Subcellular localization of DJ-1 in human HL-60 leukemia cells in response to diallyl disulfide treatment

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Abstract. Diallyl disulfide (DADS) has been demonstrated to exert potent anticancer effects *in vitro* and *in vivo*. Previous studies indicate that DADS may induce the differentiation and/or apoptosis of human leukemia cells *in vitro*. However, the mechanisms underlying these anticancer effects remain elusive. The aim of the present study was to investigate alterations in the subcellular localization of protein deglycase DJ-1 (also known as Parkinsonism associated deglycase-7, PARK-7) in the cytoplasm, nucleus and mitochondria of human leukemia HL-60 cells induced by DADS, in order to provide novel experimental evidence for the molecular mechanisms underlying the anticancer mechanisms of DADS in leukemia cells. HL-60 cells induced by DADS were collected at different time points, and proteins from the cytoplasm, nucleus and mitochondria of the cells were isolated using specific cellular component isolation kits. The protein expression levels of DJ-1 in these subcellular fractions of HL60 cells following exposure to DADS for varying lengths of time, were determined using western blotting, immunocytochemistry and immunofluorescence techniques. Following exposure of HL-60 cells to 1.25 mg/l DADS for 8 h, the protein expression levels of DJ-1 were significantly decreased in the cytoplasm, while nuclear fractions exhibited a significant increase in DJ-1 expression when compared with untreated controls. The protein expression levels of DJ-1 in mitochondria of HL-60 cells were significantly decreased following treatment with 5 and 10 mg/l DADS. These results demonstrate that exposure of HL-60 cells to low concentrations of DADS may promote DJ-1 protein translocation from the cytoplasm to the nucleus, which suggests that DJ-1 may function as a transcription factor or cofactor binding protein in the process of cell differentiation. The expression of DJ-1 in mitochondria may be associated with induction of apoptosis in HL-60 cells treated with moderate doses of DADS.

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Introduction

Acute myeloid leukemia (AML) is the most common type of acute leukemia diagnosed in adults. Although most patients achieve complete remission following induction chemotherapy treatment, the majority of patients relapse after achieving clinical remission (1,2). Differentiation therapy is an alternative AML treatment method based on the induction of leukemic blast cells to mature beyond the differentiation block, which may restore a normal cellular phenotype and lead to cell cycle arrest (3). The present study was performed in the HL-60 cell line, which was originally isolated from an acute promyelocytic leukemia patient, and is a well-characterized model for studying terminal differentiation events (4).

Diallyl disulfide (DADS) is a major sulfur compound present in garlic, which exerts anti-inflammatory and immune-modulatory effects and enhances sympathetic nervous system activity (5). Previous studies have demonstrated that DADS exerts multiple antitumor effects in a number of different tumor types by activating metabolic enzymes that detoxify carcinogens, suppressing DNA synthesis, inhibiting cell proliferation, angiogenesis or invasion, and preventing the induction of cell cycle arrest, apoptosis and differentiation (6-8).

In a previous study, DADS increased the reduction ability of nitroblue tetrazolium in a time-dependent manner in human leukemia HL-60 cells (9). In addition, DADS inhibited the growth and induced the differentiation of these cells (9). Furthermore, it was reported that DADS induced apoptosis in HL-60 cells (10). DADS-induced apoptosis involved cell cycle arrest in G2/M phase, increased histone acetylation and endoplasmic reticulum (ER) stress, and activation of the Fas-mediated cell death pathway (11). Using proteomic-screening technology, a previous study demonstrated a significant reduction in the protein expression levels of protein deglycase DJ-1 (also known as Parkinsonism associated deglycase-7, PARK-7) in HL-60 cells following DADS treatment (12).

DJ-1, originally identified as an oncogene, appears to be involved in a number of diverse biological processes (13), including regulation of transcription (14), oxidative stress mechanisms (15,16), mitochondrial regulation, fertilization and TNF-related apoptosis-inducing ligand-mediated apoptosis in tumor cells (17). At the subcellular level, DJ-1 is primarily localized to the cytosol, but is expressed at low levels in the mitochondria and nucleus (18). Under oxidative stress conditions, DJ-1 preferentially translocates to the mitochondrial matrix and the intermembrane space in order to initiate protective mechanisms (19). Despite the known association between DJ-1 and cancer (20,21), the precise association between the biochemical function of DJ-1 and its subcellular localization remains unclear.

