Effects of Perillyl Alcohol on Melanoma in the TPras Mouse Model[1]

Maria Lluria-Prevatt, Jeanne Morreale, Jacie Gregus, David S. Alberts, Fiona Kaper, Amato Giaccia, and Marianne Broome Powell[2]

Stanford University, Department of Radiation Oncology, Stanford, California 94305 [J. M., F. K., A. G., M. B. P.], and Arizona Cancer Center, University of Arizona, Tucson Arizona 85724 [M. L-P., J. G., D. S. A.]

Abstract

This study evaluates the chemopreventive effects of topically applied perillyl alcohol on the development of melanoma in TPras transgenic mice. Our strategy was to target critical pathways in the development of melanoma, in particular, the ras pathway. Ras has been shown in our experimental mouse model, as well as others, to be important in the development and maintenance of melanomas. Perillyl alcohol (POH), a naturally occurring monoterpene, inhibits the isoprenylation of small G protein, including Ras. POH (10 mM) was applied to the shaved dorsal skin of TPras mice starting 1 week before five treatments of dimethylbenz[a]anthracene (50 μg) and was continued for 38 weeks. We observed a delay in the appearance of tumors and a 25–35% reduction in melanoma incidence. POH treatment of melanoma cells *in vitro* reduced the levels of detectable Ras protein and inhibits the activation of downstream targets, mitogen-activated protein kinases and Akt. POH only minimally induced apoptosis in this system. Pretreatment but not post-treatment of the melanoma cells with POH, however, markedly reduced levels of UV-induced reactive oxygen species. These studies suggest that POH inhibition of the Ras signaling pathway may be an effective target for chemoprevention of melanoma.

Introduction

Melanoma accounts for only 10% of all skin cancers, but it is responsible for 80% of all skin cancer deaths. The incidence of melanoma is increasing at a rate that exceeds that of any solid tumor (1). Melanoma is the most common cancer in young adults and accounts for more years of life lost than any other malignancy, including breast and lung cancer (1, 2). The limited promise of most current treatments has prompted a search for effective chemoprevention strategies in melanoma.

We have shown previously that we can induce melanoma in transgenic mice that express a human activated Ha-ras gene driven by a mouse tyrosinase promoter (3, 4). These TPras mice do not spontaneously develop cutaneous melanoma; however, 12% spontaneously develop ocular melanoma. Cutaneous melanoma can be induced in these mice with topical treatment of DMBA,[3] once a week for 5 weeks. Only the TPras mice in these experiments develop melanoma, whereas none of the negative littermates do. These DMBA-treated mice have an increased number of nevi and a melanoma incidence rate of >85%, with no carcinoma and a low incidence of papillomas (3). Thus, the TPras transgenic mouse is a suitable model for testing potential chemoprevention and therapeutic agents for melanoma.

POH and its precursor, limonene, have been studied for their chemoprevention properties, in many types of cancers, other than melanoma. POH is a cyclic monoterpene found in the essential oils of numerous plants, including citrus fruit, cherries, mint, and other edible plants. Limonene has been shown to reduce the incidence of spontaneous lymphomas in $p53^{-/-}$ mice and inhibit the development of chemically induced rodent mammary, skin, liver, lung, and forestomach tumors (5). Limonene also inhibits the development of Ras oncogene-induced mammary carcinomas in rats (6). POH has chemopreventive activity in liver cancer in rats (7), pancreatic cancer in hamsters (8), nonmelanoma skin cancer in mice (9), and rat mammary tumors (10). Phase I clinical trials of the chemotherapeutic activity of limonene and POH with breast cancer patients and patients with refractory solid tumors are in progress (5, 11).

The mechanisms of action of these monoterpenes are under investigation. Their chemopreventive activity may be because of inhibition of tumor cell proliferation, acceleration of the rate of tumor cell death, induced tumor cell differentiation (12), and/or increased apoptosis of tumor cells (7). POH has been shown to inhibit protein isoprenylation (5). One important prenylated protein identified as a suspected target of POH is Ras (13), specifically Ha-ras (14). Ras farnesylation is necessary for Ras to be localized to the cytoplasmic membrane, which is critical for its growth and transforming activities. Inhibition of this isoprenylation could account for the antitumor effects of POH.

Ras is an important branch point for multiple signaling pathways that regulate many cellular functions. Ras downstream effectors include Raf serine/threonine kinases, PI3Ks, and RalGDS and related proteins (15). Ras activates these effectors by promoting their translocation to the plasma membrane. Raf phosphorylates and activates the MEK 1/2 kinases that in turn phosphorylate and activate the ERK 1/2 MAPKs.

Received 3/30/01; revised 2/15/02; accepted 3/8/02.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] Supported by NIH Grant CA 27502 and a generous gift from the Jane Cuzner Charitable Lead Trust.
[2] To whom requests for reprints should be addressed, at Stanford University, Department of Radiation Oncology, Division of Radiation and Cancer Biology, CCSR–South, Room 1230, 269 Campus Drive, Stanford, CA 94305-5152. Phone: (650) 498-5874; Fax: (650) 723-7382; E-mail: mbp@stanford.edu.

[3] The abbreviations used are: DMBA, 7,12-dimethylbenz(*a*)anthracene; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3′-kinase; MEK, mitogen-activated protein/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase; POH, perillyl alcohol; ROS, reactive oxygen species; DCFH-DA, 2′,7′-dichlorofluorescin-diacetate; PI, propidium iodine; UV, ultraviolet light.

The activated ERKs are translocated to the nucleus where they activate various transcriptional factors, including ones that promote cell proliferation. Ras interacts with a catalytic subunit of PI3K to stimulate its lipid kinase activity that results in the production of phosphatidylinositides, PtdIns(3,4,5)P_3. These in turn activate Akt, a protein that promotes cell survival. PI3K can also activate the Rac GTPase (16). Ras may also affect signaling pathways that are responsive to increased levels of ROS. Irani *et al.* (17) have reported that expression of an activated Ras, H-Ras^{V12}, in 3T3 fibroblasts can lead to the production of ROS. A farnesyltransferase inhibitor and expression of dominant negative Ras or Rac1 suppressed the ROS production.

