

Anti-tumor Substance from *Panax ginseng* Roots

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Abstract □ Antitumor polyacetylenic alcohol, panaxytriol (C₁₇H₂₆O₃), was isolated and purified from a powder of the root of *Panax ginseng* C.A. Meyer. Panaxytriol possesses unusual property of being soluble in both water and organic solvents. Panaxytriol inhibited the growth of various kinds of human cultured cell lines in dose-dependent fashion in vitro. The *in vivo* effects of panaxytriol were tested against C57BL/6 mice transplanted with B16 melanomas. Panaxytriol (8 and 40 mg/kg) administered intramuscularly(im) produced significant tumor growth delays in mice. Although a detailed mechanism of growth inhibition by panaxytriol is unknown, preliminary results appear to implicate a surface membrane site of action. And its action seems to be more dose-dependent than time-dependent. Finally, panaxytriol pharmacokinetics was evaluated in mice given single 8 mg/kg doses intraperitoneally(ip) or im. Serum panaxytriol content was measured using both tumor growth inhibitory assay and a gas chromatographic method. The maximum serum panaxytriol content after ip and im administration was 35.0 and 1.6 μg/ml respectively. These results indicate that the compound may act as cytotoxic substance even in patients.

Keywords □ *Panax ginseng*, panaxytriol, tumor growth inhibition

Introduction

For thousands of years, the roots of *Panax ginseng* has been used as an analeptic, stomachic and erythropoietic agent in Asian countries. We also know, by experience, *Panax ginseng*'s anti-tumor effect as its salient therapeutic manifestation¹⁾. However, it is only recently that the chemical, pharmacological and biological studies of this substance have been begun^{2,3)}. Thus, the anti-tumor activity of one of its saponins, ginsenoside Gh₂, was reported by Odashima *et al.*^{4,5)}

We shall report in what follows on the isolation of a polyacetylenic alcohol, panaxytriol (C₁₇H₂₆O₃), from roots of *Panax ginseng* and on the anti-tumor activity which this substance exhibits.

Materials and Methods

Cell line and culture

Nude mouse-transplantable human gastric adenocarcinoma cells (MK-1 cells and MK-3 cells), human colonic adenocarcinoma cells (SW620 cells), human pulmonary adenocarcinoma cells (PC3 cells), human malignant melanoma cells (M14 cells), human uterus carcinoma cells (HeLa cells), human erythroleukemic cells (K562 cells), mouse melanoma cells (B16 cells), and mouse fibroblast-derived tumor cells (L929 cells) were maintained in RPMI 1640 containing 10% fetal calf serum (FCS).

Human embryo-derived fibroblast (MRC-5 cells) and ascites-derived mesothelial cells (MK-1-M cells and MK-5-M cells) were maintained in RPMI 1640 10% FCS as the control.

Preparation of antitumor substance

The target substance was isolated from a powder of heat-treated roots of *Panax ginseng* C.A. Meyer, which is used in Japan as a commercial medical drug by the name of Korean Red Ginseng Powder

(Nikken Korai Ninjin Co., Ltd, Kobe, Japan). Korean Red Ginseng Powder (100 g) was subjected to a 12-hour extraction at room temperature with ethyl acetate (AcOEt). AcOEt extracts were gradually purified by preparative thin layer chromatography (Si gel) using 100% AcOEt, AcOEt-hexane (1:1) and chloroform-methanol (99:1) as developer to colorless oil (1.2 mg), as shown in Fig. 1. Finally, crystallization from distilled water resulted in 600 μg of crystals (Table 1).

Chemical analysis of anti-tumor substance

Infrared absorption (IR) spectra were determined on a Hitachi 270-30 spectrophotometer (Hitachi, Ltd., Tokyo, Japan). Proton and carbon-13 nuclear magnetic resonance (^1H - and ^{13}C -NMR) spectra were determined on a JMS-GSX 400 (JOEL, Tokyo, Japan) in deuteriochloroform solution with tetramethylsilane as internal standard. Mass spectra (MS) were recorded with a JMS-D300 spectrometer (JOEL, Tokyo) by the electron impact (EI) and chemical ionization (CI) methods.