Previous studies have demonstrated that DADS inhibits the proliferation of HL-60 cells by downregulating the expression of DJ-1 protein (22). In addition, siRNA-mediated suppression of DJ-1 expression enhanced the production of mature granulocyte cell lines and promoted the differentiation of HL-60 cells induced by DADS (22).

The aim of the present study was to investigate the expression of DJ-1 in the cytoplasm, nucleus and mitochondria of HL-60 cells induced by DADS, in order to determine whether the subcellular location of DJ-1 is altered following exposure to DADS for varying lengths of time. These results provide novel experimental evidence for the molecular mechanisms underlying the antitumor effects of DADS in human leukemia cells.

Materials and methods

Cell culture and treatments. HL-60 cells, obtained from The Cancer Research Institute, Xiangya Medical College, Central South University (Changsha, China), were maintained in L-glutamine-containing RPMI 1640 medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA), supplemented with 10% fetal bovine serum (Zhejiang Tianhang Biotechnology Co., Ltd., Hangzhou, China) and without antibiotics. The cells were incubated at 37˚C in 5% CO_2 for 2 days before they were harvested. DADS (purity 80%; the remaining 20% being diallyl trisulfide and diallyl sulfide) was purchased from Sigma-Aldrich; Merck Millipore (Darmstadt, Germany), dissolved in Tween 80 at 8 mg/ml and stored at -20˚C. HL-60 cells ($2x10^6$ cells/ml) in the logarithmic growth phase were placed in fresh medium prior to use in experiments. DADS was diluted to 1.25, 2.5, 5 and 10 mg/l with culture medium and added to cell cultures. Following 4, 8 or 12 h DADS treatment, cells were collected for analysis.

Cytoplasmic, nuclear and mitochondrial protein extraction. HL-60 cells ($2x10^6$ cells/ml) in the logarithmic growth phase were harvested and washed with ice-cold phosphate-buffered saline (PBS). Nuclear and cytoplasmic proteins were isolated from cells using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. Briefly, cells were transferred to a 1.5 ml microcentrifuge tube and pelleted by centrifugation at 500 x *g* for 5 min, 4˚C before a pipette was used to remove the supernatant. Ice-cold Cytoplasmic Extraction Reagent (CER) I was subsequently added to the cell pellet, and the tube was vortexed vigorously for 10 sec before incubating on the ice for 20 min. Ice-cold CER II, was added to the solution, and the tube was vortexed for 5 sec and then centrifuged for 10 min, 4˚C at maximum speed in a microcentrifuge (160,000 x *g*). The resulting supernatant was the cytoplasmic extract, and the insoluble fraction, containing the cell nuclei, was dissolved in ice-cold Nuclear Extraction Reagent. The sample was subsequently vortexed for 15 sec and then incubated on ice for 10 min. This step was repeated 4 times over the course of 40 min and was followed by centrifugation at maximum speed (160,000 x *g*) in a microcentrifuge for 30 min at 4˚C, to obtain the nuclear protein extract.

Mitochondrial proteins were isolated using a Cell Mitochondria Isolation kit (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's instructions. Briefly, cells ($2x10^6$ cells/ml) in the logarithmic growth phase were harvested and washed twice with ice-cold PBS. Cells were incubated with cell lysis buffer (1 ml cell lysis buffer/$2x10^7$ cells) for 10 min at 4˚C, before they were homogenized with a glass homogenizer. The cell lysate was centrifuged at 600 x *g* for 10 min at 4˚C to remove any remaining whole cells, and the supernatant was further centrifuged at 11,000 x *g* for 10 min at 4˚C. The supernatant was removed and the remaining mitochondrial pellet was resuspended in mitochondrial lysis buffer at 4˚C, and centrifuged at 24,000 x *g* for 15 min at 4˚C to remove the cell nuclei. Mitochondrial extracts were stored at -80˚C until required. The concentration of cytoplasmic, nuclear and mitochondrial protein extracts was determined using a bicinchoninic acid assay kit (Pierce; Thermo Fisher Scientific, Inc.).