In this study, we have investigated the chemoprevention effect of topical application of POH on DMBA-induced melanomas in the TPras mice. In addition, we have examined the effect of POH on Ras and Ras downstream targets *in vitro* to elucidate potential mechanisms by which POH may inhibit tumor formation in this system.

Materials and Methods

TPras Mice

The development of the TPras mice was described previously (4). The transgenic mouse line contains a mutated T24 Ha-ras gene driven by a 2.5-kb promoter region from the mouse tyrosinase gene. These mice were backcrossed with C3He/N mice.

In Vivo Experiments

All mice were housed in an AALAC-approved University Animal Facility with 12-h light cycles. Food and water were provided *ad libitum*. TPras mice were randomly set up in groups of 10–13. Studies began when the mice were 5–6 weeks old. The backs of the mice were shaved and treated topically with 10 mM POH (Sigma, St. Louis, MO) starting 1 week before 5 weekly treatments with 50 μg of DMBA (Sigma). DMBA was dissolved in 100 μl of acetone, and POH was diluted in acetone. The DMBA was applied on Thursdays. POH was given three times per week (Monday, Wednesday, and Friday) throughout the study. The POH treatments were continued for 38 weeks. The mice were observed weekly. The presence of melanocytic lesions was recorded and measured. Mice were sacrificed after 40 weeks. In experiment 1, 13 mice were in the control group, and 12 mice were in the POH-treated group; in experiment 2, 10 mice were in the control group, and 11 mice were in the POH-treated group. The number of mice in the groups (10–13) was determined, given our observation of $\geq$90% melanoma incidence in DMBA-treated mice, using standard sample size formulas and assuming a two-sided statistical test at the 5% significance level, with the hypothesized proportion for 80–90% statistical power. The time to tumor development for the mice treated with and without POH was compared using the Log-rank test. This test accounted for the observation that some mice did not develop tumors during the follow-up period.

Cell Lines

Cell lines from DMBA-induced melanomas and untreated skin of TPras mice were established as described previously (18). Cells (1984-1) were derived from a cutaneous melanoma. The RMM-A cell line was derived from melanocytes isolated from the dorsal skin of a TPras mouse. These mouse melanocytes no longer express detectable levels of the TPras transgene. Cells were maintained in either melanocyte media with 5 ng/μl 12-*O*-tetradecanoylphorbol-13-acetate (LC Services, Woburn, MA) or in M15 media with the addition of 3.5% fetal bovine serum and 3.5% newborn calf serum (Life Technologies, Inc.; Ref. 15).

Apoptosis and Cell Death Analysis

Cells (1984-1; 5×10^5) were plated in T-25 flasks. After an overnight incubation, the cells were treated with 0, 0.7, or 1 mM POH for 2, 5, 12, 19, 24, 30, 36, or 48 h. Apoptosis was measured using the Apoptosis Detection Kit (R&D Systems, Inc., Minneapolis, MN). Cell death was measured by the uptake of PI. Briefly, cells were harvested with trypsin, centrifuged, and washed with PBS containing calcium and 2% BSA. Cells were kept in the presence of 2% BSA during processing. Cell pellets of 1×10^5-1×10^6 cells were collected and then resuspended in 1 $\times$ binding buffer, 0.25 μg/ml AnnexinV-FITC, and 5 μg/ml PI and incubated for 15 min in the dark at room temperature. Cell suspensions were then diluted in 1 $\times$ binding buffer and analyzed by flow cytometry. Controls were established with cells unstained and cells stained either with Annexin V-FITC or PI alone. Cellular fluorescence was analyzed using a FACScan flow cytometer with CELL Quest software (Becton Dickinson, San Jose, CA). Each sample was analyzed in duplicate, and $\geq$10,000 events were collected to maximize the statistical validity of the compartmental analysis.

Detection of UV-induced ROS

Cells (8×10^4) were seeded in 24-well plates (Falcon) and incubated overnight. Cells were treated with 1 mM POH for 1, 6, or 19 h before UV irradiation or post-treated with 1 mM POH. DCFH-DA (Molecular Probes, Eugene, OR) in medium with 0.5% FCS was added to the cultures for a final concentration of 20 μM. After 30 min, the medium was removed, the wells were rinsed with PBS, and 500 μl of PBS were added back to each well. Cells were then UV irradiated with a FS40T12/UVB bulb (National Biological Corp., Twinsburg, OH), which primarily emits in the UVB (290–320 nm) range as determined by a spectrophotometer (model 440; Spectral Instruments; Tucson, AZ). Scans of the bulbs used for these studies indicated $<$1% of the output was in the UVC range. The cells were treated with 500 J/m^2. Doses were determined using a UVX Radiometer with a 310-nm probe (UV Products; Upland, CA). Control wells included cells with or without POH treatment that were not irradiated, and wells with DCFH-DA alone were included to correct for background fluorescence. Fluorescent readings were taken at an excitation of 492 nm and emission of 517 nm on a Molecular Devices Gemini fluorescent plate reader (Sunnyvale, CA). All readings were normalized to a percentage of relative fluorescent units (RFU) by setting the UV-alone readings at 100%. Statistical analysis was done using the Student *t* test ($\alpha = 0.05$).