In vivo tumor growth inhibitory activity

The tumor inhibitory activity of the substance extracted from powdered *Panax ginseng* roots was measured as follows. The crystallized substance was dissolved in RPMI 1640 immediately before assay. Fifty microliters of tumor cell suspension ad-

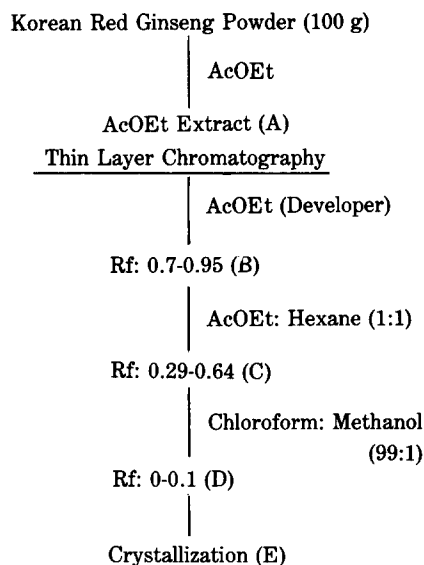


Fig. 1. Isolation of anti-tumor substance from *Panax ginseng* root. Anti-tumor substance was isolated from a powder (100 g) of heat-treated roots of *Panax ginseng* C.A. Meyer, known as Korean Red Ginseng Powder in Japan. Rf means ratio of flow.

justed to 2×10^5 cells/ml and 50 μl of the anti-tumor substance solution were plated in flat-bottomed microtiter wells (Coster, Cambridge, Mass., USA) and incubated for 48 hours at 37°C in a humidified atmosphere of 5% CO_2 air. After culture, the culture medium was gently removed; tumore cells that attached to the plate were collected by trypsinization.

Table 1. Summary of isolation of the anti-tumor substance from *Panax ginseng*

Purification step	Weight (μg)	LD_{20} ($\mu\text{g}/\text{ml}$) ^a	Total activity (ml) ^b	Yield in activity (%)
(A) AcOEt extract	570,000	62.0	9,194	100
Thin layer chromatography				
(B) AcOEt	16,800	3.2	5,250	57.1
(C) AcOEt: Hexane (1:1)	3,600	1.6	2,250	24.5
(D) Chloroform: Methanol (99:1)	1,200	0.6	1,935	21.0
(E) Crystallization	600	0.3	1,875	20.4

Anti-tumor substance was isolated and purified from heat-treated *Panax ginseng* (100 g) as described in Materials and Methods. Anti-tumor activity was measured by assay as described in Methods section using MK-1 cells as target.

^a LD_{20} shows concentration of the substance required to obtain 20% growth inhibition.

^b Total activity (ml) = $\frac{\text{weight of dried substance } (\mu\text{g})}{\text{LD}_{20} (\mu\text{g}/\text{ml})}$

Results show representative data.

Dead cells were determined by trypan blue dye exclusion. Tumor growth inhibitory activity is expressed as follows: % growth inhibitory activity =

$$\left(1 - \frac{\text{no. of viable cells in anti-tumor substance-containing RPMI1640}}{\text{no. of viable cells in RPMI1640}} \right) \times 100$$

***In vivo* experiments**

Mael C57BL/6 mice weighing 20-24 g were kept away from food but not water for 18 hours prior to experiments. C57BL/6 mice were inoculated im with B16 melanoma cells (2×10^5) in the right hind thigh on day 0, and panaxytriol (8 or 40 mg/kg) was injected im in the left hind thigh on days 1,2,3,4,5,6, 7,8,9 and 10. Thickness of bilateral hind thighs was measured with calipers every other day. Tumor size was expressed as thickness of the right hind thigh.

Purified panaxytriol was administered ip or im at a dose of 8 mg/kg to mice. Blood samples were collected by cardiac puncture from each 3 mice at varying intervals after the administration. Serum was separated by centrifugation and kept at -20°C before use. Serum panaxytriol content was measured using tumor growth inhibitory assay and a gas chromatography method.

