Concentration-dependent Increase of Murine P388 and B16 Population Doubling Time by the Acyclic Monoterpane Geraniol

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ABSTRACT

Geraniol, an acyclic end product of a plant isoprene pathway and a pyrophosphorylated intermediate in plant and animal pathways, caused a concentration-dependent increase in the population doubling time of murine P388 leukemia cells in suspension culture and of B16 melanoma cells in monolayer culture. The suppression of the growth of P388 cells by geraniol (0-0.9 mM) and by mevinolin (0-0.25 μM), a competitive inhibitor of mevalonate biosynthesis, was reversed by the addition of 0.5 mM mevalonolactone to the growth medium. Flow cytometry of asynchronous B16 cells grown with geraniol (0-0.15 mM) revealed a population characterized by larger cells with altered nuclear characteristics. Over the course of four studies, dietary geraniol increased the 50% survival time of mice by 10, 29, 33, and 50% following the i.p. transfer of P388 cells. The results of the latter study showed that, following the i.p. transfer of 1 x 10⁷ P388 cells, the control group of female C57BL x DBA/2 F₁ mice had a 50% survival time of 24 days and a maximum survival of 27 days. Mice fed a diet containing 0.1% geraniol for 14 days prior to and following the P388 cell transfer had a 50% survival time of 36 days, and 20% of the mice remained free of tumors during the 50-day trial. These studies support the possibility that monoterpenes and other isoprenoid products of plant metabolism are in part responsible for the anticarcinogenic actions of diverse fruits, vegetables, and cereal products.

INTRODUCTION

Although its role is just now being delineated (1), there is clearly a requirement for a nonsterol, mevalonate-derived product in normal cell cycling (1-14). Inhibition of 3-hydroxy-3-methylglutaryl-CoA reductase arrests the cell cycle at the G₁-S boundary (2-7). This arrest of animal (6-9) and plant (10) cell division can be reversed by the addition of mevalonate but not by the addition of intermediates or nonsterol and sterol end products of the mevalonate pathway. Mevalonic acid is required for the posttranslational modification of soluble (15) and nuclear structural proteins (16), including lamin B, a protein isoprenylated prior to the onset of the S phase (11-13, 16-18), and lamin A (19) or its precursor (20).

An elevated 3-hydroxy-3-methylglutaryl-CoA reductase activity, resistant to cholesterol feedback, is characteristic of many neoplastic tissues (21-23). Because of this and of the requirement for an end product of mevalonate metabolism at the G₁-S boundary in the cell cycle, mevinolin and other inhibitors of reductase activity have potential application as antineoplastic agents (8, 9, 22-27). This potential is further indicated by the recent finding that all p21⁰⁰⁰ proteins are posttranslationally modified by isoprenylation (25-27).

Received 5/25/90; accepted 10/8/90.

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1 Supported in part by USPHS Grants HL 33893 and CA 09451 and by the College of Agricultural and Life Sciences, University of Wisconsin-Madison.

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3 The abbreviations used are: PDT, population doubling time; MEM, minimal essential medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline.

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were held in the staining solution for 1 h at room temperature. Prior to 5 ml propidium iodide solution (50 µg/ml in PBS) (Aldrich). Cells were pelleted and resuspended in 1 ml RNase A (bovine pancreas; Sigma; 1 mg/ml in PBS) for 20 min at 37°. This was followed by the addition of 1 ml PBS. DNA histograms were generated using a Becton/Dickinson FACScan. Aliquots of 10,000 cells were measured for fluorescence by an argon laser.

**Murine B16 Melanoma Cells**

**In Vitro Studies.** B16 cells were grown in monolayer culture (25 cm² flasks, VWR Scientific) in 5 ml RPMI 1640 (GIBCO) supplemented with 10% newborn calf serum (GIBCO), 25 mM 4-(2-hydroxyethyl)-1-piperazinieethanesulfonic acid buffer (Sigma), 80 µg/ml gentamycin, and 1.8 mM l-glutamine (GIBCO). Cultures seeded with 10 x 10⁶ cells/ml were maintained at 37° in a 5% CO₂/95% air atmosphere. A stock solution of geraniol (40 mM) was prepared in absolute ethanol. Geraniol was added to experimental flasks at final concentrations of 0.05-0.3 mM. Control flasks contained a volume of ethanol equivalent to that in experimental flasks containing 0.3 mM geraniol. Cells were harvested for counting at 24, 48, and 72 h by exposing the monolayers to trypsin (GIBCO). Trypsin was inactivated by suspending the cells in medium containing 10% FBS. Cell death was determined by uptake of 10% trypan blue; cell counts were made with a hemacytometer.

