Deuterium-depleted water inhibits human lung carcinoma cell growth by apoptosis

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Abstract. To investigate the *in vivo* and *in vitro* inhibitory effects of deuterium-depleted water (DDW) on human lung cancer and the possible mechanisms underlying these effects, we cultured and treated human lung carcinoma cell line A549 and human embryonic lung fibroblasts HLF-1 with various concentrations of DDW from 2 to 72 h. Cellular growth inhibition rates were determined using the 3-(4, 5-dimethyldiazol-2-yl)-2, 5-diphenyltetrazolium-bromide) (MTT) proliferation assay. A549 cells were treated with 50±5 ppm DDW, and the morphology and structure of cells were observed by scanning electron microscopy (SEM). We observed alterations in the cellular skeleton by transmission electron microscopy (TEM) and changes in cell cycle by flow cytometry. Our data showed that DDW significantly inhibited the proliferation of A549 cells at a specific time point, and cells demonstrated the characteristic morphological changes of apoptosis under SEM and TEM. The length of the S phase increased significantly in cells treated with 50 ppm DDW, whereas the G0 to G1 phase and G2 to M phase were decreased. We observed DDW-induced cellular apoptosis using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and DNA fragment analyses. In addition, we established a tumor transplantion model by injecting H460 tumor cells into subcutaneous tissue of BALB/c mice treated with DDW for 60 days. We determined the tumor inhibition rate of treated and control groups and found that the tumor weight was significantly decreased and the tumor inhibition rate was approximately 30% in the DDW group. We conclude that DDW is a promising new anticancer agent with potential for future clinical application.

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Introduction

Lung cancer is one of the leading causes of death by cancer worldwide; approximately 80% of lung cancers can be histologically classified as non-small cell lung cancers (NSCLCs). Most patients present with locally advanced (37%) or metastatic (38%) disease at the time of diagnosis (1). Despite advances in chemotherapy, the average 5-year survival rate for patients with advanced NSCLC remains extremely poor (2), thus new agents are needed to establish an effective therapeutic strategy against NSCLC. There is great interest in developing new preventive and anti-tumor agents that are more effective and less toxic. It has recently been suggested that deuterium-depleted water (DDW) may play a potentially beneficial role in cancer prevention (3).

In nature, the ratio between deuterium and hydrogen (D/H) in ordinary water is approximately 1:6600 (4). It has been known for decades that the mass difference between hydrogen and deuterium leads to differences in the physical and chemical behavior of the two stable isotopes (5,6). In biological systems, the effect of replacing hydrogen with deuterium has also been well documented (7,8). Early studies revealed that the life span of mice with ascites tumors was prolonged by drinking 25-30% deuterium water (deuterated water) (9), and the mortality caused by ^{60}Co irradiation in mice was significantly decreased by drinking 30% deuterated water (10). Gross and Spindel discovered that high concentrations of deuterium in water induced stagnation mitosis (11). Although the high concentration of deuterium in water was able to inhibit cell proliferation by mitosis arrest and to protect the cell from radiation, it also reduced the life span of mice and even resulted in death (12,13), which limits its clinical application.

To date, research into the effects of deuterium in organisms has focused primarily on deuterated water; little research has been conducted on DDW. The possible role of naturally occurring deuterium in biological systems was first investigated in the early 1990s. DDW was shown to significantly decrease the growth rate of L_{929} fibroblast cell lines *in vitro*, and also inhibit tumor growth in xenotransplanted mice (14). Scientists have recently reported the anti-tumor characteristics of DDW when the deuterium volume fraction in normal water was

reduced by 65% (15-17); however, the mechanism underlying the anti-tumor effect of DDW is still unknown. In this study, we investigated the *in vivo* and *in vitro* effects of DDW on the growth of human lung cancer and the possible mechanisms of these effects.

Materials and methods

Materials. DDW was provided by Shanghai Chitian DDWater Bioengineering Co., Ltd. (Shanghai, China). A549 and H460 cells were purchased from the Cell Research Institute of the Chinese Medical Research Academy (Shanghai, China), and human embryonic lung fibroblasts (HLF-1 cells) were purchased from the cell bank of the Chinese Academy of Science (Shanghai, China).

Cell culture. The human lung carcinoma A549 and H460 cell lines were maintained in RPMI-1640 medium (Gibco, USA) containing 10% fetal bovine serum (FBS; Si Jiqing, HangZhou, China) at 37˚C in 5% CO_2. For *in vitro* studies, the cells were seeded in 25 ml cell culture bottles and grown in complete medium to 90% confluence. Then, cells were washed with phosphate-buffered saline (PBS) and incubated for 48 h at 37˚C in 6 ml of serum-free medium containing DDW.

