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ORIGINAL ARTICLE

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A possible mechanism for the cytotoxicity of a polyacetylenic alcohol, panaxytriol: inhibition of mitochondrial respiration

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Abstract A polyacetylenic alcohol, panaxytriol, isolated from *Panax ginseng* C. A. Meyer inhibits both tumor cell growth in vitro and the growth of B16 melanoma transplanted into mice. Our preliminary studies indicated that panaxytriol localizes to the mitochondria in human breast carcinoma cells (Breast M25-SF). This study focused on the effects of panaxytriol on mitochondrial structures and function in Breast M25-SF. The results indicate that panaxytriol rapidly inhibits cellular respiration and disrupts cellular energy balance in Breast M25-SF. At concentrations between 11.3 and 180 μM, panaxytriol causes a dose-dependent inhibition of the conversion of the tetrazolium (MTT assay) by mitochondrial dehydrogenase within 2 h. A 1-h treatment with 180 μM panaxytriol causes a significant loss of rhodamine-123 from cells with mitochondria prestained with rhodamine-123 (by flow cytometry). Specific toxic changes were observed by electron microscopy in the mitochondria of Breast M25-SF within 1 h after treatment with more than 180 μM panaxytriol. These data indicate that 180 μM panaxytriol rapidly disrupts cellular energy balance and respiration in Breast M25-SF and suggest that panaxytriol may lower cellular ATP concentrations. After treatment with 180 μM panaxytriol, cellular ATP levels were 40% of those in control cells after 1 h. ATP depletion preceded the loss of cellular viability. Neither ATP depletion nor cytolysis was found in human erythrocytes that have no mitochondria. Thus, ATP depletion resulting from a direct inhibition of mitochondrial respiration is a critical early event in the cytotoxicity of panaxytriol.

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Mitochondrial respiration · MTT assay · Rhodamine-123
ATP level

Introduction

For thousands of years, the roots of *Panax ginseng* C. A. Meyer have been used as an analeptic, stomach toning, and erythropoietic agent in Asian countries. *Panax ginseng* also has an antitumor effect [15]. We demonstrated that a polyacetylenic alcohol, panaxytriol, which was isolated from *Panax ginseng* C. A. Meyer, caused an antiproliferative activity effect on several kinds of tumor cells [11, 12, 17, 18]. Panaxytriol also suppressed the growth of B16 melanoma that were transplanted into mice [12]. Although it is not yet known in detail how it affects growth inhibition, preliminary results have implicated a surface membrane as its site of action [19]. Furthermore, its action seems to be more dose-dependent than time-dependent [12, 17–19]. At concentrations greater than 36 μM, tumor cells may be destroyed by panaxytriol [11, 12, 17, 18]. Recently, we found that panaxytriol may have a selective affinity for the mitochondria in tumor cells. This finding led us to postulate that panaxytriol causes critical damage to the mitochondria in tumor cells.

In the studies presented here, the effects of panaxytriol on mitochondrial structures and function were examined by four different assays: (1) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay; (2) retention of rhodamine-123; (3) electron microscopy; and (4) cellular ATP levels. The MTT assay was used to measure the immediate inhibition of tetrazolium conversion after the addition of panaxytriol [21]. This is a specific enzymatic reaction that directly reflects the activities of the cellular NADH and NADPH dehydrogenases [21, 25], and these dehydrogenases are located primarily in mitochondria.

Rhodamine-123 is a unique fluorescent probe that localizes to mitochondria and can thus be used to assess their functioning [6, 9, 10, 24, 26]. The ability of functional

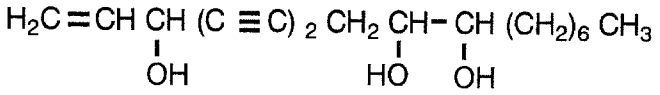

Fig. 1 Chemical structure of panaxytriol

mitochondria to retain rhodamine-123 was measured by flow cytometry.

In order to determine whether ATP depletion is involved in the cell death induced by panaxytriol, cellular ATP levels were measured. The mitochondria are responsible for most cellular oxidation and produce most of the ATP in animal cells [2], and the depletion of ATP concentrations below a certain level results in cell death [1]. Cell death due to ATP loss has been described previously in hepatocytes [4, 16, 29].

