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Preclinical report

Mechanisms of cytotoxic effects of heavy water (deuterium oxide: D_2O) on cancer cells

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Heavy water (deuterium oxide: D_2O) contains a neutron and a proton in its hydrogen atoms and shows a variety of biologic activities different from normal light water. In the present study the cytotoxic and cytostatic activity of D_2O was assessed using a BALB/c-3T3 fibroblast cell line and four human digestive organ cancer cell lines, i.e. HepG2 hepatic, Panc-1 pancreatic, KATO-3 gastric and Colo205 colonic cancer cell lines. Against four cancer cell lines, D_2O showed significant cytotoxic and cytostatic effects in a MTT assay and a Trypan blue dye exclusion assay, at concentrations higher than 30% D_2O. These effects were time and dose dependent, and the IC_{50} after 72 h of culture ranged from 20 to 30% D_2O in the Trypan blue dye exclusion assay and from 30 to 50% D_2O in the MTT assay. By contrast, IC_{50} for the 3T3 fibroblast cell line after 72 h of culture was about 15% in the Trypan blue dye exclusion assay and 50% inhibition was not achieved in the MTT assay. Furthermore, D_2O was found to significantly inhibit the invasion of tumor cells in a Matrigel invasion chamber assay at concentrations higher than 10% D_2O. Incubation with D_2O resulted in enlargement of cells, nuclear pyknosis and vacuolization, and immunostaining studies demonstrated that D_2O treatment resulted in an increase in nuclear nick-end-labeling, which indicates DNA fragmentation, in KATO-3 and HepG2 cell lines. Furthermore, the nucleic acids and protein synthesis inhibition assay suggested that the inhibition of DNA synthesis may be one of the mechanisms responsible for the antitumor effects of D_2O. Furthermore, oral administration of D_2O resulted in a significant inhibition of the growth of Panc-1 tumor xenografted s.c. in nude mice, but survival was not prolonged. In conclusion, D_2O has cytotoxic and cytostatic activities against human digestive organ cancer cell lines, and D_2O may be a potential anticancer agent. [© 1998 Lippincott Williams & Wilkins.]

Key words: Anticancer agent, chemotherapy, deuterium oxide, heavy water.

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Introduction

Heavy water (deuterium oxide: D_2O) includes a neutron and a proton in its hydrogen atoms. D_2O is different from ordinary light water in many of its properties such as viscosity and specific gravity, etc. D_2O contained in natural water is reported to be essential for the normal growth of various cells,[1] whereas D_2O at high concentrations shows a variety of biologic activities: D_2O acts like a calcium channel blocker and effectively normalizes the uptake of calcium into vascular smooth muscle, resulting in prevention of hypertension[2], and D_2O exerts radioprotective effects.[3,4] On the other hand, D_2O has also been reported to be cytotoxic at high concentrations. To our knowledge, the toxic effect of D_2O on cell growth was first reported by Fischer *et al.*,[5] who reported that the growth rate of cultured chick fibroblasts was decreased by D_2O at concentrations higher than 20%. Manson *et al.*[6] studied the effects of D_2O on the growth of various cell lines including a human uterine carcinoma HeLa cell line, a murine fibroblast L cell line and a murine leukemia cell line L-5178Y, and reported that 10% D_2O had little effect on growth rate, but 40 or 50% D_2O was toxic against these lines.[6] Siegel also studied the cytopathology of HeLa cells, which were cultured in medium containing D_2O, and found that the characteristic changes in cultured cells included clumping of cells, cell enlargement, multiple nuclei, nuclear pyknosis and vacuolization.[7] Furthermore, D_2O has also been reported to inhibit *in vivo* tumor growth; oral administration of D_2O at 30% or more concentration into nude mice, bearing human tumor xenograft, results in retardation of tumor growth and prolongation of survival.[8] It has been reported that the mechanism responsible for these growth inhibitory effects of D_2O is that D_2O causes the arrest of

cell mitosis.[9] However, previous reports have involved mainly morphological studies and the mechanisms responsible for the cytotoxic effects of D_2O remain to be fully clarified. The present study was designed to evaluate the antitumor effects of D_2O against human digestive organ cancer cell lines and the mechanisms responsible for these effects.

Materials and methods

Cell lines

A BALB/c-3T3 fibroblast cell line and four human digestive organ cancer cell lines, i.e. HepG2 hepatic, Panc-1 pancreatic, Colo205 colonic and KATO-3 gastric cancer cell lines, were employed. Each cell line was maintained by culturing in complete medium: RPMI 1640 medium (Gibco, Glasgow, UK) containing 10% heat inactivated fetal bovine serum (FBS; Gibco), 50 U/ml penicillin (Gibco), 50 μg/ml streptomycin (Gibco), 1% non-essential amino acids solution (Gibco), 1% L-glutamine (Gibco) and 1% sodium pyruvate solution (Gibco) on 25 cm^2 plastic flasks (Corning, Corning, NY) at 37 C in humidified air containing 5% CO_2. KATO-3, Colo205 and PANC-1 cells are plastic non-adherent floating cell lines. 3T3 and HepG2 cells are plastic adherent, and for experiments they were removed from plastics by incubation with a 0.25% trypsin-EDTA solution (Gibco) for 5 min at 37 C followed by tapping of the culture flasks. After washing three times in Hanks' balanced salt solution (HBSS; Gibco), cells were suspended in complete medium. When cells were about 80–90% confluent on plastic, they were used for experiments.