HLF-1 cells were maintained in α-MEM medium (Genom, HangZhou, China), containing 10% FBS at 37˚C in 5% CO_2.

Analysis of cytotoxicity. The cytotoxicity of DDW was measured in A549 and HLF-1 cells every 2 h for 24 h by the 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT; Kai Ji Co. Ltd., Nan Jing, China) colorimetric assay. A preliminary study was conducted to determine the optimal concentration of DDW and the length of treatment. A549 and HLF-1 cells were cultured in DDW (25, 50 or 105 ppm) and normal water in 96-well plates at $2x10^4$ cells/100 μl well or $1x10^4$ cells/100 μl well, respectively. Cytotoxicity was determined 24, 48 and 72 h after treatment. The MTT proliferation assay is based on the ability of mitochondrial dehydrogenase in viable cells to convert the MTT reagent into a soluble blue formazan dye. At the end of the culture period, 50 μl of the MTT reagent was added, and cells were incubated for 4 h at 37˚C. After removal of the culture medium, cells were lysed with dimethyl sulfoxide (DMSO) to determine the amount of formazan product. The dishes were placed on a shaking platform until the formazan crystals were dissolved. Absorption was measured by a microplate reader (Multiskan MK3; Shanghai, China) at 550 nm, and the results were expressed as percent decrease in cell viability compared to the controls (3). Each cell sample was measured three times, and the mean was reported.

Transmission electron microscopy (TEM). A549 monolayer cells were treated with DDW at 50±5 ppm for 10 h, 72 h or 40 days and subsequently collected and fixed with 25% glutaraldehyde in 0.2 M PBS (pH 7.4) at 4˚C for 2 h. A549 cells were fixed with osmic acid and dehydrated in graded ethanol solutions before embedding. After staining, samples were analyzed using TEM (CM 120; Philips, The Netherlands).

Microscopy. After incubation with 50±5 ppm DDW for 40 days, changes in morphology and structure of A549 cells were observed by fluoroscope microscopy (Olympus, Japan). In addition, membrane morphology was observed by SEM (Multimode Nanoscope IIIa; Digital Instrument Co., USA). For SEM, A549 cells were collected and seeded on a glass overnight and then fixed for 15 min with 0.25% glutaric dialdehyde. Cells were dried and sprayed with gold after dehydration.

Flow cytometric analysis. Cells were harvested by trypsinization after DDW treatment (10 or 72 h), washed twice with PBS and then re-suspended in ice-cold PBS and fixed with 70% ethanol. After the ethanol was removed, the cells were washed once in PBS. The cells were then centrifuged, and cell pellets were re-suspended in 1 ml propidium iodide (PI)/Triton X-100 staining solution (0.1% Triton X-100 in PBS, 0.2 mg/ml RNase A and 10 μg/ml PI) and incubated at least 30 min at room temperature. The stained cells were analyzed using a FACScan flow cytometer in combination with BD Lysis II software (BD Co., USA).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. Cells were treated with 50 ppm DDW for 48 or 72 h, seeded on polylysine-coated slides, fixed with 4% paraformaldehyde in 0.1 M PBS for 1 h at 25˚C, and then permeabilized with 1% Triton X-100 in 0.01 M citrate buffer (pH 6.0). DNA fragmentation was detected using a TUNEL detection kit (Kai Gene, Nan Jing, China), which specifically labeled the 3' hydroxyl terminus of DNA strand breaks using fluorescein isothiocyanate (FITC)-conjugated dUTP. DNA was also labeled with FITC DNA-binding dye for 5 min. FITC labels were observed with a fluorescence microscope. As a positive control, A549 cells were incubated in culture media without DDW for 48 h and treated with DNase I. The percentage of apoptotic cells was calculated as the number of apoptotic cells divided by the total number of cells.

Electrophoresis of DNA fragments. A549 cells were treated with 50 ppm DDW for 10, 24, 48 or 72 h. DNA and the DNA marker TrackIt 1 kb Plus Ladder (Kai Gene) were separated on 1.5% agarose gels.

Animal experiment. Male BALB/c nude mice (weight 20±2 g) were purchased from the ShangHai SLAC Laboratory Animal Co. Ltd. (Shanghai, China). The 8-week-old mice used in this study were maintained in a specific pathogen-free environment. Animal care and maintenance were carried out in accordance with the Guide for the Care and Use of Laboratory Animals by Long Hua Hospital, Shanghai, China.