Materials and methods

Reagents

Panaxytriol was isolated and purified from a powder of heat-treated roots of *Panax ginseng* C. A. Meyer, red ginseng (Nikkan Korai Ninnjin, Kobe, Japan) by the method previously reported [11, 12, 18]. The chemical structure of panaxytriol is shown in Fig. 1. MTT was purchased from Wako Pure Chemical (Osaka, Japan), and rhodamine-123 was purchased from Sigma (St. Louis, Mo.).

Cells

A human breast carcinoma cell line, Breast M25-SF, was maintained in serum-free SF-02 medium (Sanko Junyaku Chemical, Tokyo, Japan) containing human insulin and transferrin. The cultures were transferred at 3- to 5-day intervals.

Clonogenic assay

The in vitro cytotoxicity of panaxytriol was determined by a clonogenic assay with Breast M25-SF cells as the target cells. Briefly, the Breast M25-SF cells were pretreated for 1 h with various concentrations of panaxytriol. One hour later, these cells were rinsed with phosphate-buffered saline (PBS) and resuspended in drug-free media. The suspensions were plated (1000 cells/well, 34.6 × 17 mm) and incubated at 37 °C for 10–12 days, after which the surviving colonies (containing more than 50 cells) were stained with methylene blue and counted. The survival ratio of Breast M25-SF was calculated from the formula:

Survival ratio = (Number of colony pulse-treated with panaxytriol / Number of colony pulse-treated with drug free medium) × 100.

Isolation of nuclei and mitochondria

The Breast M25-SF lysates were divided into two fractions, nuclear and mitochondrial, by a differential centrifugation [7]. Briefly, the Breast M25-SF cells (1.8×10^7 cells) were homogenized at 0–4 °C in homogenizing medium containing 10 mM Tris-HCl and 2 mM $MgCl_2$ at pH 7.5, using a homogenizer (Tokyo Rikakiki, Tokyo, Japan) set at 2500 rpm. The homogenates were centrifugated at 1000 × *g* for 5 min. The supernatants and pellets were collected separately. The pellets were then resuspended in sucrose medium containing 0.25 M sucrose, 10 mM Tris-HCl, 2 mM $MgCl_2$ and 0.1% bovine serum albumin at pH 7.5 and centrifuged once more. The resulting pellets were resuspended in a small volume (2 ml) of sucrose medium (nuclear fraction). The supernatants were centrifuged at 9000 *g* for 10 min to harvest the mitochondrial pellets. These were then resuspended in sucrose medium and centrifuged once more. The resulting pellets were resuspended in 2 ml of sucrose medium (mitochondrial fraction).

Measurements of panaxytriol

The panaxytriol concentration was determined by an enzyme-linked immunosorbent assay (ELISA) as described previously [23]. The nuclear or mitochondrial fractions were exposed to 100 ng/ml of panaxytriol for 1 h at 37 °C. After incubation, each of these supernatants was collected by ultracentrifugation at 105000 × *g* for 1 h. Panaxytriol uptake by the nuclear or mitochondrial fractions was calculated by subtracting the supernatant values from the total panaxytriol values. Panaxytriol uptake by these fractions was expressed in nanograms per unit of weight.

MTT assay

For the measurement of the inhibition of tetrazolium conversion by panaxytriol, an MTT assay [21] was performed. Briefly, 50 µl of Breast M25-SF (5×10^4 cells/ml) and 50 µl of a panaxytriol solution were plated in flat-bottomed microtiter wells and incubated for 2 h at 37 °C in a humidified atmosphere of 5% CO_2 in air. Following rinsing, the cells were resuspended in fresh medium. Ten microliters of MTT (5 mg/ml) was added to the microculture wells. After a 1-h incubation at 37 °C, 100 µl was removed from each well, to which 100 µl of 100% dimethyl sulfoxide (DMSO) was added to solubilize the MTT-formazan product. The absorbance at 590 nm was measured with a UVmax Kinetic Microplate Reader (Molecular Devices, Menlo Park, Calif.).