Extraction and determination of panaxytriol from serum samples

A 1.0 ml of serum sample or culture medium was mixed with 5 ml of AcOEt and vigorously shaken for 10 min. The AcOEt-phase was collected and evaporated to dryness under a stream of nitrogen. The dry residue was dissolved in 1 ml of chloroform. A 1.0 ml of the solution was placed in a Bond Elut 1-ml silica gel column (Analytichem International, Harbor City, CA, USA), which was hung in a centrifuge tube, and centrifuged at $90 \times g$ for 5 min. The Bond Elut column was washed with 1 ml of chloroform. Finally, 1 ml of AcOEt-hexane (1:1) solution was added for sample elution, and the column was centrifuged at $90 \times g$ for 5 min. A 1.0 ml of the eluent was divided into two tubes. Each of eluents was evaporated to dryness under nitrogen.

One of the dry residue was dissolved in RPMI 1640 culture medium, and the growth inhibitory ac-

tivity of the residue was measured using MK-1 cells as a target.

The other dry residue was used for determination of panaxytriol content by a gas chromatographic method⁶. This method allows us to detect panaxytriol at concentration as low as 12.5 ng.

Statistical analysis

Difference between experimental groups were examined by Student's t-test; one-tailed probability limit was set at $p < 0.05$.

Results

Isolation and purification of anti-tumor substance

An anti-tumor substance was isolated from *Panax ginseng* as described in Materials and Methods above (Fig. 1). Since it was soluble in both water and organic solvents such as AcOEt, methanol and chloroform, the substance at each stage of extraction was dissolved in RPMI 1640, and the growth inhibitory activity of the substance was measured using MK-1 cells as target. Table 1 illustrated representative data. LD20 shows the concentration of substance ($\mu\text{g/ml}$) required to obtain 20% growth inhibition. LD20 of the initial (A) and final (E) products were 62 and $0.32 \mu\text{g/ml}$, respectively.

Molecular structure of anti-tumor substance

All chemical analyses were performed using the final product (Table 1). The molecular skeleton of the anti-tumor substance was recognized by ^{13}C -NMR⁷.

Data from the experimental analysis were identical with those of authentic panaxytriol, heptadeca-1-ene-4,6-diyne-3,9,10-triol, previously described by other investigators^{8,9}. Fig. 2 shows the molecular structure of panaxytriol.

Effect of panaxytriol on *in vitro*-cell growth

We examined the effect of panaxytriol on the growth of various kinds of cells. Panaxytriol inhibited the growth of human gastric carcinoma MK-1 and MK-3 cells, human colonic carcinoma SW620 cells, human pulmonary carcinoma PC3 cells, hu-

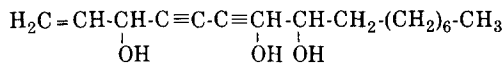


Fig. 2. Molecular structure of panaxytriol.

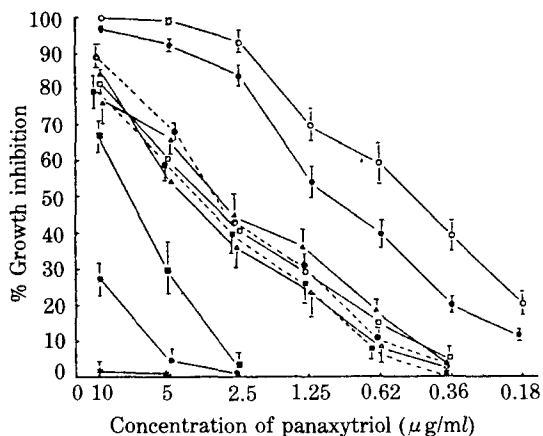


Fig. 3. Dose-effect relationship of panaxytriol on growth of tumor cells. Fifty microliters of cell suspension ($2 \times 10^5/\text{ml}$) and $50 \mu\text{l}$ of panaxytriol containing medium were plated in flat-bottomed microtiter wells and incubated for 48 hours in 5% CO_2 incubator at 37°C . % growth inhibition = $\times 100$. Results are the mean \pm SD of four parallel wells. \circ , human gastric adenocarcinoma MK-1 cells; \bullet , human gastric adenocarcinoma MK-3 cells; \blacktriangle , human pulmonary adenocarcinoma PC3 cells; \square , human malignant melanoma M14 cells; \triangle , human colonic adenocarcinoma SW620 cells; \blacksquare , human erythroleukemia K562 cells; \odot , human uterus carcinoma HeLa cells; \ominus , mouse melanoma B16 cells; \boxplus fibroblast-derived tumor L929 cells; $*$, human embryo-derived fibroblast MRC-5 cells.

