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SHORT COMMUNICATION

Inhibition of *N*-methyl-*N*-nitrosourea- and 7,12-dimethylbenz[*a*] anthracene-induced rat mammary tumorigenesis by dietary cholesterol is independent of Ha-*ras* mutations

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Dietary cholesterol has previously been shown to inhibit rat mammary tumorigenesis but the mechanisms remain unclear. Uptake of serum low density lipoprotein cholesterol by tissues leads to down-regulation of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, the rate limiting enzyme in cholesterol biosynthesis that catalyzes the formation of mevalonate. In addition to being a precursor of cholesterol, mevalonate is necessary for DNA synthesis and cell proliferation. Isoprenoids, also derived from mevalonate, are required for the post-translational modification of Ras proteins that are mutated in a number of carcinogen-induced rat mammary tumors. The purpose of this study, therefore, was to determine whether inhibition of tumorigenesis by cholesterol is dependent on the frequency of mutations in the Ha-*ras* gene. Female Sprague–Dawley rats (30/group) were given a single dose of either *N*-methyl-*N*-nitrosourea (MNU, 50 mg/kg i.p.) or 7,12-dimethylbenz[*a*]anthracene (DMBA, 100 mg/kg intragastrally), carcinogens that produce tumors with either a high (MNU) or low (DMBA) frequency of Ha-*ras* mutations in codon 12 or 61, respectively. Rats were fed either a control AIN-93G diet or the control diet supplemented with 0.3% cholesterol for 14 weeks. Dietary cholesterol significantly decreased the final tumor incidence in rats given DMBA (83 versus 100%, $P < 0.05$) or MNU (53 versus 77%, $P < 0.05$). HMG-CoA reductase activity was higher in mammary tumors than in normal mammary glands, but the activity of this enzyme was reduced by cholesterol feeding only in mammary glands and not in tumors. Tumors induced by MNU had a high frequency of Ha-*ras* mutations in both the control (65%) and cholesterol-fed (68%) groups. Tumors induced by DMBA had a low frequency of Ha-*ras* mutations that also did not differ between the control (21%) and cholesterol-fed (18%) groups. These findings show that dietary cholesterol inhibits mammary tumorigenesis induced by either MNU or DMBA and that the inhibition is independent of the type or extent of mutations in the Ha-*ras* gene.

The effects of dietary lipids on mammary tumorigenesis are dependent on both the type and amount of the lipid (1). Although fatty acids have been studied extensively (2), the effects of sterols remain unclear (3). We have previously shown that dietary cholesterol inhibits the development of *N*-methyl-*N*-nitrosourea (MNU)-induced rat mammary tumors (4) and proposed that elevated serum low density lipoprotein (LDL) cholesterol in animals fed cholesterol may be associated with the inhibition of tumorigenesis. Cholesterol in the serum can enter cells via the LDL receptor and act as a negative feedback inhibitor of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (5). This rate limiting enzyme in the cholesterol biosynthesis pathway converts HMG-CoA into mevalonate, which is required for DNA synthesis and cell proliferation (6,7). Thus, exogenous cholesterol may inhibit mevalonate synthesis in preneoplastic mammary epithelial cells, thereby inhibiting their proliferation and subsequent development into neoplasms. Indeed, competitive inhibitors of HMG-CoA reductase (statin drugs) inhibit the development of rat and mouse mammary tumors *in vivo* (8,9) and the proliferation of human breast cancer cells *in vitro* (10,11). The mevalonate-derived product required for cell proliferation has not yet been identified, but is known not to be cholesterol (12).

The *ras* gene family of protooncogenes encode a group of heterotrimeric G-proteins that are commonly overexpressed or mutated in a variety of animal and human tumors (13,14). Post-translational modification of Ras proteins by isoprenoids derived from mevalonate is required for their translocation from the cytosol to the plasma membrane (15,16). Since the statin drugs cause a build-up of cytosolic Ras (non-functional) and a decrease in membrane-bound Ras (functional), it was postulated that inhibitors of mevalonate synthesis exert their inhibitory effects on cell proliferation and tumorigenesis via the Ras pathway (17). In support of this notion, inhibitors of Ras farnesylation inhibit colony formation of cells transformed with the *ras* oncogene but not those transformed with other oncogenes (18), suppress growth of Ras-transformed cells (19) and induce regression of mammary tumors in *ras* transgenic mice (20). Furthermore, there is evidence from studies in *Caenorhabditis elegans* that the activity of the mutant Ras protein is affected more than the wild-type protein to decreases in farnesylation (21).

