Effect of diallyl disulfide on cell cycle arrest of human colon cancer SW480 cells

Qian-Jin Liao,1 Jian Su,2 Jie He,1 Ying Song,1 Hai-Lin Tang1 and Qi Su1,*

1Cancer Research Institute; 2Department of Pathology; The Second Affiliated Hospital; University of South China; Hengyang, Hunan P.R. China

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Background and Objective: Our previous study revealed that diallyl disulfide (DADS) significantly inhibited cell proliferation and induced cell cycle arrest at G2/M phase of human colon cancer SW480 cells. However, the molecular mechanism of cell cycle arrest remains unclear. This study was to investigate the role and the molecular mechanism of DADS in the induction of cell cycle arrest of human colon cancer cell line SW480.

Methods: Proliferation of SW480 cells after DADS treatment was measured by MTT assay and cell counting. Phase distribution of cell cycle arrest of human colon cancer cell line SW480. This study was to investigate the possible mechanism of DADS in inducing cell cycle arrest in human colon cancer cell line SW480.

Materials and Methods

Main drugs and reagents. DADS and propidium iodide (PI) were purchased from Sigma-Aldrich Inc. (Shanghai, P.R. China). The mouse anti-human proliferating cell nuclear antigen (PCNA), p53, cyclin B1 and p21WAF1 monoclonal antibodies were products of Santa Cruz Company (USA). The immunohistochemistry kit was bought from Maixin Company (Fuzhou, Fujian P.R. China).

Cell culture. The human colon cancer cell line SW480 was obtained from China Center for Type Culture Collection (Wuhan, Hubei P.R. China). Cells were cultured in RPMI-1640 medium containing 10% fetal calf serum (FCS) and incubated at 37°C in 5% CO2. Cells during the logarithmic phase of growth were selected for experiments.

MTT. Cells during the logarithmic phase of growth were harvested and digested using trypsin into single cell suspension. Cells were seeded into the 96-well plate at a density of 2.0 × 104 per well. Cells were divided into three groups: blank control group (culture medium only), control group (SW480 cells without DADS treatment) and treatment group (SW480 cells treated with different concentrations of DADS). After 24 h, different doses of DADS were added and incubated with SW480 cells. After another 24 h, 20 μL 5 mg/mL MTT solution was added into each well and incubated for 4 h. DMSO (100 μL) was added following the removal of the supernatants. The crystals were thoroughly dissolved, and the absorbance value of each well was measured at 570 nm by enzyme-linked immunosorbent assay (ELISA). The inhibition rate (IR) was calculated according to the following formula: IR= (1-average absorbance value of the treatment group)/ average absorbance value of the control group) × 100%.

Cell count. SW480 cells during the logarithmic phase of growth were harvested, and seeded in the 24-well plate at a density of 2.0 × 104 per well. After 24 h of culture, cells were counted using trypan blue exclusion assay (N0). Then cells were incubated with...
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Table 1  Distribution of SW480 cell cycle after diallyl disulfide (DADS) treatment

<table>
<thead>
<tr>
<th>Group (μg/mL)</th>
<th>G0/G1 (%) 24 h</th>
<th>S (%) 48 h</th>
<th>G0/G1 (%) 72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>50.8±3.2</td>
<td>54.4±1.1</td>
<td>49.3±6.2</td>
</tr>
<tr>
<td>10</td>
<td>32.2±0.6</td>
<td>25.8±4.1</td>
<td>31.8±1.7</td>
</tr>
<tr>
<td>20</td>
<td>16.0±3.5</td>
<td>19.8±0.8</td>
<td>14.9±4.1</td>
</tr>
<tr>
<td>30</td>
<td>21.3±1.6</td>
<td>23.2±0.9</td>
<td>28.9±2.4</td>
</tr>
<tr>
<td>40</td>
<td>30.2±1.0*</td>
<td>34.7±0.3*</td>
<td>40.2±4.3*</td>
</tr>
<tr>
<td>50</td>
<td>36.9±0.8*</td>
<td>46.6±1.7*</td>
<td>53.5±3.3*</td>
</tr>
<tr>
<td>60</td>
<td>42.3±2.4*</td>
<td>50.0±0.7*</td>
<td>62.1±3.8*</td>
</tr>
</tbody>
</table>

* p < 0.05, vs. 0 μg/mL, °p < 0.05, vs. among each group. Results are presented as mean ± SD.

Different concentrations of DADS for 48 h. The cell number was counted (Nt) after centrifugation. The cell population doubling time (TD) was calculated as follows: TD = (t × \lg2)/\lg(Nt/N0).

