Anticancer Effect of Deuterium Oxide on a Bladder Cancer Cell Related to Bcl-2 and Bax

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Abstract: To evaluate the potentiality, as a drug for an intravesical instillation after a transurethral resection of a bladder tumor, we studied the anticancer effects of deuterium oxide (D\textsubscript{2}O) related to bcl-2 and bax. Bladder cancer cell T-24 was used and culture media were prepared with H\textsubscript{2}O and D\textsubscript{2}O at different concentrations (D\textsubscript{2}O v/v), 0 (control), 75, and 100 %. Cells were exposed to each D\textsubscript{2}O for 2, 2.5, 3, and 3.5 h. The anti-proliferative effects were measured by a quantitative colorimetric assay (MTT assay) and a hemocytometer. Invasion study was implemented with a modified reconstituted basement membrane after an exposure to D\textsubscript{2}O. Immunohistochemical staining and Western blot analysis for bcl-2 and bax were implemented to evaluate the relation between D\textsubscript{2}O and apoptosis. Marked cytoreduction was observed at an exposure to both 75 and 100 % of D\textsubscript{2}O. The cellular proliferation in 75 % D\textsubscript{2}O was 68.95, 46.77, 24.65, and 14.41 % when compared to the control at an exposure time of 2, 2.5, 3, and 3.5 h, respectively. The comparative ratios in 100 % were 37.93, 4.30, 1.00, and 0.18 % at each exposure group. An invasion of the D\textsubscript{2}O exposed cells into the reconstituted basement membrane did not occur. Morphologic cellular findings suggested an apoptosis. The bcl-2 and bax proteins were noted from most cells regardless of an exposure to D\textsubscript{2}O. The expressions of the bcl-2 were decreased with an increased exposure time in both the 75 and 100 % D\textsubscript{2}O. The expressions of the bax were increased with an increased exposure time for both concentrations. D\textsubscript{2}O has high antiproliferative, and anti-invasive effects at 75 and 100 % D\textsubscript{2}O with an exposure time longer than 2.5 h. We concluded that the mechanism of the antiproliferative effect of the D\textsubscript{2}O would be mediated by an apoptosis. And the cytotoxic results related to D\textsubscript{2}O show the potential for an application as an agent for a post TUR-BT intravesical instillation in a superficial bladder cancer.

Keywords: antiproliferation, deuterium oxide, bladder cancer cell

Introduction

Bladder cancer is one of the two most common urological cancers in many countries and bladder mucosa is covered by urothelium, mainly transitional epithelium. The biological characteristics of bladder cancers are a high rate of recurrence and superficiality [1,2]. The causes of a high recurrence rate [3-7] are not simple but a high rate of superficiality has a life threatening effect [8-10]. Urothelia in each organ has similar causative or risk factors for cancers [11,12].

The replacement of hydrogen (\textsuperscript{1}H) by deuterium (\textsuperscript{2}H) at
H$_2$O results in deuterium oxide (D$_2$O, heavy water). Although D$_2$O is the primary coolant and moderator in nuclear reactors, it has been used for the measurement of some physiologic statuses in humans. D$_2$O has some toxic biological effects on both benign and malignant cells and tissues [13-15]. The toxicity of D$_2$O in experimental animals was not significant when the amount administered to the animal was less than 25 % of the total amount of the fluid intake [16]. The mechanism of a D$_2$O toxicity would be the induction of a disturbance in the DNA and RNA synthesis [14] and a cell cycle arrest with an impairment of the mitosis and membrane functions [17,18]. The study on the cytotoxic and cytostatic effects [14,15] related to cancerous cells has a long history, however, a toxicity in humans has not been clearly verified. A small amount of D$_2$O intake, when administered orally or infused intravenously, had no side effects in humans [19,20].

The fate of a bladder cancer is determined by alterations in the activities of various genes which are involved in the regulation of a cell proliferation and death. A decreased or increased in apoptotic rate facilitates an expansion or shrinkage of cancers. Some proteins, such as bcl-2 and bcl-XI, are blockers on cell death, while bax and bcl-Xs are promoters. Bax and Bcl-2 have important effecter genes involved in the apoptotic pathways. Bax is a 21-KDa protein with a homology to bcl-2 at a highly conserved domain I and II [21]. Bcl-2 over-expression suppresses the induction of an apoptotic cell death [22,23]. Overexpression of the bcl-2 protein protects against a wide variety of apoptotic attacks including a radiation and nearly all chemotherapeutic drugs [26]. This overexpression of bcl-2 in cancer cells has been reported to interfere with the therapeutic effect of cisplatin through an inhibition of the apoptotic pathway [27]. The bax protein acts as a determinant of an intrinsic chemosensitivity [28], and a gradual enrichment for the bax-negative tumor cells confers a selective survival advantage rendering them more resistant to a chemotherapy-induced apoptosis [23].