Rhodamine-123 retention study

Rhodamine-123 was made fresh before use and used at a final concentration of 5 µg/ml in SF-02. Growing Breast M25-SF cells were collected and incubated in SF-02 containing rhodamine-123 (5 µg/ml) for 30 min at 37 °C. The cells stained with rhodamine-123 were rinsed twice with PBS. Following rinsing, the stained cells were added to SF-02 with or without panaxytriol and incubated further for 1 h at 37 °C. The cells were then washed three times with PBS and resuspended in PBS containing 10% fetal calf serum (FCS). The percentage of labeled cells and the relative mean of the fluorescence were determined by flow cytometry (ORTH Cytoron Absolute, Tokyo, Japan).

Electron microscopy

The Breast M25-SF were incubated for 1 h at 37 °C in SF-02 with or without panaxytriol and then washed twice with PBS. The cells were fixed in 3% glutaraldehyde and 1% osmium tetroxide solutions. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a JEOL 1200 EX electron microscope (Nippon Denshi, Tokyo, Japan).

ATP and ADP analysis

The Breast M25-SF (2×10^6 cells) were incubated in SF-02 with or without panaxytriol for 1 h at 37 °C in a 5% CO_2 and water-saturated air atmosphere. After incubation, the cells were collected by centrifugation at 6000 *g* for 10 s and rinsed with cold PBS. Following rinsing, the cells were lysed with 0.5 ml of 0.2 M phosphate buffer (pH 7.0), and then 0.5 ml of 6% perchloric acid were added. The mixtures were centrifuged at 6000 *g* for 5 min, and the cell-free supernatants were collected. The ATP and ADP concentrations in the supernatants were determined by a high-performance liquid chromatography (HPLC) method as described previously [27]. HPLC analysis was performed using a Shimadzu Model LC-6A pump, Shimadzu SPD-6A UV detector (with a 260 nm fiber), a Shima-pack WAX-1 (4 mm I. D. × 5 cm) column and a Shima-pack Diol (4 mm I. D. × 5 cm) precolumn (Shimadzu, Kyoto, Japan). ATP and ADP were eluted with a mobile phase of 0.5 M phosphate buffer (pH 7.0).

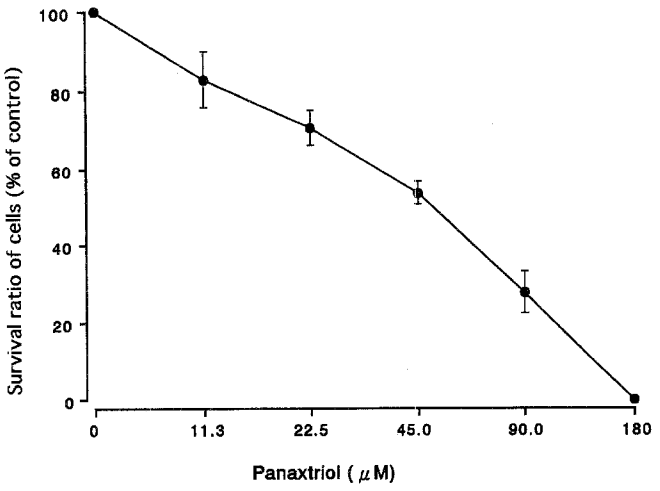

Fig. 2 Cytotoxicity of panaxytriol against Breast M25-SF. The in vitro cytotoxicity of panaxytriol was determined by a clonogenic assay as described in "Materials and methods". Mean ± SD of three experiments

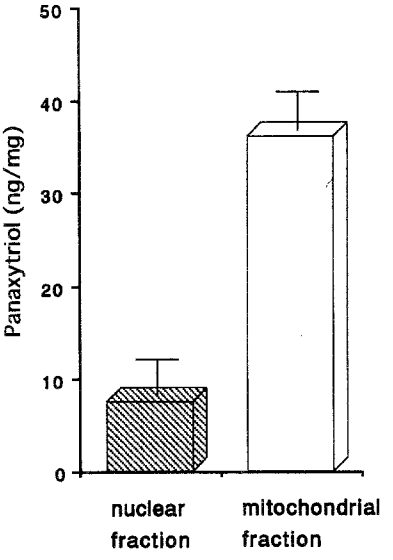

Fig. 3 Panaxytriol uptake by the nuclear and mitochondrial fractions of Breast M25-SF. The nuclear (▨) or mitochondrial (□) fractions were incubated with panaxytriol for 1 h at 37 °C. These supernatants were collected and the panaxytriol concentrations were determined by ELISA as described in Materials and methods. Mean ± SD of three experiments

Results

Cytotoxicity of panaxytriol against Breast M25-SF

The cytotoxic effect of panaxytriol was determined by a clonogenic assay using Breast M25-SF as targets as described in the Materials and methods section. Panaxytriol produced a dose-dependent cytotoxicity (Fig. 2). The concentration of panaxytriol required to obtain 50% colony growth inhibition of Breast M25-SF was 50 μM.