Western Blot Analysis

Ras Immunoprecipitation. Cells (1984-1) were treated for 19 h with 100 μM Lovastatin (Merck Research Lab, Rahway, NJ), 1 mM POH (Sigma), and 20 μM SR45023 A (Apomine; ILEX Oncology, San Antonio, TX) at 37°C. Total proteins were extracted with lysis buffer containing 50 mM Tris-HCl, 250 mM NaCl, 0.5% NP40, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml *N*-tosyl-L-phenylalanine chloromethyl ketone, 20 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, and 0.66 trypsin inhibitory units of aprotinin. The

lysates were sonicated, and the proteins were collected in a microcentrifuge at 14,000 rpm. Protein concentrations were determined using bicinchoninic acid protein assay reagent (Pierce; Rockford, IL). Protein concentrations were measured three times for each sample. Each cell lysate (400 μg) was immunoprecipitated with Ha-ras agarose conjugate antibody (Santa Cruz Biotechnology; Santa Cruz, CA). Proteins were separated on 16% SDS-PAGE and transferred onto polyvinylidene difluoride membrane. The membranes were first blocked with 5% nonfat dry milk in TBST buffer [20 mM Tris-HCl (pH 7.6), 137 mM NaCl, and 0.1% Tween 20] and then incubated with primary antibody (Anti-ras; Santa Cruz Biotechnology) at 2 μg/ml, followed by a secondary horseradish peroxidase-conjugated goat antirat antibody at 0.8 μg/ml (Santa Cruz Biotechnology) and visualized using New England Nuclear Renaissance chemiluminescence reagents (DuPont, Boston, MA).

MAPK and Akt. RMM-A and 1984-1 cell lines were cultured in M15 with low serum (0.5%) and no insulin for 24 h. Cells were then treated with POH (1 mM) for 10–12 h. For experiments examining serum stimulation, serum (7%) was added to half the plates for an additional hour before protein extracts were prepared. For insulin stimulation, the cells were treated with POH as described above and stimulated with insulin (10 μg/ml) for the last hour of incubation. In addition to POH, an inhibitor of PI3K, LY294002 (50 mM; Bio Mol Res Lab, Plymouth Meeting, PA), or an inhibitor of MEK kinase, U0126 (10 mM; Bio Mol Res Lab), was incubated with the cells 10–12 h before insulin stimulation. Proteins were extracted as described above and electrophoresed through a 10% polyacrylamide gel (50 μg of protein/lane). Antibodies used to detect MAPKs, p42, and p44 were ERK1 (K-23; Santa Cruz Biotechnology) and p-ERK (E-4; Santa Cruz Biotechnology). Akt was detected using anti-pKBα/AKT (Transduction Labs, Lexington, KY) and phospho-Akt (ser 473; Cell Signaling, Beverly, MA) antibodies. SYPRO Ruby Protein Blot Stain (Molecular Probes) was used to monitor the amount of protein that was loaded into each lane and transferred onto the nitrocellulose membranes. The staining procedure was performed according to the manufacturer guidelines. Ruby stain has a sensitivity limit of 2–8 ng/band.

Results

Reduction of Melanoma Incidence by Topically Applied POH. To evaluate the prevention of melanoma by POH, TPras mice were treated topically with 10 mM POH starting 1 week before five weekly treatments of DMBA (50 μg). Control mice received DMBA or POH alone. POH was applied three times a week for 38 weeks. The results of two separate studies are presented in Fig. 1. In the mice treated with DMBA alone, 100% tumor incidence was observed in two separate studies (Fig. 1). In the first study, the DMBA-only mice developed tumors starting at 13 weeks; >50% had tumors by 18 weeks, and 100% had tumors at 33 weeks. In study 1, there was a significant reduction, 25%, in the number of mice with tumors in the POH treatment group ($p = 0.0258$), not seen until 16 weeks. By 24 weeks, 33% of the mice had tumors; at 30 weeks, 75% of the mice had tumors with no additional incidences ≤38 weeks. Three mice died in the POH/DMBA group before the end of the DMBA treatments and were not included in the study. No tumors had developed on these mice, and there was no obvious cause for the deaths. In the second study, the DMBA-only group began developing melanoma at 18 weeks, and 100% of the mice developed tumors by 24 weeks. In the POH/DMBA group from study 2, 1 mouse developed a tumor at 19 weeks, and by 29 weeks, 64% of the mice had melanoma. No additional mice developed tumors, and the study was terminated after 40 weeks. In the second study, the melanoma incidence was reduced by 36% with POH treatment ($p < 0.0001$). No tumors developed on mice treated with POH alone. In the DMBA-only groups, the tumors ranged in size from 2 to 15 mm; on the POH/DMBA mice, tumors measured 2–10 mm in size. Mice from both groups had from one to three cutaneous tumors. During these experiments, we observed the development of small flat pigmented nevi on both the DMBA- and POH/DMBA-treated mice. These small lesions appeared 2–3 weeks before measurable (2 mm) pigmented lesions that developed into melanoma. Histopathology evaluations performed previously (3) confirmed that the tumors were melanomas. During the studies, only minimal skin irritation was observed in the mice with POH treatment or acetone alone. The irritation appears to be because of dry skin and scratching.

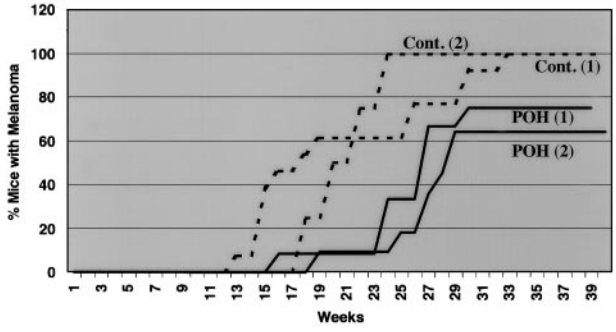

Fig. 1. POH reduces the incidence of DMBA-induced melanoma on TPras mice. TPras mice (5–6 weeks old; 10–13/group) were treated with POH (10 mM) for 1 week before DMBA treatment (50 μg, 1 × week/5 weeks). POH was given three times a week for 38 weeks. In experiment 1, a 25% reduction and in experiment 2, a 35% reduction in the number of mice that developed melanoma was observed. As reported previously, only melanoma was observed on these mice (3). Log-rank tests were used to show significance of the reduction: exp. 1, $p = 0.0258$; exp. 2, $p < 0.0001$.