For *in vivo* studies, the mice were randomly divided into two groups of 8 animals each; the model group and DDW-treated group. The model group mice and DDW-treated group mice drank tap water and DDW, respectively. To construct the H460 xenograft model, cells were harvested after 14 days by trypsinization, washed twice with PBS and re-suspended at $1x10^7$ cells/ml. Approximately $2x10^6$ H460 cells were injected subcutaneously into the right hind flank of all mice. Animals were provided with DDW or tap water continually until the end of the experiment.

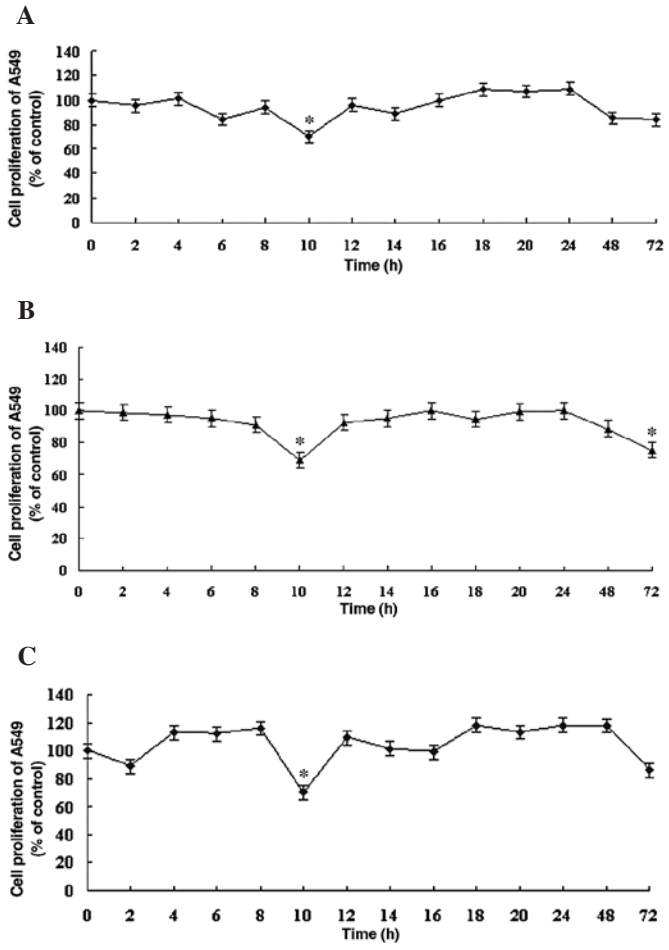

Figure 1. DDW inhibited the growth rate of A549 cells. Cells were treated with DDW for different times and at various concentrations: (A) 25, (B) 50 and (C) 105 ppm. The cell growth was determined using the MTT cell proliferation assay. Results are expressed as the percentage of cell growth relative to the untreated control cells. The data are presented as mean ± SD. *P<0.05 vs. control.