Animals

Congenital athymic BALB/c nude mice (*nu+/nu+*), 6–8 weeks old, were purchased from CLEA Japan (Tokyo, Japan). They were bred and housed under specific pathogen-free conditions at the Shimane Medical University Laboratory Animal Center.

Deuterium oxide (D_2O)

D_2O (99.9%) was purchased from Isotec (Miamisburg, OH). Deuterated medium was prepared by dilution of powdered RPMI 1640 with distilled water including D_2O at concentrations ranging from 5 to 50%, as indicated.

Protocols of *in vivo* drug administration

The mice were randomized into two groups of five mice each: D_2O-treated and control groups. The mice of the D_2O-treated group were orally given water containing 30% D_2O and the control group was orally given ordinary water, freely until their death, according to the previous report.[8] Seven days after initiation of D_2O or ordinary water intake 10^7 tumor cells were inoculated s.c. into the backs of nude mice, the size of growing tumor was measured serially with calipers. The toxicities of D_2O were evaluated in terms of the loss of body weight and the mortality. To estimate the tumor volume (V), the following formula was used:[10] $V = L \times W^2 \times 1/2$, where L indicates the length and W indicates the width of the tumor.

Morphological observation

The tumor cells were cultured overnight in a 100 mm plastic dish (Corning) with culture medium containing D_2O. After incubation with D_2O for the indicated period, the resulting morphological changes in the cells were observed under a phase microscope and photographed. Furthermore, the cells were cultured in a Lab-Tek Chamber Slide (Nunc, Naperville, IL) with D_2O at various concentrations. After incubation for the indicated period, the slides were washed in phosphate buffered saline (PBS; Gibco), dried at room temperature, fixed in 100% methanol for 30 min, then stained with hematoxylin, and then observed under a microscope and photographed.

TdT-mediated dUTP-biotin nick-end-labeling (TUNEL staining)

TUNEL staining[11] was performed using apopDETEK (Enzo Diagnostics, Farmingdale, NY), according to the instructions with minor modifications. The cells were incubated with D_2O in eight-well Lab-Tek Chambers (Nunc) at 4×10^5/0.2 ml/well. After incubation, the cells were fixed with neutral formalin solution, washed with PBS, fixed again with a mixture of ethanol:acetic acid (2:1) for 5 min and then washed again with PBS. The cells were treated with 20 μg/ml of Proteinase K (Boehringer, Mannheim, Germany) for 20 min and then with 0.3% H_2O_2 for 7.5 min at room temperature. Next, cells were rinsed with TdT buffer and reacted with terminal deoxy transferase (0.3 U/μl, Gibco) and then with biotin-16-2′-deoxyuridine-5′-triphosphate (Boehringer). The resulting cell morphology was observed with an optical microscope after

staining using the ABC method. For evaluation of the positive cell ratios, a total of at least 300 cells in one optical field were counted three times and the mean values were taken as percent positive. All experiments were set up in triplicate (three slides) and the mean values were used for calculation.

Trypan blue dye exclusion test

For determination of the cell proliferation activity, 5×10^5 cells were cultured for 24–72 h in 10 ml of culture medium containing D_2O at various concentrations in 25 cm^2 plastic flasks (Corning) at 37°C under 5% CO_2 and the number of growing cells was measured. After culture for the indicated periods, the cells were treated with 0.25% trypsin-EDTA solution (Gibco) for 5–10 min, and the cells were removed from the plastic by tapping and shaking the flasks. The recovered cells were washed twice and resuspended in 1 ml of culture medium, and the live and dead cells were counted using a hemocytometer after staining with Trypan blue. All experiments were set up in triplicate (three flasks) and mean values were used to calculate the inhibition rate. The results were expressed as percent inhibition relative to the control value.

MTT assay

Cell damage was assessed in a 24–72 h culture using the MTT [3-(4,5-dimethyl-2-thiazolyl) 2,5-diphenyl-2H-tetrazolium bromide; Sigma St Louis, MO] method.[12] MTT was dissolved in Hanks' balanced salt solution (HBSS; Gibco) at 5 mg/ml. The cells were incubated in 96-well flat-bottom microtiter plates (Corning) at 5×10^4/0.2 ml/well in the presence of D_2O at various concentrations at 37°C under 5% CO_2. After culture with D_2O for 72 h, MTT solution (25 μl/well) was added and the mixture was incubated for 4 h. The supernatant was carefully taken up with a pipette and the cells were stained by treatment with 0.1 ml/well of propanol for 1 h. The number of cells was measured with a microplate reader (Model 450; Nippon Bio-Rad, Osaka, Japan) at 570 nm. All experiments were set up in quadruplicate and mean values were used to calculate the cytotoxicity rate. The results were expressed as percent cytotoxicity of the control value.

