ABSTRACT

Dietary administration of the monocyclic monoterpene d-limonene causes complete regression of both dimethylbenz[a]anthracene- and N-nitroso-N-methylurea-induced rat mammary carcinomas. Carcinomas regress when limonene is added to the diet either when the tumor is small and still capable of spontaneously regressing or when it is large and progressed beyond the stage when it is susceptible to spontaneous regression. The limonene dose-tumor regression response relationship is steep. Significant regressions are not observed at 5% dietary level, while a majority of tumors completely regress above a 7.5% dietary level. Limonene appears to act in a cytostatic fashion. Its removal from the diet results in a significant number of tumor recurrences. Regressing tumors have a unique histopathological appearance that is not associated with gross cytotoxicity, immune cell involvement, or apoptosis. Preliminary analysis suggests a remodeling/redifferentiation event underlying regression. The underlying mechanism of action of limonene in causing tumor regression is unknown. However, it should be noted that limonene can selectively inhibit the isoprenylation of small G proteins. Monoterpenoids such as limonene represent a novel class of anticancer drugs with the potential to cause tumor regressions with limited toxicity.

INTRODUCTION

Limonene, a monocyclic monoterpen, has been shown to have both chemopreventive and therapeutic efficacy in rodent models. Thus far, most efforts have been directed at characterizing its ability to prevent carcinogen-induced cancer. We have shown that limonene inhibits rat mammary carcinomas induced by both the indirectly acting carcinogen DMBA and the directly acting carcinogen NMU. Limonene is effective at both the initiation and promotion/progression stages for DMBA-induced cancer (1) but is only effective at the promotion/progression stage for NMU-induced mammary cancer (2). In addition to preventing DMBA-initiated mammary cancer, we have shown in a preliminary study that limonene could cause the regression of palpable mammary tumors (3).

The mechanism underlying limonene’s prevention of DMBA-initiated tumors is likely to be induction of hepatic DMBA detoxification enzymes. This enzyme induction results in a systemic reduction in DMBA-DNA adducts (5). This mechanism of action, however, does not explain the activity of limonene in preventing NMU-induced tumors or in causing the regression of DMBA-induced tumors.

Recently, we have shown that limonene could selectively inhibit the isoprenylation of a subset of proteins (6). Isoprenylation is a posttranslational modification in which an isoprene group is covalently attached to the carboxyl end of a protein. Most of the isoprenylated proteins affected by limonene have a molecular weight of 21,000–26,000 (6) and are small G proteins such as the members of the p21 ras family (7). The development of inhibitors of protein isoprenylation has been suggested as an important goal in cancer therapeutics (reviewed Ref. 8). Limonene is the first agent to our knowledge to both specifically inhibit protein isoprenylation in cells and have antitumor activity in vivo. The relationship between these 2 endpoints still requires clarification. Here we report a detailed characterization of the antitumor effects of limonene on DMBA- and NMU-induced rat mammary carcinomas.

MATERIALS AND METHODS

Tumor Induction. Wistar-Furth female rats were obtained from Harlan Sprague-Dawley, Inc. (Madison, WI). All rats, arriving at 43–48 days of age, were housed 4 rats/cage in wire-bottomed metal cages, and all were maintained with a light/dark cycle of 12 h. Rats were provided Teklad Lab Blox chow and acidified water ad libitum.

After 1 week of acclimation, at 50–55 days of age, all rats were randomly assigned to groups and carcinogens were administered. DMBA (Eastman Kodak, Rochester, NY) was given as a single gastric intubation of 50 mg/kg body weight. The DMBA was dissolved in a stock solution of 20 mg DMBA/ml sesame oil, heated, and allowed to cool to room temperature before administration. NMU (Ash-Stevens, Inc., Detroit, MI) was given as a single i.v. (tail vein) injection of 50 mg/kg body weight. The NMU was dissolved in a stock of 10 mg NMU/ml of a 0.9% NaCl solution acidified to pH 5.0 with acetic acid. Fresh solutions of NMU were prepared every 20 min.