man malignant melanoma M14 cells, human erythroleukemia K562 cells, mouse L929 tumor cells, and mouse B16 melanoma cells in a dose-dependent fashion (Fig. 3). Although the growth of human uterus carcinoma HeLa cells was also inhibited by panaxytriol, high concentrations of panaxytriol, over $10 \mu\text{g}/\text{ml}$, were required for significant inhibition. On the other hand, panaxytriol did not significantly inhibit the growth of human fibroblast MRC-5 cells, and human ascites-derived mesothelial cells (MK-1-M and MK-5-M) at concentrations of $10 \mu\text{g}/\text{ml}$.

When human MK-1 cells were used as a target, panaxytriol not only inhibited the cell growth, but

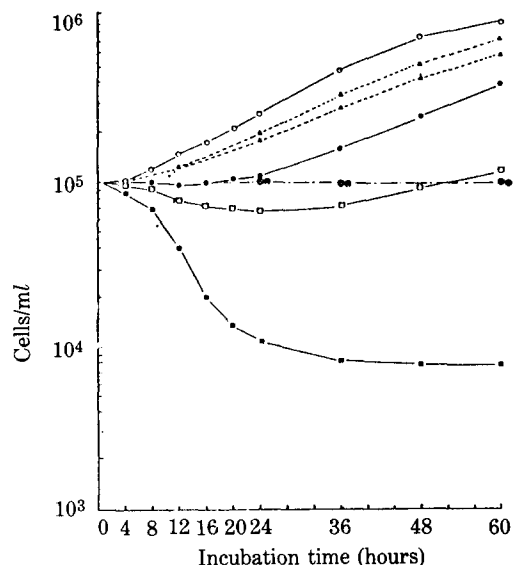


Fig. 4. Time-effect relationship of panaxytriol on growth of various kinds of cells. Fifty microliters of cells suspension ($2 \times 10^5/\text{ml}$) and $50 \mu\text{l}$ of panaxytriol containing medium were seeded in duplicate cultures and viable cells were counted at indicated time intervals. Results are the mean of 2 independent experiments. \circ , MK-1 cells (panaxytriol: $0 \mu\text{g}/\text{ml}$); \bullet , MK-1 cells ($0.3 \mu\text{g}/\text{ml}$); \square , MK-1 cells ($0.6 \mu\text{g}/\text{ml}$); \blacksquare , MK-1 cells ($5 \mu\text{g}/\text{ml}$); \triangle , MRC-5 cells ($0 \mu\text{g}/\text{ml}$); \blacktriangle , MRC-5 cells ($20 \mu\text{g}/\text{ml}$); \ominus , human peripheral lymphocytes ($20 \mu\text{g}/\text{ml}$); \odot , human peripheral lymphocytes ($\mu\text{g}/\text{ml}$).

also caused cell destruction at concentrations of over $0.6 \mu\text{g}/\text{ml}$ (Fig. 4). However, panaxytriol did not cause such a cell destruction against MRC-5 cells, human peripheral red blood cells, neutrophils and lymphocytes even at concentrations of over $20 \mu\text{g}/\text{ml}$.

In order to see if cell destruction caused by panaxytriol is related to a rapid growth of MK-1 cells, MK-1 cells were treated with an inhibitor of DNA synthesis, mitomycin C, at 37°C for 30 min. Although cell viability was not affected in this treatment, the cell growth was completely inhibited. Cell destruction by panaxytriol was found not only in rapid growing MK-1 cells but also in mitomycin C-treated cells (Fig. 5).

