 for 2.5 h then washed with PBS. The cells then were stained with secondary antibody with fluorescence for 30 min. The percentage of STAT1 was assayed using Western blotting as described elsewhere (17).

Results

Effect of DADS on the production of mRNA STAT1 from colo 205 cells. Representative gel of differential display RT-PCR showing changes on the levels of STAT1 mRNA in colo 205 cells after exposure to 0, 0.5, 5 or 50 μM DADS (Figure 1A). The results indicated that 50 μM DADS promoted the levels of mRNA STAT1 while other low doses did not show significant effects on mRNA STAT1 expression in colo 205 cells. Figure 1B shows the sequence of STAT1 of colo 205 cells.

Effect of DADS on the production of STAT1 and apoptosis of colo 205 cells. The results from colo 205 cells after exposure to different concentration of DADS demonstrated that DADS induced STAT1 expression and these effects were dose-dependent (Figure 2A). The treatment with 50 μM DADS induced significantly increased STAT1 and these effects were also time-dependent (Figure 2B). However, increased time of incubation from cells exposed to 50 μM DADS led to an increase in the levels of STAT1 and apoptosis (Figure 2B and C).

Effect of STAT-1 decoy oligonucleotides on DADS-induced STAT1 and apoptosis of colo 205 cells. After cells were pretreated with STAT-1 decoy oligonucleotides, followed by 50 μM DADS, levels of STAT1 and the percentage of apoptosis was lower than without decoys as presented in Figure 3A and B. The result clearly demonstrate that STAT1 is associated with the apoptosis even though it did not completely block the induction of apoptosis. This result also showed that DADS induced apoptosis in these cells through multiple pathways.

Discussion

We used the differential display mRNA RT-PCR to demonstrate that DADS induced STAT1 expression and these results were also confirmed by flow cytometric analysis using anti-STAT1 antibody stained (data not shown) and Western blotting (Figures 1 and 2). The results also showed that DADS-increased the mRNA levels of STAT1 analyzed by DDRT-PCR (Figure 1). Apparently STAT1 is involved in DADS induced apoptosis, because STAT1 has been directly implicated in apoptotic cell death (Figure 2). The importance of STAT1 as a pro-apoptotic factor has been reported previously using a variety of cell systems. The interesting point is that lymphocytes derived from mice deficient in STAT1 showed reduced apoptosis and enhanced proliferation (18). Other investigators have demonstrated that STAT1-deficient human U3A fibrosarcoma cells are less susceptibility to tumor necrosis factor α-induced cell death than parental cells containing STAT1 (18). It has also been reported that U3A STAT1-deficient cells are resistant to hypoxia-induced cell death (19). Many studies have also demonstrated that STAT1 promoted apoptosis in cardiac myocytes exposed to ischemia/reperfusion injury (20-23).

The results of this study are in agreement with other reports which showed STAT1 phosphorylation on serine 727 but not tyrosine 701 is required for STAT1-induced apoptosis in colo 205 cells with DADS treatment and Western blot studies (data not shown). Although cancer cells were stimulated with interferon or epidermal growth factor, the