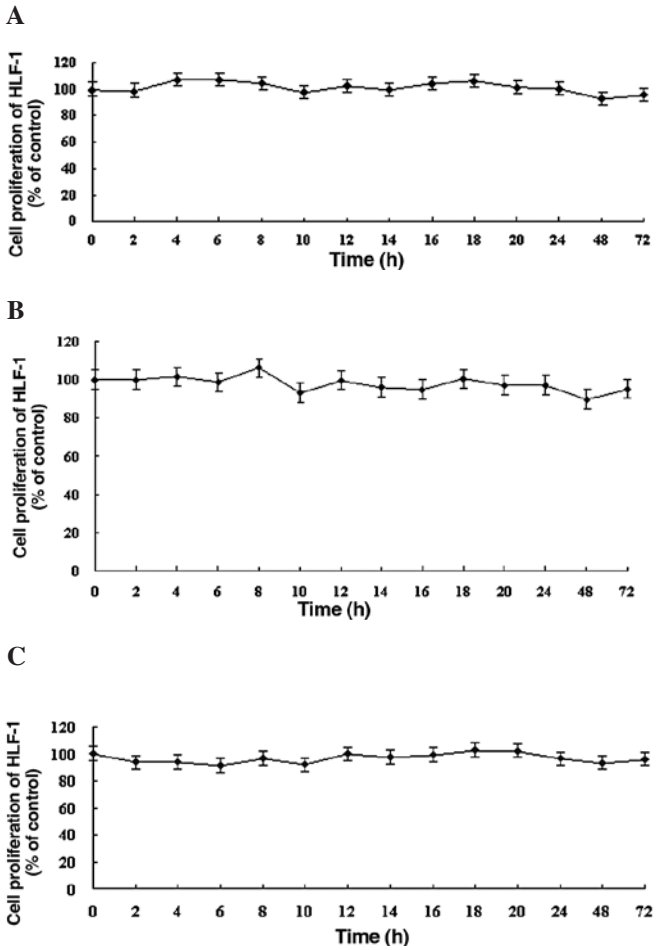

Figure 2. The effect of DDW on the growth of HLF-1 cells. Cells were treated with DDW for different times and at various concentrations: (A) 25, (B) 50 and (C) 105 ppm. The cell growth was determined using the MTT cell proliferation assay. Results are expressed as the percentage of cell growth relative to the untreated control cells. The data are presented as mean ± SD.

Specimen preparation. Experimental pulmonary metastases were established by inoculation of $2x10^6$ H460 cells. Two months later, the H460 xenograft model mice were sacrificed, and the tumors were weighed. The tumor inhibition rate was calculated using the following formula: tumor inhibition rate = (tumor weight of control group - tumor weight of treatment group)/tumor weight of control group x 100%.

Statistical analysis. Data are expressed as the mean ± SD. Differences between groups were analyzed by analysis of variance (ANOVA) or the Student's t-test. Analyses were performed with SPSS software version 13.0 (SPSS Inc., IL, USA). A P-value <0.05 was considered statistically significant.

Results

DDW inhibits the growth rates of A549 and HLF-1 cells. To determine the optimal DDW treatment in the A549 human lung carcinoma cell line, the effect of DDW was assessed using the MTT cell proliferation assay. The effects of DDW on the growth and functional integrity of A549 cells are shown in Fig. 1. There were no significant changes in the growth rate until 10 h of exposure to DDW. At 10 h, cell viability of the treated cells (25, 50 or 105 ppm DDW) decreased significantly to 70.39, 68.93 and 69.90%, respectively, compared to the untreated controls (Fig. 1). The cell growth rate subsequently returned to the level of the controls at 48 h. After 72 h, cell viability at different DDW concentrations decreased to 84.11, 75.23 and 86.44% of control cells, respectively.

In contrast, DDW did not significantly alter the growth of human embryonic lung fibroblast HLF-1 cells compared to controls during the 72 h treatment (Fig. 2). Based on these results, we chose to use 50 ppm DDW and A549 cells for subsequent experiments.

DDW treatment alters A549 cell morphology and structure. We observed the morphology and structure of A549 cells by TEM. Untreated A549 cells showed a flattened profile of cell morphology. There were no alterations to mitochondria, rough or smooth endoplasmic reticulum, Golgi apparatus, lamellar bodies or karyon (Fig. 3A). To observe DDW-induced morphological changes, we incubated A549 cells with 50±5 ppm DDW for 10 or 72 h. After a 10-h treatment, a few myelin bodies