Western blot analysis. Sample protein extracts were denatured by boiling at 100˚C for 5 min in 5X SDS-PAGE Sample Loading buffer (Beyotime Institute of Biotechnology), at a ratio of 4:1. Proteins were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes by electroblotting. Membranes were blocked using 5% non-fat dry milk in tris-buffered saline with 0.1% Tween 20 (TBST) buffer for 2 h at room temperature. Membranes were subsequently incubated with mouse monoclonal anti-DJ-1 (dilution, 1:3,000; catalog no. 05-828; EMD Millipore, Billerica, MA, USA), mouse anti-β-actin (dilution, 1:2,000; catalog no. A5441; Sigma-Aldrich; Merck Millipore), rabbit polyclonal anti-TATA-box binding protein (TBP; dilution, 1:2,000; catalog no. 22246-1-AP; ProteinTech Group, Inc., Chicago, IL, USA), or rabbit polyclonal anti-voltage-dependent anion channel 1 (VDAC1; dilution, 1:1,000; catalog no. 10866-1-AP; ProteinTech Group, Inc.) primary antibodies overnight at 4˚C. β-actin, VDAC1 and TBP were used to normalize protein loading in cytoplasmic, mitochondrial and nuclear protein extracts, respectively. Membranes were then washed with TBST and incubated for 1 h with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, (dilution, 1:2,000; catalog no. CW0102M; Beijing ComWin Biotech Co., Ltd., Beijing, China) or HRP-conjugated goat anti-rabbit IgG, (dilution, 1:1,000; catalog no. CW0103M; Beijing ComWin Biotech Co., Ltd.). After washing the membranes in TBST, antibody binding was detected using Ponceau S staining solution (Beyotime Institute of Biotechnology). Densitometry was performed using AlphaImager software version 3.3.0 (ProteinSimple Bioscience and Technology Co., Ltd., Shanghai,