**Flow Cytometry.** Cultures for DNA staining were grown in media with final concentrations of 0.05-0.15 mM geraniol. Experimental cultures seeded with 9 x 10⁴ cells/ml were grown for 5 days, with replacement of fresh medium on Day 3. Monolayers were harvested by trypsinization, pelleted by centrifugation (10 min, 1,000 x g), and resuspended in 6 ml ice-cold 70% ethanol containing 0.5% Tween 20 for 30 min. The cells were pelleted by centrifugation, resuspended in 1 ml PBS (0.02 M sodium phosphate, pH 7.2, in 0.15 M NaCl) with 0.5% Tween 20, and incubated with 1 ml RNase A (bovine pancreas; Sigma; 1 mg/ml in PBS) for 20 min at 37°. This was followed by the addition of 5 ml propidium iodide solution (50 µg/ml in PBS) (Aldrich). Cells were held in the staining solution for 1 h at room temperature. Prior to flow cytometric analysis (42), cells were pelleted and resuspended in 1 ml PBS. DNA histograms were generated using a Becton/Dickinson FACScan. Aliquots of 10,000 cells were measured for fluorescence distribution. Propidium iodide was excited with 30 mW of 488-nm light from an argon laser.

**RESULTS**

A summary of the results of six studies of the suppression of P388 cell growth at 24, 48, and 72 h by geraniol is shown in Fig. 1. The PDTs (10.6 h) increased proportionately with incremental increases in the concentration of geraniol in the medium.

Geraniol, at the concentrations used in these trials, was not lethal. In a representative experiment, P388 cells were grown in media containing 0 (n = 2), 0.4 (n = 4), or 0.7 (n = 4) mM geraniol. At 20 h, cells grown in media containing 0.4 (n = 2) and 0.7 (n = 2) mM geraniol were washed with 82 ± 6% (SD) recovery and resuspended in geraniol-free medium. PDTs of the cultures during the ensuing 24-h period were calculated. The PDTs of control cells and of cells continued in 0.4 and 0.7 mM geraniol were 10.5, 14.8, and 18.0 h, respectively. The PDTs of cells grown for 20 h in media containing 0.4 and 0.7 mM geraniol prior to transfer to geraniol-free media were 11.1 and 12.2 h, respectively. Also, the viability of cells grown with 0.9 mM geraniol was checked by confirming that cell counts of living cells corresponded to counts with acridine orange and ethidium bromide.

The rescue of P388 cells from the growth-suppressive action of geraniol was also accomplished by adding supplemental mevalonate to the cultures (Fig. 2). Cultures with initial populations of 3.5 x 10⁴ cells/ml were incubated for 24 h in media containing 0.3 (not shown), 0.6 (A, D), or 0.9 (O, C) mM geraniol. Mevalonate (0.5 mM) was added to cultures (open symbols) at 24 h.
Geraniol was fed before and after the transfer of P388 cells as indicated.

Table 1  **Summary of in vivo studies**

<table>
<thead>
<tr>
<th>Study</th>
<th>Time of prior feeding (days)</th>
<th>Control</th>
<th>Geraniol</th>
<th>n</th>
<th>Dietary geraniol (%)</th>
<th>Cell load</th>
<th>50% survival time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-1</td>
<td>14</td>
<td>6</td>
<td>9</td>
<td></td>
<td>0.10</td>
<td>1 x 10^6</td>
<td>18</td>
</tr>
<tr>
<td>A-2</td>
<td>14</td>
<td>6</td>
<td>9</td>
<td></td>
<td>0.10</td>
<td>1 x 10^6</td>
<td>24</td>
</tr>
<tr>
<td>B-1</td>
<td>4</td>
<td>12</td>
<td>12</td>
<td></td>
<td>0.50</td>
<td>5 x 10^6</td>
<td>14</td>
</tr>
<tr>
<td>B-2</td>
<td>4</td>
<td>12</td>
<td>12</td>
<td></td>
<td>0.50</td>
<td>1 x 10^6</td>
<td>19</td>
</tr>
</tbody>
</table>

* 33% survival: control, 20 days; geraniol, 25 days.
* 33% survival: control, 25 days; geraniol, 38 days.
* 25% survival: control, 19 days; geraniol, 19 days.
* 25% survival: control, 23 days; geraniol, >42 days.
DISCUSSION

These studies provide evidence that geraniol, an early end product of a plant mevalonate pathway, increases the population doubling times of mammalian leukemic and melanoma cells. Under our conditions, geraniol effected a concentration-dependent increase in the PDT of cells, its effect in suspended culture was reversed by supplemental mevalonate, and cells resumed a normal doubling time after transfer to geraniol-free medium. At high concentrations of geraniol (>1.0 mM), the monoterpenone was lethal to P388 cells, perhaps because of its membrane-fluidizing action (43).