Effect of purified panaxytriol on tumor growth suppression *in vivo*

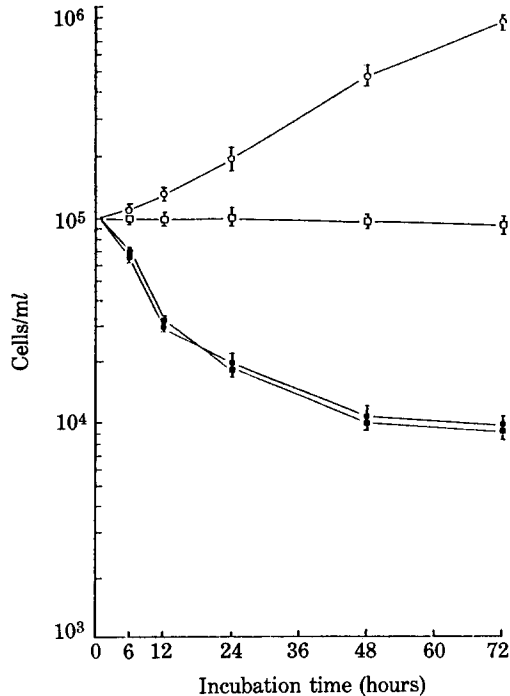


Fig. 5. Effect of pretreatment by mitomycin C on cell destruction by panaxytriol. MK-1 cell ($10^6/ml$) were treated with $10\mu g$ of mitomycin C/ml or medium at $37^\circ C$ for 30 minutes. After treatment, MK-1 cells were washed three times with medium. Washed MK-1 cells ($2 \times 10^5/ml$) were cultured in medium containing panaxytriol. Viable cells were counted at indicated time intervals. Results are the mean \pm SD of four parallel wells. ○, medium-treated cells ($0\mu g/ml$); ●, medium-treated cells ($5\mu g/ml$); □, mitomycin C-treated cells ($0\mu g/ml$); ■, mitomycin C-treated cells ($5\mu g/ml$).

To test the effect of panaxytriol on suppression of tumor growth *in vivo*, panaxytriol was administered im into B16 melanoma-transplanted C57BL/6 mice. Im inoculation of 2×10^5 B16 cells produced a visible black nodule within 5 days in all the mice. Tumor size was expressed as thickness of tumor transplanted limb. The suppressive effect was determined by three indicators, the incidence of tumor, the latency period, and tumor size. Mice receiving panaxytriol developed palpable tumors. But, panaxytriol produced significant palpable tumor growth delays in a dose-dependent manner (Fig. 6).

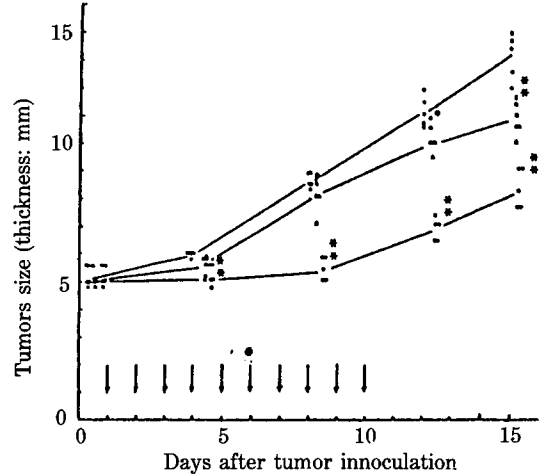


Fig. 6. C57BL/6 mice were inoculated im with B16 melanoma cells (2×10^5) in the right back thigh on day 0, and various concentrations of panaxytriol (○, $0 mg/kg$; △, $8 mg/kg$; ■, $40 mg/kg$) were injected im in the left back thigh on days 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10. Tumor size was expressed as thickness of the right back thigh. There were 5 mice in each group. *, $p < 0.05$ compared with control mice.

Absorption of panaxytriol by target cells

To investigate the mechanism of cell growth inhibition by panaxytriol, the following experiments were performed. Cell-free supernatants were collected at varying intervals from the mixed culture of panaxytriol and MK-1 cells. A remaining panaxytriol content in the supernatants was measured with a specific gas chromatographic methods. Eight microgram of panaxytriol were added to 1 ml of MK-1 cell suspension (10^6 cells/ml), and the suspension was incubated at $37^\circ C$. Panaxytriol content in the culture supernatant decreased gradually to 5.6, 4.0 and $0.6 \mu g/ml$ at 1, 2 and 6 hours, respectively (Fig. 7).