Flow cytometry. Cultured cells were collected, centrifuged at 1000 × g for 5 min, and washed by pre-cooled PBS solution at 4°C. Then cells were centrifuged again at 1000 × g for 5 min. The cell concentration was adjusted to 1 × 10^5/mL, and cells were fixed in 70% ethanol at 4°C. Cells were stained with PI at room temperature. The cell cycle distribution of SW480 cells was analyzed by the Epics XL flow cytometer provided by Beckman Coulter (Fullerton, CA USA). All experiments were repeated three times.

Immunocytochemistry. SW40 cells were trypsinized, collected and fixed in ethanol. Then cells were embedded. After the sections were dewaxed and hydrated, PBS was used to rinse the cells for three times, five min each time. A drop of peroxidase blocking solution (Reagent A) was added on each section, followed by incubation at 37°C for 10 min. Following incubation with PBS, non-immune serum (Reagent B) was added and incubated at 37°C for 5 min. After non-immune serum was removed, the primary antibody was added and incubated at 4°C overnight. The sections were rinsed three times with PBS. The secondary antibody (Reagent C) was added and incubated at 37°C for 10 min. The sections were rinsed three times again with PBS before adding HRP-streptavidin (Reagent D). Then the sections were incubated at 37°C for 10 min, followed by washing three times for 10 min with PBS.

Western blot. Total proteins of SW480 cells were extracted using protein lysis buffer (100 mmol/L Nacl, 10 mmol/L Tris-Hcl pH 7.6, 1 mmol/L EDTA pH 8.0, 1 μg/mL aprotinin and 100 μg/mL PMSE) and quantified by biocinchonic acid (BCA) assay. The concentration of each sample was adjusted. An equal amount of protein was separated by SDS-PAGE gel electrophoresis and transferred to a membrane. The membrane was blocked in non-fat milk, followed by incubation with the primary antibody at 4°C overnight. After washing with three times with TBST, 15 min each time, the secondary antibody was added and incubated at room temperature for 1 h. The protein signals were detected by electrochemical luminescence (ECL). The thin-layer chromatogram scanner (TLC) CS-930 (Japan) was used to measure the light density of each blotted band.

Statistical analysis. All data were analyzed by T-test or Chi-square test of SPSS 10.0 Statistical Package. The results are presented as means ± standard deviation. p < 0.05 was considered significantly different.

Results

Inhibition effect of DADS on proliferation of SW480 cells. After treated with 30 μg/mL, 40 μg/mL, 50 μg/mL, 60 μg/mL and 70 μg/mL DADS for 48 h, the proliferation of SW480 cells was inhibited by 23.8%, 43.7%, 49.7%, 60.9% and 66.0%, respectively (p < 0.05). The inhibition effect was in a dose-dependent manner, and the half maximal inhibitory concentration (IC50) was 49.39 μg/mL.

Effect of DADS on the population doubling time of SW480 cells. The population doubling time of SW480 in normal culture was 34.50 h. When the concentration of DADS was increased from 30 μg/mL to 70 μg/mL, the population doubling time of SW480 cells was increased from 35.99 h to 94.74 h (p < 0.05).

Effect of DADS on cell cycle of SW480 cells. As shown in Table 1, the cell cycle distribution of SW490 cells was not significantly altered at different time points in the control group, while was significantly changed in groups treated with different concentrations of DADS. Compared with the control cells, treatment with DADS significantly decreased the percentage of cells in G0/G1 phase and increased at G2/M phase in a time- and dose-dependent manner (p < 0.05), but did not apparently change the percentage of S phase cells. The apoptotic rate was significantly higher in the treatment groups than in the control group (p < 0.05). The apoptosis was most apparent at 48 h after DADS treatment.

Effect of DADS on expression of cell cycle associated proteins in SW480 cells. After incubation with 50 μg/mL DADS for 48 h, cells with positive expression of p53 or PCNA were stained pale yellow, yellow or brown in the cytoplasm. Expressions of p53 and PCNA were significantly weaker in the treatment groups (111.3 ± 49.39 μg/mL).

Effect of DADS on expression of Cyclin B1 and p21WAF1 in SW480 cells. Cells were incubated with DADS (50 μg/mL) for...
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DADS is a low molecular weight liposoluble extract from garlic, and is also the main component of garlic. We showed that DADS significantly inhibited cell proliferation of SW480 cells and induced G2/M cell arrest in a dose- and time-dependent manner. We propose that DADS may prolong the cell cycle by inducing G2/M arrest to inhibit proliferation of SW480 cells.