Nucleic acids and protein synthesis inhibition assay

The nucleic acids and protein syntheses were assessed by measuring incorporation of [^{3}H]thymidine ([^{3}H]TdR) for DNA synthesis, [^{3}H]uridine ([^{3}H]UdR) for RNA synthesis and [^{3}H]leucine ([^{3}H]Leu) for protein synthesis as previously described.[13,14] [^{3}H]TdR, [^{3}H]UdR and [^{3}H]Leu were purchased from Amersham (Tokyo, Japan). Cells were cultured with D_2O (30%) in 96-well flat-bottom microtiter plates at 5×10^4/0.2 ml/well for 72 h. Cells were labeled with [^{3}H]TdR, [^{3}H]UdR and [^{3}H]Leu at 0.5 μCi for the last 18 h of the culture period. After culture, cells were harvested onto glass fiber filters (Titertek; Flow, Rickmansworth, UK) with a semiautomatic cell harvester (Titertek). After drying at room temperature, each glass fiber sample was put into scintillation vials (Packard, Meriden, CT) and immersed in scintillation cocktail (PCS; Amersham) for 12 h. The radioactivity of each sample was measured as isotope uptake rate (c.p.m.) in a liquid scintillation counter. Each experiment was set up in quadruplicate and the mean values were used for calculation of inhibition rate, which was calculated as follows: % inhibition = (control c.p.m. − test c.p.m.)/control c.p.m. × 100 (%).

Matrigel invasion chamber assay

The invasive activity of tumor cell lines was determined by a Matrigel invasion chamber (Becton Dickinson, Bedford, MA) assay according to the instructions.[15,16] An aliquot of 500 μl of chemoattractant (the supernatant of a 3T3 fibroblast cell culture incubated for 12 h from the semiconfluent state) was added to the external fluid in each well of a Falcon 24-well plate (Becton Dickinson) and 10^4/0.2 ml of the tumor cells was added to the interior of the chamber (Falcon cell culture insert with an 8 μm pore size PET membrane coated with Matrigel). Matrigel basement membrane matrix is a solubilized basement membrane preparation extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma. It contains laminin, collagen type IV, heparin sulfate proteoglycan, entactin and growth factors, including transforming growth factor-β, basic fibroblast growth factor and others which occur naturally in the EHS tumor. After a 24 h incubation, the cells that passed through the Matrigel-coated artificial membrane were counted to determine their invasive activity. D_2O was added to the chamber at various concentrations and incubated as described above. The chamber was taken off, the culture medium was removed by suction and the non-invasive cells attached to the upper surface of the membrane were removed with a cotton swab. The chamber was dipped in formalin solution, and the

cells were fixed and stained with hematoxylin. Then the artificial basal membrane was removed using a scalpel and mounted on a glass slide. The cells that had invaded the underside of the membrane were counted using a phase microscope at ×200 magnification in four fields and the mean values were used for calculations. Each experiment was set up in triplicate (three chambers for each test group).

Statistics

All results were expressed as means ± SD and the p values were determined by Student's t-test using Medical Plan II computer software (Sankyo).

Results

Cytostatic and cytotoxic effects of D_2O

The *in vitro* cytostatic effects of D_2O on the 3T3 fibroblast line and four human digestive organ cancer cell lines were assessed by the Trypan blue dye exclusion assay and the results are summarized in Figure 1. During 72 h of culturing, untreated 3T3, KATO-3, Colo205, Panc-1 and HepG2 showed 3.3-, 10.2-, 7.3-, 6.0- and 8.1-fold increases in growth, respectively. When D_2O was added to the culture medium, the growth of each cell line was dose-dependently inhibited and 50% D_2O was associated with a more than 70% inhibition rate on all cell lines cultured for 72 h. The IC_{50} (concentration to obtain 50% inhibition) was 15% for 3T3, 26% for KATO-3, 37% for Colo205, 17% for Panc-1 and 27% for HepG2 lines in a 72 h culture (Table 1). Panc-1 was most sensitive to D_2O, and at a low concentration of D_2O (5%), D_2O showed more than 20% inhibition in a 72 h culture.

The cytotoxicity curves for the five cell lines based on the MTT assay (3 day culture) are shown in Figure 2. D_2O alone showed significant cytotoxic effects on five lines, and the cytotoxicity increased time and dose dependently. The cytotoxic effect was seen later than the cytostatic effect and its magnitude was also less than that of the cytostatic effects. The IC_{50} of D_2O for the four lines was 45% for KATO-3, 38% for Colo205, 34% for Panc-1 and 42% for HepG2, but 50% inhibition was not achieved for the 3T3 line in the MTT assay (Table 1). D_2O at a low concentration (5%) showed less than 10% cytotoxicity against four cell lines except for Colo205.