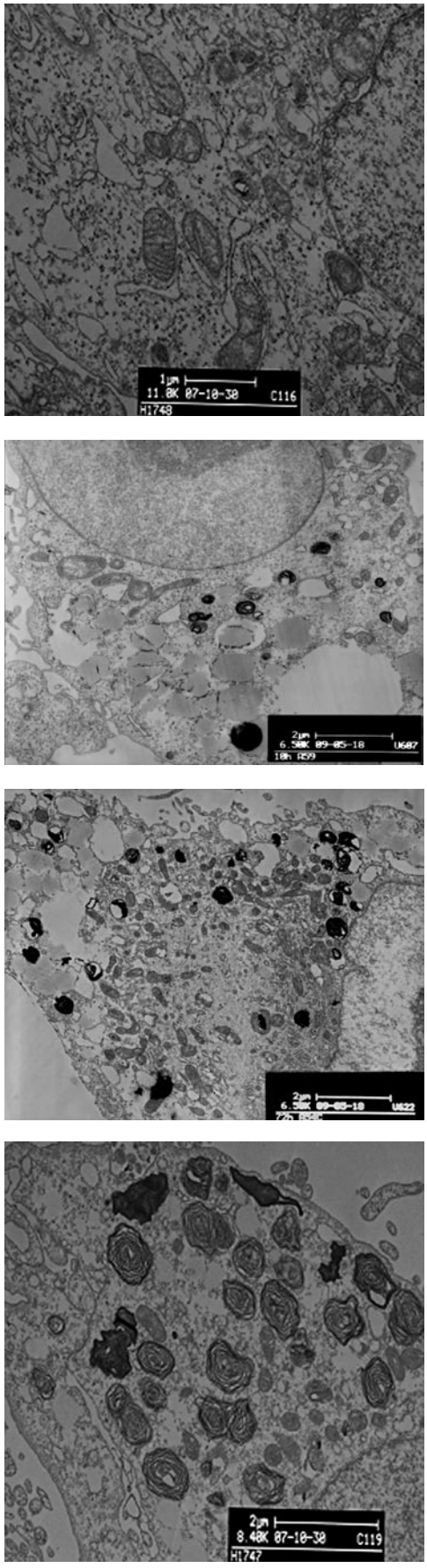

Figure 3. Representative transmission electron microscopy images of A549 cells treated with DDW for 10 h, 72 h or 40 days. (A) Control group (magnification x11,800), (B) 10-h DDW treatment (magnification x6500), (C) 72-h DDW treatment (magnification x6500) and (D) 40-day DDW treatment (magnification x8480) (A, bar=1 μm; B, C and D, bar=2 μm).

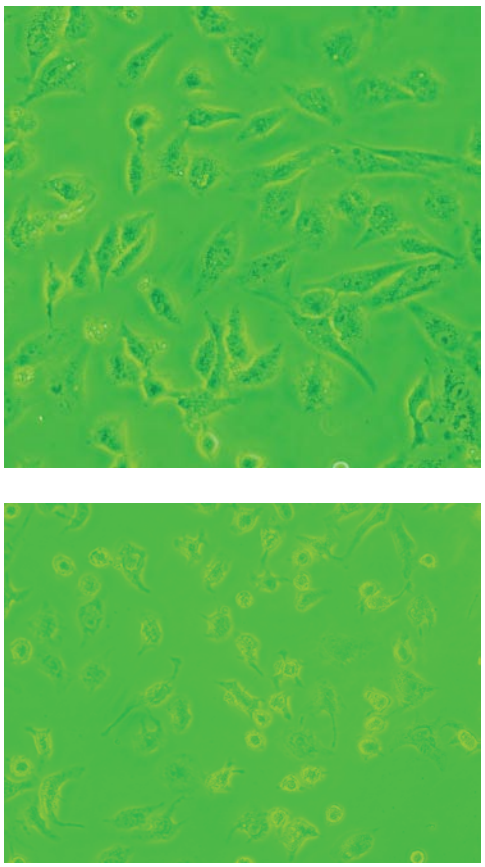

Figure 4. Representative microscopy images of A549 cells treated with DDW for 40 days. Compared to untreated control cells cultured in RPMI-1640, cell morphology was modified by incubation with 50±5 ppm DDW. Magnification x640.

and physalides were observed in the cytoplasm (Fig. 3B), and more myelin bodies and physalides were apparent after 72 h of treatment.

Cells exposed to DDW exhibited modified morphology, which was more pronounced after 40 days of treatment. Untreated control cells were shuttle-shaped or kidney-shaped (Fig. 4A), but they changed to an amorphous polygon when treated with DDW (Fig. 4B). Using SEM and TEM, we observed submicroscopic changes in the morphology. Under SEM, untreated control cells showed a smooth profile and more extracellular matrix than those exposed to DDW (Fig. 5A and B). In contrast, A549 cells exposed to DDW had a rough profile and numerous microvilli on the cell surface (Fig. 5C and D). Under TEM, DDW-treated cells showed numerous myelin bodies in the cytoplasm (Fig. 3D); however, these changes were not observed in the control cells.

DDW treatment alters the cell cycle. To determine whether DDW alters the cell cycle in A549 cells, we stained the cells with PI and used flow cytometry to assess the sub-G1 population. Whereas no significant changes were found in the proportion of cells in the sub-G1 phase between the control cells and DDW-treated cells at 10 h (3.24 and 4.41%, respectively), a significant increase in the proportion of cells in the sub-G1 phase was observed in cells treated for 72 h (6.24 vs. 3.24%; Fig. 6). Meanwhile, cell cycle alterations in

