Naturally occurring deuterium is essential for the normal growth rate of cells

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The role of naturally occurring D in living organisms has been examined by using deuterium-depleted water (30–40 ppm D) instead of water containing the natural abundance of D (150 ppm). The deuterium-depleted water significantly decreased the growth rate of the L\textsubscript{529} fibroblast cell line, and also inhibited the tumor growth in xenotransplanted mice. Eighty days after transplantation in 10 (59\%) out of 17 tumorous mice the tumor, after having grown, regressed and then disappeared. We suggest that the naturally occurring D has a central role in signal transduction involved in cell cycle regulation.

Deuterium-depleted water; Na\textsuperscript{+}/H\textsuperscript{+} antiport system; Isotope effect; Hydrogen isotope discrimination; Cell growth

1. INTRODUCTION

In nature the ratio of deuterium to hydrogen (D/H) is about 1:6600; this means that the natural concentration of D is about 150 ppm (0.015 atom\%). A worldwide survey of hydrogen isotopes in precipitation revealed\textsuperscript{[1,2]} that the D content covers a range of 120 and 160 ppm depending mainly on the site of sampling and there are several indications that the D/H ratio is not constant in living organisms either\textsuperscript{[3]}. The effect of the replacement of hydrogen with deuterium in biological systems is well documented (e.g.\textsuperscript{[3,4]}), however, the possible role of naturally occurring D in the living organism has never, so far as we are aware, been investigated.

Our work was initiated by the hypothesis that the naturally occurring variations of deuterium concentration may influence cellular activity or cell growth. The supposition was that in living organisms the D/H ratio changes during the cell cycle. We assumed that the activation of H\textsuperscript{+} extrusion membrane transport systems generates an increase in the D/H ratio in the intracellular space by preferentially eliminating the lighter isotope (e.g. H\textsuperscript{+}-ATPase\textsuperscript{[5]}). This increase reflects the metabolic activity of the cell and may also serve as a signal that, in turn, triggers a change in enzyme activity.

This has wider implications if one considers that one of the earliest events in the response of mammalian cells to mitogens\textsuperscript{[6]}, Ha-ras protein\textsuperscript{[7]}, v-mos or Ha-ras oncogenes\textsuperscript{[8]} is the activation of the Na\textsuperscript{+}/H\textsuperscript{+} antiport system which leads to alkalinization of the cells. Similarly, when cultured NIH3T3 cells were transformed with a yeast H\textsuperscript{+}-ATPase\textsuperscript{[9]} gene the cells showed elevated intracellular pH (pH\textsubscript{i}) and exhibited transformed properties both in vitro and in vivo\textsuperscript{[10]}. These experiments support the generally accepted idea that the elevated pH\textsubscript{i} may be a proliferative trigger even though the regulatory mechanism involved is not known. Corroboration was obtained by experiments which showed that if the increase in pH\textsubscript{i} in vitro is prevented it generally inhibits proliferation\textsuperscript{[11]}. However, the results do not explain why the artificial increase of pH\textsubscript{i} is not sufficient to stimulate proliferation\textsuperscript{[12]} if the elevated pH\textsubscript{i} is indeed the proliferative trigger.

We show here, by using deuterium-depleted water, that naturally occurring deuterium is essential for maintaining the normal cell growth rate.

2. MATERIALS AND METHODS

Cell lines (MDA-MB-231, MCF-7, breast adenocarcinoma; L\textsubscript{529} mouse fibroblast) were maintained as stocks in DMEM (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum. For the preparation of media, water samples with different D content were used. Deuterium-depleted water (30–40 ppm D) was obtained from tap water (150 ppm) by electrolysis, the heavy water (99.78 at\%) from ISOTOP (Moscow).

L\textsubscript{529} cells were allowed to grow till confluence and were then treated with trypsin; approximately 2000 cells were aliquoted (in 20–30 \(\mu\)l) into 35 mm Petri dishes containing 4 ml regular medium (150 ppm)
or medium which had been prepared with deuterium-depleted water (30 ppm). The seeded cells resulted in 2-3 cells/mm² density. Cell proliferation was followed by counting the cells in 15-18 separate one-mm² areas, first for 4 h after seeding, then daily.

For the XTT-microculture tetrazolium assay 8000 cells per well were seeded into micro well plates. After 4 h the medium was replaced by media with different D content and incubated for 20–24 h at 37°C in 5% CO₂. The XTT (Sigma, St. Louis, MO; Catalogue No. X4251) assay for cell growth was applied [13].

Small fragments (2-3 mm in greatest linear dimension) of two different human breast adenocarcinoma (MDA-MB-231, MCF-7) were implanted subcutaneously in each flank of female CBA/Ca mice which were immunosuppressed before transplantation [14].