Evaluation of Apoptosis in a TPras Melanoma Cell Line Treated with POH. A key mechanism that has been reported to contribute to the antitumor activity of POH is the induction of apoptosis (7). The 1984-1 mouse melanoma cell line was used to examine the induction of apoptosis by POH. 1984-1 was derived from a DMBA-induced melanoma on a TPras mouse. 1984-1 cells were treated with 0.7 or 1 mM POH for 1–48 h and analyzed for annexin binding by flow cytometry. Simultaneous staining of the cells with PI was used to detect POH-induced necrosis. As shown in Fig. 2, we observed a small increase in the population of cells that bound only annexin, after 24 (4.1%) and 48 h (11.3%) when compared with untreated cells (2.9%). During the first 19 h, no significant difference in the treated and untreated was observed. This assay was repeated three times. The percentage of cells in the top right quadrant, cells that bind both annexin V and PI, which is indicative of necrotic cells or cells in a late apoptotic stage, was also increased after 24 and 48 h exposure to 1 mM POH. A control group of cells was treated with etoposide (2 μM). This agent has been shown to induce apoptosis in other cell types (19). A portion (25%) of the cells treated with the etoposide for 48 h was stained with annexin only. These results suggest that POH can induce apoptosis in a small percentage of melanoma cells exposed to 1 mM POH. Similar results were observed with cells treated with 0.7 mM POH (data not shown). Little or no cell death, however, was observed with 0.5 mM POH.

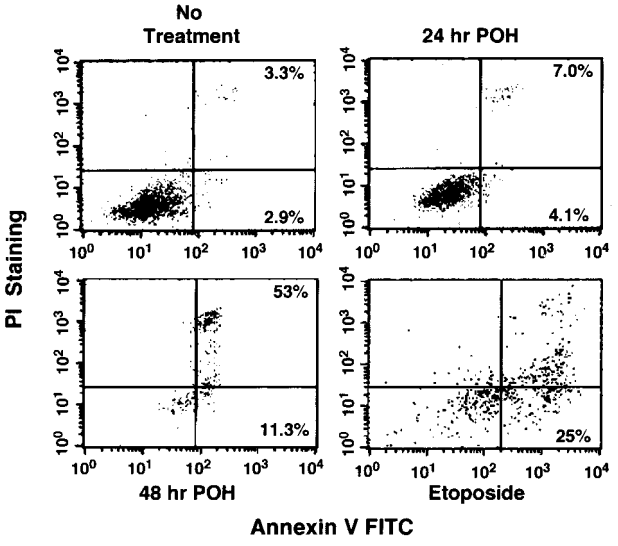

Fig. 2. POH-induced apoptosis or cell death in the TPras melanoma cells. Melanoma cells (1984-1) were treated with 1 mM POH for 0, 24, and 48 h, stained with Annexin V and PI, and analyzed by flow cytometry analysis. The *X* axis of the scatter dot plots reflect log Annexin V-FITC fluorescence, and *Y* axis reflects the PI fluorescence. Unstained viable cells are in the *bottom left quadrant*. Apoptotic cells stain Annexin V+ and PI− and are found in the *bottom right*. Necrotic cells stain PI+ (*top right quadrant*). Treatment with etoposide was used as a control for apoptosis; 25% of the cells were apoptotic after 48 h. Each treatment was assayed in duplicate, and the assay repeated three times.

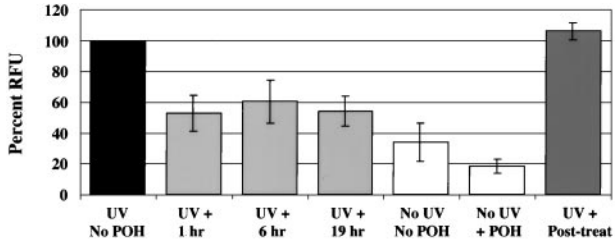

Fig. 3. POH reduces ROS production in UV-B-stimulated and unstimulated TPras melanoma cells that express an activated Ha-ras. Cells were pretreated with 1 mM POH for 1, 6, or 19 h before UV exposure (500J/m2) or post-treated with 1 mM POH. DCFH-DA (20 μM; Molecular Probes) was added to the cultures 30 min before UV irradiation. Control cells were not irradiated but incubated with POH or in media alone. Fluorescent readings were taken at an excitation of 492 nm and emission of 517 nm and normalized to percentage of RFU by setting the UV-alone readings at 100%. Statistical analysis was done using the Student *t* test ($*\alpha \leq 0.05$). Each measure was done in quadruplicate, and the assay repeated three times.

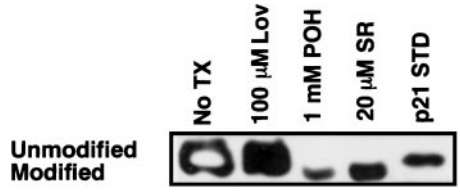

Fig. 4. POH reduced the amount of detectable Ha-ras protein in 1984-1 melanoma cells. Cells were treated for 19 h with 100 μM Lovastatin, 1 mM POH, or 20 μM SR45023A. Each cell lysate (400 μg) was immunoprecipitated with Ha-ras agarose-conjugated antibody. Immunoprecipitants were analyzed by Western blotting. Ras standard (*right lane*) is a nonprenylated p21ras. This experiment has been performed four times with similar results for each assay.

POH Effect on UV-induced ROS. As an alternative mechanism for the antitumor activity of POH in melanoma, we tested it for antioxidant properties. Intracellular levels of ROS in UV-stimulated and unstimulated 1984-1 cells were measured using DCFH-DA. Our assay to detect ROS uses DCFH-DA, an intracellular probe, which is cleaved by esterases on entering the cell. Several studies have used this assay for the analysis of ROS, specifically, hydrogen peroxide (20–22). Cells were irradiated at 500 J/m^2 with a UVB source to induce ROS. This resulted in a 3-fold increase in ROS levels when compared with cells not exposed to UV (Fig. 3). Cells pretreated with 1 mM POH for 1, 6, or 19 h showed a 40–45% reduction in UV-induced ROS production compared with POH untreated cells (Fig. 3). This result cannot be explained by direct quenching of ROS or UVB irradiation by POH because POH was removed from the cells, and the cells were washed before UV irradiation. POH reduced the levels of ROS in cells not stimulated by UV by ~45% as well. When the cells were treated with POH immediately after UV exposure, no reduction in ROS levels was detected. These results suggest that POH may inhibit the ras signaling pathways in premelanoma cells or lesions by reducing the intracellular level of ROS.