Limonene Administration. In the first experiment (pair-feeding study), groups of 60 rats were each treated with either DMBA or NMU. Beginning at 4 weeks after carcinogen administration, rats were weighed and palpated biweekly. Upon palpation of first mammary tumor(s) (diameter, ≥3 mm), rats were randomly assigned to control or 10% (w/w) limonene diet and pair-fed. In the second experiment (dose response study), 200 rats were given DMBA. Upon palpation of first mammary tumor(s) (diameter, ≥3 mm), rats were randomly assigned to 0, 2.5, 5, 7.5, or 10% limonene diets and fed ad libitum. In the third study (large tumor regression study), groups of 50 rats each received either DMBA or NMU. Upon palpation of the first mammary tumor(s) with a minimum diameter of 10 mm, rats were randomly assigned to 10% limonene or control diet and fed ad libitum. An additional independent group of limonene-treated tumors in this protocol was collected during tumor regression for histopathological analysis.

Limonene (>99% pure by gas-chromatographic analysis; Aldrich, Milwaukee, WI) and Teklad 4% mouse/rat diet meal were thoroughly mixed and stored at −20°C. Fresh diets were made every 7–10 days. All rats were provided fresh diets daily to minimize evaporation of limonene. For pair-feeding, the quantity of diet consumed by the 10% limonene-fed rat was measured every 24 h and the assigned partner was pair-fed accordingly.

Tumor Regression Evaluation. All palpable mammary tumors were classified as either “primary” tumors, i.e., the first tumor(s) palpated in a rat with a minimum diameter of 3 mm, or “secondary” tumors, i.e., any palpable tumor in a rat arising after initial diet assignment. At diet assignment, some rats had more than one primary tumor. All carcinogen-exposed rats not bearing a first palpable tumor by week 18 after carcinogen were removed from the experiment. Complete regression of a tumor was defined as nonpalpability for a minimum of 3 consecutive weeks.

All rats in the first 2 studies were followed for a minimum of 15 weeks after the diet assignment for tumor growth or regression at primary tumor sites and all other mammary glands. From these studies, a subset of rats was removed from limonene diets and followed for the regrowth of regressed tumors. Rats in the third study were followed a...
minimum of 11 weeks after diet assignment. Rats were necropsied if moribund. Complete necropsies were performed on all rats at the termination of the study. Greater than 95% of tumors remaining at the autopsy date were diagnosed as mammary carcinomas based on gross and histopathological criteria (9).

Statistical Methods. All proportions were compared using the approximate unconditional test for differences in binomial proportions (10). Where proportions of secondary tumors were compared, the within-rat correlation was estimated using the method of Generalized Estimating Equations (11). (The computer program was supplied by Zeger and Karim at The Johns Hopkins University Department of Biostatistics.) The correlations were low enough (<0.16) to allow the assumption of independence among tumors. Differences in the number of secondary tumors were estimated and compared using a generalized linear model assuming Poisson data. Differences in time to regression were tested using the log-rank test. Further differences between regression patterns of limonene and control rats were investigated using a nonparametric estimate of the hazard function (12).

RESULTS

Pair-Feeding Study. In the initial experiment, rat mammary carcinomas were induced by DMBA or NMU. At the doses used, the tumor latencies for rats treated with either carcinogen were not significantly different \( (P = 0.49) \). The median time to first tumor was 10.5 weeks for DMBA and 12 weeks for NMU (Fig. 1).

DMBA-treated rats were assigned to limonene diet at an average of 10.7 weeks \( \pm 0.6 \) (mean \( \pm \) SEM). At the time of assignment to the limonene group, the average tumor diameter was 4.8 \( \pm 0.3 \) mm. Rats were assigned to the control group 10.9 weeks \( \pm 0.6 \) after DMBA treatment. Their average tumor diameter at assignment was 5.0 \( \pm 0.2 \) mm. NMU-treated rats were assigned to the limonene diet at 11.9 weeks \( \pm 0.6 \) after NMU. At this time, their average tumor diameter was 4.5 \( \pm 0.3 \) mm. The pair-fed controls were assigned at 11.4 weeks \( \pm 0.6 \) having an average tumor diameter of 4.5 \( \pm 0.3 \) mm.

