Induction of Apoptosis in Liver Tumors by the Monoterpene Perillyl Alcohol

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Abstract

The monoterpenes d-limonene and perillyl alcohol (POH) inhibit the growth of mammary tumors. In this investigation we tested whether POH is also effective in reducing liver tumor growth. Diethylnitrosamine was used to induce liver tumors in male Fischer 344 rats. Two weeks after diethylnitrosamine exposure was discontinued, the animals were divided into POH-treated and untreated groups. The mean liver weight for the POH-treated rats after 19 weeks of POH treatment was 10-fold less than that for the untreated animals. POH did not influence tumor cell proliferation but increased the apoptotic index approximately 10-fold. The mRNA levels for the mannose 6-phosphate/Insulin-like growth factor II receptor and the transforming growth factor type II receptor were increased in the liver tumors from the POH-treated animals when compared to the corresponding receptor mRNA levels in the normal tissue surrounding the tumors and in the tumors of untreated animals. These results demonstrate that POH does not promote the formation of liver tumors, but rather inhibits their growth by enhancing tumor cell loss through apoptosis.

Introduction

The monoterpenes d-limonene and POH are naturally occurring substances derived from orange peels and lavender, respectively. Recently it has been shown that these two compounds are not only potent breast cancer chemopreventive agents but also effective chemotherapeutic agents against advanced mammary tumors (1—3). Consequently, d-limonene is presently in Phase I clinical trials (4). The mechanism of action of these monoterpenes may involve a reduction in the isoprenylation of G proteins including ras (5) and an enhancement in both the production and activation of the potent growth inhibitor TGFβ (2). Interestingly, the antiestrogen, TAM, has also been suggested to cause breast cancer regression partly through a TGFβ-dependent mechanism (6).

Three structurally related isoforms of TGFβ are secreted from mammalian cells as biologically inactive complexes. Each of these heteromeric complexes is composed of the mature TGFβ homodimer, a LAP that is ionically associated with the mature molecule (7), and a TGFβ-binding protein that is covalently bound to the TGFβ-LAP (8). The TGFβ latent complex contains phosphomannosyl residues and consequently binds to the M6P/IGFII receptor (9). M6P/IGFII receptors function principally to mediate the endocytosis and intracellular trafficking of lysosomal enzymes; however, this receptor also binds a variety of growth factors, including IGFII and the latent complex of TGFβ (10). The binding of the TGFβ latent complex to the M6P/IGFII receptor results in either TGFβ degradation in the lysosomes or plasmin-induced extracellular TGFβ activation (10, 11).

Active TGFβ released from the latent complex can then bind to the TGFβ receptors (12—16). TGFβ signal transduction begins with the binding of TGFβ to the TGFβ type II receptor and the subsequent formation of a heteromeric complex between the TGFβ type I and type II serine/threonine kinase receptors (15). The type III receptor is not directly involved in signal transduction but rather binds TGFβ for presentation to the TGFβ signaling receptors (16). Thus, changes in the cellular membrane concentrations of either the M6P/IGFII receptor or the TGFβ type I, II, and III receptors may alter the ability of cells to both activate and respond to TGFβ. Accordingly, only mammary tumors that exhibit elevated levels of the M6P/IGFII receptor regress in response to d-limonene (2).

Because of the potential of using monoterpenes to treat breast cancer (3, 4), we investigated whether POH also influences the growth and development of liver tumors. This is particularly important because although TAM is effective in treating breast cancer, it also promotes the formation of rat liver tumors (17). Furthermore, we examined the effect of POH on the expression of the M6P/IGFII receptor and TGFβ receptors in rat liver tumors since monoterpenes-induced mammary tumor regression can be explained in part through a TGFβ-dependent mechanism (2). The results of this investigation demonstrate that POH significantly inhibits liver tumor growth by markedly increasing the frequency of apoptosis. In addition, the increased level of apoptosis in the liver tumors is associated with an elevated expression of the M6P/IGFII receptor and TGFβ type I, II, and III receptors.