Panaxytriol uptake by mitochondrial fractions

Panaxytriol uptake by the nuclear and mitochondrial fractions was examined as described in Materials and methods. Panaxytriol uptake by the nuclear and mitochondrial fractions was 7.56 ± 3.67 and 36.27 ± 4.05 ng/mg, respectively (Fig. 3). This result suggests that panaxytriol preferentially localizes to the mitochondrial fraction.

Effect of panaxytriol on tetrazolium conversion

The MTT assay measures the conversion of a tetrazolium salt to a colored formazan product by mitochondrial enzyme activity. Breast M25-SF were exposed to various concentrations of panaxytriol for 2 h at 37 °C, and then washed with PBS. The washed cells were resuspended in fresh medium, and then MTT was added to the cells and the incubation was continued for another 1 h. Panaxytriol rapidly reduced the conversion of the tetrazolium dye during the 1-h incubation period in a dose-dependent fashion (Fig. 4). The cells, in which the conversion of tetrazolium is inhibited, were poorly stained by the trypan blue solution, indicating that these cells were not dead. The reduced production of formazan crystals in the mitochondria of Breast M25-SF can be seen by light microscopy. The formazan crystals deposited in the mitochondria of non-treated Breast M25-SF after 1 h in the MTT assay solution are shown in Fig. 5A. Reduced formazan formation in the Breast M25-SF pretreated with 180 μM panaxytriol for 1 h are shown in Fig. 5B.

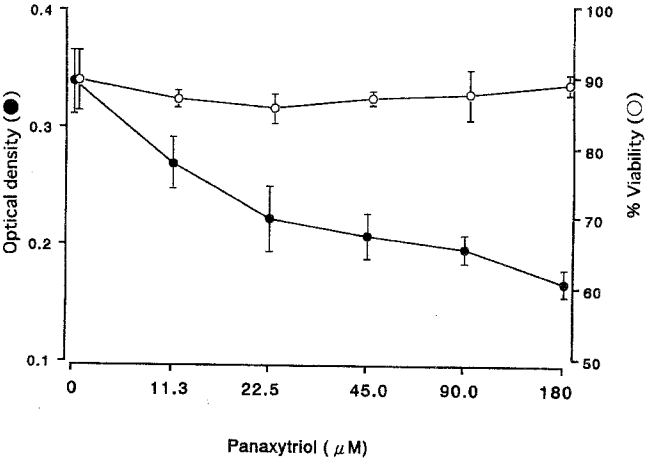

Fig. 4 Rapid reduction in tetrazolium conversion by panaxytriol. Fifty microliters of a suspension of Breast M25-SF (5×10^4 cells/ml) and 50 μl of a panaxytriol solution were plated in microtiter wells. After a 2-h incubation, cells were washed with PBS, resuspended with fresh medium, and then a 1-h MTT assay (●) and the trypan blue dye exclusion assay (○) were performed. Mean ± SD of three experiments

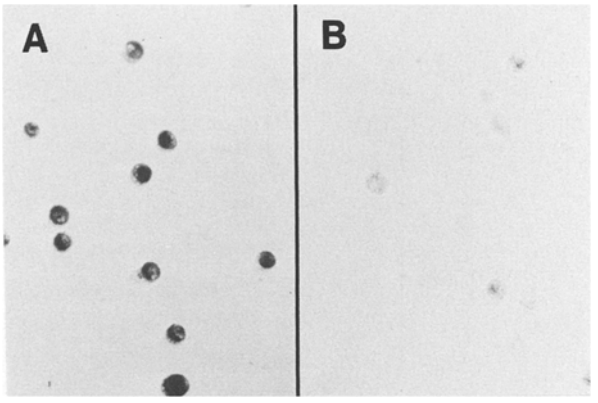

Fig. 5 Light micrographs of panaxytriol-treated cells and MTT assay. Light micrographs of Breast M25-SF after the 1 h incubation for the MTT assay without (A) and with 180 μM (B) panaxytriol