DMBA-induced primary carcinomas in limonene-treated rats had a complete regression rate of 68% differing significantly from the pair-fed controls \( (P < 0.001) \) (Table 1). Ninety-six % of NMU-induced primary tumors completely regressed in limonene-fed rats, again differing significantly from the controls \( (P < 0.001) \) (Table 1). The time required for a primary tumor to regress to a nonpalpable mass in the limonene-treated groups (DMBA and NMU) was significantly shorter than the time for spontaneous regressions in the pair-fed control group \( (P < 0.001) \) (Table 1). These differences can be best illustrated using a nonparametric estimate of the hazard function (11). Fig. 2 shows that limonene-induced regressions occur earlier than spontaneous regressions. In addition to these rapidly regressing tumors, limonene-treated rats had a subset of tumors that regressed more slowly with kinetics similar to those of spontaneously regressing cancer. Finally, this analysis also suggests that the kinetics of spontaneous tumor regression differ in DMBA- and NMU-induced carcinomas.

Not only was limonene effective in causing the complete regression of primary rat mammary tumors, but it is also prevented the development of secondary tumors arising after initial diet assignment. There was an approximately 2-fold reduction in the average number of secondary tumors per rat for both carcinogens (Table 1; Figs. 3 and 4). Limonene was also effective in causing the regression of these secondary tumors. Sixty-three % of DMBA-induced secondary tumors and 100% of NMU-induced tumors completely regressed in limonene-fed rats. These rates were significantly greater than their respective controls \( (P < 0.01) \) (Table 1).

Toxicity was limited to minor weight losses in limonene-fed rats in this study (Fig. 5). Limonene-fed rats experienced weight loss during the first week, which was likely due to food aversion. Weight gain occurred over the next 5 weeks, followed by a plateau for the remainder of the experiment. For DMBA-treated rats at week 5 after diet assignment, there was a minor but significant difference in weights between limonene- and pair-fed controls \( (P = 0.003) \); however, by week 10 no statistical difference in weight was detected \( (P = 0.2231) \). NMU-treated rats showed the same pattern of weight gain for both treated and pair-fed groups (data not shown).

Dose Response Study. A dose response study was conducted to determine the minimum dose of dietary limonene required to cause a significant increase in the complete regression of DMBA-induced rat mammary tumors. In this experiment, primary tumor regression rates for controls and 2.5% and 5% limonene diet-fed rats were similar \( (P > 0.6) \) (Table 2). In contrast, 7.5 and 10% limonene diets were effective in causing complete tumor regression of primary tumors \( (P < 0.001) \) (Table 2). The time to complete regression of the primary tumors treated with 7.5 and 10% limonene diets was significantly shorter than that of the control \( (P < 0.001) \) (Table 2).

In addition, the 7.5 and 10% limonene diets were effective in reducing the numbers of secondary tumors arising in situ following initial diet assignment (Table 2). The complete regression rate of secondary tumors induced by DMBA is also related to the limonene dose administered. In control and 2.5% limonene diet groups, secondary tumors regressed at similar frequencies, while 5, 7.5, and 10% limonene diet groups had complete regression rates that were greater than the control \( (P < 0.001) \) (Table 2).

In this study, all rats fed dietary limonene expressed some initial food aversion (Table 3). At day 0, there was a statistically significant difference in amounts of limonene diet consumed.
LIMONENE TUMOR REGRESSION

Table 1 Complete regression of DMBA- and NMU-induced rat mammary carcinomas by dietary limonene-pair-feeding study