We further investigated changes in cell cycle associated proteins at G2/M phase. p21WAF1 is the downstream gene of p53, which belongs to the cyclin-dependent kinase inhibitor (CDKI) family. It can inhibit CDK1, CDK2, CDK4 and CDK6 to induce cell cycle arrest. After binding to PCNA, p21WAF1 inhibits PCNA-dependent DNA synthesis to suppress cell proliferation. Recent studies indicate that p21WAF1 participates in regulation at G2/M phase. PCNA could interact with several CDK-cyclin complexes and form a tetramer with Cdc25C and CDK1/cyclin B1, which

Discussion
Cell cycle abnormality is one of the specific characteristics of malignant tumors, which results in continuous proliferation of tumor cells. Currently, cancer treatment focuses on how to intervene the cell cycle of tumor cells, in order to slow down cell proliferation or induce apoptosis. Studies have revealed that many antitumor drugs can specifically inhibit cell cycle. DADS is a low molecular weight liposoluble extract from garlic, and is also the main component of garlic. We showed that DADS significantly inhibited cell proliferation of SW480 cells and induced G2/M cell arrest in a dose- and time-dependent manner. We propose that DADS may prolong the cell cycle by inducing G2/M arrest to inhibit proliferation of SW480 cells.

Figure 1. Expression of PCNA and p53 protein after diallyl disulfide (DADS) treatment in SW480 cells (SP x 400). (A) A large amount of granules (brown yellow) of positive PCNA staining appear in the nucleus of control cells. (B) Granules (yellow brown) of positive PCNA staining are barely seen in the DADS group. (C) large amount of granules (yellow brown) of positive p53 staining appear in the nucleus of control cells. (D) Granules (yellow brown) of positive p53 staining are barely seen in the DADS group.

Figure 2. Expression of cyclin B1 and p21WAF1 protein in SW480 cells after diallyl disulfide (DADS) treatment.

24 h, 48 h and 72 h, respectively. The expression of cyclin B1 was significantly decreased in a time-dependent manner. At 72 h after treatment, the expression of cyclin B1 was decreased to 14.5% of that in the control group (p < 0.05), while that of p21WAF1 was increased to 3.2 times of that in the control group (p < 0.05) (Fig. 2).
would promote cells to enter M phase from G2. When it is induced, p21WAF1 competes with Cdc25C to bind PCNA, so that CDK1/cyclin B1 could not be activated by Cdc25C, thus to lead G2/M cell cycle arrest.8,9 When G2/M arrest was induced by Genistein in immortalized breast epithelial cells, the expression of p21WAF1 was significantly increased, which upregulated the combination of p21WAF1 with CDK1. The results imply that induction of p21WAF1 expression is one of the mechanisms that genistein decreases the activity of CDK1 and induces G2/M cell cycle arrest.10 We found that after SW480 was treated with DADS, the expression of p21WAF1 was upregulated, while that of PCNA was downregulated, which may be associated with DADS induced G2/M in SW480 cells.

p53 gene is one of the anti-oncogenes closely related with human cancer. The wild-type p53 could up-regulate p21WAF1 to induce G2/M cell cycle arrest.12 Mutant p53 promotes cell proliferation and malignant transformation. DADS could induce the expression of wild-type p53.13,14 We found that the expression of mutant p53 was downregulated after SW480 cells were treated with DADS.

Cyclin B1 is a key regulatory factor promoting cells entering M phase from G2 phase. When cells enter G2 phase, the content of cyclin B1 is dramatically increased. During mitosis metaphase, the expression and activity of cyclin B1 reach the peak and are decreased quickly in the anaphase.15 The expression of cyclin B1 directly influences the activity of CDK1. Combination of cyclin B1 and CDK1 is essential to initiate and facilitate cells to go into M phase.16 Cyclin B1 is found closely correlated to G2/M cell cycle arrest and the down-regulated expression of cyclin B1 would induce G2/M cell cycle arrest.17 When G2/M cell cycle arrest was induced by Genistein in PC-3 cells, the expression of cyclin B1 was decreased significantly.18 We noticed that DADS-induced G2/M cell cycle arrest was accompanied by a significant decrease in cyclin B1.

In summary, DADS significantly inhibits cell proliferation, and induces G2/M cell cycle arrest of human colon SW480 cells, which may correlate to the upregulation of p53, PCNA and cyclin B1 and the downregulation of p21WAF1.

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References