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Limonene Chemoprevention of Mammary Carcinoma Induction following Direct *in Situ* Transfer of v-Ha-*ras*[1]

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ABSTRACT

Monoterpenes, including limonene and its *in vivo* rat plasma metabolites, have been shown to be inhibitors of protein isoprenylation of small G proteins, including p21 *ras*. In addition, dietary limonene has been shown to be capable of preventing the development and causing the regression of chemically induced mammary carcinomas, many of which contain activated *ras* oncogenes. On the basis of these observations, it was hypothesized that a possible mechanism by which limonene exerts its effects on the chemoprevention and regression of mammary tumors involves the inhibition of protein isoprenylation of the small G protein p21. In the first study, we asked whether dietary limonene was able to prevent the development of mammary carcinomas which were induced using direct retroviral gene transfer of v-Ha-*ras* into the mammary parenchyma *in situ*. Limonene modified neither the rate of gene transfer nor the stability of gene expression. However, limonene did greatly inhibit the formation of mammary carcinomas induced by the insertion of activated *ras*.

In a follow-up study, we asked whether chemoprevention by limonene was preferentially effective against a subset of chemically induced mammary carcinomas with activated *ras*. Rats were fed limonene to prevent the development of *N*-nitroso-*N*-methylurea-induced mammary tumors, a majority of which contain the activated Ha-*ras* oncogene. As expected, limonene administration increased the latency period and lowered the frequency of mammary carcinoma development as compared to controls. However, tumor characterization revealed that limonene treatment did not alter the percentage of carcinomas with activated *ras*. These studies are consistent with the above studies in that limonene is effective in preventing mammary carcinomas with activated *ras*. Interestingly, carcinomas without activated *ras* were prevented to the same extent as those with the activated oncogene.

INTRODUCTION

The posttranslational modification of cellular proteins by isoprenylation is an important mechanism by which the subcellular localization of specific proteins is specified. The subset of isoprenylated proteins include many small G proteins, nuclear matrix components, and other important molecules (1). The isoprenylation of proteins is accomplished by the enzymatically catalyzed covalent linkage of either farnesyl or geranyl-geranyl to the carboxyl terminus via a cysteine residue (1). Interfering with this posttranslational protein modification has been shown to perturb cellular physiology. For example, when the isoprenylation of the small G protein-activated *ras* was prevented by site-specific mutation of the cysteine at the prenylation site, this oncogenic protein could not associate with the plasma membrane and subsequently was unable to cause cellular transformation (2). On the basis of observations of this sort, the protein prenyltransferases have been suggested as potential targets for both cancer prevention and therapy (3, 4).

Several agents have been identified that specifically block protein prenylation. Among these are a series of monoterpenes including limonene and its *in vivo* metabolites which block both protein farnesylation and geranyl geranylation (5, 6). More recently, compounds which selectively block protein farnesylation have also been reported (4). The monoterpenes, however, are the first specific inhibitors of protein prenylation which have been shown to be efficacious in preventing and treating cancer *in vivo*. For example, limonene was shown to prevent both 7,12-dimethylbenz(*a*)anthracene- (7, 8) and NMU[3]-initiated (9) rat mammary carcinomas. Limonene is also capable of causing the complete regression of the majority of advanced primary rat mammary carcinomas without significant toxicity (10–12). A Phase I trial of limonene in patients with advanced cancer is currently under way (13).

In addition to selectively blocking the isoprenylation of small G proteins, monoterpenes have also been shown to have additional cellular effects, including the inhibition of CoQ synthesis (14) and the induction of various growth factors and growth factor receptors (12). It is thus possible that the monoterpenes may act through a select subset or a combination of these or other cellular activities. Here we ask whether limonene can prevent the formation of mammary carcinomas that are specifically initiated by the retroviral vector transfer of activated *ras* to *in situ* mammary cells (15).