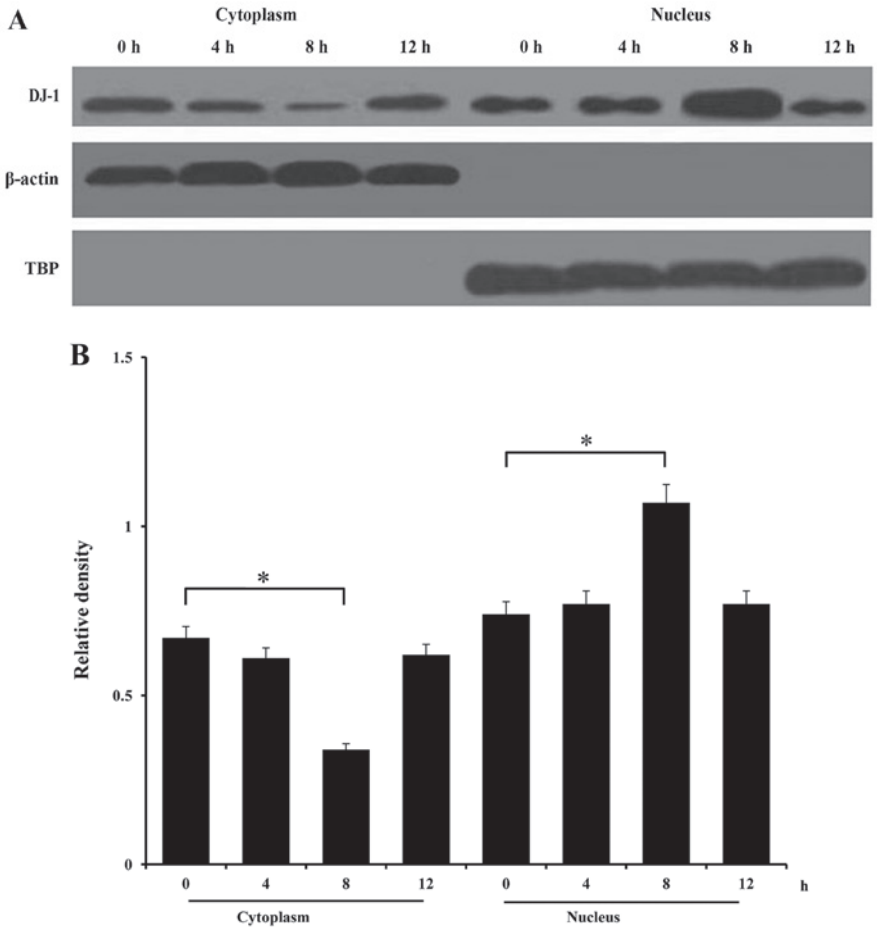

Figure 1. (A) Representative western blot and (B) quantification of band densities of DJ-1 protein expression in the cytoplasm and nucleus of HL-60 cells treated with 1.25 mg/l DADS for 0, 4, 8 or 12 h. β-actin was used as a loading control for DJ-1 expression in the cytoplasm. *P<0.05 vs. 0 h untreated controls. DADS, diallyl disulfide.

China), and target protein expression levels were normalized to the reference protein expression levels.

Immunocytochemistry and immunofluorescence. Immunocytochemical analysis of DJ-1 expression in HL-60 cell specimens was performed using a DAB Detection Kit (Fuzhou Maixin Biotech, Co., Ltd., Fuzhou, China). Cells (20 μl/coverslip; 1x10^6 cells/l) were first seeded on coverslips and fixed with 95% ethanol for 30 min at room temperature. Endogenous peroxidase activity was inhibited by incubating cells in 0.3% (v/v) hydrogen peroxide in methanol for 10 min, followed by washing three times with PBS for 5 min each time. Samples were then blocked with 10% (v/v) normal goat serum diluted in PBS for 30 min, before incubating with the anti-DJ-1 antibody [diluted, 1:200 in PBS containing 3% (wt/vol) bovine serum albumin; catalog no. 05-828; EMD Millipore] overnight at 4˚C. After washing three times with PBS for 5 min each time, cells were incubated with a biotinylated goat anti-mouse IgG antibody (dilution, 1:100; catalog no. bs-0296G-Bio; BIOSS, Beijing, China) for 15 min at room temperature, followed by three additional 5-min wash steps with PBS. Cells were subsequently incubated with streptavidin-avidin-HRP for 20 min at room temperature, and then washed with PBS as described above. Samples were visualized by incubating cells in 3,3'-diaminobenzidine at room temperature for 2 min. After counterstaining with hematoxylin for 30 sec and rinsing with tap water, samples were immediately dehydrated by sequential immersion in gradient ethanol solutions. Images were obtained using a light microscope (Olympus Corporation, Tokyo, Japan).

Immunofluorescence analysis of DJ-1 expression in HL-60 cells was achieved by first seeding cells (20 μl/coverslip; 1x10^6 cells/l) on coverslips and fixing with 4% paraformaldehyde for 20 min at room temperature. Samples were then blocked in PBS containing 0.2% Triton X-100 and 5% normal goat serum for 1 h at room temperature. Cells were subsequently incubated with mouse monoclonal anti-DJ-1 (diluted, 1:200 in PBS containing 0.2% Triton X-100 and 1% normal goat serum; catalog no. 05-828; EMD Millipore) at 4˚C overnight. After washing three times with PBS, cells were incubated with a Texas Red-conjugated goat anti-mouse IgG (dilution, 1:50; catalog no. SA00005-1; ProteinTech Group, Inc.) secondary antibody at room temperature for 1 h. Nuclei were stained with 4',6-diamidino-2-phenylindole for 5 min at room temperature, before the cells were washed twice with PBS and analyzed using an EVOS FL Auto fluorescence microscope (Thermo Fisher Scientific, Inc.).

Statistical analysis. Experiments were repeated a minimum of three times. Data are presented as the mean ± standard deviation. Differences between treatment groups were analyzed