We examined if the disappearance of panaxytriol from culture medium is due to absorption or metabolic inactivation by target cells. MK-1 cells (10^6 cells/ml) were incubated with panaxytriol at a concentration of $8 \mu g/ml$ at $4^\circ C$ and $37^\circ C$. Decrease of panaxytriol was more rapid when the incubation was done at $4^\circ C$ (Fig. 7). This finding suggests that the disappearance may be due to absorption by target cells because cell metabolisms are generally

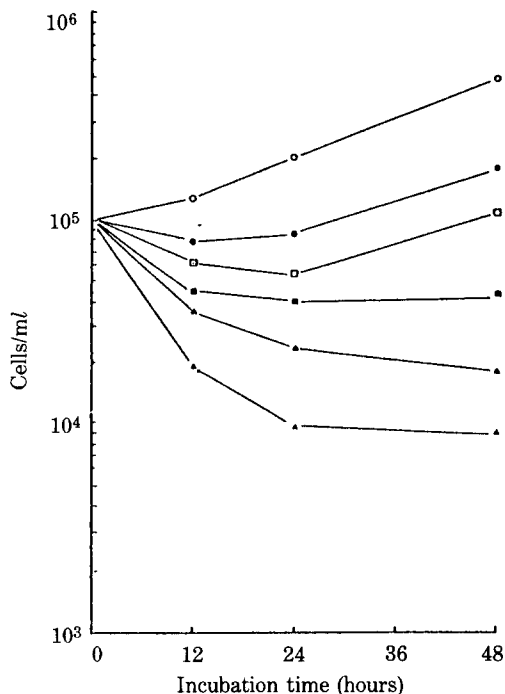


Fig. 7. Absorption of panaxytriol by MK-1 cells. MK-1 cells ($10^6/\text{ml}$) were incubated with panaxytriol at a concentration of $8\mu\text{g}/\text{ml}$ at 4°C and 37°C . Cell-free supernatants were collected at varying durations from the cultured. Panaxytriol content in the supernatants was determined by a gas chromatographic method described in METHODS section. Results are the mean of two experiments. \circ , 37°C ; \bullet , 4°C .

inhibited at 4°C .

Next, we examined whether or not the absorption of panaxytriol by target cells is related to the sensitivity of target cells to panaxytriol. Several kinds of target cells (10^6 cells) were incubated with $8\mu\text{g}$ of panaxytriol for 3 hours at 37°C . After culture, remaining panaxytriol contents was measured. Absorption of panaxytriol by sensitive cells was generally larger than that by resistant cells (Table 2).

Effect of length of exposure to panaxytriol on growth

Rapid disappearance of panaxytriol from culture medium also suggests that continuous contact between panaxytriol and target cells is not necessarily

Table 2. Absorption of panaxytriol by target cells

Target cells	Cell no.	Panaxytriol (μg)
None	0	8
Sensitive cells ^a		
MK-1 cells	10^6	1.7 ± 0.8
L929 cells	10^6	1.4 ± 0.7
B16 cells	10^6	3.5 ± 0.9
Sensitive cells ^b		
HeLa cells	10^6	7.1 ± 0.9
MRC-5 cells	10^6	7.6 ± 0.5
RBC ^c	10^8	7.4 ± 0.4
PBL ^d	10^7	7.0 ± 0.2

Eight microgram of panaxytriol were added to 1 ml of target cell suspension (10^6 - 10^8) and the suspension was incubation for 3 hours at 37°C . After culture, cell-free supernatants were collected and panaxytriol content in the supernatants was measured by a gas chromatographic method described in Methods section. Results are the mean \pm SEM of the three parallel tubes.

^a LD_{50} is below $2.5\mu\text{g}/\text{ml}$.

^b LD_{50} is over $5\mu\text{g}/\text{ml}$.

^c Human peripheral red blood cells.

^d Human peripheral lymphocytes.

required for growth inhibition. MK-1 cells were incubated with $5\mu\text{g}/\text{ml}$ of panaxytriol for various durations. After the respective incubation times, cells were washed three times with culture medium to eliminate panaxytriol. Washed MK-1 cells were cultured in panaxytriol-free culture medium for an additional 48 hours. Although cell viability was not affected in this treatment, the cell growth was significantly inhibited by a transient contact, i.e. 30 minutes, with panaxytriol. When cells were exposed to panaxytriol over for 60 min, cell destruction was also observed (Fig. 8).