MATERIALS AND METHODS

Animals and Limonene Administration. Female Wistar-Furth rats (Harlan-Sprague-Dawley, Inc., Madison, WI) housed under a 12-h light/12-h dark cycle were fed a modified AIN-76A diet (TD No. 85821; Teklad, Madison, WI) *ad libitum* upon arrival. Rats which were to receive retroviral infusions were randomly assigned to groups fed either control or 5% limonene-supplemented diet. The limonene diet was fed to these rats from 2 weeks prior to infusion through the end of the experiment. The rats which were to receive NMU were assigned to either control or 5% limonene diet 1 week post-NMU administration and continued on these diets until the end of the experiment. All rats in both studies were fed fresh diet three times/week. Limonene (Aldrich, Milwaukee, WI; >99% pure by gas chromatography analysis) was directly added to the AIN-76A diet (w/w ratio) and allowed to mix for approximately 20 min. Fresh diets were made every 7–10 days and stored at −20°C.

v-Ha-*ras* Mammary Carcinogenesis Study. The details for pJR-*gal* and pJR-*ras* construction have been described previously (15, 16). These vectors contain the coding sequences for β-galactosidase or the v-Ha-*ras* oncogene, with expression driven by the Moloney murine leukemia virus long terminal repeat. In addition, these vectors contain a neomycin resistance (*neo*^{r}) gene driven by an internal SV40 early promoter to provide a G418 selection marker.

pJR-*gal* and pJR-*ras* were transfected into ψ2 cells by calcium phosphate precipitation. Ecotropic virus was harvested from these cells and used to infect the amphotropic packaging cell line PA317. The virus stocks from PA317 were concentrated by centrifuging at 34,000 × *g* for 6 h through a 20% sucrose cushion and resuspending the pellets in 1/100 of the original volume, leading to a 20–50-fold increase in virus titer.

Mammary glands of Wistar-Furth rats consuming either control or 5% limonene diet were infused i.d. (15) with either pJR-*gal* or pJR-*ras* at 50–60 days of age. Starting 2 days before virus infusion, all rats received 3 daily s.c. injections of an indirectly acting mammary mitogen, perphenazine (3 mg/kg, Sigma Chemical Co.) (17). Immediately before mammary i.d. infusion, freshly thawed virus stocks were mixed with 80 μg/ml polybrene and 2 mg/ml Indigo

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[3] The abbreviations used are: NMU, *N*-nitroso-*N*-methylurea; i.d., intraductal.

carmine (a vital tracking dye). The perphenazine-treated rats were anesthetized with ether and the central duct of each gland was cannulated with a 27-gauge blunt-ended needle. Approximately 15 μl of virus suspension were infused into the luminal spaces. This volume was sufficient to fill the mammary gland without disrupting the mammary ductal structure.

The first retroviral infusion study was designed to determine whether the mammary cell genome of limonene-fed rats incorporated the virally encoded sequences at a similar frequency and with a similar stability as observed in control rats. Two groups of six rats each were fed either 5% limonene diet or control diet. After 2 weeks on the diets, mammary glands from rats in both groups were infused i.d. with pJR-*gal* vector at a virus titer of 5×10^7 colony-forming units/ml following perphenazine treatment. The rats continued on their respective diets and mammary glands from 3 control and 3 limonene-fed rats were removed at either 3 days or 1 month after virus infusion. The rat mammary epithelial cells were isolated, allowed to adhere to plastic dishes, stained with X-*gal*, and scored for β-galactosidase activity by methods described previously (15).

For the v-Ha-*ras* carcinogenesis study, 44 rats were randomly assigned to either 5% limonene or control diet groups. Two weeks later, all the rats were infused i.d. with pJR-*ras* vector at a virus titer of 5×10^7 colony-forming units/ml. All rats continued to receive limonene or control diet for the entire experiment and were palpated for mammary tumors weekly.