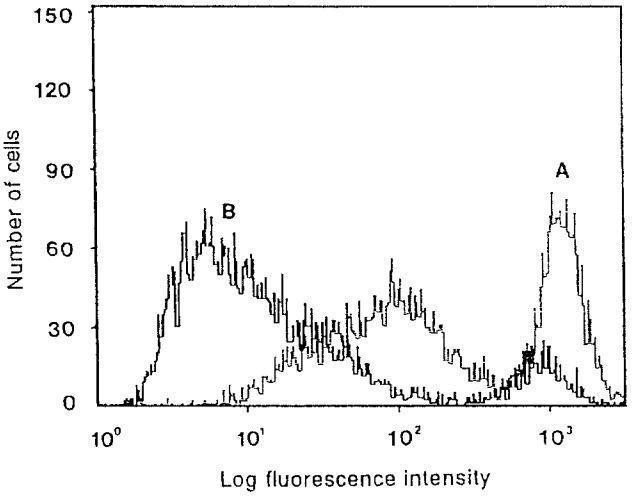

Fig. 6 A, B Decreased rhodamine-123 retention in Breast M25-SF exposed to panaxytriol. The effect of panaxytriol on rhodamine 123 retention in Breast M25-SF prestained with rhodamine 123 was measured by flow cytometry. Breast M25-SF were prestained with rhodamine 123 and then the stained cells were exposed to panaxytriol (A: 0 μM and B: 360 μM) for 1 h. A total of 5000 cells were assessed to obtain each spectrum. A decrease in the fluorescence intensity in panaxytriol-treated cells is depicted by a shift to the left on the abscissa. $p < 0.01$ (A vs B)

Table 1 Effect of panaxytriol on rhodamine-123 retention in Breast M25-SF prestained with rhodamine-123 (mean ± SD of three experiments)

Condition	Fluorescence intensity
Control	173.10 ± 4.29
Panaxytriol (180 μM)	160.43 ± 6.14*
Panaxytriol (360 μM)	87.60 ± 4.03**

* $P < 0.05$; ** $P < 0.01$

Effect of panaxytriol on rhodamine-123 retention

In order to further examine the ability of panaxytriol to inhibit mitochondrial function, the effect of panaxytriol on rhodamine-123 retention by functional mitochondria was studied. Breast M25-SF were stained with rhodamine-123 by incubation in a solution containing 5 μg/ml of rhodamine-123. These prestained cells were exposed to 180 or 360 μM panaxytriol for 1 h, and then the fluorescence intensity of rhodamine-123 in the panaxytriol-treated cells was compared with that in the untreated cells. A significant decrease in the fluorescence intensity in the panaxytriol-treated Breast M25-SF was observed and is shown in Table 1 and Fig. 6. These results indicate impairment of the ability of the treated mitochondria to retain rhodamine-123.