***In Vitro* Inhibition of Ras by POH.** POH has been reported to inhibit farnesylation or decrease farnesylated Ras proteins (23). The effect of POH on Ha-Ras protein expression in 1984-1 melanoma cells was assessed. Cells were incubated with 1 mM POH, 100 μM Lovastatin, 20 μM SR45023A, or media alone for 19 h. Extracted proteins were immunoprecipitated with a Ha-ras antibody and analyzed by Western blot (Fig. 4). In the lane with protein from untreated cells, there are two bands that correspond to unfarnesylated (unmodified) and farnesylated (modified) Ras. The band present in the p21 Ras standard lane represents unmodified Ras. With the Lovastatin treatment, we observed a large increase in the slower migrating band that corresponds to unfarnesylated Ras. Lovastatin competitively inhibits β-hydroxy-β-methylglutaryl coA reductase and impairs Ras farnesylation. No overall decrease in the amount of detectable Ras was seen. With POH-treated cells, the amount of Ha-ras detected was significantly reduced. The faster migrating band is indicative of farnesylated Ras. The SR45023A agent, like POH, has been proposed to interfere with protein farnesylation (24). With SR45023A, a decrease in the amount of detectable Ha-ras was also observed, and the band that was detected appeared to be farnesylated Ras.

POH Inhibits Activation of Akt and MAPKs. Because of the striking effect of POH on Ha-ras, we next investigated the effect of POH on effectors downstream of the Ras/PI3K pathway and the Ras/Raf pathway. Levels of phosphorylated and unphosphorylated Akt and MAPKs, Erk 1 and Erk 2, were examined in POH-treated and untreated cells. The cell lines used in these experiments were the TPras melanoma line, 1984-1, and a melanocyte cell line developed from untreated skin of the TPras mouse, RMM-A, which no longer expresses the activated form of Ha-ras. The cells were cultured for 24 h in M15 media with 0.5% serum to induce quiescence, and then POH was added for an additional 12 h. During the last 50 min of the POH treatment, serum was added to a final concentration of 7%. Control wells contained cells grown in M15 media with 0.5% serum and no POH for 26 h followed by serum stimulation, as described above. Western blot analyses revealed that serum stimulated phosphorylation of p42 and p44 MAPK in both the 1984-1 and RMM cells (Fig. 5). POH reduced the amount of detectable phosphorylated p42/p44 MAPK in serum-

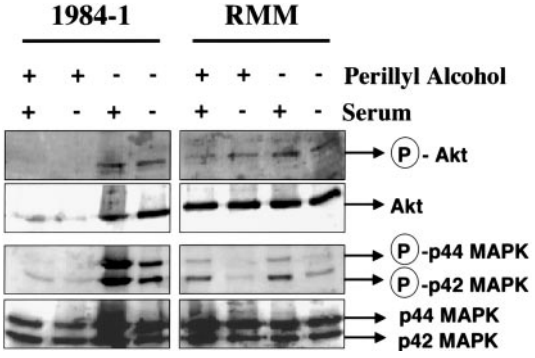

Fig. 5. Effect of POH on MAPKs and Akt in serum-stimulated TPras melanoma cells and mouse melanocytes. Western blot hybridization of protein lysates (50 μg/lane) from 1984-1 and RMM-A cells treated with POH for 12 h and then stimulated with serum (7%) for 1 h. MAPKs, phospho-ERKs, Akt, and phospho-Akt were detected with antibody (see "Materials and Methods"). These experiments were repeated three times.

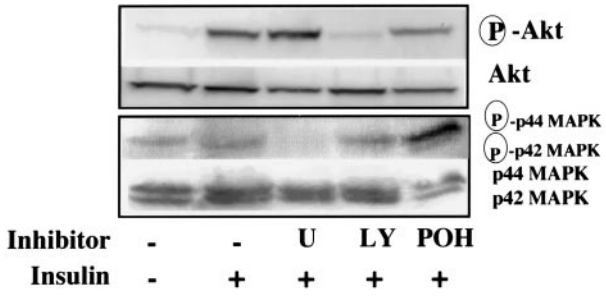

Fig. 6. POH reduces insulin-stimulated phosphorylation of Akt in 1984-1 TPras melanoma cells. Western blot hybridization analysis of protein lysates (50 μg/lane) from cells treated with POH (1 mM), a PI3K inhibitor, LY294002 (50 mM), and a MEK kinase inhibitor, U0126 (10 mM), for 12 h before insulin (10 μg/ml) stimulation for an additional hour. These studies were repeated four times.

stimulated cultures in the 1984-1 cells and reduced the levels of phosphorylated MAPKs in the unstimulated RMM-A and 1984-1 cells. A key difference in these two cell lines is that 1984-1 expresses an activated Ha-ras, and RMM-A cells do not. The phosphorylation of Akt was inhibited by POH in the 1984-1, but not the RMM-A, cells. Curiously, it appears that POH also reduced the expression of Akt but did not affect the levels of expression of nonphosphorylated p42/p44 MAPKs. Serum stimulation of Akt in either cell line was slight or negligible.