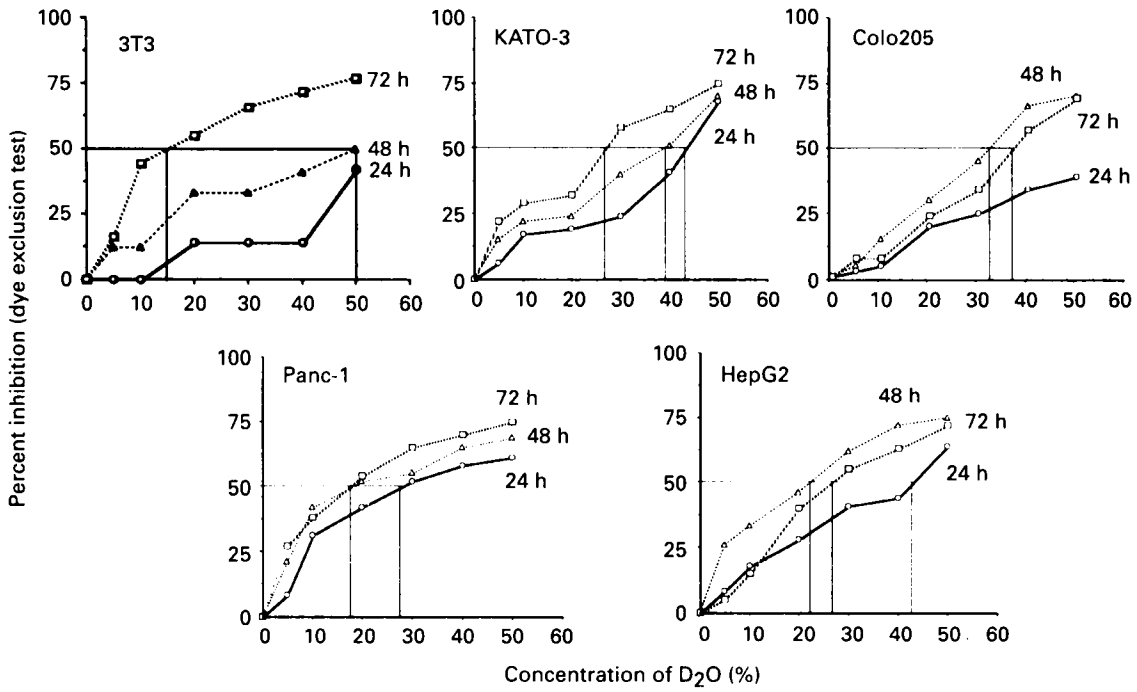

Figure 1. The in vitro cytostatic effect of D_2O on BALB/c-3T3 fibroblast and four human digestive organ cancer cell lines. The cytostatic effects of D_2O were assessed in a 24–72 h culture using the dye-exclusion test, as described in Materials and methods.

Inhibitory effects of D_2O on DNA, RNA and protein synthesis

The IC_{50} of D_2O in the Trypan blue dye exclusion test ranged from 18 to 33%, and the effects of 30% D_2O on DNA, RNA and protein synthesis were assessed in three floating lines, i.e. KATO-3, Colo205 and Panc-1, by measuring the incorporation of [^{3}H]TdR, [^{3}H]UdR and [^{3}H]Leu into the cells. D_2O at 30% inhibited isotope uptakes in a concentration- and culture duration-dependent manner until 72 h. Figure 3 summarizes the inhibition rate of incorporation of [^{3}H]TdR, [^{3}H]UdR and [^{3}H]Leu in the three cell lines by 30% D_2O. DNA synthesis was mainly inhibited in the KATO-3 and Colo205 lines, whereas protein synthesis was most highly inhibited in the Panc-1 line.

Table 1. IC_{50} of D_2O for BALB/c-3T3 fibroblast and four human cancer cell lines in the trypan blue dye exclusion test and MTT assay

	Cell line	Culture period (h)		
		24	48	72
Trypan blue dye exclusion test	3T3	NE	50	15
	KATO-3	43	38	26
	Colo205	NE	33	37
	Panc-1	27	17	17
	HepG2	43	22	27
MTT assay	3T3	NE	NE	NE
	KATO-3	NE	NE	45
	Colo205	NE	NE	38
	Panc-1	NE	39	34
	HepG2	NE	NE	42

Numbers indicate the percent of D_2O in culture medium. The Trypan blue dye exclusion test and MTT assay were performed as described in Materials and methods.
NE, not evaluated due to inhibition rate or cytotoxicity rate lower than 50%.

Effect of D_2O on the cell invasivity

Effects of D_2O on invasivity of tumor cells were assessed in three cell lines, i.e. KATO-3, Colo205 and HepG2, by the Matrigel invasion chamber assay. Panc-1 attached poorly to the basal membrane and it could not be used for the Matrigel invasive chamber assay. The invasivity of tumor cell lines was assessed after 24 h of incubation, at which time the invasivity reached its maximum. Figure 4 summarizes the effects of D_2O on the invasivity of the three cell lines and D_2O showed significant inhibition on invasiveness of tumor cells. This inhibitory effect was not, however, dose