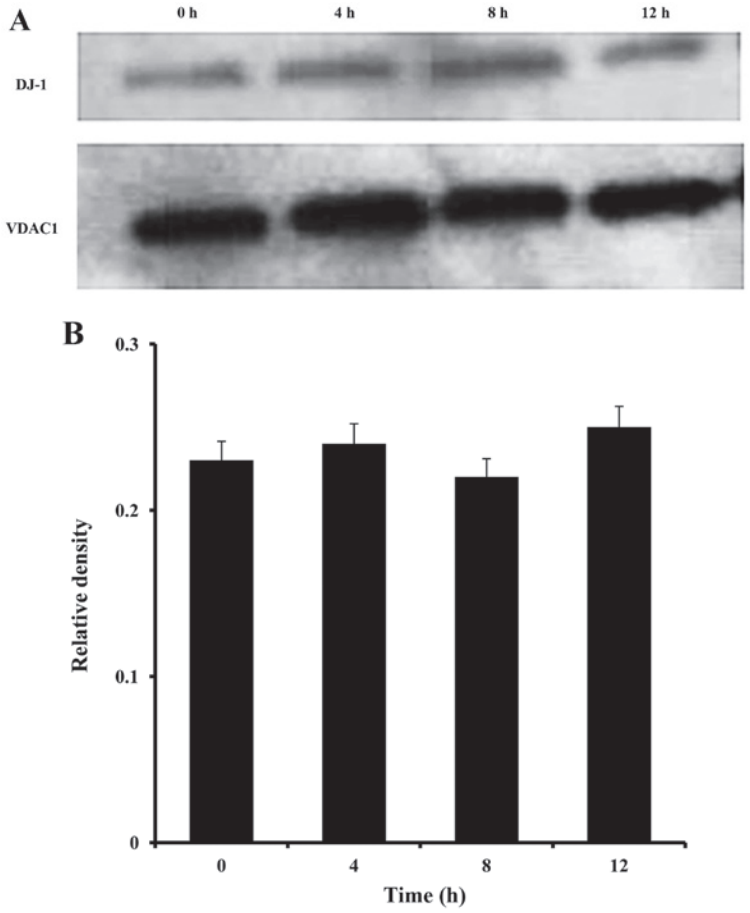

Figure 2. (A) Representative western blot and (B) quantification of band densities of DJ-1 protein expression in the mitochondria of HL-60 cells following treatment with 1.25 mg/l DADS for 0, 4, 8 or 12 h. DADS, diallyl disulfide.

with the Student's t-test using GraphPad Prism software version 5.01 (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

DADS promotes DJ-1 protein translocation from the cytoplasm to the nucleus in HL-60 cells. As shown in Fig. 1, western blot analysis demonstrated that the protein expression levels of cytoplasmic DJ-1 in HL-60 cells treated with DADS (1.25 mg/l for 8 h) were significantly reduced (P=0.013), while the nuclear expression levels of DJ-1 were significantly increased (P=0.032).

DADS decreases the expression of DJ-1 in the mitochondria of HL-60 cells. As shown in Fig. 2, western blot analysis indicated that the protein expression levels of DJ-1 in the mitochondria of HL-60 cells following treatment with by DADS (1.25 mg/l) were not significantly different at 4, 8 and 12 h when compared with untreated controls (P=0.178). By contrast, exposure of HL-60 cells to 5 and 10 mg/l DADS for 8 h was associated with a significant decrease in mitochondrial DJ-1 protein expression levels (P=0.029; Fig. 3).

Increased levels of DJ-1 in the nucleus of HL-60 cells following exposure to DADS as determined by immunocytochemical analysis. As presented in Fig. 4A, immunocytochemical analysis demonstrated that DJ-1 protein expression was detected primarily in the cytoplasm, and low levels were detected in the nucleus. By contrast, following exposure of HL-60 cells to 1.25 mg/l DADS for 8 h, an increase in the expression of DJ-1 protein was observed in the nucleus (Fig. 4B).

Increased expression of DJ-1 in the nucleus of HL-60 cells following exposure to DADS as determined by immunofluorescence analysis. As demonstrated in Fig. 4, DJ-1 expression was observed primarily in the cytoplasm, with low levels observed in the nucleus. In order to confirm the observed increase in DJ-1 expression in the nucleus following exposure of cells to DADS, the intracellular distribution DJ-1 in HL-60 cells was analyzed by immunofluorescence. As shown in Fig. 5, an increase in the number of DJ-1-positive nuclei were observed in HL-60 cells following exposure to DADS (1.25 mg/l) for 8 h (Fig. 5F), when compared with untreated controls (Fig. 5C). These results provide additional evidence demonstrating that DJ-1 translocated from the cytoplasm to the nucleus following DADS treatment.