We next examined the effect of POH on insulin-induced Akt phosphorylation. Insulin has been shown to stimulate the PI3K cascade, which then leads to Akt phosphorylation and activation (25). Cells (1984-1) were treated with POH as described above, and insulin (10 μg/ml) was added for 1 h before protein extracts were prepared. As controls, additional cultures were treated with two other inhibitors, LY-294002, an inhibitor of PI3K (Biomol, Inc.), or U 0126, an inhibitor of MEK (Biomol, Inc.). Insulin stimulated the phosphorylation of Akt in 1984-1 cells (Fig. 6). POH reduced the amount of phosphorylated Akt by >50%; however, a POH-mediated decrease in total Akt was not observed. The PI3K inhibitor, LY-294002, completely blocked phosphorylation of Akt with no effect on Akt protein expression, and the MEK inhibitor, U 0126, had no inhibitory effect on activation of Akt as expected. No increased stimulation of Erk 1/2 above background was observed with insulin (data not shown). These studies demonstrate that POH treatment blocks the activation of effectors of the Ras/PI3K and the Ras/Raf pathways in melanoma cells, which express an activated Ras.

Discussion

The experiments presented in this manuscript demonstrate that topically applied POH is a potentially effective chemopreventive agent. We have observed a 25–35% reduction of DMBA-induced melanoma in the TPras mice when they are treated with 10 mM POH and a delay in onset of tumor development. To investigate the mechanisms of the chemoprevention activity, we examined the effect of POH on a melanoma cell line established from a tumor on the TPras mice that has been described previously (18). In addition to transgenic expression of an activated human Ha-ras, the tumors and cultured tumor cells have complete or partial loss of p16, an alteration that has been found in some human melanoma cells as well (18).

Monoterpenes, including POH, have been shown to act through: (*a*) inhibition of isoprenylation of small G proteins; (*b*) induction of apoptosis through the induction of mannose 6-phosphate/insulin-like growth factor 2 receptor and transforming growth factor B1 genes; and (*c*) modulation of AP-1 activity (7, 23, 26). In this study, we have examined the effect of POH on Ha-ras. POH and other monoterpenes have been reported to alter p21 ras by decreasing overall levels of Ras or by inhibiting farnesylation of the protein (13, 27). In our system, we have observed that POH decreases the overall level of Ha-ras expression. We did not observe an accumulation of cytosolic p21 ras that would indicate an inhibition of ras farnesylation (data not shown). With Lovastatin, as shown in Fig. 2, we did observe an increase in the amount of unmodified p21 ras, and in fractionated samples, there was also an increase of ras in the cytosolic fraction (data not shown). These observations agree with the finding of Hohl *et al.* (13, 23). Their studies showed that POH decreases the levels of [^{35}S]methionine-labeled Ras. The Ras decrease was to a greater extent than decreases in the levels of radiolabelled methionine into total cellular protein, thus indicating some degree of specificity of POH to depress Ras levels.

The reduction of Ras could affect numerous signal transduction pathways that regulate proliferation, differentiation, migration, and survival. We looked at two major pathways that could be affected by the reduction in activated Ras. One of these pathways is the Raf/MEK/MAPK pathway. Activation of MAP kinases has been shown to be important for the transcription of the *cyclin D1* gene and entry into the DNA synthesis phase of the cell cycle (28, 29). In the 1984-1 cells, we observed that POH reduced both constitutive levels of phosphorylated p42/p44 Erks and serum-induced levels. POH could be blocking melanoma cell proliferation by inhibiting activation of the Ras/Raf/MAPK pathway.

A second downstream event that we examined was Akt activation, which is thought to be important for cell survival (5, 16, 30). Akt is a direct target of PI3K, although it can be activated by PI 3K-independent pathways (31). Activated Akt has been reported to phosphorylate multiple targets that contribute to an antiapoptotic signal. These include Bad, caspase 9, cyclin D, members of the Forkhead family of transcription factors, GSK3, HIF-1, and IKK, leading to NF-Kβ release (30, 32–34). In our studies of the melanoma cell line 1984-1, we observed a low constitutive level of phosphorylated Akt, which

was markedly increased by the addition of insulin, but not serum, to quiescent cells. Insulin stimulates the PI3K/Akt pathway through insulin-like growth factor 1 receptors on melanoma cells (25). POH treatment reduced the constitutively phosphorylated Akt in the serum stimulation studies and partially inhibited phosphorylation of Akt by insulin stimulation. Curiously, we observed that POH treatment also reduced the amount of Akt that was detected with antibodies to the unphosphorylated Akt. A similar decrease in expression of unphosphorylated Erks was not observed. Experiments are in progress to investigate the effect of POH on Ras and Akt mRNA levels. On the basis of our findings that POH could reduce activation of Akt and the report by Mills *et al.* (7) suggesting that POH leads to an apoptotic response in liver tumors, we expected POH to induce apoptosis in our melanoma cells. After treating our cells with POH for times $\leq$48, we observed only a minimal induction (11%) of apoptosis and an increase in binding of PI over Annexin V. These observations indicated that the POH-treated melanoma cells were dying of necrosis before there was any substantial apoptosis.

Irani *et al.* (17) have demonstrated that expression of an activated Ras or Rac1 results in increased levels of ROS in cells. It has been proposed that activation of Rac and downstream oxidases can occur through Ras activation via the PI3K system (16). Our results demonstrate that POH reduced the levels of ROS in unstimulated 1984-1 cells, as well as cells stimulated primarily with UVB. We saw a 40–45% inhibition of UV-induced ROS in cells treated with POH for 1, 6, or 19 h. When the cells were given POH immediately after UV, there was no apparent ROS reduction for $\leq$45 min. The latter result suggests that POH antioxidant effect may be indirect. There is little published data that investigates direct antioxidant properties of terpenes. One study has reported that one diterpenoid, Tanshinone I, had some antioxidant properties because of a furan ring structure, which is not present in the monoterpenes we discuss here (35). An indirect antioxidant activity of POH reported by Elegbede *et al.* (36) is the induction of ROS scavengers, such as glutathione-S-transferase. POH-mediated reduction in ROS may also affect levels of protein-tyrosine phosphatases that are regulated by H_2O_2 levels. Reduced levels of H_2O_2 could prevent inactivation of phosphatases, thus reducing receptor protein tyrosine phosphorylation and subsequent cell stimulation (37).