In addition to MNU, 7,12-dimethylbenz[*a*]anthracene (DMBA) is frequently used to induce rat mammary tumors (22). One of the major differences between tumors produced by these carcinogens is the frequency and type of mutations in the *ras* gene. MNU-induced tumors generally have a high frequency (~85%) of Ha-*ras* mutations whereas tumors induced by DMBA have a low frequency of mutations in this gene (~20%) (23–25). Moreover, mutations found in MNU-induced tumors are GGA→GAA transition mutations in codon 12 while those found in DMBA-induced tumors are CAA→CTA transversion mutations in codon 61.

Since cholesterol can inhibit mevalonate synthesis that may, in turn, affect Ras protein modification (26), we hypothesized

Abbreviations: DMBA, 7,12-dimethylbenz[*a*]anthracene; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; LDL, low density lipoprotein; MNU, *N*-methyl-*N*-nitrosourea.

that dietary cholesterol selectively inhibits the development of tumors harboring Ha-*ras* mutations. The objective of this experiment, therefore, was to determine whether inhibition of tumorigenesis by cholesterol is dependent on the frequency of Ha-*ras* mutations by comparing the effect of cholesterol on mammary tumorigenesis induced by MNU and DMBA.

Pathogen-free female Sprague–Dawley rats (43 days old) purchased from Charles River Laboratories (St Constant, Quebec, Canada) were housed at 23°C and 50% humidity with a 12 h light/dark cycle. They were acclimatized for 1 week on the control AIN-93G diet (27) with free access to food and water. At 50 days of age, half of the animals were given 50 mg/kg MNU i.p. and the other half given 100 mg/kg DMBA intragastrally, doses that have previously been shown to give similar tumor incidences and latency periods (28). Three days after carcinogen treatment the animals were randomized into four groups ($n = 30$) and fed either a control AIN-93G diet or the control diet supplemented with 0.3% cholesterol (Dyets, Bethlehem, PA). The experiment was terminated when tumor incidence in one of the experimental groups reached 100% (14 weeks). An additional group of animals ($n = 18$) received no carcinogen and were used for biochemical analysis of normal tissues. These animals were fed either the control or 0.3% cholesterol diet for 1 week starting at 50 days of age. Animals were weighed and palpated for mammary lesions weekly. Moribund animals, those with tumors >15 mm or those remaining after 14 weeks were anaesthetized, blood samples were taken by cardiac puncture and the rats were then killed by cervical dislocation. Tissues were dissected, immediately frozen in liquid nitrogen and stored at –80°C. All tissue preparations were performed over ice or at 4°C. Tissues were homogenized in buffer A (20 mM Tris–HCl, pH 7.2, 0.25 M sucrose, 70 mM KCl, 5 mM EDTA, 5 mM EGTA, 1 mM dithiothreitol, 50 μM leupeptin) and centrifuged for 10 min at 500 *g*. Supernatants were centrifuged for 15 min at 9000 *g* followed by a final spin for 1 h at 100 000 *g*. Microsomes were assayed for HMG-CoA reductase activity as described below. The concentration of total microsomal protein was determined using the Bio-Rad protein–dye binding assay according to the manufacturer's protocol with bovine serum albumin as the standard. HMG-CoA reductase activity was determined using a radiochemical assay as previously described and expressed as pmol mevalonolactone formed/min/mg microsomal protein (29).

Mutations in the Ha-*ras* gene at codon 12 or 61 were analyzed by PCR procedures that have been previously described (30) and using the published rat sequence (31). DNA was isolated from frozen tissue according to standard procedures (32). An aliquot of 1 μg of DNA was amplified using a Perkin-Elmer 480 DNA thermal cycler and Ready-To-Go PCR beads (Pharmacia, Baie d'Urfe, Quebec, Canada) with 250 nM primers (Table I). The PCR conditions were an initial denaturing step for 5 min at 94°C followed by 30 cycles of denaturation (94°C for 1 min), annealing (62°C for 2 min) and extension (72°C for 3 min + 5 s/cycle). Control PCR reactions were carried out using no DNA, rat liver DNA (negative for Ha-*ras* mutations) and DNA from tumors with known mutations in codon 12 or 61. PCR products were digested overnight with either 12 U *Xmn*I (for codon 12) or 12 U *Xba*I (for codon 61) then hybridized for 2 h at 55°C with the corresponding probes (Table I) that were end-labeled with [γ-^{32}P]ATP and 2 U T4 polynucleotide kinase as previously described (33). The GGA→GAA mutation in codon 12 and the two mismatched bases in the downstream primer produce an *Xmn*I restriction site leading to a 71 bp normal (wild-type) and 53 bp mutant band. The CAA→CTA mutation in codon 61 produces an *Xba*I restriction site leading to a 79 bp normal and 44 bp mutant band. The bands were resolved using 12% polyacrylamide gels and detected by autoradiography.