Serum panaxytriol content

In order to examine if panaxytriol administered presents tumor growth inhibitory activity *in vivo*, growth inhibitory activity in serum samples was measured by growth inhibition assay using MK-1 cells as a target. Thus, the inhibitory substance content was determined on the assumption that it is panaxytriol. When panaxytriol was administered ip

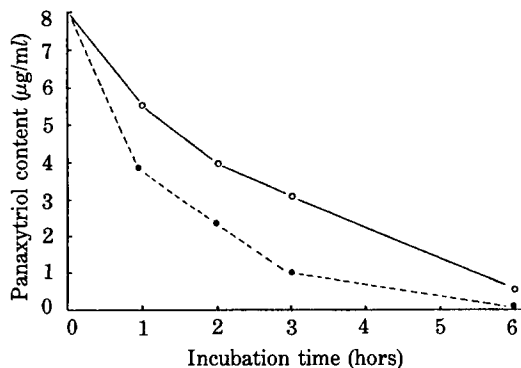


Fig. 8. Effect of length of exposure to panaxytriol on growth inhibition. MK-1 cells ($2 \times 10^5/\text{ml}$) were incubated with or without $5 \mu\text{g}/\text{ml}$ of panaxytriol for various durations at 37°C . After the respective incubation times, cells were washed three times with culture medium. Washed cells ($10^5/\text{ml}$) were cultured in panaxytriol-free culture medium for additional 48 hours and counted at indicated time intervals. Results are the mean of 2 independent experiments. ○, 0 hour; ●, 30 minutes; □, 60 minutes; ■, 120 minutes; △, 180 minutes; ▲, 48 hours.

at a dose of $8 \text{ mg}/\text{kg}$ to mice, inhibitory substance content in serum was $45.3 \pm 6.1 \mu\text{g}/\text{ml}$ at 5 min and decreased rapidly until 30 min after administration (Fig. 9).

When panaxytriol was administered im at a dose of $8 \text{ mg}/\text{kg}$ to mice, inhibitory substance content in serum rose gradually and reached its maximum at a concentration of $1.6 \pm 0.4 \mu\text{g}/\text{ml}$ one hour after administration. Then it decreased gradually to $0.43 \pm 0.06 \mu\text{g}/\text{ml}$ at 4 hours (Fig. 10).

In order to confirm if the inhibitory substance in serum is panaxytriol, serum panaxytriol content was determined by a specific gas chromatographic method described above. When panaxytriol was administered ip at $8 \text{ mg}/\text{kg}$ to mice, panaxytriol content in serum was $35.0 \pm 3.0 \mu\text{g}/\text{ml}$ at 5 min and decreased rapidly to $1.4 \pm 0.9 \mu\text{g}/\text{ml}$ at 15 min. The half-time was 12.3 min (Fig. 9). When panaxytriol was given im at $8 \text{ mg}/\text{kg}$ to mice, panaxytriol content reached its maximum at a concentration of $1.6 \pm 0.4 \mu\text{g}/\text{ml}$ one hour later and decreased to $0.3 \pm 0.05 \mu\text{g}/\text{ml}$ at 4 hours (Fig. 10). The half-time is still unknown because it was not able to prepare a

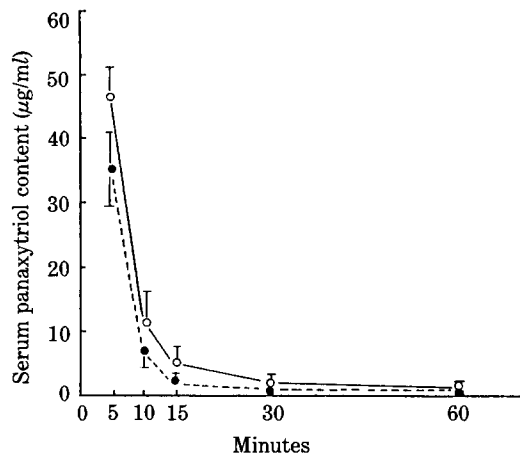


Fig. 9. Serum panaxytriol content after ip administration of panaxytriol to mice. Panaxytriol was administered ip at a dose of $8 \text{ mg}/\text{kg}$ to C57BL/6 mice. Panaxytriol content in serum was determined both by a tumor cell growth inhibition assay (○---○) and by a specific gas chromatographic method (●---●) described in MATERIALS AND METHODS. Results are the mean \pm SEM of three mice.