<table>
<thead>
<tr>
<th></th>
<th>No. of rats</th>
<th>Primary tumor regression (%)</th>
<th>Wks(^a) to regress</th>
<th>No. of secondary tumors/rat</th>
<th>Secondary tumor regression (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DMBA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% limonene diet</td>
<td>25</td>
<td>19/28(^a)(68)</td>
<td>3.25(^b)</td>
<td>1.08(^b)</td>
<td>17/27(^b) (63)</td>
</tr>
<tr>
<td>Pair-fed control</td>
<td>25</td>
<td>6/26 (23)</td>
<td>14.5</td>
<td>1.92</td>
<td>9/48 (19)</td>
</tr>
<tr>
<td><strong>NMU</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% limonene diet</td>
<td>25</td>
<td>26/27(^a)(96)</td>
<td>2.5(^b)</td>
<td>0.32(^b)</td>
<td>8/8(^b) (100)</td>
</tr>
<tr>
<td>Pair-fed control</td>
<td>24</td>
<td>14/24 (58)</td>
<td>4.0</td>
<td>0.71</td>
<td>7/17 (41)</td>
</tr>
</tbody>
</table>

\(^a\) Kaplan-Meier estimate of the time when 25% of tumors will have regressed.
\(^b\) Significantly different from controls (\(P < 0.01\)).

Fig. 2. Hazard analysis of tumor regression. The hazard function for tumor regression versus time after tumor appearance is given for tumors induced by DMBA (A) and NMU (B). Tumor regressions in limonene [dark (upper) line] and controls [light (lower) line] are compared.

(overall test, \(P < 0.001\)); however, by day 4 food levels consumed among all the groups were approximately equal (overall test, \(P = 0.9171\)). All limonene-fed rats had some initial weight loss followed by weight gain (Fig. 6). Although rats consuming 7.5 and 10% limonene diets did not achieve weights similar to controls, they were capable of gaining weight and stabilizing following the initial loss.

**Limonene Withdrawal Studies.** Initial data, from the first 2 experiments presented above, demonstrated that limonene could induce the complete regression of mammary tumors. In extensions of these 2 studies, rats that consumed 7.5 or 10% limonene diets and that had primary tumors that completely regressed were randomly assigned to either continue on the limonene diet or switch to the control diet. In the pair-feeding study, primary DMBA- and NMU-induced tumors from the limonene group that had completely regressed were followed for 3 weeks after regression of the primary tumor. At this time, rats were randomly assigned to continue on the 10% limonene diet or switch to control diet and subsequently followed for 15 additional weeks. In both the DMBA- and NMU-induced tumor groups, rats continuing on the 10% limonene diet did not re-develop palpable tumors at the primary site of regression (0 of 9 DMBA; 0 of 14 NMU). However, 4 of 6 DMBA-induced tumors recurred after limonene removal (\(P = 0.0034\)), while 4 of 10 NMU-induced primary tumors had recurred by 15 weeks after limonene removal (\(P = 0.0067\)).

Next, an additional study was conducted in which the limonene treatment period following regression was extended from 3 to 15 weeks. Rats with DMBA-induced primary tumors that completely regressed following the consumption of 7.5 or 10% limonene diets were maintained on their respective diets for an additional 15 weeks. They were then randomly assigned to either continue on the limonene diet or switch to control diet. Within 15 weeks after randomization, rats continuing on the 7.5 or 10% limonene diets had palpable regrowth in 2 of 12 and 0 of 16 cases, respectively, at primary tumor locations. Rats switched from 7.5% limonene diet to control diet had regrowth in 6 of 16 primary tumor locations (\(P = 0.2628\)), while those switched from 10% limonene diet had recurrences at 10 of 15 sites (\(P < 0.001\)). Primary tumors that spontaneously regressed in control animals were also followed. No regrowth was observed in 10 tumors followed for 30 weeks.

**Large Tumor Regression Study.** Primary tumors treated in the first 2 experiments were small, having an average diameter of 4-5 mm. Tumors in this size range had significant levels of spontaneous regression. In this experiment, large tumors having a low spontaneous regression rate were induced by either DMBA or NMU. Rats with tumors growing to a minimum diameter of 10 mm were randomly assigned to 10% limonene or control diet and fed ad libitum.