Discussion

Previous studies have reported that DADS exerts a number of beneficial effects by decreasing the formation of carcinogen-induced cancers and inhibiting the proliferation of

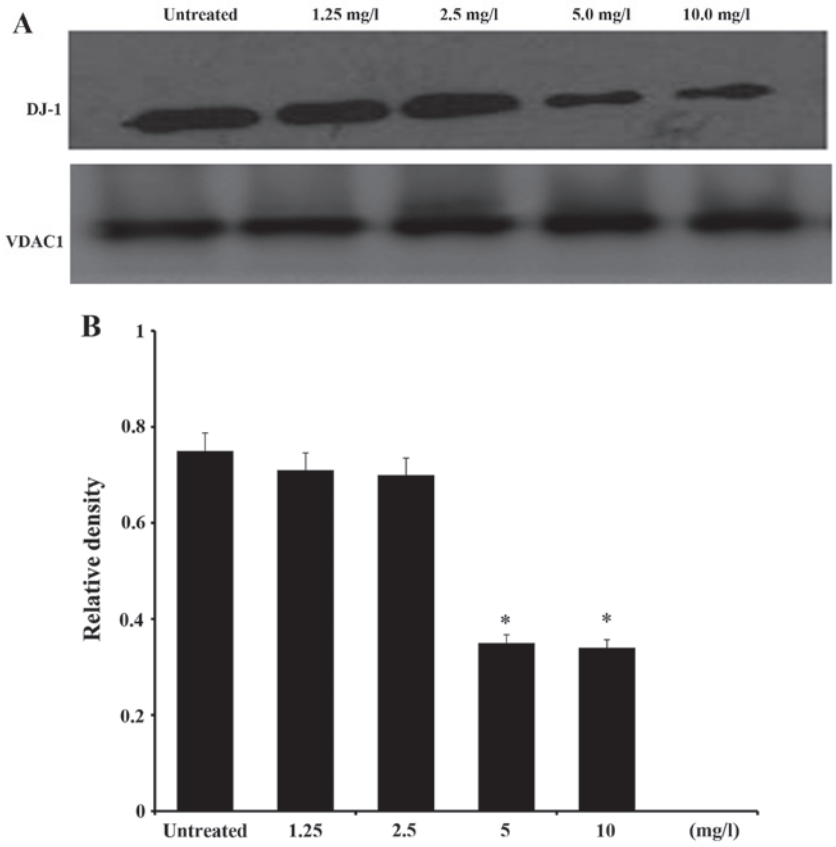

Figure 3. (A) Representative western blot and (B) quantification of band densities of DJ-1 protein expression in the mitochondria of HL-60 cells following treatment with 0, 1.25, 2.5, 5, and 10 mg/l DADS for 8 h. VDAC1 was used as a loading control. *P<0.05 vs. untreated controls. DADS, diallyl disulfide; VDAC1, anti-voltage-dependent anion channel 1.

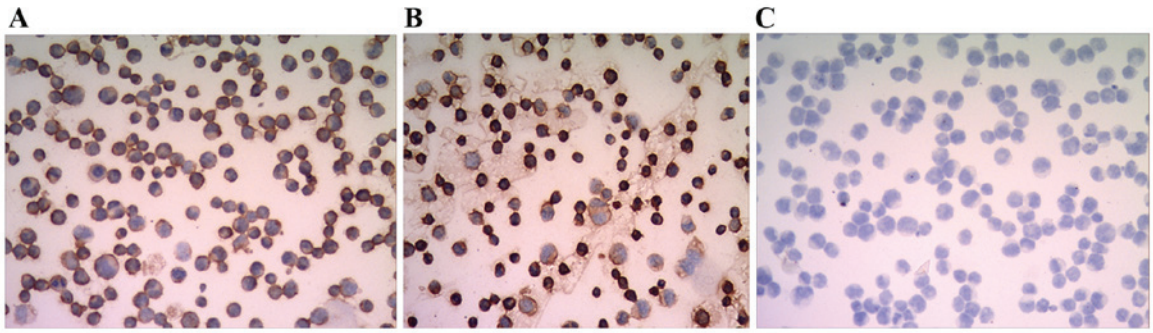

Figure 4. Effect of DADS on the expression and localization of DJ-1 in HL-60 cells as determined by immunocytochemistry. Microscope images (magnification, x40) of DJ-1 expression in HL-60 cells following treatment with 1.25 mg/l DADS for (A) 0 and (B) 8 h. (C) HL-60 cells that were stained with hematoxylin alone. DADS, diallyl disulfide.