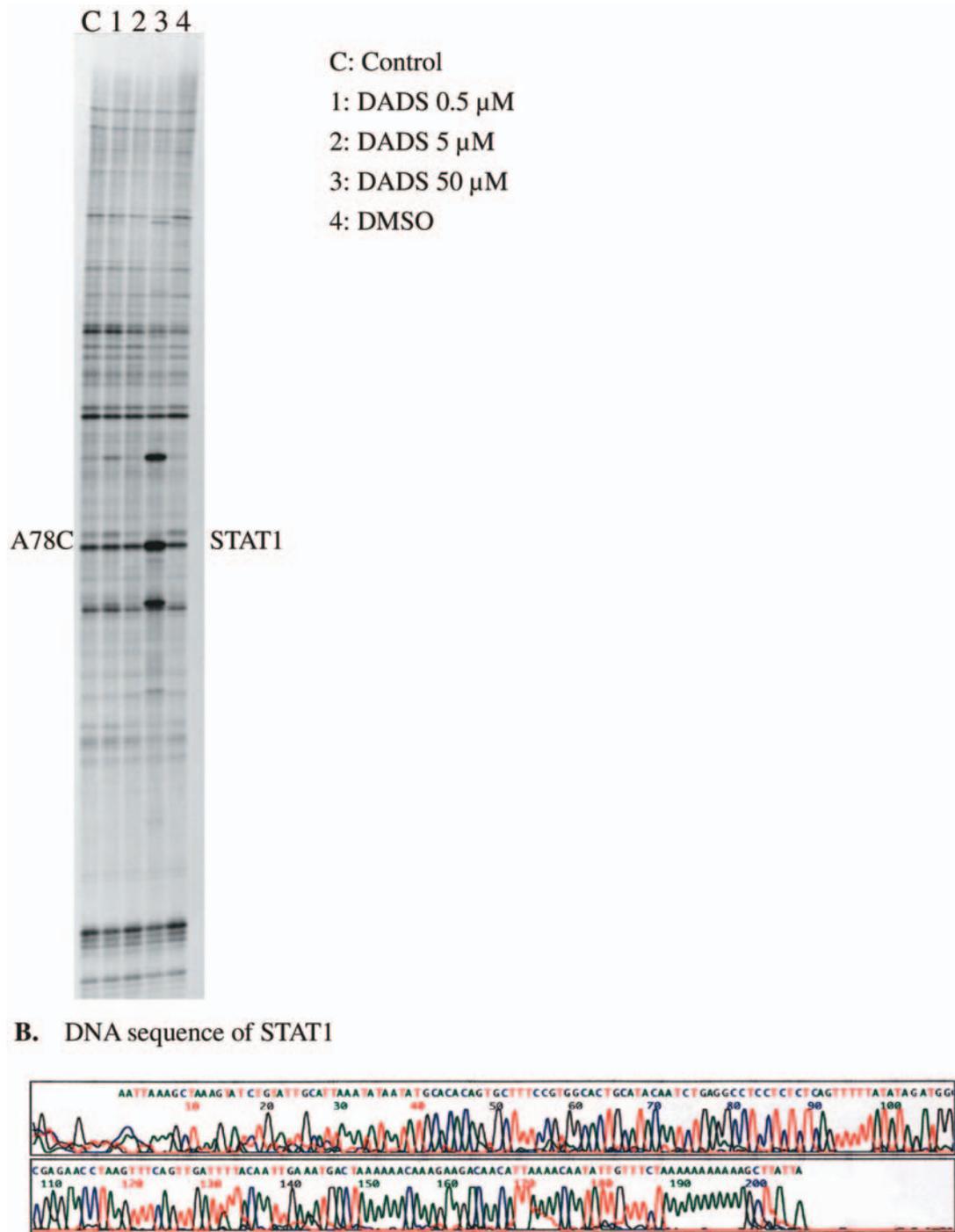


Figure 1. Representative differential display RT-PCR showing changes in the levels of STAT1 mRNA in colo 205 cells after exposure to DADS. Cells ($5 \times 10^6/ml$) were treated with 0, 0.5, 5 and 50 μM DADS for 24 h before total RNA was isolated, DDRT-PCR was performed (A) and the STAT1 sequenced (B) as described in the Materials and Methods section.

STAT1 activation was generally accepted to be initiated by tyrosine phosphorylation at a single site (Tyr701), which is a carboxyl of the SH2 domain (24). Targeting STAT-1 might thus provide an interesting and novel way to interfere with the

development of apoptosis. We also used STAT-1 decoy oligonucleotides (CATGTTATGCATATTCCTGTAAGTG) (ODN) added to the colo 205 cells as in the protocol by Quarcoo *et al.*, 2004 (25) before DADS was added to the same