Mevinolin suppresses mevalonate availability by competitively inhibiting 3-hydroxy-3-methylglutaryl-CoA reductase activity (1). On the other hand, the decrease in mevalonate biosynthesis after treatment with monoterpenes (28–31) reflects a decrease in the number of reductase molecules (29). This action is consistent with that of another yet to be identified mevalonate-derived product that inhibits the translation of a class of 3-hydroxy-3-methylglutaryl coenzyme A reductase mRNA transcribed independently of sterol feedback regulation (1). The mevalonate-derived product is the significant feedback inhibitor of mevalonate synthesis in proliferating cells (1, 21), whereas cholesterol plays the major regulatory role in sterologenic tissues (44). Mevinolin and geraniol reduce the flow of mevalonate into the cellular pool that supports the synthesis of sterols, ubiquinone, dolichol, and isopentenyl adenine, as well as the synthesis of the isoprenoid products, farnesyl pyrophosphate (1, 15, 18, 27) and geranylgeraniol pyrophosphate (45, 46), required for the posttranslational modification of ras proteins (25–27) and lamins A and B (12, 16–20). Conversely, cholestyramine, a bile acid sequestrant, enhances the flow of mevalonate into the cellular pool (47) and concomitantly promotes chemically induced colon (48) and mammary (49) carcinogenesis.

Asynchronous (11) and synchronized cells arrested at the G1–S interface of the cell cycle by mevalonate deprivation (2–7) exhibit an altered morphology that is reversed by mevalonate supplementation (6, 9, 11). Among the regulation-related events occurring prior to the onset of the S phase is the enhanced synthesis of 3-hydroxy-3-methylglutaryl-CoA reductase (3, 7, 50, 51) resistant to sterol feedback regulation (44). Cells in asynchronous cultures arrested by mevalonate deprivation exhibit an altered morphology (11, 51–53) that is reflected in our finding (Fig. 6) of a 20% increase in cell size. DNA histograms of asynchronous cultures arrested by mevalonate deprivation (53) (Fig. 7) differ from DNA histograms of similarly arrested synchronous cultures (4, 7). Hence, mevalonate might also be
required for the traverse of cells through the G2 or M phase of the cell cycle (53). Most likely, the DNA histogram shown in Fig. 7 reflects the incomplete arrest of cell division effected by geraniol. At present, two classes of animal proteins, both of which play central roles in tumor growth, are reported to be anchored by covalently linked isoprenoid moieties to subcellular membranes (1). The first to be identified, lamin B (11–13, 16–18), requires farnesylation and anchorage to the nuclear membrane during the G1 stage of the cell cycle. We propose that the limits imposed on the mevalonate pool of the proliferating cells by the geraniol-mediated suppression of 3-hydroxy-3-methylglutaryl-CoA reductase activity (31) become rate-limiting for the synthesis of farnesyl pyrophosphate, leading to an increased PDT for both P388 and B16 cells. Previously noted was our finding of a 5-fold increased sensitivity of the latter to geraniol. This difference in sensitivity might be related to the much shorter PDT of the B16 cells. Alternatively, the difference in sensitivity may have roots in the second class of isoprenylated proteins. The p21" proteins are anchored to the inner surface of the plasma membrane by hydrophobic farnesyl moieties (25–27). The major oncogene in the B16 melanoma is the Ki- ras (54), whose product, independent of the need for palmitoylation, requires farnesol modification for transforming activity (55).

Monoterpenes have been shown to be effective anticarcinogens under experimental conditions using directly and indirectly acting chemical carcinogens (38–41). d-Limonene, a widely studied cyclic monoterpene, reduced the average number of rat mammary carcinomas that developed in 7,12-dimethylbenz[a]anthracene-treated rats when the terpene was fed only during the initiation or during the promotion/progression stage of carcinogenesis (40). This monoterpene also caused the regression of mammary tumors when fed to rats only after the appearance of frank tumors (56). Further, d-limonene suppressed the development of spontaneous mammary tumors (57). We now report that the first product of the diverse monoterpenoid pathways, geraniol, increases the population doubling time of murine B16 and P388 cells. Moreover, dietary geraniol provided some resistance to the growth of P388 cells transplanted to the peritoneal cavities of mice. The action underlying these diverse anticarcinogenic activities may be resolved by observations of the effect of the monoterpenes on mevalonate biosynthesis (28–31), an action initially noted in studies of their influences on serum cholesterol level (50, 51, 58).

Dietary patterns recommended for lowering the risk of cancer and cardiovascular disease emphasize the value of fruits, vegetables, and cereals (59). These foods are the primary dietary sources of the isoprenoid products of plant secondary metabolism. This leads us to suggest that the protective actions attributed to ascorbic acid, the carotenoids, selenium, glutathione, the tocopherols, and the fiber constituents, as well as to inducers of microsomal detoxification systems present in plant products, are in part due also to the influence of one or more classes of the isoprenoids on the pool of mevalonic acid (30).

REFERENCES
ARREST OF CELLS BY GERANIOL


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