various types of cancer cells (23,24). The results obtained in a previous preliminary study demonstrated that DADS inhibited the proliferation of leukemia cells and induced the differentiation of HL-60 cells following exposure to a low dose (<1.25 mg/l) (25). At this dose, HL-60 cells were inhibited in G_1 phase of the cell cycle; at which point the synthesis of RNA and proteins occurs and DNA synthesis in inhibited, leading to inhibition of cell proliferation and induction of differentiation (25). Treatment of HL-60 cells with a moderate dose of DADS (>1.25 mg/l) has been demonstrated to induce apoptosis of HL-60 cells by inhibiting cell proliferation at the G_2/M phase, and by inhibiting the ERK and activating the p38 signaling pathways (12,26). The aim of the present study was to investigate the subcellular localization of the DJ-1 protein in HL-60 cells induced by DADS, and provide a basis for further studies into the potential molecular mechanisms underlying the effects of the subcellular localization of the DJ-1 protein in HL-60 cells.

In the current study, HL-60 cells were incubated with 1.25 mg/l DADS for 0, 4, 8 and 12 h, and the cytoplasmic, nuclear and mitochondrial subcellular fractions of the cells were isolated. Western blot analysis revealed that HL-60 cells treated with 1.25 mg/l DADS for 8 h exhibited a significant decrease in DJ-1 protein expression levels in the cytoplasm, and

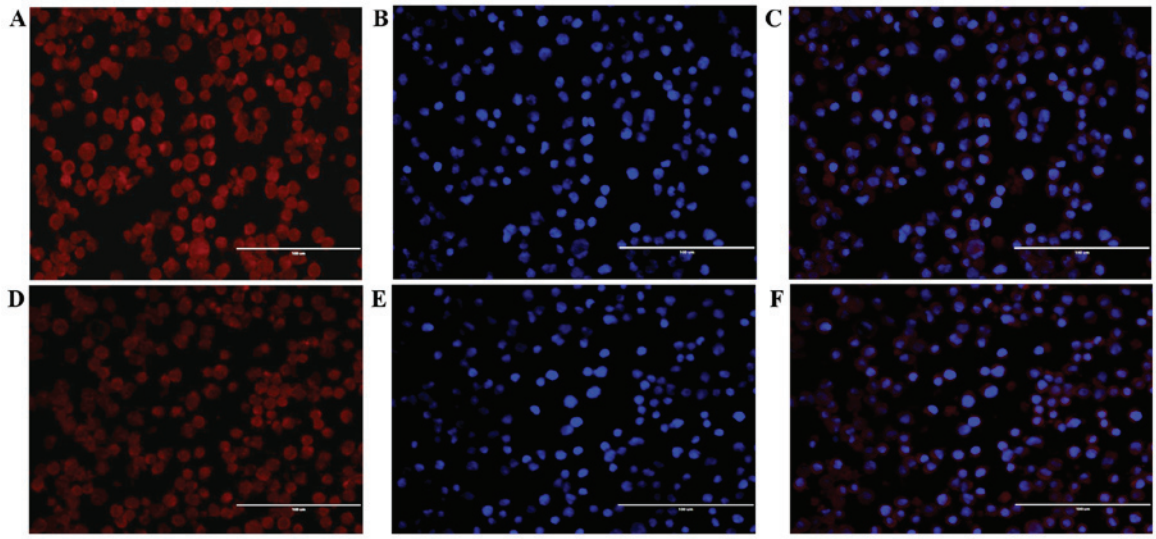

Figure 5. Effect of DADS on the expression of DJ-1 in HL-60 cells as determined using immunofluorescence techniques. Fluorescence microscope images (magnification, x40; scale bars, 100 μm) of (A) DJ-1 expression and (B) the nucleus of untreated HL-60 cells, with (C) DJ-1 and nuclear positioning images merged. Fluorescence microscope images (magnification, x40; scale bars, 100 μm) of (D) DJ-1 expression and (E) the nucleus of HL-60 cells following treatment with 1.25 mg/l DADS for 8 h, with (F) DJ-1 and nuclear positioning images merged. Nuclear positioning was determined by staining cells with 4',6-diamidino-2-phenylindole.