The low systemic toxicity of D$_2$O in experimental animals and its cytotoxicity against human cancer cells suggests the possibility of an application of D$_2$O for the treatment of urological cancers. In this research, we will study the D$_2$O effects related with a cell proliferation, bcl-2 and bax to access the potentiality of D$_2$O for the prevention of a recurrence or treatment of early superficial bladder cancer after a transurethral resection.

**Experimental**

Authors studied the potentiality of deuterium oxide (D$_2$O) as an agent for a management of a bladder cancer. We used a transitional cancer cell, T-24 that was purchased from the ATCC. We prepared a powdered culture media base, RPMI-1640 (Sigma, USA). The culture medium was made with pure 3rd degree distilled water (DW) and filtered D$_2$O (Aldrich, USA) according to the protocol. The media used for the control was prepared with water only and the media used for the experiments was composed of water and D$_2$O in solutions of both 75 and 100 % D$_2$O (D$_2$O in vol./vol.). Each medium was supplemented with 10 % fetal calf serum, L-glutamine, HEPES and antibiotics (Sigma, USA). After thawing, the T-24 cells were cultured with media composed of water and an amplified enough for a further study. At the time of an experiment, the media comprised of water only was changed according to the protocol at a post-subculture of 24 h, different concentrations and exposure times. After an exposure to D$_2$O, the cells were washed gently twice with PBS and fresh media with water only was supplied. Further culture was maintained for more than 6 days and the cells were harvested. For a detachment of the cell, flasks were first washed gently with PBS twice and then the 0.025 % trypsin-1mM EDTA solution was applied [24,25].

**Grouping**
The cells were grouped into two according to the media as mentioned above: control, cells cultured in the media with water, and cells cultured in the media with water and D$_2$O in 75 and 100 % D$_2$O (D$_2$O in vol./vol.). Cells in the 24 well culture plates were used for the MTT tests. Each 75 and 100 % group was divided into subgroups at an exposure time of 2, 2.5, 3, and 3.5 h.

**Cellular Morphologic Change and a Cellular Proliferation**

Morphologic changes were checked everyday from the D$_2$O treatment of 24 h. Cell growth was measured with the MTT test and a hemocytometer. In the MTT test, briefly, for the 24 well plates, the cells were cultured in a normal medium for one day and exposed to the planned concentration of D$_2$O and time, and then the medium was removed gently and cultured for 3 more days with a normal media. At the 3rd day, the cells were treated with MTT (0.1 mg/mL) for 3.5 h, and the medium was removed and DMSO was applied for a solubilization of the cell. Changes in the absorbance at a wavelength of 570 nm (OD$_{570}$) were measured by using a microplate reader (Bio-Rad model 550). Evaluated absorbance data was calculated and compared to 100 % of the control. In the hemocytometer, the cell numbers were measured under a light microscope.
Invasiveness of a Cancer Cell into a Reconstituted Basement Membrane

Effect of D₂O on the invasiveness of bladder cancer cells into a reconstituted basement membrane was studied with a modified Boyden chamber (Transwell®, 8 µm pore, Costar USA). The upper surface of the filter at the upper chamber was covered with Matrigel (100 µL, 500 µg/mL) and the lower surface was covered with type IV collagen (50 µL, 50 µg/mL). The media filled into the upper chamber was complete RPMI-1640 (100 mL), which was the mixture with the cancer cells (1.5 × 10⁶/mL) that were exposed to D₂O (75 % for two hours) and the control. The media in the lower chamber was filled with complete RPMI-1640 (600 mL) which was supplemented with bovine serum albumin (0.1 %) without a cell. The Boyden chamber was incubated for 12 h. After the incubation, the upper chamber was removed, all the cells in the upper chamber were sucked out and the upper surface of the filter was completely cleaned. The filter was cut sharply and stained with hematoxylin and eosine. The lower surface of the filter was examined by a microscope (×400).