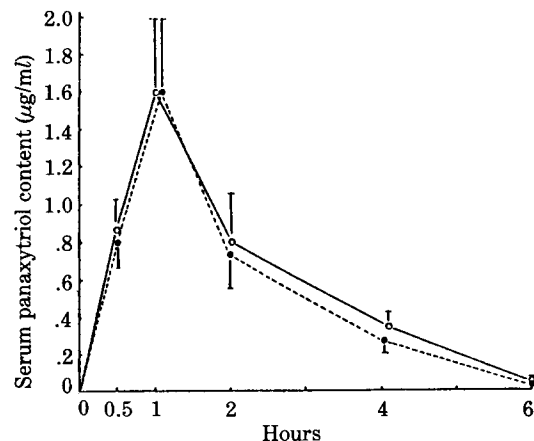


Fig. 10. Serum panaxytriol content after im administration of panaxytriol to mice. Panaxytriol was administered im at a dose of $8 \text{ mg}/\text{kg}$ to C57BL/6 mice. Panaxytriol content in serum was determined both by a tumor cell growth inhibition assay (○---○) and by a specific gas chromatographic method (●---●). Results are the mean SEM of three mice.

sufficient amount of purified panaxytriol. Panaxytriol content determined by tumor growth inhibition assay was very similar to that determined by a

specific gas chromatography.

Discussion

A new type of cell growth inhibitory substance has been isolated and purified from the root of *Panax ginseng* C.A. Meyer. Data from IR, ^1H - and ^{13}C -NMR, and MS spectra are identical with those of panaxytriol, a polyacetylenic alcohol, that have been described previously^{8,9)} (Fig. 2). Four kinds of polyacetylene compound have so far been isolated from *Panax ginseng*, but, to the best knowledge of the authors, there are no reports concerning a detailed biological activity of polyacetylenic alcohol^{10,11)}

In our investigation, we have tried elemental experiments of panaxytriol in order to specify this agent's *in vitro* antitumor activity. Panaxytriol inhibits the growth of several human and murine tumor cells in a dose-dependent fashion (Fig. 3). The inhibition, however, dose not necessarily require a sustained contact between target tumor cells and panaxytriol. An exposure of tumor cells to panaxytriol for a mere 30 minutes induces a significant growth inhibition, as seen in Fig. 7. These results indicate that panaxytriol's mode of action is more dose-dependent than time-dependent.

One sees that, under certain conditions, tumor cells may be destroyed by panaxytriol (Fig. 4). On the other hand, panaxytriol dose not cause measurable cell damage against human peripheral red blood cells, lymphocytes, neutrophils, mesothelial cells and fibroblasts. The fact that tumor cells treated with mitomycin C, which inhibits DNA synthesis, were also destroyed by panaxytriol clearly suggests that cell destruction by this agent is not related to DNA metabolism (Fig. 5). Panaxytriol is absorbed by target cells *in vitro*. And the degree of absorption by malignant cells is generally larger than that by normal cells (Fig. 6 and Table 2). Panaxytriol, an aliphatic alcohol, has amphipathic properties. These findings suggest a panaxytriol's selective action to malignant cells. The results appear to implicate a surface membrane site of action.

In order to confirm this compound to be an anti-

tumor substance, we should show data of some *in vivo* experiments that the compound is surely active *in vivo* system. For this purpose, at first, we tried to clarify that panaxytriol administered ip and im to animals is, at least partly, not metabolically inactivated and that it presents cell growth inhibitory activity in blood stream (Fig. 9,10). Secondary, the *in vivo* effects of panaxytriol was assessed on B16 melanomas transplanted in mice (Fig. 6).

This preliminary result indicates that panaxytriol may play a significant role in tumor growth even in patients. Anyway, to confirm a clinical significance of panaxytriol, we must wait for results from more detailed animal experiments, including toxicological studies and antitumor experiments.

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