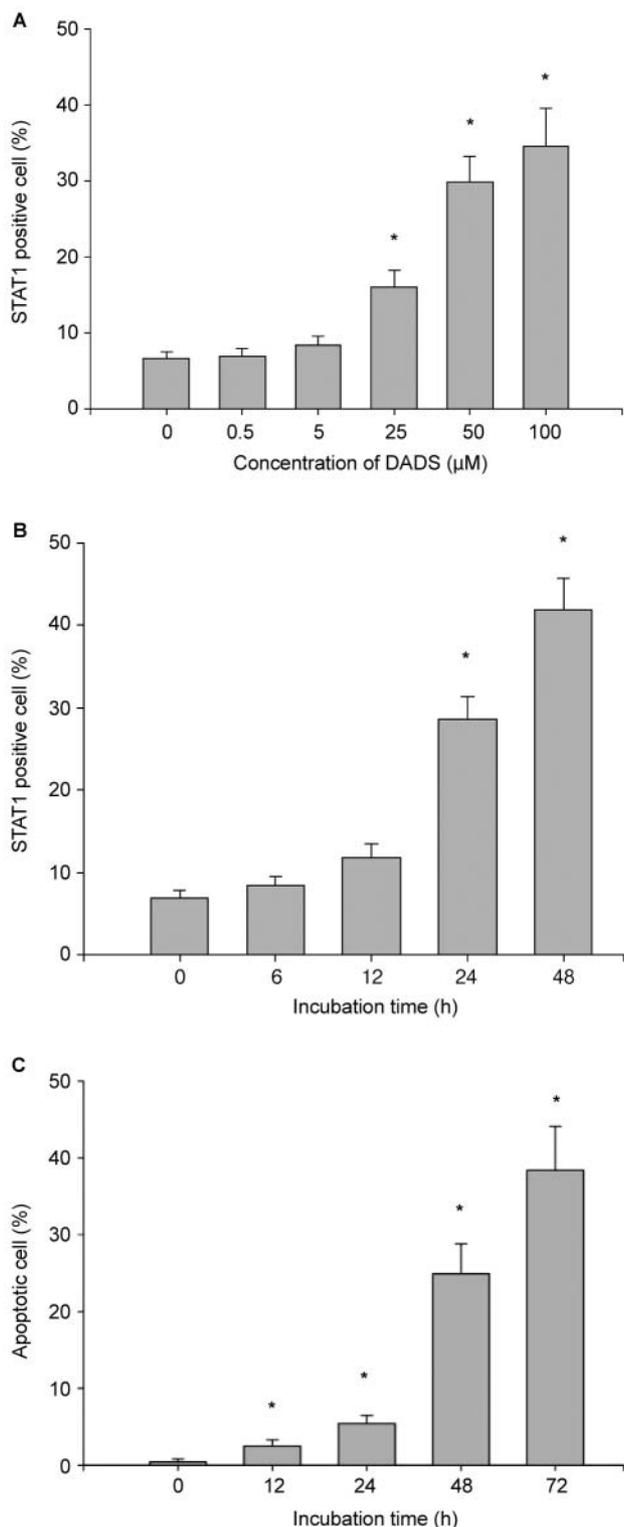


Figure 2. Effect of DADS on the levels of STAT1 and apoptosis of colo 205 cells. Cells (5×10^5 cells/ml) were treated with various concentrations of DADS for different time periods. Then cells were harvested for staining with anti-STAT1 (panel A and B) and determination of apoptosis (panel C). The stained cells were determined with flow cytometry as described in the Materials and Methods section.

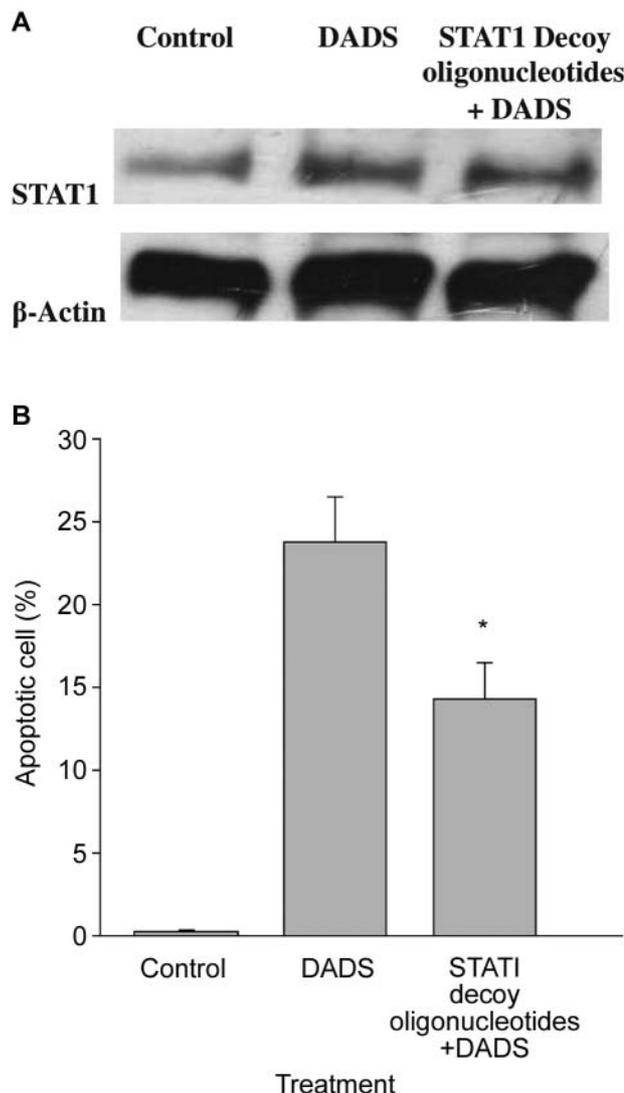


Figure 3. Effect of STAT1 decoy oligonucleotides on the production of STAT1 and apoptosis of colo 205 cells after treatment with DADS. Cells (5×10^5 cells/ml) were treated with 50 μM DADS or pretreated with STAT1 decoy oligonucleotides and were incubated for 3 h followed by 50 μM DADS treatment for 24 h. The harvested cells and the percentage of STAT1 (A) and apoptosis (B) were assayed as described in the Materials and Methods section.

cells for 48 h we observed that apoptosis was reduced (Figure 3), which is in agreement with the reports from Quarcoo *et al.*, 2004 who demonstrated that the disruption of STAT-1 signaling by local application of STAT-1 decoy ODN inhibited the development of allergy (25). Our data also showed that DADS promoted the levels of STAT1 which led to a decrease in the levels of Bcl-2 (data not shown), which is in agreement with the reports that demonstrated that STAT-1 is known to induce down-regulating of the expression of anti-apoptotic genes such as Bcl-2 and Bcl-x promoters (26).

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