At 11 weeks after diet assignment, limonene was effective in causing the complete regression of 53% (8 of 15) DMBA-induced large tumors and 78% (7 of 9) of NMU-induced large tumors. In controls, no spontaneous regression to a nonpalpable mass was observed (0 of 14 DMBA and 0 of 8 NMU; \(P < 0.001\)). Of the 7 DMBA-induced limonene-treated tumors that
LIMONENE TUMOR REGRESSION

Fig. 3. Prevention of secondar) tumors arising in limonene-fed rats. The average number of secondary DMBA-induced tumors per rat is plotted versus weeks on therapy for 10% limonene diet (-----, n = 25 rats) and pair-fed control (----, n = 25 rats).

Fig. 4. Prevention of secondary tumors arising in limonene-fed rats. The average number of secondary NMU-induced tumors per rat is plotted versus weeks on therapy for 10% limonene diet (-----, n = 25 rats) and pair-fed control (----, n = 24 rats).

We previously demonstrated that dietary limonene could prevent the development of mammary carcinomas in the rat (1–3). A preliminary study also suggested that limonene could induce mammary carcinoma regression (4). Here we extend this initial observation by clearly demonstrating that dietary limonene causes the complete regression of both small and large mammary tumors induced by either DMBA or NMU. Limonene was previously shown to prevent DMBA-induced tumors at dietary levels of 1% and below (1). NMU-induced tumors could be prevented by diets containing 5% limonene (2). Here we established that the minimum dose of limonene required for the induction of complete regression of established mammary tumors was a dietary level of 7.5%, suggesting that therapy required a greater dose than did prevention.

Limonene was very effective at inducing and maintaining the regression of mammary carcinomas. However, we have demonstrated that if limonene administration is terminated as late as 15 weeks after regression, a substantial number of carcinomas recurred. This regrowth was not seen with continuous 10% limonene administration. This observation suggests that limonene may have a cytostatic rather than cytotoxic mechanism.
LIMONENE TUMOR REGRESSION

Table 2 Complete regression of DMBA-induced rat mammary carcinomas by dietary limonene

<table>
<thead>
<tr>
<th>% limonene in diet</th>
<th>No. of rats</th>
<th>Primary tumor regression (%)</th>
<th>Wks* to regress</th>
<th>No. of secondary tumors/rat</th>
<th>Secondary tumor regression (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>34</td>
<td>10/39(26)</td>
<td>12</td>
<td>2.38</td>
<td>18/80(23)</td>
</tr>
<tr>
<td>2.5</td>
<td>34</td>
<td>11/39(28)</td>
<td>13</td>
<td>2.91</td>
<td>28/97(29)</td>
</tr>
<tr>
<td>5</td>
<td>32</td>
<td>10/33(30)</td>
<td>11</td>
<td>2.53</td>
<td>45/81(56)</td>
</tr>
<tr>
<td>7.5</td>
<td>33</td>
<td>30/37(81)</td>
<td>5b</td>
<td>1.18b</td>
<td>28/38(74)</td>
</tr>
<tr>
<td>10</td>
<td>34</td>
<td>33/37(89)</td>
<td>3b</td>
<td>0.71b</td>
<td>24/26(92)</td>
</tr>
</tbody>
</table>

* Kaplan-Meier estimate of the time when 25% of tumors will have regressed.
* Significantly different from control (P < 0.01).

Table 3 Effects of dietary limonene on food consumption

<table>
<thead>
<tr>
<th>% Limonene in diet</th>
<th>No. of rats</th>
<th>Day 0*</th>
<th>Day 4</th>
<th>Day 8</th>
<th>Day 12</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>19</td>
<td>12.5 ± 1.5b</td>
<td>14.7 ± 0.7</td>
<td>16.1 ± 1.2</td>
<td>14.9 ± 0.1</td>
<td>16.8 ± 1.2</td>
</tr>
<tr>
<td>2.5</td>
<td>19</td>
<td>8.0 ± 1.6</td>
<td>16.2 ± 1.0</td>
<td>17.2 ± 0.2</td>
<td>16.5 ± 0.8</td>
<td>16.5 ± 1.2</td>
</tr>
<tr>
<td>5</td>
<td>21</td>
<td>5.5 ± 0.9</td>
<td>15.1 ± 1.7</td>
<td>14.6 ± 0.4</td>
<td>15.2 ± 0.7</td>
<td>15.6 ± 1.2</td>
</tr>
<tr>
<td>7.5</td>
<td>18</td>
<td>2.7 ± 0.7</td>
<td>14.1 ± 1.9</td>
<td>16.9 ± 1.4</td>
<td>15.6 ± 2.8</td>
<td>17.6 ± 1.6</td>
</tr>
<tr>
<td>10</td>
<td>22</td>
<td>3.6 ± 0.6</td>
<td>14.7 ± 1.6</td>
<td>16.5 ± 2.2</td>
<td>11.7 ± 2.7</td>
<td>14.8 ± 1.3</td>
</tr>
</tbody>
</table>