NMU-induced Mammary Carcinogenesis Study. Rats (50–55 days of age) were injected i.v. with a single dose (30 mg/kg) of NMU (Ash-Stevens, Inc., Detroit, MI). The NMU was dissolved in 0.9% NaCl acidified to pH 5.0 with acetic acid. Fresh solutions were prepared every 20 min. One week after NMU administration, 48 rats were randomly assigned to either limonene or control diet groups. These diets were fed until the end of the experiment.

The rats were palpated weekly for mammary tumors beginning 4 weeks after NMU injection. Tumors were surgically resected when they reached a size of approximately 1 cm^3 and the rats were returned to the experiment. All remaining mammary tumors were removed at the termination of the experiment. Tumors were rapidly removed and divided for histopathology and molecular analysis. Samples for molecular analysis were flash frozen in liquid nitrogen and stored at −80°C.

DNA was purified from 77 tumor samples by phenol-chloroform extraction and ethanol precipitation (18). Genomic DNA was analyzed for codon 12 second base G→A mutation by previously published methods (19). Briefly, the DNA was first digested with *MnlI* to reduce the intact wild-type *ras* sequence and then amplified by polymerase chain reaction. The polymerase chain reaction products were resolved on agarose gels and detected by Southern analysis with a radiolabeled mutation-specific oligonucleotide under stringent conditions.

RESULTS

pJR-*gal*/Limonene Study. In order to determine whether limonene was able to modify the incorporation rate or stability of a retroviral vector, a vector containing β-galactosidase was used to infect limonene-treated or control rat mammary glands. Mammary epithelial cells were then examined for β-galactosidase activity at either 3 days or 1 month after vector infusion. The proportion of cells expressing β-galactosidase activity at 3 days after infusion was 5.6×10^{-3} for limonene-treated cells and 6.7×10^{-3} for controls. At 1 month after infusion, the proportions were 10.9×10^{-3} and 8.7×10^{-3} for limonene-fed rats and controls, respectively. The data from this study thus indicate that approximately 0.6% of the mammary epithelial cells of the infused glands expressed the β-galactosidase in both the limonene-fed and control groups. This expression was stable for at least 1 month, suggesting that limonene does not affect the rate of incorporation or the stability of retrovirally inserted genes. This level of incorporation and stability is consistent with that reported previously (15).

pJR-*ras*/Limonene Study. For the rats infused with the pJR-*ras* retrovirus, body weight data was collected at two time points in this experiment. On the day of infusion, limonene-fed rats ($n = 21$) had an average body weight of 134.3 ± 7.7 (SD) g as compared to controls ($n = 22$), the average body weight of which was 174.0 ± 26.9 g. This weight difference was mostly likely due to an initial food aversion observed in rats fed limonene. By 41 days after infusion, limonene-fed rats weighed 188.0 ± 9.8 g, while controls weighed 198.0 ± 11.3 g.

Mammary tumors (≥3 mm in diameter) were detected by palpation within 5 weeks postinfusion in the control rats. For rats fed limonene, the latency time to first tumor development was 135.0 days as compared to a control group tumor latency of 83.5 days ($P = 0.04$, survival analysis/log rank test). Decreases were also observed in the percentage of limonene-treated rats with one or more tumors (Fig. 1). The median number of mammary tumors detected by palpation in limonene-treated rats was 1.0 tumors/rat as compared to 4.0 for control rats ($P = 0.01$, Mann-Whitney nonparametric test) (Fig. 2). At 18 weeks postinfusion, all rats were removed from the study and complete necropsies were performed. Many additional tumors were