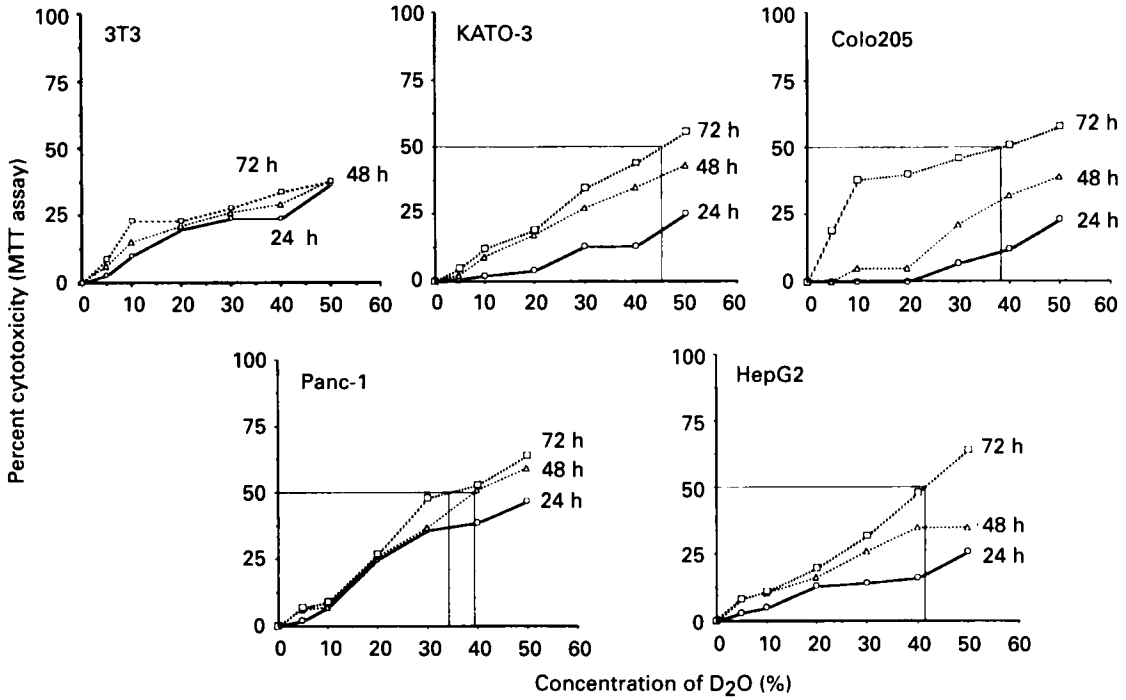

Figure 2. The in vitro cytotoxic effects of D_2O on BALB/c-3T3 fibroblast and four human digestive organ cancer cell lines. The cytotoxic effects of D_2O were assessed in a 24–72 h culture using the MTT method, as described in Materials and methods.

dependent, and there was no difference in inhibition rates between 10, 30 and 50% D_2O.

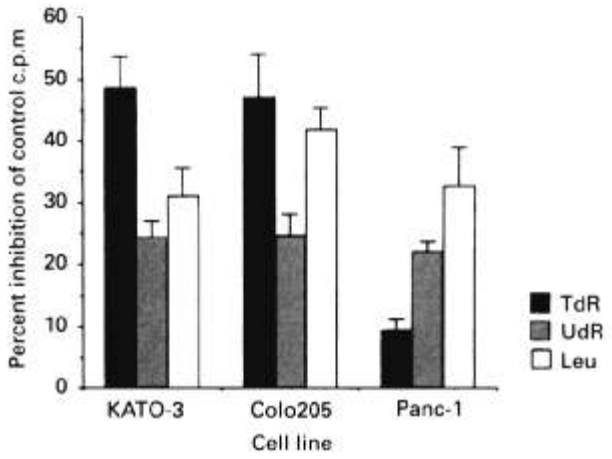

Figure 3. The inhibitory effects of D_2O on DNA, RNA and protein synthesis in human digestive organ cancer cell lines. The inhibitory effects of D_2O on DNA, RNA and protein syntheses were assessed by incorporation of [^{3}H]TdR, [^{3}H]UdR and [^{3}H]Leu, respectively in a 72 h culture, as described in Materials and methods.

Morphological changes to the cells after D_2O treatment

Figure 5(a and c) shows pictures of the KATO-3 gastric cancer cell line obtained by phase microscopy after incubation with 30% D_2O for 48 h. The incubation with D_2O resulted in enlargement of cells, nuclear pyknosis and vacuolization, which were also demonstrated by H&E-staining (Figure 5b and d). The same morphological changes were also observed in the other three lines.

TUNEL staining after incubation with D_2O

In order to investigate the relationship between cell damage and apoptosis caused by D_2O, TUNEL staining for evaluation of apoptosis was applied, because it has been shown that an increase in TUNEL staining indicates DNA fragmentation caused by apoptosis.[11] Figure 6 shows TUNEL staining in HepG2 cells and increases in nuclear TUNEL staining were seen in D_2O-treated cells in comparison with the control cells, which were cultured with medium alone. Similar results were also seen in KATO-3 cell lines and after culturing cells with D_2O for 72 h the number of cells

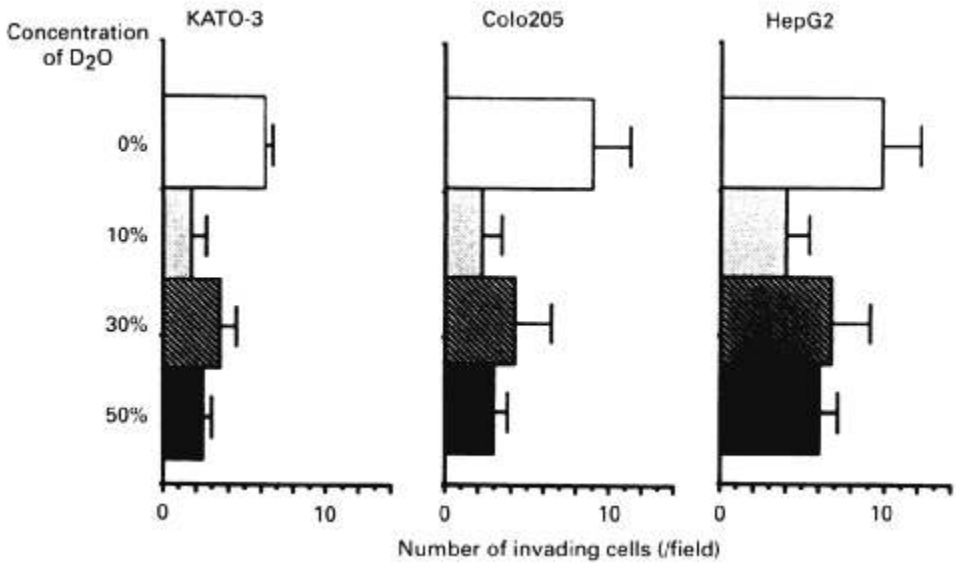

Figure 4. The effects of D_2O on in vitro invasivity of human digestive organ cancer cell lines. The invasive activity of tumor cell lines was determined in a 24 h culture by a Matrigel invasion chamber assay, as described in Materials and methods.