Immunohistochemical Staining of the T-24 Cell on Bel-2 and Bax

We localized the bax and bcl-2 proteins in the cells by an immunohistochemistry. After an embedding of cell pellets with the OCT compound (A.O.), they were sectioned by 10 µm. Sections were dried and washed with a 0.02 M phosphate buffered saline (PBS) for 15 min. Normal goat serum was treated (1:20) for 90 min at room temperature for a reduction of the non-specific binding. And then the primary antibodies were treated (rabbit-derived anti-bax and bcl-2, 1:500) for 24 h at 4 °C. Then washed in TBS, TTBS and incubated with secondary antibodies (1:200, biotinylated goat anti-rabbit IgG) for 2 h. After a treatment with 0.5 % periodic acid, these were incubated with the avidine-biotinylated peroxidase complex (Vector, 1:50) for 90 minutes at room temperature. Then stained with 3,3’-diaminobenzidine-H₂O₂ in PBS for 5 min and washed. Finally, the slides were observed under a bright field microscope, and photographed.

Western Blot Analysis

Western blot analysis was used. Detached cells were homogenized and the protein content was measured with the Bio-Rad protein assay, with bovine serum albumin as a standard. The samples (total 50 µg/lane protein) were analyzed by 12.5 % sodium dodecylsulphate - polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. And the proteins were transferred to the PVDF membrane by using a transfer-buffer system and a semidry electrophoretic transfer cell (15 V, 45 min.). The membrane being transferred was blocked with a blocking solution (Tris buffer saline (TBS) 0.1 % Tween 20, 6 % non-fat dry milk, TTBS) to reduce the nonspecific binding. They were incubated in a primary antibody (rabbit-derived anti-bax and bcl-2, 1:500) for 24 h at 4 °C. Then washed in TBS, TTBS and incubated with a secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG, Bio-Rad 1:1000) for 2 h. The proteins were detected by the ECL Western blotting detection system (Amersham Pharmacia Biotech). Western blots were analyzed by densitometry using the computer-based Sigma Gel (SPSS Inc. Chicago, USA) system. Density values were expressed as mean ±SEM. One-way ANOVA analysis followed by Tukey-Kramer multiple-comparisons test was performed to determine the significance of differences between relevant treatment groups. In every case, the acceptance level for statistical significance was P < 0.05.

Results and Discussion

Shape of the Cells

D₂O treated bladder cancer cells showed marked changes in their shape when compared to the control. Many cells did not attach onto the bottom of culture flask and the other which remained attached had lost their polygonal shape with an ovoid to round swelling, increased vacuolization within the cell and clumping of the nucleus. And the outline of some swollen cells was indistinct and some were irregular which suggests an apoptotic process but some were shrunk, too. Some had a clumped nucleus at one side of the cytoplasm or next to the cell wall. Control showed nearly a confluent, typical polygonal shape but the margin of the outline was clear with some processes (Figures are not presented).

Proliferation of the Cells

The ratio of the cellular proliferation after a D₂O exposure was measured by a quantitative colorimetric assay (MTT assay) and compared to the control. The mean comparative ratios of the cellular proliferation in 75 % D₂O were 68.95, 46.77, 24.65, and 14.41 % when compared to the control at an exposure time of 2, 2.5, 3, and 3.5 h, respectively. The comparative ratios in 100 % were 37.93, 4.30, 1.00, and 0.18 % at each exposure group (Figure 1). 100 % showed a considerable cytoeduction from longer than 2.5 h, with a survival cancer cell rate of less than 5 % when compared to the control. In 75 %, although the cytoeduction was not as much as the 100 %, with an increased exposure time, there was a considerable reduction of the cellular survival rate.
Figure 1. Proliferation of the bladder cancer cell T-24 after an exposure to D\textsubscript{2}O, measured by a quantitative colorimetric assay (MTT assay). All three groups were seeded 5 x 10\textsuperscript{4} cells and they were exposed for more than two hours and cultured for 6 days more. The cells that were exposed for more than 2.5 h showed a reduced cellular number at less than half of the control (46.77 in 75 % group and 4.3 in 100 % group).

Figure 2. Immunohistochemical staining for bcl-2 and bax. All positive staining appear as a brown color. The positive cell density for bax (A and B) appears higher than bcl-2 (C and D). Scale bar = 200 µm (A and C) and 50 µm (B and D).

Invasion Study
Most of the D\textsubscript{2}O exposed cells in the upper chamber were floating and round shaped, without an adhesion and only a few were adhered to the upper surface of the filter at a post-seeding of 15 h. The cells in the experimental group that were exposed to D\textsubscript{2}O showed no invasion into the reconstituted basement membrane. The control showed 95.4 cells at a high power field.