* Mean ± SEM.

of action. If so, limonene, like anticancer cytostatic drugs such as tamoxifen, would have to be administered continuously for maximal effectiveness.

A limited histopathological analysis suggests that limonene induces regression by a unique cellular mechanism. Limonene-treated regressing mammary carcinomas tend to lose their epithelial elements and eventually resemble benign fibroadenomas. This cell loss appears not to involve either an immunological or apoptotic mechanism. However, it is interesting to point out that limonene induced partially regressed tumors resemble the histopathological appearance of some spontaneously regressing tumors.4 Limonene-induced regression, however, differs from spontaneous regression in mammary tumors in several aspects. Limonene-induced regressions occur more rapidly than spontaneous regressions. Their hazard functions also are both quantitatively and qualitatively different. In addition, spontaneously regressed tumors do not recur in contrast to limonene-induced regressions that often recur after the withdrawal of limonene. Finally, limonene is effective in the induction of regression in large tumors that have progressed beyond the stage at which spontaneous regressions are observed.

Another important finding of these studies is that at levels of dietary limonene required to induce complete tumor regression, little or no toxicity is observed. The underlying mechanism responsible for limonene's extremely favorable therapeutic ratio can only be speculatively discussed at this time. We have recently reported that in cultured fibroblasts and mammary epithelial cells, limonene and its major circulating major metabolites selectively inhibit the isoprenylation of cellular proteins. Only isoprenylated proteins in the molecular weight range of 21,000–26,000 are affected by limonene (6). Most of these are small G proteins that are likely involved in signal transduction (7). Isoprenylation of these proteins consists of...
Fig. 8. Histopathology of a regressing limonene-treated mammary carcinoma. Histopathology of a regressing mammary tumor in a rat fed 10% limonene diet starting when this tumor was 10 mm in diameter. The area of tumor (upper right) is that of a typical growing rat mammary carcinoma. The other more central area shows histopathological changes that are typically found in limonene-treated regressing tumors.

Fig. 9. Histopathology of a site at which a mammary tumor completely regressed. This site previously supported a mammary tumor that was treated with 10% limonene after it reached a diameter of 10 mm.

covalently adding either farnesyl or geranyl-geranyl to the carboxyl end of the protein. Inhibiting this hydrophobic posttranslational modification prevents the protein from assuming its correct subcellular location and thus interferes with its function. We thus speculate that this very selective but partial inhibition of the isoprenylation of small G proteins may be involved with the regression of certain tumors. Current efforts in our laboratory are directed at evaluating this hypothesized mechanism for the induced regression of carcinomas by monoterpenes.

We have also shown that more potent monoterpene inhibitors of protein isoprenylation than limonene exist. For example, both major rat serum metabolites of limonene, perilllic acid and dihydoperilllic acid, are more potent than limonene in inhibiting protein isoprenylation (6). Because of the large dose of limonene required to induce tumor regression, we are currently identifying more potent monoterpene inhibitors of protein isoprenylation and testing them for activity in vivo. It is also important to extend in vivo evaluation to other tumor types in addition to mammary carcinomas. At this time, there is no evidence to suggest that breast is a unique organ-tumor site for this therapeutic approach. The data presented here suggest the hypothesis that the cellular process of protein isoprenylation is a potential cancer therapeutic target.

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Limonene-induced Regression of Mammary Carcinomas

Jill D. Haag, Mary J. Lindstrom and Michael N. Gould


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