Table I. Primer and probe sequences for PCR–RFLP/liquid hybridization assay

Primer/probe	Sequence
Codon 12	
Upstream primer	5′-ACCCCTGTAGAAGCGATGAC-3′
Downstream primer[a]	5′-AGGGCACTCTTTCGAACGCC-3′
Probe	5′-CAAGCTTGTGGTGGTGGGCG-3′
Codon 61	
Upstream primer	5′-TTGATGGGGAGACGTGTTTA-3′
Downstream primer	5′-ATGTACTGGTCCCGCATGGC-3′
Probe	5′-TACTGGACATCTTAGACACA-3′

[a]Mismatched bases are underlined.

Serum was prepared by centrifuging blood samples at 2500 *g* for 10 min and stored at –80°C prior to analysis for total cholesterol using a colorimetric assay as described by the manufacturer (Boehringer Mannheim, Laval, Quebec). Tumor incidence data were analyzed by χ^2 test. Time to tumor appearance was determined using the Mantel–Haenzel procedure and is expressed as the number of weeks for half of the animals to develop a tumor. Enzyme activity and serum cholesterol values were determined using one-way ANOVA followed by Student's *t*-test with the Bonferroni *post hoc* test for multiple comparisons (GraphPad Prism, San Diego, CA).

At the end of the 14 week experiment, cholesterol feeding resulted in higher serum cholesterol levels in rats treated with either MNU (6.02 ± 1.44 versus 2.85 ± 1.00 mmol/l, $P < 0.01$) or DMBA (6.04 ± 1.37 versus 2.02 ± 0.37 mmol/l, $P < 0.01$). As expected, cholesterol significantly decreased the final tumor incidence in the MNU group (53 versus 77%, $P < 0.05$) (Figure 1). In rats treated with DMBA, cholesterol also decreased the final tumor incidence (83 versus 100%, $P < 0.05$). Similarly, cholesterol reduced the average time for half of the animals to develop tumors in both the MNU group (10 versus 14 weeks, $P < 0.03$) and the DMBA group (7 versus 9 weeks, $P < 0.01$).

We next determined whether a decrease in mevalonate synthesis in mammary glands or tumors could explain the inhibitory effects of cholesterol on tumorigenesis. HMG-CoA reductase activity was greater in mammary tumors induced by MNU or DMBA than in normal mammary glands, whether or not cholesterol was present in the diet (Figure 2). Dietary cholesterol, however, significantly decreased HMG-CoA reductase enzyme activity in normal mammary glands but not in mammary tumors.

The frequency of tumors harboring Ha-*ras* mutations is summarized in Table II and representative blots are shown in Figure 3. Tumors induced by MNU had a high frequency of Ha-*ras* codon 12 mutations in both the control (65%) and cholesterol-fed (68%) groups. Tumors induced by DMBA had a low frequency of Ha-*ras* mutations in codon 61 in both the control (21%) and the cholesterol-fed (18%) groups. None of the MNU-induced tumors harbored codon 61 mutations and none of the DMBA-induced tumors harbored codon 12 mutations.

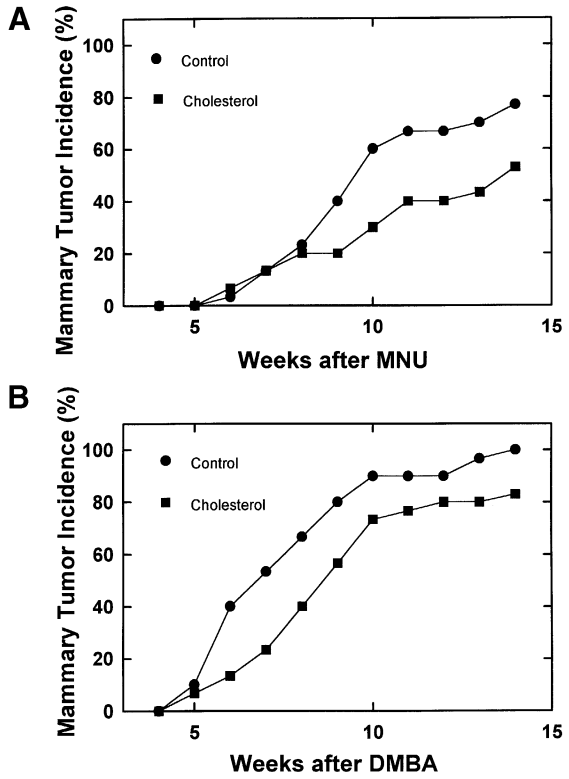

Fig. 1. Incidence of mammary tumors over time following treatment of Sprague–Dawley rats with either MNU (**A**) or DMBA (**B**).