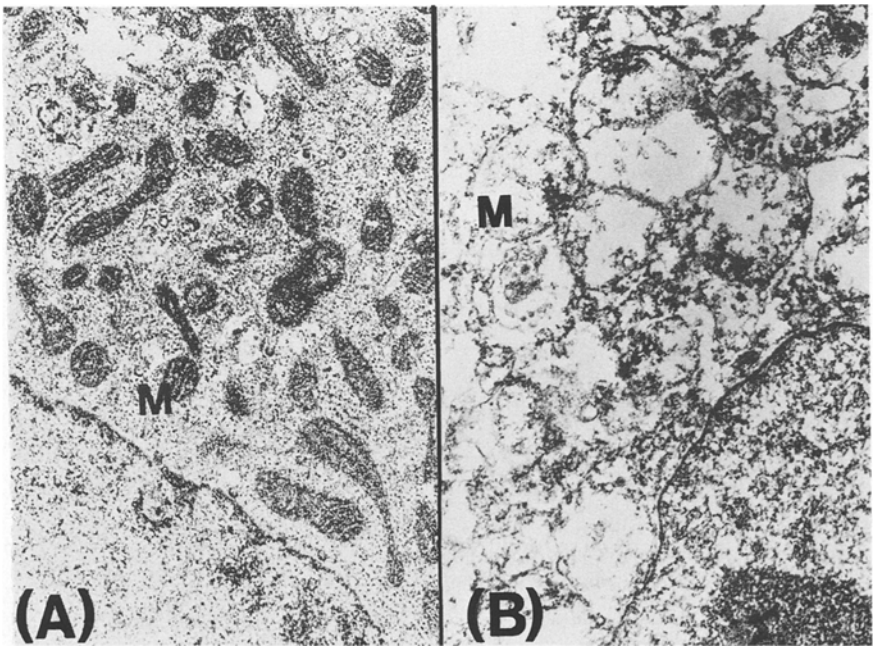

Fig. 7 A, B Electron micrographs of panaxytriol-treated Breast M25-SF. **A** untreated control cells at 1 h. Mitochondria (M) have normal cristae (× 13000). **B** cells treated with 180 μM panaxytriol at 1 h. Swollen mitochondria are present, and there is extensive loss of cristae in most mitochondria (× 13000)

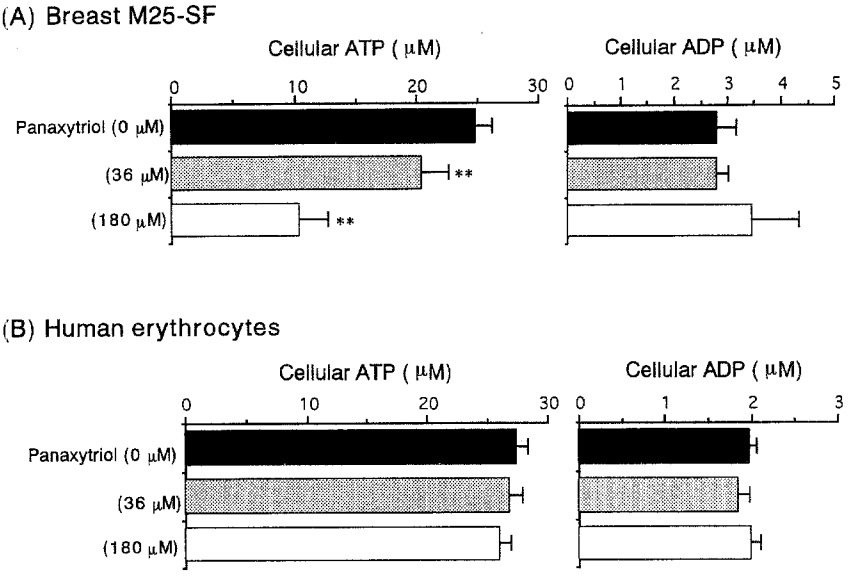

Fig. 8 A, B Effect of panaxytriol on cellular ATP and ADP levels in Breast M25-SF and human erythrocytes. Breast M25-SF (2×10^6 cells) and human erythrocytes (2×10^8 cells) were incubated without panaxytriol or with 36 μM or 180 μM panaxytriol for 1 h at 37 °C. These cells were collected and washed with cold PBS. The cellular concentrations of ATP and ADP were determined by HPLC analysis as described in Materials and methods. Mean ± SD of three experiments. ** $P < 0.01$

Morphological characteristics in panaxytriol-treated Breast M25-SF

When Breast M25-SF were treated with a concentration of panaxytriol greater than 180 μM for 1 h, significant toxic changes were found in the mitochondria but not in the nuclei (Fig. 7). In Breast M25-SF treated with panaxytriol, the mitochondria were swollen and had lost their cristae (Fig. 7B).

Effect of panaxytriol on cellular ATP and ADP levels

As presented above, panaxytriol causes functional and structural damage to the mitochondria of Breast M25-SF. Since mitochondria carry out most of the cellular oxidation and produce the majority of the ATP in animal cells, panaxytriol may thus lower cellular ATP. The cellular concentrations of ATP and ADP in Breast M25-SF incubated with 36 or 180 μM panaxytriol for 1 h are shown in Fig. 8A. The cellular ATP concentrations of panaxytriol-treated Breast M25-SF declined in a dose-dependent manner. No significant difference in the cellular levels of ADP was found.