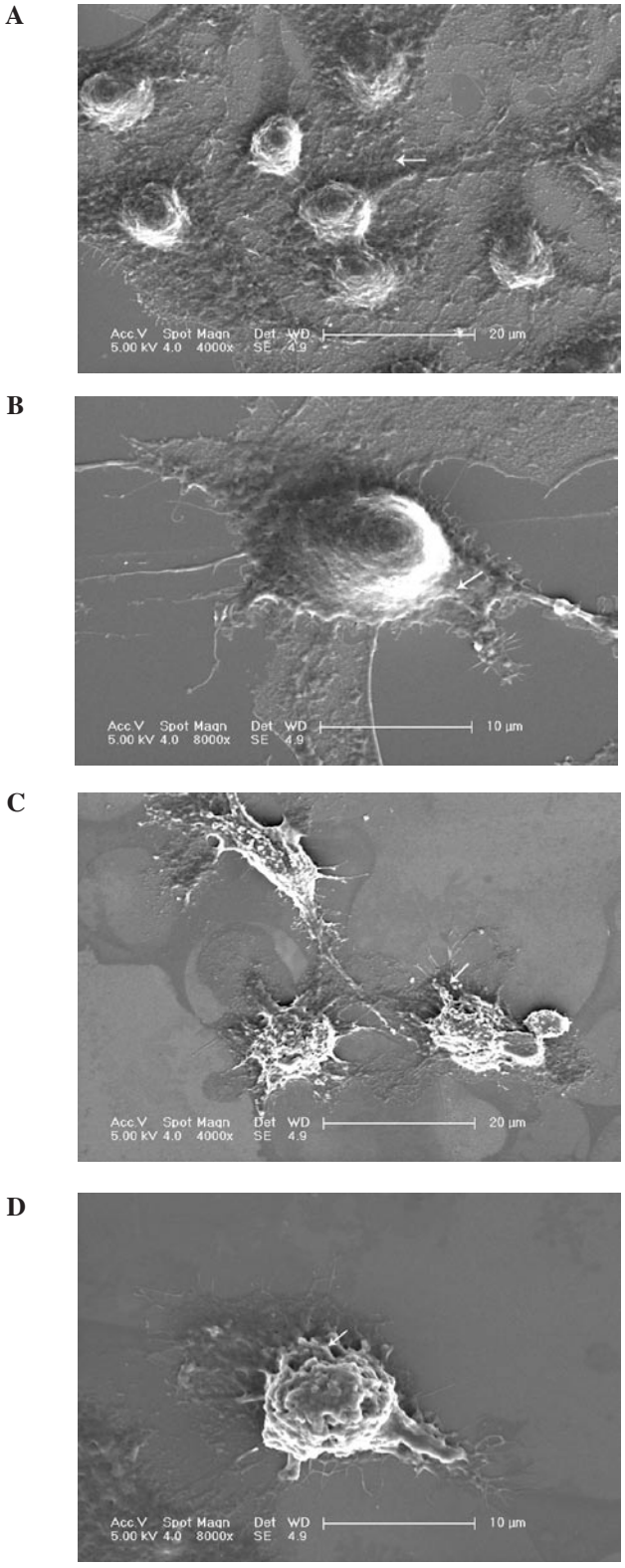

Figure 5. Representative scan electron microscopy images of A549 cells. A and B (magnification x4000 and x8000, respectively) display the shape of normal A549 cells. C and D (magnification x4000 and x8000, respectively) display the cells modified by treatment with DDW for 40 days (A and C, bar=20 μm; B and D, bar=10 μm). Arrows point to microvilli.

DDW-treated cells were analyzed by flow cytometry. The S phase increased whereas the G0 to G1 phase and G2 to M phase were reduced in DDW-treated cells compared to the control cells (Table I; Fig. 6).