Immunohistochemical Staining
The immuno-positive cells for bcl-2 and bax were stained in brown. The cell density for the stained bcl-2 (Figures 2C and D) was less than bax (Figures 2A and B). The staining density of both the bcl-2 and bax were different from each other.

Western Blot Analysis
Western blot analysis on bcl-2 and bax was implemented separately based on the concentration of D\textsubscript{2}O. In 75 % D\textsubscript{2}O (Figure 3), the bcl-2 expression was highest in the control (1) and reduced with an increased exposure time. The bax expression was lowest in the control (1) and an increased with an increased exposure time. The trends of the expression of bcl-2 and bax with an increased exposure time are directly opposite (1: Control, 2: Exposure 2 h, 3: Exposure 2.5 h, 4: Exposure 3 h, 5: Exposure 3.5 h) ($P < 0.01$) when compared with the respective normal group ($n = 5$).
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Figure 4. Western blot analysis of bcl-2 (A, B) and bax (C, D) in protein extracted from cells exposed to 100% D₂O. The bcl-2 expression was highest in the 2 h exposure (1) and with an increased exposure time, the expression was reduced. The bax expression was the lowest in the 2 h exposure (1) and the expression was increased with increased exposure time. The trends of expression of bcl-2 and bax were similar to the 75% D₂O exposure and the expressions were directly opposite as time progressed (P < 0.01) when compared with the respective normal group (n = 5).

time, the expression was reduced. The bax was the lowest in 2 h exposure (1) and the expression was increased with increased exposure time.

In general, the expression of bcl-2 was reduced with an increased exposure time but the expression of bax was increased with an increased exposure time in both concentrations of D₂O.

D₂O is a fluid which has an anticancer effect that has a low toxicity regardless of its efficacy or selectivity. The anticancer effect of D₂O had been studied from several decades and its response against various cancer cells or xenografted cancer tissues have been impressive. Hepatic, pancreatic, gastric and colonic cancer cells or tissues were studied for a D₂O treatment [13,29] and its cytostatic or cytotoxic effects against cultured or xenografted cancer cells have been identified. Although the complete mechanism of D₂O has not yet been defined, it has the drug metabolic switching effect and it reduces the genotoxicity of some anticancer drugs [30]. The cytotoxicity might be caused by an impairment of the mitosis by a cell cycle arrest [18], interference of a cell division, impairment of a membrane function [17], and a depression of the uptake of the DNA-precursors in cells [15]. In vivo studies with a rodent show that the systemic toxic effects in an experimental animal were not observed when the D₂O contents were less than 25–30% of the total volume of the water intake. In some human volunteers, D₂O was taken orally [19,31] or infused intravenously in small amounts [20] but humans did not notice any toxic effects. Some volunteers on this research team did not experience any disorder although they drank a mixture containing up to 50 mL of D₂O, including 75 and 100% solutions, with more than one week interval and several times. The degree of tolerance to D₂O is different according to the species or tissues [30].

In this study, we set only 8 groups of experiments according to the duration of the exposure time and the concentration of the D₂O. We found a tendency that the proliferation of the bladder cancer cells was reduced gradually but markedly, almost none in the 100 D₂O, at more than a 2.5 h exposure (Figure 1).

The cell line T-24 which we used was derived from infiltrating a bladder cancer. So, we studied the effect of D₂O on the invasiveness of this cell through a reconstructed basement membrane. The results showed that D₂O prevents an adhesion and invasion of cells when compared to the control, which is necessary for metastasis, although we did not study the specific genes related with these events. This result would be beneficial for cancer patients.

A study related with bladder and D₂O is very rare. We studied [32] the morphologic changes and growth patterns of the cancer cells exposed to D₂O in three major urological organs: prostate, bladder and kidney. Among them, bladder cancer cells were the most sensitive to D₂O and the prostate cancer cells were the least. D₂O showed a dose and exposure time dependent cytotoxicity regardless of their origin. The morphologic changes were progressive, from a normal to appearances indicating a DNA fragmentation.