On the other hand, neither ATP depletion nor ADP depletion was found in human erythrocytes with no mitochondria (Fig. 8B).

Discussion

Panaxytriol, which was isolated from *Panax ginseng* C. A. Meyer, is a new type of antitumor substance [11, 12, 17, 18]. Panaxytriol possesses the unusual property of being soluble in both water and organic solvents. Our preliminary studies indicate that panaxytriol has a strong affinity for mitochondria in cultured tumor cells (Fig. 3). It has been reported that the impairment of mitochondrial function and toxic cell death are closely related events [3, 4, 8, 13, 20, 22, 26, 28, 29]. In this paper we have demonstrated the possibility that the damage to mitochondria caused by panaxytriol contributes, at least partially, to its cytotoxicity against target tumor cells when these cells are exposed to high concentrations of panaxytriol for relatively brief periods (12–24 h). The effects of panaxytriol on mitochondrial structures and function were examined by the four different assays mentioned in the Introduction.

The conversion of tetrazolium to the formazan product requires reduction with NADH or NADPH. Rago et al. [22] demonstrated that the antiparasitic drug suramin rapidly and reversibly reduced the conversion of MTT-tetrazolium dye during 4 h and that the rapid inhibition of tetrazolium conversion is a result of the inhibition of dehydrogenase activity, a direct reflection of diminished cellular respiration and mitochondrial activity. The inhibition of tetrazolium conversion by panaxytriol (>11.3 μM) is a rapid event, taking less than 2 h (Fig. 4). The cells in which tetrazolium conversion is inhibited do not appear to be dead, as suggested by the trypan blue dye exclusion assay. The rapid inhibition by panaxytriol of tetrazolium conversion in Breast M25-SF indicates a disruption of respiration in mitochondria. The inhibition of mitochondrial respiration may be the result of a direct action of panaxytriol, but this effect is not lethal, since Breast M25-SF treated with panaxytriol (<22.5 μM) for 2 h gradually recover their ability to convert the tetrazolium (data not shown).

The fluorescent compound rhodamine-123 selectively accumulates in mitochondria [9, 10]. A rapid release of rhodamine-123 from mitochondria implies that there is some impairment of the energy-dependent transmembrane potential of the mitochondria [24]. The cellular content of rhodamine-123 can be measured by flow cytometry [6, 24, 26]. When Breast M25-SF cells prestained with rhodamine-123 were exposed to high concentrations of panaxytriol (180–360 μM) for 1 h, the fluorescence intensity of

rhodamine-123 significantly decreased (Table 1, Fig. 6). These findings also support the other data indicating that panaxytriol causes functional damage to mitochondria.

The ultrastructural damage caused by panaxytriol (180 μM) appears to be confined to the level of the mitochondria with swelling and alterations of the double membrane as well as loss of cristae (Fig. 7). Several investigators have pointed out that the enlargement of mitochondria may represent a crucial pathologic event and can lead to cell death [3, 8, 13, 22, 28].

The rapid conversion of ADP to ATP in mitochondria helps to maintain a high ratio of ATP to ADP in cells [5, 14]. The concentration of ATP in a cell is about 10 times that of ADP. Accordingly, any disruption in mitochondrial activity induces a rapid decrease in cellular ATP levels. In fact, substances that cause functional and structural damage to mitochondria often lower cellular ATP levels [28, 29]. The cellular ATP levels in Breast M25-SF after a 1-h incubation with 36 or 180 μM panaxytriol fell to approximately 80% or 40% of the levels in control cells, respectively (Fig. 8 A). Yet, there is little change in the cellular ADP levels. Panaxytriol induced neither ATP nor ADP depletion in human erythrocytes, which have no mitochondria (Fig. 8 B). In human erythrocytes, glycolysis is a major source of the cell's ATP. Thus, ATP depletion in Breast M25-SF exposed to panaxytriol may be a result of the direct inhibition of mitochondrial respiration caused by panaxytriol. These results indicate that ATP depletion resulting from an impairment of mitochondrial structures and function is a critical early event in the cytotoxicity of high concentrations of panaxytriol.

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