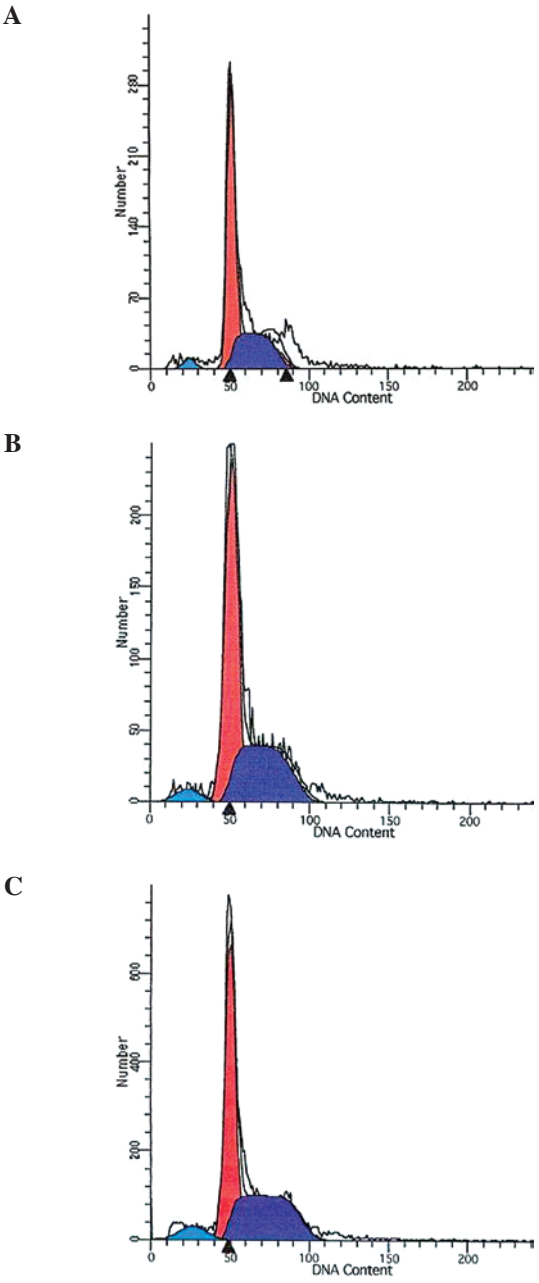

Figure 6. Cell cycle analysis of DDW-treated A549 cells by flow cytometry. The percentage of non-apoptotic and apoptotic cells in each cycle was observed by flow cytometry. The data is presented as mean ± SD (n=3). (A) Control group, (B) 10-h DDW treatment and (C) 72-h DDW treatment.

DDW-induced cell apoptosis. To ascertain whether DDW induces apoptosis in A549 cells, we treated cells with 50 ppm DDW and observed DDW-induced apoptosis with the TUNEL assay (Fig. 7A). Apoptosis was evident in 31.39±2.54% of cells at 48 h and 25.38±3.90% at 72 h. The increased apoptosis was significant compared with the untreated control group (10.87±1.11%; $P<0.05$, Student's t-test). DNA was extracted and analyzed by electrophoresis. As shown in Fig. 7B, fragmented DNA was observed in cells treated with DDW for 48 or 72 h.

DDW influences tumor inhibition rates in vivo. We investigated whether DDW inhibits the growth of transplanted

Table I. Cell cycle population in A549 cells (mean ± SD).

Cycle	Control	10 h	72 h
Sub-G1	3.24±0.78	4.41±0.37[a]	6.24±0.55[a]
G0-G1	60.11±2.15	58.63±2.40	55.23±1.47
S phase	32.65±0.78	38.47±0.29[a]	44.03±0.35[a]
G2-M	7.24±1.37	2.90±0.08[a]	0.75±0.01[a]

[a]P<0.05 vs. control; t-test.

Table II. Tumor weight and inhibition rates of nude mice.

Group	n	Tumor weight (g)	Inhibition rates (%)
Control	8	10.64±0.83	-
DDW	8	7.36±0.78[a]	30.80

[a]P<0.05; t-test.

tumors in mice. After drinking DDW for 60 days, tumor growth in nude mice was considerably reduced. We observed a significant decrease by 30.80% on tumor inhibition rates in the DDW group (Table II).

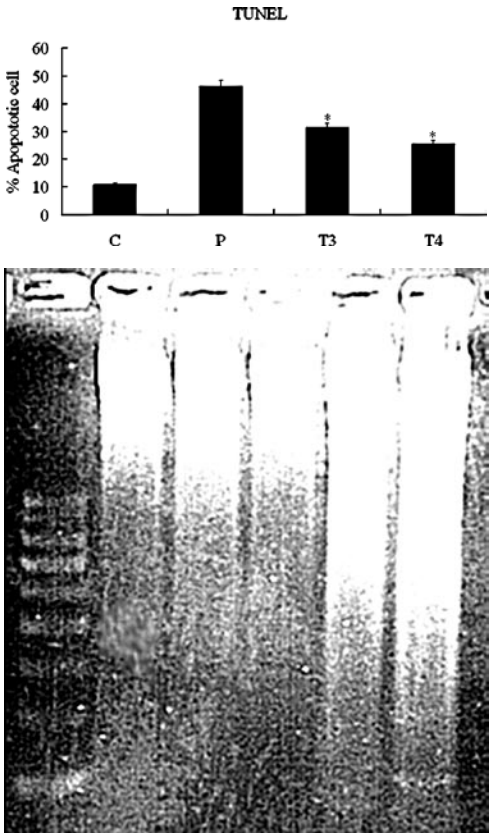

Figure 7. Assessment of cell apoptosis by TUNEL (A) and DNA fragment (B) analyses. A549 cells were treated with 50 ppm DDW for 10 (T1), 24 (T2), 48 (T3) or 72 h (T4). C, control group; P, DNase I-treated A549 cells as positive control (*P<0.05 vs. control).