Materials and Methods

Animals. Male Fischer 344 (F344) rats weighing 80 to 100 g were obtained from Charles River Laboratories (Raleigh, NC). They were fed Purina rodent chow No. 5010 (PMI Feeds, St. Louis, MO) and water ad libitum and were maintained in a temperature- and humidity-controlled room under a 12-h light/dark cycle. Following 1 week of acclimatization, the rats were treated with DEN (Sigma, St. Louis, MO) at 50 ppm in the drinking water for 1 month. Two weeks after the removal of DEN, the animals were randomly placed in cages (3/cage) and given either Purina No. 5001 powdered food (PMI Feeds) or powdered food containing POH (Aldrich, Inc., Milwaukee, WI). POH was administered at 1% (w/w) in the food for the first week and at 2% (w/w) thereafter. Rats were fed POH and control diet ad libitum because previous studies demonstrated that the degree of breast tumor regression was independent of whether the animals were pair-fed or received their diets ad libitum (3). POH diet was prepared weekly and stored in sealed containers at 4°C; both the POH and control diets were replaced daily.

After 19 weeks of exposure to POH, both groups of rats were killed. Livers were removed and total liver weight, total tumor weight, and number of tumors were recorded. Pieces of the tumors and the normal liver tissue surrounding the tumors were frozen in liquid nitrogen for RNA extraction. In addition, portions of the liver tissue and tumors were fixed overnight in OmniFix (Xenetics Biomedical, Tustin, CA) for immunohistochemical staining. All animal use was in full compliance with NIH guidelines for humane care and was approved by the Duke University Medical Center Animal Use Committee.

RNA Preparation and RNase Protection Assay. Total RNA was extracted by the single step acid guanidinium thiocyanate-phenol-chloroform
method (18). cDNA constructs of the receptors were made from full length rat cDNA for the type I, II, and III TGFβ receptors. The type I receptor (12) BamHI-BamHI (amino acids 519—864) fragment was subcloned into pBLUESCRIPT KS (Stratagene, La Jolla, CA); the type II receptor (19) Swal-Xhol (1345—1634) fragment and type III receptor (14) PstI-BamHI (1771—2039) fragment were subcloned into pGem 3B and pGem 7Zf+ (Promega, Madison, WI), respectively. A 308-base pair PstI-NarI fragment of rat M6P/IGF-II receptor cDNA from the plasmid p45 (20) was subcloned into pBLUESCRIPT KS was used as an internal standard (21). These constructs were used to prepare [α-32P]CTP (DuPont NEN, Boston, MA) labeled antisense RNA probes using the Ambion Maxiscript in vitro transcription kit (Ambion, Inc., Austin, TX) according to the manufacturer’s recommended procedure. Gel-purified TGFβ receptors and M6P/IGFII receptor probes (250,000 cpm) and the GAPDH probe (150,000 cpm) were hybridized with 30 μg of total RNA at 48°C for 16 h. RNase A (1 mg/ml) and RNase T1 (20,000 units/ml) (Ambion, Inc.) were added to digest single-stranded RNA. Following digestion, the RNA was precipitated and resuspended in gel loading buffer, heated to 95°C for 5 min, and resolved on a 6 M urea-7% denaturing polyacrylamide gel. The gel was run for 2 to 3 h at 60 W. dried on Whatman filter paper (Whatman, Inc., Maidstone, United Kingdom), and placed on a phosphorimager screen (Molecular Dynamics, Sunnyvale, CA) for quantitation analysis. GAPDH was used as an internal standard to determine RNA integrity and the uniformity of gel loading (data not shown). Volume integration of the protected RNA probe was normalized to the mean volume integration of the protected bands of three age-matched controls to ensure consistency within each and between experiments.

DNA Labeling Index. The nuclear DNA labeling index was determined by the incorporation of BrdUrd, as described previously (22). Briefly, the animals were given BrdUrd via i.p. injection 2 h prior to sacrifice. Liver tissues were removed, fixed overnight in OmniFix, embedded in paraffin, and serially sectioned at 6 μm intervals. The sections were placed on glass histology slides. The incorporation of BrdUrd into cell nuclei was observed using the Amer- sham cell proliferation kit (Amersham Corp., Arlington Heights, IL) with immunoperoxidase staining and metal enhanced diaminobenzidine (Pierce, Rockford, IL) as the chromagen. Tissue sections were lightly counterstained with hematoxylin to facilitate the visualization of unlabeled nuclei. Hepatic tumors were classified according to size. Nodules <500 μm in diameter were classified as small tumors, and those >500 μm in diameter were classified as large tumors. At least 500 cells were counted in several randomly chosen fields to determine the DNA labeling index (fewer cells were counted in small tumors containing less than 500 cells). All slides were coded and scored blindly by one person.