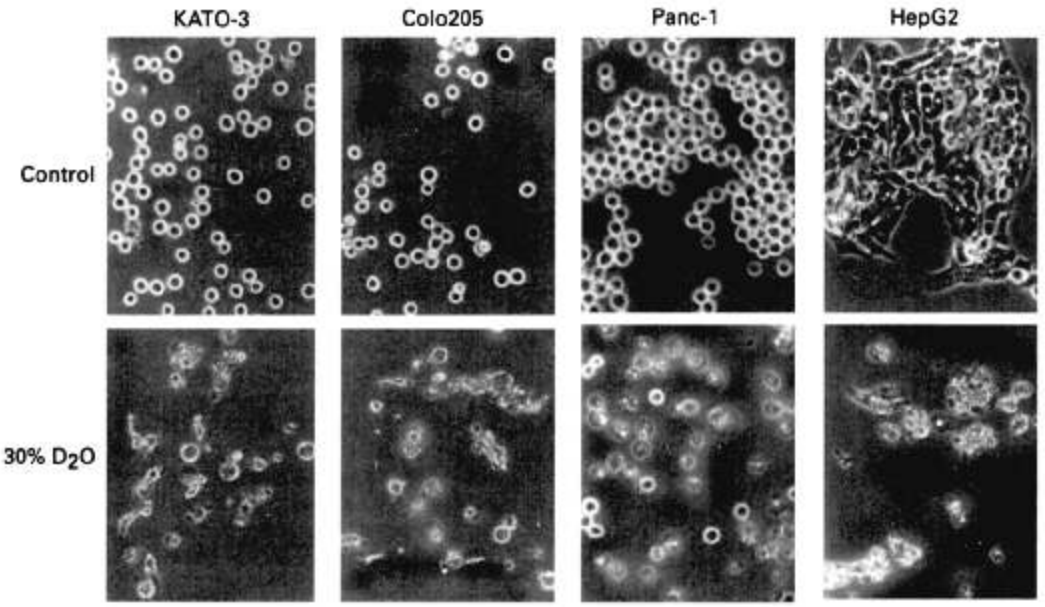

Figure 5. Morphological changes to the cells after D_2O treatment. After incubation with D_2O for 48 h, the resulting morphological changes in the cells were observed under a phase microscope and photographed.

with positive TUNEL staining increased, as summarized in Table 2.

In vivo antitumor effect of D_2O on Panc-1 tumor xenografted s.c. in nude mice

After the mice were deuterized by oral intake of 30% D_2O in water for 7 days, 10^6 Panc-1 cells were s.c. inoculated and the tumor size was compared with those of the control mice, which were given ordinary water. The tumor size of the D_2O group was significantly smaller than that of the control mice on day 11 after tumor inoculation. However, this effect was transient and all mice died between day 15 and day 21 after tumor inoculation, and there was no difference in survival between the two groups. There were no significant differences in body weight and mortality of the mice between the two groups (Table 3).

Discussion

In the present study, when human cancer cell lines were cultured in medium containing D_2O at concentrations higher than 10%, morphological changes including enlargement of cells and vacuolization of nuclei were seen 24 h after culture initiation. These results were compatible with a previous report, in which the characteristic changes caused by D_2O in cultured cells were clumping of cells, cell enlargement, multiple nuclei, nuclear pyknosis and vacuolization.[7] These observations suggest that D_2O may cause nuclear damage.

The present study demonstrates that D_2O exerts cytotoxic and cytostatic activity against various human digestive organ cancer cell lines. First, it was demonstrated that D_2O inhibited *in vitro* cell growth as evaluated by Trypan blue dye exclusion in a time- and dose-dependent manner. At concentrations of D_2O greater than 20%, the growth of all lines tested was significantly inhibited by D_2O, and 30% D_2O showed about 30–50% inhibition of growth and 50% D_2O showed about 70% inhibition in comparison with control cultures grown in D_2O-free medium for 72 h. A similar result was also observed in 3T3 fibroblasts which were used as normal cell controls. The cytotoxic activity of D_2O evaluated by the MTT assay was also dose and time dependent, and the cytotoxic activity of D_2O at a concentration of 50% was about 20, 30 and 55–60% after 24, 48 and 72 h culture, respectively, in cancer cell lines tested, but it was about 40% in the 3T3 cell line, even after 72 h culture. The magnitude of the cytotoxic effect was, however, lower than that of the cytostatic effect in all the lines tested and the cytostatic effect was seen earlier in culture than the cytotoxic effect. These observations suggest that the antitumor activity of D_2O is mainly cytostatic. In the present study, 3T3 fibroblast cells