3. RESULTS

3.1. Effect of D on the growth rate of animal cell lines in vitro

The effect of the D concentration of the medium on the growth of L299 fibroblast cell lines in tissue culture was investigated by comparing the growth rates in DMEM prepared with deuterium-depleted water and normal water. The differences between cell growths in the two media were most pronounced within the first 20 h, when the cells were derived from a culture in which they were allowed to grow till confluence (Table I). Over 300 single cells were individually monitored, 32% of which were found to have completed the division in the medium prepared with deuterium-depleted water; this contrasts with the value of 46% in the normal medium.

The effect of D on cell growth was also monitored by measuring the optical density of XTT [13]. Fig. 1 illustrates that a ‘shortage’ of D inhibits the cell growth but a slight excess of D enhances cell proliferation.

In a further demonstration of the difference in cell growths between the two media with different D contents (30 and 150 ppm), the cells were synchronized by arresting them in the G₂/G₁ phase by a 24-h incubation in serum-free DMEM [15] prior to transferring them to the two media. Fig. 2 shows that deuterium is crucial to the start of the cell proliferation as the lag period is 6-8 h longer in the medium with low D content.

3.2. Effect of D concentration on tumor formation in mice

The results obtained in vitro suggested that by reducing the D content of the body water it may be possible

<table>
<thead>
<tr>
<th>D concentration (ppm)</th>
<th>Cell number*</th>
<th>% of the seeded cells with completed division within</th>
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<tr>
<td></td>
<td>0 h</td>
<td>20 h</td>
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<tr>
<td>30</td>
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<td>489</td>
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<tr>
<td>150</td>
<td>352</td>
<td>513</td>
</tr>
</tbody>
</table>

*The numbers represent the cell counts in 5 independent experiments.
to inhibit tumor growth. To test this possibility human breast adenocarcinomas MDA-MB-231 and MCF-7 were transplanted into CBA/Ca mice. The drinking water of the animals in the treated groups was replaced with deuterium-depleted water (30 ppm) one day after transplantation. The number of tumorous mice was recorded and the results summarized, see Table II. Tumors were first detected three weeks (20th day) after transplantation by palpation. At that time no differences were observed between the treated and control groups. Thirty days later (50th day) there was no detectable tumor in 7 (41%) formerly tumorous mice in the two treated groups. Two weeks later (65th day) 72% of the tumorous mice in the control groups had already died, but only 12% of the animals in the treated groups had died. Eighty days after transplantation all mice in the two control groups had died except for one, in which the tumor spontaneously disappeared. At the same time 70% of the animals were still alive in the treated groups. Finally, in the two treated groups in 10 (59%) out of the 17 tumorous mice the tumor after having grown, regressed and then disappeared and one mouse with a tumor lived two weeks after the last of the tumorous mice had already died in the control group.

### 4. DISCUSSION

The results reveal that the growth of different animal cell lines in tissue culture is definitely inhibited in deuterium-depleted water. This striking observation suggests that the natural abundance of D is essential to trigger and maintain the normal growth rate of the cell. In the in vivo experiments the longer survival and the fact that in 59% of the tumorous mice the tumor disappeared because of the treatment with deuterium-depleted water also suggest that the presence of D in a certain concentration is necessary for normal cell growth, especially for the proliferation of tumorous cells.

It is known that the binding site for protons to be transported by plasma membrane H⁺-ATPase of yeast does not accept deuterons with the same ease as H⁺ or perhaps not at all [5]. It is therefore reasonable to assume that when the cell eliminates the H⁺ to govern the pH, by activating the Na⁺/H⁺ antiport system [7,8,11] the D/H ratio increases in the intracellular space.

We suggest that the cell cycle regulating system is somehow able to recognize the changes in the D/H ratio and when this ratio reaches a certain threshold this will trigger the molecular mechanism which causes the cell to enter the S phase. Our assumption provides explanations for the following observations: (i) the growth of animal cells in tissue culture in deuterium-depleted water slows down because the water with low D content extends the time necessary for the cell to reach the threshold of D/H; (ii) the yeast ATPase gene might behave as an oncogene in mammalian cells [9] because the specific elimination of H⁺ from the cell generates the high D/H ratio required for cell proliferation; (iii) the absence of Na⁺/H⁺ antiport activity precludes cell growth at neutral and acidic pH [16] because cells are not able to increase the intracellular D concentration; (iv) the artificial increase of pH, is not sufficient to stimulate proliferation [12] because it does not modify the internal D/H ratio; (v) a slight increase in the D concentration in the external space stimulates growth because it makes it easier for the cells to elevate the intracellular D concentration up to the threshold level.

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### REFERENCES


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### Table II

<table>
<thead>
<tr>
<th>Cell lines: MDA-MB-231</th>
<th>MCF-7</th>
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<tbody>
<tr>
<td>Incidence&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Days&lt;sup&gt;b&lt;/sup&gt;</td>
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<sup>a</sup>The column shows the days after transplantation.
<sup>b</sup>Results are expressed indicating the number of mice developing tumor/total number of animals.