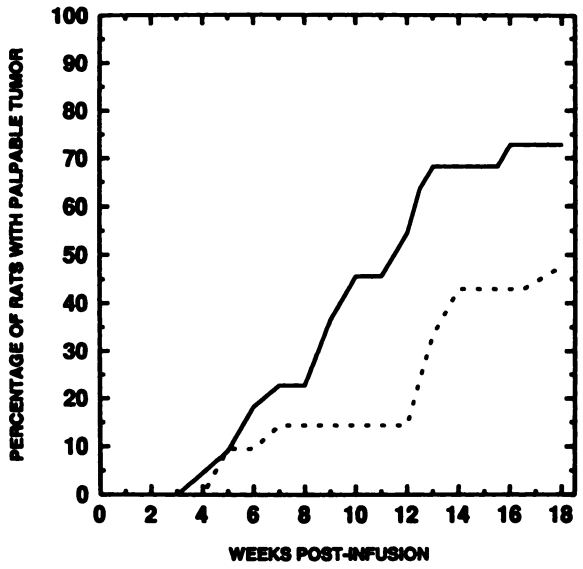

Fig. 1. *ras*-initiated mammary tumor latency. The percentage of Wistar-Furth rats fed either 5% limonene diet (- - - -, $n = 21$ rats) or control diet (——, $n = 22$ rats) developing at least one mammary tumor is plotted *versus* the time postinfusion of the JR-*ras* vector that the tumors were first detected by palpation.

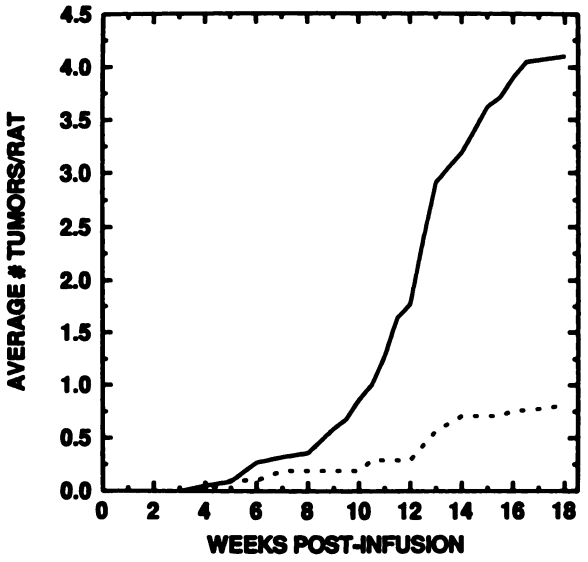

Fig. 2. *ras*-initiated mammary tumor frequency. The average number of mammary tumors/rat for limonene-treated (- - - -, $n = 21$) rats and control (——, $n = 22$) rats is plotted *versus* weeks postinfusion of the retroviral vector JR-*ras*.

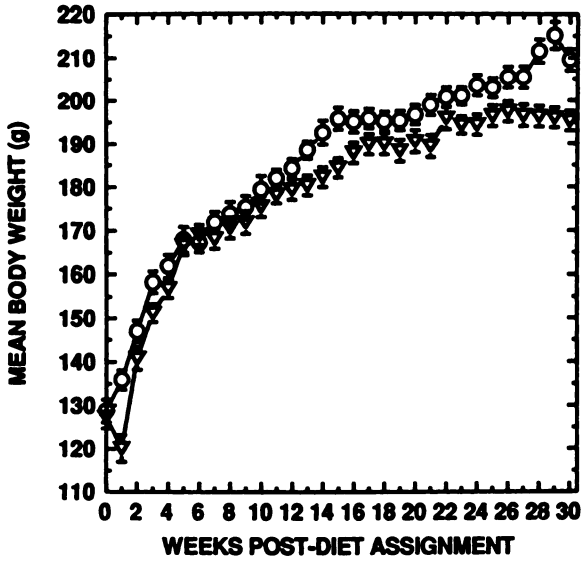

Fig. 3. Effects of dietary limonene on weight gain. Mean body weight in grams ± SEM (*bars*) is plotted *versus* weeks after diet assignment: 5% limonene diet (▽, n = 24 rats) and control diet (○, n = 24 rats).