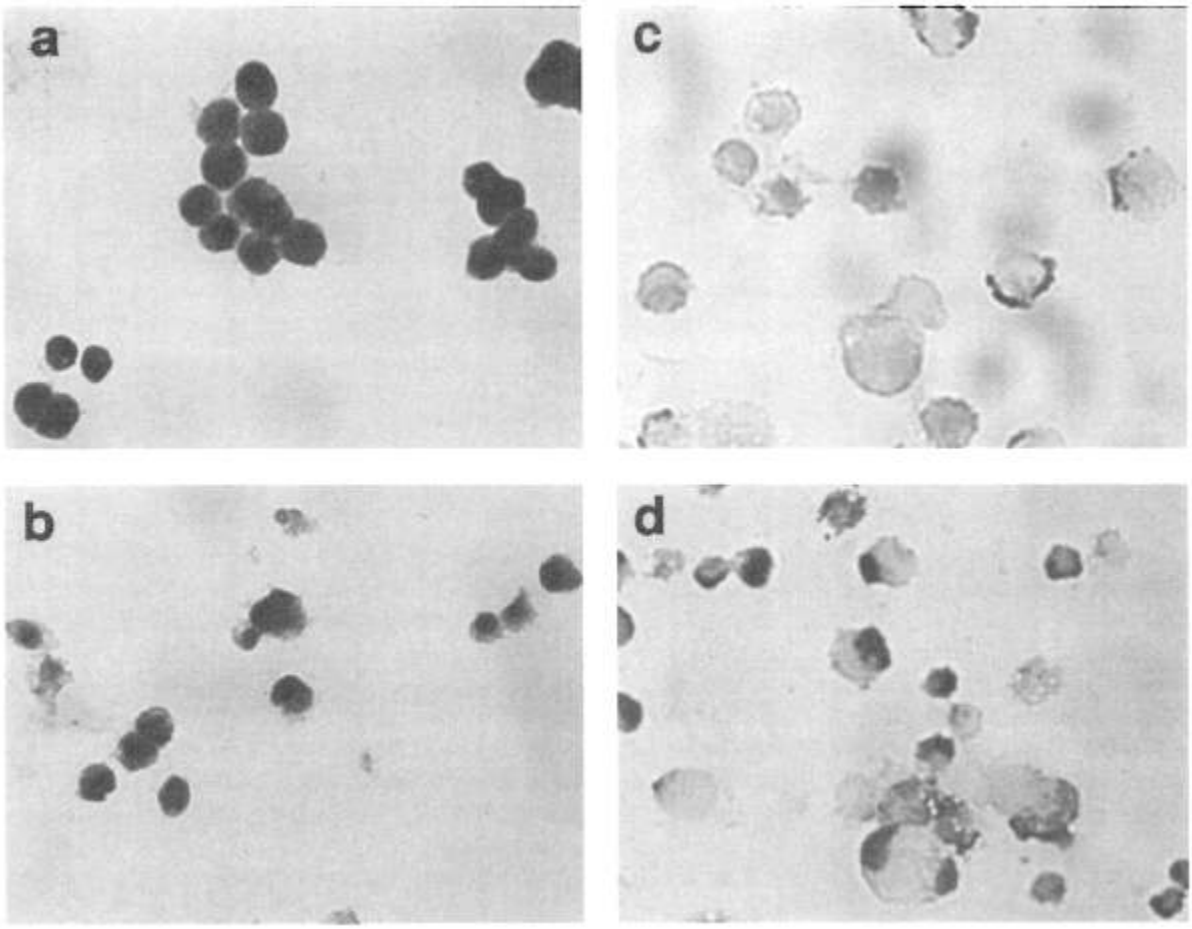

Figure 6. TUNEL (nick-end-labeling) staining in HepG2 cells. After cells were cultured in medium alone (control) or medium containing 30% D_2O for 72 h, labeling was performed, as described in Materials and methods. (a) Control (hematoxylin staining); (b) 30% D_2O (hematoxylin staining); (c) control (nick end labeling); (d) 30% D_2O (nick-end-labeling).

Table 2. Nick-end-labeling of human cancer cell lines

Cell line	Percent positive cells	
	Control (0% D_2O)	30% D_2O
KATO-3	1.1±0.9	7.4±3.6
Colo205	0.0	3.9±7.5
Panc-1	0.0	0.0
HepG2	0.7±0.6	38.5±12.7

After being cultured in medium containing 30% D_2O for 72 h the cells were stained as described in Materials and methods.

were employed to compare the effect of D_2O on cancer cell lines, and the results demonstrated that fundamentally there was no difference between fibroblast cells and cancer cells.

The mechanisms responsible for the cytotoxic or cytostatic activities of D_2O are not fully understood, but several authors have demonstrated that D_2O inhibits or arrests the cell cycle. Schroeter *et al.*[17] reported that D_2O disturbed the cell cycle of PtK2 cells by blocking initial (pro-) metaphase, by slowing down the rate of DNA synthesis followed by successive accumulation of cells in S/G_2 phases, or by arrest of the cell cycle at the S/G_2 boundary or in the G_2 phase. Lamprecht *et al.*[9] researched the disorganization of mitosis in HeLa cells caused by D_2O, and reported that 10–25% D_2O induced changes in the proportions of mitotic phases, 50% D_2O strongly inhibited the cell cycle, and 75% D_2O blocked the cell cycle before prophase and at (pro-) metaphase. These results suggest that D_2O exerts its antitumor activity through inhibition of DNA synthesis or damage to cellular DNA. In the present study, we analyzed the effects of D_2O on DNA, RNA and protein synthesis, and inhibition of DNA synthesis may be the major mechanism responsible for the antitumor activity of D_2O in two lines, i.e. KATO-3 and Colo205. To our knowledge, there is only one publication on the

Table 3. In vivo inhibitory effect of D_2O on the growth of xenotransplanted Panc-1 tumor