Another mechanism that has been reported for POH or limonene is the inhibition of the metabolic activation of carcinogens by Phase I and II carcinogen-metabolizing enzymes, resulting in the detoxification of carcinogens (36, 38). Maltzman *et al.* (38) reported that the anticarcinogenic activity of limonene during the initiation stage of DMBA-induced mammary tumors was not because of changes in DMBA activation mediated by Phase I hepatic enzymes, such as cytochrome P-450. They observed an increase in the proximate carcinogen DMBA-3,4 dihydrodiol in rats fed a diet containing 5% limonene. Other studies by these researchers have shown that a 5% limonene dietary supplement did increase Phase II hepatic metabolizing enzymes, including glutathione-S-transferase and uridine diphosphoglucuronosyl transferase (36). They proposed that Phase II detoxification enzymes contributed to a delay in onset of the mammary tumors when limonene was fed during the initiation stage of carcinogenesis (39). The delay in onset of melanoma in our study may also be because of POH induction of Phase II enzymes. Additional studies in which TPras mice are treated with POH at the end of the 5-week DMBA treatments will address this issue.

In conclusion, we have demonstrated that POH used topically can reduce melanoma incidence. Limonene and POH are metabolized extensively when given p.o. This may explain a Phase I clinical trial showing only marginal antitumor activity with p.o. doses of POH (11). The authors proposed that their results could be because of reduced activity of the POH metabolites (11). However, this conclusion is not supported by the studies of Gould and Hardcastle (40, 41). Our present study in melanoma and a recent study by Barthelman *et al.* (9), showing a reduction in UVB-induced nonmelanoma tumors, indicate that topical application of POH is effective in skin cancer models. Perhaps topical application may prove to be more effective as a skin cancer chemoprevention agent with considerably less toxicities.

Acknowledgments

We thank Drs. Margaret Briehl and G. Tim Bowden for critical reading of the manuscript. We also thank Dr. Zhong Yun for his technical advice.

References

1. Eggermont, A. M. M., and Kirkwood, J. M. Introduction. Eur. J. Cancer, *34* (Suppl.)*:* S1–S2, 1998.

2. Kosary, C. L., Ries, L. A. G., Miller, B. A., Hankey, B. F., Harras, A., and Edwards, B. K. (eds.). SEER Cancer Statistics Review, 1973–1992. NIH Publication No. 96-2789. Bethesda, MD: National Cancer Institute, 1996.

3. Powell, M. B., Gause, P. G., Hyman, P., Gregus, J., Prevatt-Lluria, M., and Bowden, T. G. Induction of melanoma in TPras transgenic mice. Carcinogenesis (Lond.), *20:* 1747–1753, 1999.

4. Powell, M. B., Hyman, P., Bell, O. D., Balmain, A., Brown, K., Alberts, D., and Bowden, G. T. Hyperpigmentaion and melanocytic hyperplasia in transgenic mice expressing the human T24 Ha-ras gene regulated by a mouse tyrosinase promoter. Mol. Carcinog., *12:* 82–90, 1995.

5. Crowell, P. Prevention and therapy of cancer by dietary Monoterpenes. J. Nutr., *129:* 775S–778S, 1999.

6. Gould, M. N., Moore, C. J., Zhang, R. Wang, B., Kennan, W. S., and Haag, J. D. Limonene chemoprevention of mammary carcinoma induction following direct *in situ* transfer of v-Ha-ras. Cancer Res., *54:* 3540–3543, 1994.

7. Mills, J. J., Chari, R. S., Boyer, I., Gould, M. N., and Jirille, R. L. Induction of apoptosis in liver tumors by the Monoterpene perillyl alcohol. Cancer Res., *55:* 979–983, 1995.

8. Stark, M. J., Burk, Y. D., McKinzie, J. H., Ayoubi, A. S., and Crowell, P. L. Chemotherapy of pancreatic cancer with Monoterpene perillyl alcohol. Cancer Lett., *96:* 15–21, 1995.

9. Barthelman, M., Chen, W., Gensler, H., Huang, C., Dong, Z., and Bowden, T. Inhibitory effects of perillyl alcohol on UVB-induced murine skin cancer and AP-1 transactivation. Cancer Res., *58:* 711–716, 1998.

10. Haag, J. D., and Gould, M. N. Mammary carcinoma regression induced by perillyl alcohol, a hydroxylated analog of limonene. Cancer Chemother. Pharmacol., *34:* 477–483, 1994.

11. Hudes, G., Szarka, C. E., Adams, A., Ranganathan, S., McCauley, R. A., Weiner, L. M., Langer, C. J., Litwin, S., Yeslow, G., Halberr, T., Qian, M., and Gallo, J. M. Phase I pharmocokinetic trial of perillyl alcohol (NSC 641066) in patients with refractory solid malignancies. Clin. Cancer Res., *6:* 3071–3080, 2000.

12. Morse, M. A., and Stoner, G. D. Cancer chemoprevention: principles and prospects. Carcinogenesis (Lond.), *14:* 1737–1746, 1993.

13. Hohl, R., and Lewis, K. Differential effects of Monoterpenes and Lovastatin on ras processing. J. Biol. Chem., *270:* 17508–17512, 1995.

14. Stayrook, K. R., McKinzie, J. H., Barbhaiya, L., and Crowell, P. Effects of the antitumor agent perillyl alcohol on H-ras *vs.* K-ras farnesylation and signal transduction in pancreatic cells. Anticancer Res., *18:* 823–828, 1998.

15. Malumbres, M., and Pellicer, A. Ras pathways to cell cycle control and cell transformation. Front. Biosci., *3:* 887–912, 1998.

16. Shields, J., Pruitt, K. Aidan, M., Shaub, A., and Der, C. Understanding Ras: 'it ain't over 'til it's over.' Trends Cell Biol., *10:* 147–154, 2000.

17. Irani, K., Xia, Y., Zweier, J. L., Sollott, S. J., Der, C. J., Fearon, E. R., Sundaresan, M., Finkel, T., and Goldschimidt-Clermont, P. J. Mitogenic signaling mediated by oxidants in Ras-transformed fibroblast. Science (Wash. DC), *275:* 1649–1652, 1997.