Discussion

In this study, we investigated the *in vitro* effects of DDW on growth rate, morphology and structure of cells, cell cycle distribution and apoptosis. We found that DDW significantly suppressed the proliferation of A549 cells at 10 h. This inhibitory effect disappeared from 12 to 24 h, but returned during the prolonged 48- or 72-h treatment. In contrast, DDW exerted no significant effects on HLF-1 cells, indicating a cell-specific response to DDW treatment or a more rapid adaptation for HLF-1 cells compared with A549 cells.

A previous study demonstrated that 30 ppm DDW significantly decreased the growth rate of L_{929} fibroblast cells and also inhibited tumor growth in xenotransplanted mice. Deuterium is crucial to the start of cell proliferation as the lag period is 6-8 h longer in medium with low D content (14). DDW also affects seed germination; inhibition of germination was highest 5-6 days after the beginning of germination, but the inhibition was not observed after 10-12 days (18). In addition, the biological effects of DDW on plant cells were investigated in a previous study. In the first half hour of DDW treatment, plants showed biochemical changes similar to those induced by dark treatment; respiration increased, photosynthesis stopped and intracellular pH became alkaline, whereas extracellular pH became more acidic. Maximum effects were noted 30 min after treatment, and then cells gradually returned to normal (19). The results in the present study are remarkably similar to those of previous studies.

Cell division is sensitive to intracellular changes in deuterium concentration, and a normal concentration of deuterium is essential to initiate and to maintain normal cellular growth (14). Our results appear to support the hypothesis of Laskey *et al*, who hypothesized that mechanisms exist in both animal and plant cells that detect changes in deuterium concentration (19). It is necessary to reach the threshold of intracellular D/H to initiate cell division. When cells are cultured in a medium with low deuterium concentration, proliferation is inhibited due to the increased time required to reach the appropriate D/H ratio. In higher organisms, a regulatory system has developed over millions of years, which is sensitive to intracellular changes in D/H. The D/H ratio can increase more rapidly in normal than in tumor cells (19). Tumor cells have a higher growth rate than normal cells as a result of consuming a greater quantity of deuterium (20). We observed that *in vitro* proliferation of tumor cells was inhibited by DDW, whereas proliferation of normal cells was not, suggesting that DDW may influence the D/H ratio in tumor cells, which, in turn, affects the growth rate.

In the present study, we found that DDW increases the S phase cell population and inhibited the proliferation of A549 cells. A greater proportion of DDW-treated A549 cells were arrested at S phase at 72 than at 10 h, as observed by flow cytometry. The cell cycle regulatory system appeared to perceive the D/H ratio, and at the threshold level it triggered the molecular mechanism that finally caused the cell to enter into the S phase (19).

Cell apoptosis via intracellular mechanisms leads to cell death. Many agents have been discovered to treat cancers by

inducing an abnormal cell cycle and apoptosis. Thus DDW may have potential as a cancer therapy. Using TUNEL and DNA fragment analyses we showed that DDW significantly increased the number of apoptotic cells after 48 h, indicating that DDW may trigger a molecular mechanism to induce cells to apoptosis. Gyongyi and Somlyai found reduced expression of C-myc, Ha-ras and P53 in six different organs (spleen, lung, thymus, kidney, liver and lymph nodes) of nude mice in the DDW-treated group (21). They suggested that naturally occurring deuterium may be involved in the regulation of genes that play important roles in the cell cycle or tumor development (21). Therefore, future studies exploring the molecular mechanism of DDW-induced apoptosis are vital to elucidate its tumor-inhibitory effects.

Our *in vivo* results revealed that DDW significantly inhibited tumor growth. However, we do not know whether the effect was caused by cell apoptosis. Further studies are needed to elucidate the mechanism of DDW-induced tumor inhibition *in vivo*.

In summary, we found that DDW exerts effects on the cell cycle and changes in configuration and induces apoptosis *in vitro*. We also found that DDW inhibits tumor growth in xenotransplanted mice. Collectively, these findings suggest the potential for DDW as an anti-tumor drug with clinical application.

Acknowledgements

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