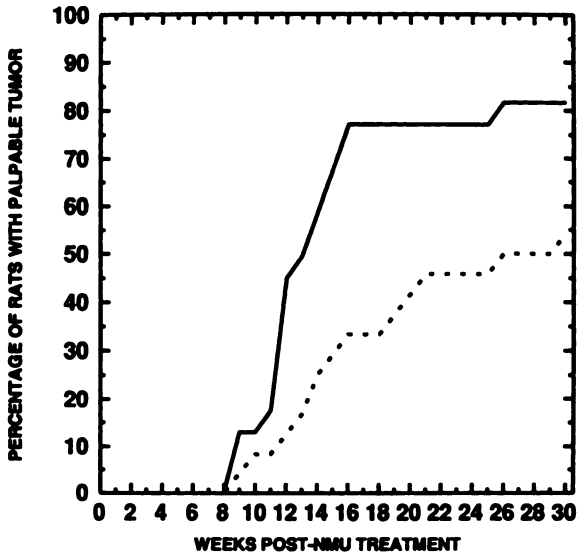

Fig. 4. Mammary tumor latency. The percentage of rats with a palpable tumor induced by NMU is plotted *versus* weeks postcarcinogen treatment for limonene-fed rats (- - - -, n = 24) and controls (——, n = 24).

detected at necropsy that were not previously palpated. The final tumor frequency in control rats was 7.6 tumors/rat whereas only 1.2 tumors/rat were found in limonene-fed rats. All mammary tumors were examined histologically and all were classified as mammary carcinomas.

NMU/Limonene Study. In this study, a single i.v injection of 30 mg/kg of NMU was used to induce rat mammary carcinomas. One week after NMU administration, all rats were randomly assigned to continue on the AIN-76A purified diet or switch to 5% limonene-AIN-76A diet until the end of the experiment at 30 weeks post-NMU administration. Body weights of the rats were determined weekly (Fig. 3). As consistently observed in limonene-fed rats, there was an initial weight loss, most likely due to food aversion, followed by weight gain comparable to rats fed control diet *ad libitum*.

At week 30, 54% of the rats fed 5% limonene diet had developed at least 1 palpable mammary tumor as compared to 82% of controls (Fig. 4). This level of dietary limonene was also capable of reducing the average number of tumors/rat by over 50% (Fig. 5). All of the tumors were histologically examined and classified as mammary carcinomas.

Analysis of c-Ha-*ras* codon 12 activation was conducted for 77 tumors, 51 obtained from control and 26 from limonene-fed rats. Of the control tumors, 25 of 51 (49%) had H-*ras* activation via a codon 12 mutation, whereas 13 of 26 (50%) of the tumors arising in limonene-treated rats had this activating mutation (Fig. 6).

DISCUSSION

We have previously reported that the monoterpene limonene can prevent and treat chemically induced rat mammary carcinomas (7–12). It has been shown that these chemically induced tumors are heterogeneous for *ras* activation. In NMU-induced tumors, 20–90% have Ha-*ras* activation, with the actual percentage being inversely related to NMU dose (19, 20). In contrast, less than 25% of 7,12-dimethylbenz(*a*)anthracene-induced tumors have activated Ha-*ras* (20). Although these tumors are classified as adenocarcinomas based

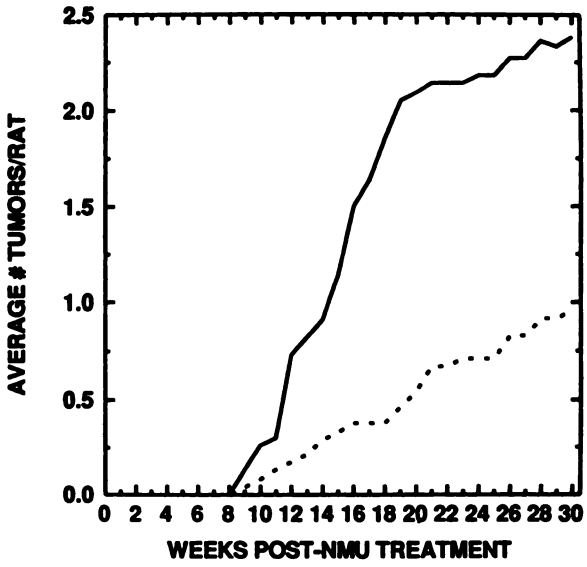

Fig. 5. Prevention of multiple tumors by dietary limonene. The average number of mammary tumors/rat is plotted *versus* time after NMU administration for limonene-fed (- - - -, n = 24) and control (——, n = 24) rats.