Apoptotic Index. Apoptotic cells were immunohistochemically stained using a TGFβ1 pre[266—278] antibody against TGFβ1-LAP which intensely stains hepatocytes undergoing apoptosis (23). OmniFixed paraffin-embedded sections cut at 6 μm were deparaffinized and rehydrated as described previously (2). Endogenous peroxidase activity was reduced by treatment with hydrogen peroxide (0.3%, v/v) in methanol followed by three washes in PBS. The slides were incubated in 3% hydrogen peroxide (0.3%, v/v) in methanol followed by three washes in PBS, normal goat serum (10%, v/v). The slides were washed 3 more times in PBS and incubated overnight at 4°C with either the TGFβ1 pre[266—278] antibody in PBS or nonimmune control IgG in PBS at the same concentration as the primary antibody. Following 3 washings in PBS, the slides were immunoperoxidase stained using a Vectastain Elite ABC kit (Vector Laboratories Inc., Burlingame, CA) with diaminobenzidine as the chromagen. Unstained nuclei were visualized by lightly counterstaining the slides with hematoxylin. The apoptotic labeling index was determined by counting at least 500 cells in randomly chosen fields (fewer cells were counted in small tumors containing fewer than 500 cells). All slides were coded and scored blindly by one person.

Results

Liver Tumor Growth. The mean tumor mass per liver was 7.0 ± 2.7 g (SEM) in the untreated rats (n = 11), and 0.8 ± 0.4 g in the POH-treated rats (n = 10) (Fig. 1). This 10-fold reduction in mean liver tumor mass demonstrates that POH significantly (P < 0.001) inhibits liver tumor growth. In contrast, POH treatment did not alter normal liver weight (12.8 ± 0.6 g for POH rats, 11.6 ± 1.0 g for control rats; P > 0.1). POH treatment caused approximately a 10% decrease in body weight compared to control rats; however, this appears to be due primarily to a decrease in body fat, inasmuch as the normal liver weight was unaltered by POH treatment.

DNA Labeling Index. The incorporation of BrdUrd into nuclear DNA during a 2-h pulse-labeling period was examined to identify hepatocytes undergoing DNA synthesis (Fig. 2). POH treatment did not significantly change (P > 0.1) the nuclear labeling indices in either the tumors or the normal liver tissue surrounding the tumors. The labeling indices in both the large and small tumors were, however,
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Fig. 3. Apoptosis in liver tumor cells from a POH-treated rat. Hepatocytes undergoing apoptosis are stained positively with the TGFβ1 pre[266-278] antibody (23). Nuclei are lightly counterstained with hematoxylin. Arrowheads, apoptotic bodies. × 400.

Fig. 4. Apoptotic index in liver tumors and the surrounding normal liver tissue from POH-treated animals (large tumor n = 6, small tumor n = 12) and untreated animals (large tumor n = 5, small tumor n = 11). Bars, SEM.

M6P/IGFII and TGFβ Receptor Expression. POH treatment had no effect on the levels of mRNA for the TGFβ type II and III receptors in normal tissue (n = 6) compared to the receptor levels in normal tissue from untreated rats (n = 6). However, the level of mRNA for the type I receptor in normal liver was reduced by 27% in POH-treated rats (P < 0.05). In contrast, mRNA levels for TGFβ type I, II, and III receptors were all significantly elevated in large tumors from POH-treated rats compared to the mRNA levels in large tumors from untreated animals (Fig. 5). TGFβ type I receptor mRNA in liver tumors from POH-exposed animals was elevated 100% (P = 0.025), and the type II and III receptors had mRNA levels which were elevated by 38% (P = 0.05) and 49% (P = 0.04), respectively.

No POH-related change in M6P/IGFII receptor expression was significantly higher (P < 0.01) than the labeling index in the surrounding normal liver. Also the labeling index was significantly greater (P < 0.05) in the large tumors than in the small tumors in both the POH-treated and untreated rats.

Apoptotic Index. The staining of apoptotic cells using a TGFβ1 pre[266-278] antibody was used to determine the apoptotic index (Fig. 3). Exposure to POH for 19 weeks caused a 5-fold increase in the apoptotic index in the large tumors (P < 0.01) and a 10-fold increase in the apoptotic index in small tumors (P < 0.001) when compared to the apoptotic indices of comparable sized tumors in untreated animals (Fig. 4). The apoptotic index was very low (<0.1%) in the surrounding normal liver of untreated animals and was not altered by POH treatment.