	Estimated tumor volume ($mm^3 \pm SD$)			Mean survival	Body weight
	Day 8	Day 11	Day 13	(days ± SD)	(g ± SD, day 13)
Control (n=7)	355 ± 252	932 ± 515[a]	1830 ± 840	17.6 ± 2.5	28.4 ± 2.1
30% D_2O (n=7)	247 ± 194	394 ± 323[a]	1191 ± 931	18.1 ± 2.8	28.1 ± 2.9

D_2O or ordinary water intake started 7 days before tumor inoculation. The mice of the D_2O-treated group were orally given water containing 30% D_2O and the control group was orally given ordinary water, freely until their death. Seven days after initiation of D_2O or ordinary water intake, 10^7 Panc-1 tumor cells were inoculated s.c. into the backs of nude mice (day 1) and the tumor size was compared. The estimated tumor volume was calculated as described in Materials and methods.
[a] $p < 0.05$.

effects of D_2O on DNA and protein synthesis by measuring incorporation of radiolabeled thymidine or leucine, and D_2O was reported to significantly inhibit DNA synthesis in sea urchin eggs after 8 h of fertilization, but protein synthesis was not inhibited.[18] Furthermore, TUNEL staining in the present study also suggested that D_2O may cause apoptosis in two cell lines and this may also be one of the mechanisms responsible for the inhibitory effects of D_2O on DNA synthesis in cells. The present results are compatible with previous reports. However, D_2O inhibited protein synthesis mainly in the Panc-1 line, which was the cell line most sensitive to D_2O. This suggests that the effects of D_2O differ between cell lines and are dependent on individual biologic characteristics.

The present study also analyzed the effect of D_2O on the invasivity of cells, which is one of the important characteristics of malignant cells. Invasivity of the cells was assessed by counting cells which passed through the artificial basement membrane in a Matrigel invasion chamber assay. The invasive ability differed between the lines tested, but the effects of D_2O on invasivity of cells were almost uniformly the same: invasivity of cells was inhibited by D_2O at a low concentration (10%). Several steps are considered to be necessary for invasion of malignant cells into the basement membrane: (i) attachment to the basement membrane, (ii) destruction of the basement membrane by release of proteases, and (iii) migration into the organ or tissue and growth of cancer cells. Previous reports have demonstrated that D_2O caused an arrest of cell mitosis in several types of cells: D_2O blocks mitosis either by immobilizing the mitotic spindle or by enhancing the assembly of spindle microtubules in sea urchin eggs;[19] D_2O affects the turn-over of microtubules in mitotic cells indirectly by impairing centrosome function in PtK2 cells.[20] Furthermore, D_2O has been reported to affect the microfilament system of cells, and to induce shape changes, movement and F-actin redistribution in human neutrophil granulocytes.[21] Moreover, D_2O induces an increase in the length and number of microtubules of the mitotic spindles in dividing eukaryotic cells through enhancement of tubulin polymerization.[22] These reports suggest that D_2O affects the cytoskeleton of living cells, resulting in inhibition of the motility and migration of cells. This may be one of the mechanisms responsible for inhibition of cell invasivity by D_2O.

The present study also evaluated the *in vivo* effects of D_2O, and tumor growth was significantly inhibited in the mice which were orally given 30% D_2O in comparison with the mice administered ordinary water, although this effect was transient and there was no difference in survival between the two groups. For *in vivo* use of D_2O, administration route, its fate after administration and dose are major concerns. When mice or rats were orally administered 15–30% D_2O in drinking water, after about 6 days an equilibrium state in the body fluid was reached.[23] After oral administration D_2O is considered to distribute the same as ordinary water, because D_2O resembles ordinary water so closely.[23] Accordingly, it is very difficult to affect a significant influence selectively on the tumor. D_2O has been reported to retard the growth of human carcinomas when tumor-bearing mice were given drinking water containing 30% D_2O,[8] and that D_2O combined with the cytostatic drugs, 5-fluorouracil and bleomycin, showed synergistic effects against an adenocarcinoma of the colon and a squamous cell carcinoma of the oral cavity.[24] Concerning the toxicity of D_2O *in vivo*, no ill effects were noted over 4 months when 0.5% of the body water was replaced with D_2O in humans,[25] but other authors have reported serious toxicity of D_2O at high concentrations, and survival of mice and rats was greatly reduced when more than one-third of their body water was replaced with D_2O.[26] High concentrations of D_2O have also been reported to be toxic to living cells and organs,[23] although 25% D_2O did not show any toxicity when administered as drinking water.[27] Accordingly, oral administration of D_2O at concentrations lower than 30% may be suitable for *in*

vivo use. Furthermore, other administration methods, especially local administration such as intraperitoneal, intrapleural or intra-arterial administration, should be also studied to improve the efficiency of its antitumor effect and reduce its systemic toxicity for clinical application of D_2O.

The present study demonstrated that D_2O exerts cytotoxic as well as cytostatic effects against human digestive organ cancer cell lines *in vitro*. The results also showed that tumor growth and cell invasivity were inhibited by D_2O at concentrations of 10–30%, at which dose D_2O may be administered without serious toxicity.[27] We are planning to administer D_2O locally, e.g. by intra-arterial infusion as therapy for the treatment of liver tumor or by intraperitoneal or intrapleural administration for treatment of malignant effusion.

Conclusion

D_2O has cytotoxic and cytostatic activities against human digestive organ cancer cell lines at a concentration of 10–30%, and D_2O may be a potential anticancer agent.

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