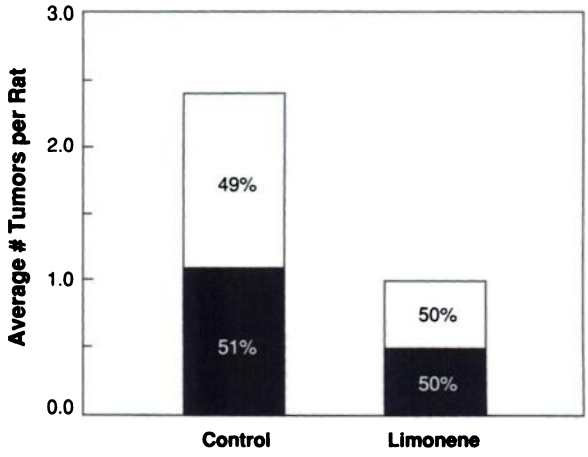

Fig. 6. Number of mammary carcinomas with *ras* activation. The average number of tumors induced by NMU in limonene-fed and control rats is plotted. □, percentage of carcinomas in each group with *ras* activation via a codon 12 mutation.

on occasional local invasion, they are, in general, less invasive and less aggressive than their human counterpart. In contrast to the chemical induction of rat mammary tumors, we have developed a rat model in which a gene of interest can be placed directly into the mammary parenchyma *in situ* using retroviral vectors (15). This methodology has several advantages over both transgenic and carcinogen-induced models for mammary carcinogenesis and chemoprevention studies (21). Using this vector model, we subcloned v-Ha-*ras* into the JR vector and infused it directly into the mammary duct. Rats receiving this vector develop clonal mammary cancer which originates from infected cells (15). The mammary adenocarcinomas that arose were more aggressive than chemically induced tumors. They more frequently invade surrounding tissue, are more readily transplantable, and also metastasize to distant sites (15, 16) as compared to chemically induced tumors.

In the present study, we asked whether limonene could prevent mammary carcinomas induced by the activated *ras* oncogene v-Ha-*ras*. We chose to study this oncogene because data from our previous studies showed that monoterpenes such as limonene and its metabolites could inhibit the isoprenylation of p21-*ras* (5, 6). Rats receiving a dietary dose of limonene that was less than 50% of a minimally toxic dose exhibited both extended latency and reduced frequency of *ras*-induced mammary carcinoma development. Thus, limonene is effective in preventing *ras*-initiated mammary carcinomas. However, it is not known if this preventative activity of the terpenes directly involves their modification of *ras* processing.

In addition to this specific result, this study represents the first demonstration of the utility of this retroviral gene transfer-mammary carcinogenesis model for chemoprevention screening and mechanistic studies. We believe that this model will be of general utility for testing chemoprevention agents and also will be adaptable to other dominant oncogenes.

We extended the above v-Ha-*ras* study to ask whether tumors with endogenous activated *ras* were selectively prevented by dietary monoterpenes. Tumors were induced with the directly acting carcinogen NMU and limonene was used to partially suppress tumor development. All tumors that occurred in the control and terpene-treated animals were analyzed for *ras* activation. We predicted that if mammary carcinomas with activated *ras* were a preferential tumor subpopulation for prevention, then limonene would be more effective at inhibiting the formation of carcinomas with *ras* activation. This would result in a lower percentage of tumors with *ras* activation in the terpene-treated group in comparison to the control group. This prediction was not realized, suggesting that limonene does not selectively prevent carcinomas with *ras* activation. This carcinoma subpopulation is not a specific tumor type at which the preventive effects of limonenes are focused.

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