Fig. 5. Expression of mRNA for TGFβ type I, II, and III and M6P/IGFII receptors in large liver tumors (T) and in the normal liver tissue surrounding the tumors (N) from POH-treated animals (n = 6) and untreated animals (n = 6). Bars, SEM.
found in normal liver tissue. In contrast, the mean level of M6P/IGFII receptor mRNA was increased more than 100% in the large liver tumors of POH-treated rats compared to the mean level in the surrounding normal tissue \((P < 0.01)\) and increased approximately 50% compared to the mean level in the large liver tumors of untreated rats \((P = 0.01)\). These results show that the POH treatment preferentially elevated liver tumor mRNA expression for the M6P/IGF-II receptor which functions to activate TGF\(\beta\) and the three types of TGF\(\beta\) receptors that mediate TGF\(\beta\) signaling.

**Discussion**

POH treatment of male F344 rats initiated through DEN exposure produced a 10-fold decrease in mean liver tumor mass when compared to the mean tumor mass of untreated animals. This result demonstrates that POH inhibits rather than promotes liver tumor development, a result similar to that observed with \(d\)-limonene (24). Analysis of nuclear DNA labeling indices showed that POH did not alter the tumor cell proliferation rate but rather markedly increased tumor cell loss through apoptosis. Thus, by elevating the death rate of tumor cells, POH may be useful therapeutically against hepatocellular carcinomas, both alone and in combination with presently utilized chemo-/radiotherapeutic agents which primarily affect the birth rate of tumor cells.

During \(d\)-limonene treatment, mammary tumors that regress up-regulate and overexpress the M6P/IGFII receptor; however, mammary tumors that do not respond to \(d\)-limonene treatment undergo no change in receptor expression (2). Our apoptotic data suggest that the small tumors in the POH-treated rats are more responsive to treatment than are large tumors. Accordingly, the tumors were predominantly small in the POH-treated rats and large in the untreated animals. Since the tumors isolated from untreated rats were primarily large, we also used the largest tumors isolated from POH-treated rats to compare the TGF\(\beta\) and M6P/IGFII receptor levels. By comparing the larger liver tumors from the POH-treated rats to those from untreated rats, we could have introduced a bias against observing a relative increase in the expression of the M6P/IGFII and TGF\(\beta\) type I, II, and III receptors. Nevertheless, despite this potential problem, a significant elevation was observed in the expression of these receptors in tumors from POH-treated rats when compared to their expression in tumors from untreated rats.

TGF\(\beta\) induces apoptosis in both normal and malignant hepatocytes (23, 25). Thus, an increased apoptotic frequency could be induced by enhancing the cellular response to TGF\(\beta\). Our results show that the mRNA for the M6P/IGFII receptor in liver tumors of POH-treated rats was elevated, as was seen previously in mammary tumors that regressed in response to \(d\)-limonene treatment (2). Additionally, the TGF\(\beta\) type I, II, and III receptors, which mediate TGF\(\beta\) signal transduction, are preferentially elevated in liver tumors from POH-treated animals. Together these results suggest that liver tumor growth in POH-treated animals is inhibited because POH selectively enhances apoptosis by increasing the ability of the tumor cell to both activate and respond to TGF\(\beta\).

Agents that promote liver tumor development (e.g., phenobarbital) do not stimulate tumor cell proliferation despite rapid focal growth (25). This result can be explained by the finding that apoptosis within the developing nodules is inhibited by these tumor-promoting agents. Recently, we also reported that phenobarbital-promoted liver tumors exhibit significantly reduced steady-state levels of mRNA and protein for the TGF\(\beta\) type I, II, and III receptors when compared to the levels in nonpromoted liver tumors (26). In contrast, this study shows that POH treatment elevates TGF\(\beta\) receptor expression in liver tumor cells. These findings support the hypothesis that POH is an anti-promoter/chemopreventive agent in the liver because it enhances rather than reduces the efficacy of TGF\(\beta\) to induce apoptosis in liver tumors. However, the mechanism by which POH enhances apoptosis in liver tumors without causing damage to various normal tissues with rapid cellular proliferation (e.g., intestine, bone marrow, skin, etc.) is presently unknown.

In conclusion, the results of this study demonstrate that POH significantly inhibits the growth of liver tumors. Thus, unlike TAM, which promotes the development of rat liver tumors (17), POH blocks mammary tumor development without promoting liver tumor formation. This study may provide important insights for evaluating ongoing clinical trials that are investigating the efficacy of monoterpenes in cancer patients (4).

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**References**

19. Tsuchida, K., Lewis, K. A., Mathews, L. S., and Vale, W. W. Molecular character-


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