a significant increase in DJ-1 expression in the nucleus when compared with untreated controls. By contrast, HL-60 cells treated with 1.25 mg/l DADS for 12 h exhibited an increase in DJ-1 expression in cytoplasm and decreased expression in the nucleus when compared with cells treated with 1.25 mg/l DADS for 8 h. These results suggest that DJ-1 protein may be translocated back from the cell nucleus into the cytoplasm following 8 h of DADS exposure. Immunocytochemistry and immunofluorescence assay analysis results confirmed the observed translocation of DJ-1 from the cytoplasm to the nucleus following exposure to DADS for 8 h. Proteins are often distributed in different subcellular locations, which is associated with their different functions (27). A previous study demonstrated that the DJ-1 protein, which is considered to be involved in the regulation of gene transcription, translocates from the cytoplasm to the nucleus during S phase of the cell cycle upon mitogen stimulation (28). Proteins may regulate gene transcription through DNA acetylation (deactivation), by altering the structure of chromatin or as transcription factors (29). The level of DJ-1 in the nucleus is increased primarily through an increase in the levels of reactive oxygen species (30). DJ-1 modulates gene transcription in combination with specific transcription factors or cofactors, rather than through direct interaction with DNA (31). For example, DJ-1 binds to the DNA-binding region of p53, resulting in repression of the DNA-binding affinity of p53, which decreases Bax expression (32).

Based on the results from previous studies, the observed translocation of DJ-1 from the cytoplasm to the nucleus in HL-60 cells following exposure to a low dose of DADS in the present study, suggests that DJ-1 may function as a binding protein involved in inducing differentiation. It is possible that DJ-1 interacts with additional target proteins and activates the transcription of specific target genes involved in inducing cell differentiation. Nuclear DJ-1 proteins have been demonstrated to inhibit the activity of signal transducer and activator of transcription (STAT) proteins by binding to activated STATs and influencing the transcriptional activity of downstream target genes (33). A previous study demonstrated that a major purpose of nuclear receptor-mediated transfer of proteins into the nucleus is to regulate the transcription of target genes (34). Following regulation, the nuclear receptor may dissociate from DNA and remain in the nucleus or traffic slowly to the cytoplasm (35).

In the majority of tumor cells, DJ-1 is primarily localized to the cell cytoplasm, but is also present in the mitochondria and nucleus (21,36). The specific mechanisms underlying the translocation of DJ-1 require further investigation and confirmation. Low levels of DJ-1 are present in the mitochondria; however, an increase in oxidative stress has been demonstrated to increase mitochondrial DJ-1 expression levels (21). DADS stabilizes the mitochondrial transmembrane potential, and maintains mitochondrial function in response to oxidative stress (25). Mitochondria-target sequence-conjugated DJ-1 has been demonstrated to exert a greater protective role against oxidative stress-induced cell death when compared with nuclear-target sequence-conjugated DJ-1 (28). Mitochondrial dysfunction occurs in DJ-1 knockout mice (37). These findings suggest that mitochondrial DJ-1 serves a role in mitochondrial homeostasis, which is associated with the regulation of cell survival and apoptosis (38,39). In the present study, mitochondrial DJ-1 protein expression levels in HL-60 cells were not significantly altered following treatment with 1.25 mg/l DADS for 4, 8 or 12 h when compared with the untreated controls. By contrast, mitochondrial DJ-1 protein expression levels were significantly decreased following exposure to 5 and 10 mg/l DADS when compared with untreated controls. These concentrations of DADS have been previously demonstrated to induce apoptosis in HL-60 cells (13,14). Therefore, these results suggest that expression of DJ-1 in the mitochondria may be associated with induction of apoptosis in HL-60 cells induced by DADS. However, the precise mechanisms involved in mediating this process require further investigation in future studies.

Acknowledgements

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