18. Gause, P. R., Prevatt-Lluria, M., Keith, W. N., Balmain, A., Linardopolous, S., Warneke, J., and Powell, M. B. Chromosomal and genetic alterations of 7,

12-dimethylbenz[a]anthracene-induced melanoma from TP-ras transgenic mice. Mol. Carcinog., *20:* 78–87, 1997.

19. Baker, A., Payne, C. M., Briehl, M. M., and Powis, G. *Thioredoxin*, a gene found overexpressed in human cancer inhibits apoptosis *in vitro* and *in vivo*. Cancer Res., *57:* 5162–5167, 1997.

20. Ubezio, P., and Civioli, F. Flow cytometric detection of hydrogen peroxide production induced by doxorubicin in cancer cells. Free Radic. Biol. Med., *16:* 509–516, 1994.

21. Narayanana, P. K., Goodwin, E. H., and Lehnert, B. E. α Particles initiate biological production of superoxide anions and hydrogen peroxide in human cells. Cancer Res., *57:* 3963–3971, 1997.

22. Samuelson, D. J., Powell, M. B., Lluria-Prevatt, M., and Romagnolo, D. F. Transcriptional activation of the gp91phox NADPH oxidase subunit by TPA in HL-60 cells. J. Leukoc. Biol., *69:* 161–168, 2001.

23. Hohl, R. (ed.). Monoterpenes As Regulators of Malignant Cell Proliferation. Dietary Phytochemical in Cancer Prevention and Treatment, pp. 137–146. New York: Plenum Publishing Corp., 1996.

24. Niesor, E., Flach, J., Lopes-Antoni, I., Perez, A., and Bentzen, C. L. Development of drugs affecting lipid metabolism and neoplastic diseases. Curr. Pharm. Des., *7:* 231–259, 2001.

25. Coulonval, K., Vandepert, F., Stein, R., Kozma, S., Lamy, F., and Dumont, J. Phosphatidylinositol 3-kinase, protein kinase B and ribosomal S6 kinases in the stimulation of thyroid epithelial cell proliferation by camp and growth factors in the presence of serum. Biochem. J., *348:* 351–358, 2000.

26. Satomi, Y., Miyamato, S., and Gould, M. N. Induction of AP-1 activity by perillyl alcohol in breast cancer cells. Carcinogenesis (Lond.), *20:* 1957–1961, 1999.

27. Crowell, P. L., Ren, Z., Lin, S., Vedejs, E., and Gould, M. N. Structure-activity relationships among Monoterpenes inhibitors of protein isoprenylation and cell proliferation. Biochem. Pharmacol., *47:* 1405–1415, 1994.

28. Webber, J. D., Raben, D. M., Phillips, P. J., and Baldassare, J. J. Sustained activation of extracellular-signal-regulated kinase 1 (ERK1) is required for the continued expression of cyclin D1 in G_1 phase. Biochem. J., *326:* 61–68, 1997.

29. Balmanne, K., and Cook, S. J. Sustained MAP kinase activation is required for the expression of cyclin D1. P12 ^{Cip1} and a subset of AP-1 proteins in CCL39 cells. Oncogene, *18:* 3085–3097, 1999.

30. Page, C., Lin, H. J., Jin, Y., Castle, V. P., Nunez, G., Huang, M., and Lin, J. Overexpression of Akt/AKT can modulated chemotherapy-induced apoptosis. Anticancer Res., *20:* 407–416, 2000.

31. Filippa, N., Sable, C. L., Floux, C., Hemmings, B., and VanAbberghen, E. Mechanism of protein kinase B activation by cylic AMP-dependent protein kinase. Mol. Cell. Biol., *19:* 4989–5000, 1999.

32. Khwaja, A. Akt is more than just a Bad kinase. Nature, *40:* 33–34, 1999.

33. Franke, T., Kaplan, D., and Cantley, L. PI3K: downstream AKTion blocks apoptosis. Cell, *88:* 435–437, 1997.

34. Zundel, W., Schindler, C., Haas-Kogan, D., Koong, A., Kaper, F., Chen, E., Gottschalk, A., Ryan, H., Johnson, R., Jefferson, A., Stokoe, D., and Giaccia, A. Loss of PTEN facilitates HIF-1-mediated gene expression. Genes Dev., *14:* 391–396, 2000.

35. Ng, T. B., Liu, F., and Wang, Z. T. Anitoxidant activity of natural products from plants. Life Sci., *66:* 709–723, 2000.

36. Elegbede, J. A., Malzman, T. H., Elson, C. E., and Gould, M. N. Effects of anticarcinogenic Monoterpenes on phase II hepatic metabolizing enzymes. Carcinogenesis (Lond.), *14:* 17508–17512, 1993.

37. Lee, S-R., Kwon, K-S., Kim, S-R., and Rhee, S. G. Reversible inactivation of protein-tyrosine phosphatase 1B in A431 cells stimulated with epidermal growth factor. J. Biol. Chem., *273:* 15366–15372, 1998.

38. Maltzman, T. H., Christou, M., Gould, M., and Jefcoate, C. R. Effects of monoterpenoids on *in vivo* DMA-DNA adduct formation and on phase I hepatic metabolizing enzymes. Carcinogenesis (Lond.), *12:* 2081–2087, 1991.

39. Elson, C. E., Maltzman, T. H., Boston, J., Tanner, M., and Gould, M. Anti-carcinogenic activity of d-limonene during the initiation and promotion/progression stages of DMBA-induced rat mammary carcinogenesis. Carcinogenesis, *9:* 331–332, 1988.

40. Gould, M. N. Prevention and therapy of mammary cancer by Monoterpenes. J. Cell. Biochem., (Suppl.)*:* 139–144, 1995.

41. Hardcastle, I. R., Rowlands, M. G., Barber, A. M., Grimshaw, R. M., Mohan, M. K., Nutley, B. P., and Jarman, M. Inhibition of protein prenylation by metabolites of limonene. Biochem. Pharmacol., *57:* 801–809, 1999.