The biological marker for the prediction of the cancer biology, other than the pathological parameters such as the size, histological type and grade of differentiation, is very limited. This is also true of the risk factors for a pro-
gression or metastasis in cancer. For a prediction of a risk factor in a bladder cancer, retrospective studies with bcl-2 families were analyzed on a clinical basis [22,23,33]. The bcl-2 and bax proteins were detected in normal cells (Figure A to D), and cells (Figure A to D) treated with D_2O. Some did not detect bcl-2 mRNA in any of the normal samples, while others were positive in 66 % of the low stage tumors and 100 % of the high stage tumors [34]. Bax was positive in 62 % of the normal, 16 % of the low stage tumors and in 14 % of the high stage [32]. Bcl-2/bax ratio was higher in the non-papillary urothelial cancers pointing to the conclusion that Bcl-2 is excessive in these tumors [23]. Some have reported that a positive bcl-2 was linked to a higher recurrence probability in Ta-T1 urothelial cancers and when the bcl-2/bax ratio was less than 1, the relapse-free time seemed to be significantly longer [22]. Bax inhibits the function of bcl-2 by inducing a bax-bcl-2 complex or by competing with other bcl-2 targets [21]. When the bax expression was higher than the bcl-2 expression, the results seemed to be protective against an early relapse at an early stage of the disease [22]. According to this study, the longer the exposure time and the higher the concentration, the greater the expression of bax and lower the expression of bcl-2 leads to a favorable ratio of bcl-2/bax which would induce a greater protection from a recurrence of a bladder cancer.

In this study we interpreted our data as sequential changes of bcl-2 and bax when instead of interpreting the static data at points in each individual as done by others [22,23,25,33-35], in the past. Both the progressive increased bax and decreased bcl-2 expressions in this study suggest that D_2O is responsible for these changes, although the mechanism has not yet been identified.

In this research, we studied the expression of bcl-2 and bax at two different concentrations. The expression of bcl-2 was lower in 100 % D_2O than in 75 % D_2O, and the expression decreased as the exposure time increased at the same concentration. The expression of the bax was lower in 75 % D_2O than in 100 % D_2O, and the expression increased as the exposure time increased in the same concentration. The general pattern of the protein in these tumors [23]. Some did not detect bcl-2 mRNA in any of the normal samples, while others were positive in 66 % of the low stage tumors and 100 % of the high stage tumors [34]. Bax was positive in 62 % of the normal, 16 % of the low stage tumors and in 14 % of the high stage [32]. Bcl-2/bax ratio was higher in the non-papillary urothelial cancers pointing to the conclusion that Bcl-2 is excessive in these tumors [23]. Some have reported that a positive bcl-2 was linked to a higher recurrence probability in Ta-T1 urothelial cancers and when the bcl-2/bax ratio was less than 1, the relapse-free time seemed to be significantly longer [22]. Bax inhibits the function of bcl-2 by inducing a bax-bcl-2 complex or by competing with other bcl-2 targets [21]. When the bax expression was higher than the bcl-2 expression, the results seemed to be protective against an early relapse at an early stage of the disease [22]. According to this study, the longer the exposure time and the higher the concentration, the greater the expression of bax and lower the expression of bcl-2 leads to a favorable ratio of bcl-2/bax which would induce a greater protection from a recurrence of a bladder cancer.

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Most superficial bladder cancers are treated by a transurethral resection (TUR). The basic principle of a TUR is a complete resection of the visible tumor. After a TUR of the bladder tumor, if a complete repair on the resection site has been done, the resorption of the irrigation fluid into the circulation would be minimal to none. Postoperative (post TUR) intravesical instillation of an anticancer agent or an immunostimulant is a common procedure to aid in the delay or prevention of the recurrence of superficial bladder cancers [35]. Mitomycin C, thiotepa, Adriamycin, epirubicin, etoglucid, bacillus Calmette-Guerin (BCG), interferon (IFN), interleukin-2 (IL-2), bropirimine, keyhole-limpet hemocyanin (KLH), etc. are used for this purpose. These agents have varying effects but they also have unwanted side effects [36] which are due to the resorption of these agents through the resection sites. If we consider past studies [19,20] and our volunteers experiences, a low or lack of toxicity of the D_2O for humans proposes the potentiality as a safe agent for an intravesical instillation after a TUR of a bladder cancer although a small resorption would occur.

Conclusions

The results of this study show that D_2O has anti-proliferative, anti-adhesive and anti-invasive effects, and it also shows that an increased concentration and exposure time leads to genetic changes which indicate the possibility of a favorable prognosis. With the above data, we consider that D_2O has the potentiality as a chemotherapeutic agent for a postoperative intravesical instillation in a superficial bladder cancer which has a low systemic toxicity.

We can expect high therapeutic results with concentrated D_2O (75 or 100 % solutions) for an exposed postoperative bladder of more than 2.5 h for a prevention of a recurrence or treatment of an early small superficial bladder cancer after surgical treatment.

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References

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