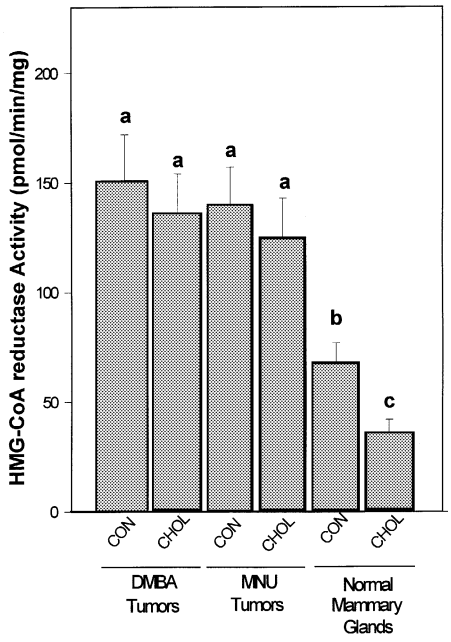

Fig. 2. Activity of HMG-CoA reductase in mammary glands and tumors from rats fed either a control diet containing no cholesterol (CON) or a diet containing 0.3% cholesterol (CHOL).

Our results show that dietary cholesterol effectively inhibits the development of mammary tumors induced by either MNU or DMBA. Cholesterol, however, did not affect the frequency of tumors with Ha-*ras* mutations, suggesting that the Ras pathway may not be the target of the inhibitory effect. These results support the evidence that the antiproliferative effects of competitive inhibitors of HMG-CoA reductase are independent of Ras function (34). We have also shown here that dietary cholesterol leads to a down-regulation of HMG-CoA reductase in the mammary glands of Sprague–Dawley rats. Thus, it seems likely that cholesterol inhibits tumorigenesis by reducing mevalonate synthesis in preneoplastic mammary epithelial cells. This may lead to a reduction in the proliferation of these cells and their subsequent development into neoplasms by a mechanism that is independent of the Ras pathway.

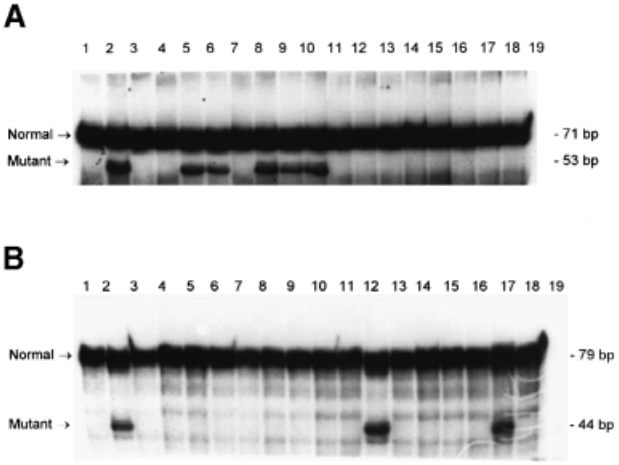

Fig. 3. Representative blot of Ha-*ras* mutation analysis by RFLP–PCR in mammary tumors induced by MNU (lanes 3–10) or DMBA (lanes 11–18). Tumors were taken from rats fed either a control diet containing no cholesterol (lanes 3–6 and 11–14) or a diet containing 0.3% cholesterol (lanes 7–10 and 15–18). Control reactions were carried out using rat liver DNA (lane 1), tumors with a known codon 12 (**A**) or 61 (**B**) mutation (lane 2) and a sample containing no DNA (lane 19).

Table II. Frequency of codon 12 and 61 Ha-*ras* mutations in rat mammary tumors

	Codon 12	Codon 61
MNU		
Control	15/23 (65%)	0/12 (0%)
Cholesterol	13/19 (68%)	0/12 (0%)
DMBA		
Control	0/12 (0%)	7/34 (21%)
Cholesterol	0/12 (0%)	4/22 (18%)

The results of the present study are consistent with a previous study that showed limonene, an inhibitor of mevalonate synthesis and mammary tumorigenesis, does not affect the proportion of tumors with Ha-*ras* mutations (35). The mammary tumor inhibitors conjugated linoleic acid and n-3 polyunsaturated fatty acids also have no effect on the frequency of tumors with mutant Ha-*ras* (36,37). A variety of tumor promoters, however, appear to selectively promote the development of tumors with normal Ha-*ras* (38–40).

The higher activity of HMG-CoA reductase that we observed in mammary tumors compared with normal mammary glands is consistent with previous reports showing elevated rates of cholesterol synthesis in mammary tumors versus normal mammary glands (41). Indeed, HMG-CoA reductase is overexpressed in a variety of tumors (42), suggesting that an increased rate of mevalonate synthesis may be necessary to maintain the high proliferative rates of cancer cells. Failure of cholesterol to down-regulate HMG-CoA reductase in mammary tumors indicates that tumor cells may lack a feedback regulatory mechanism. This observation has been demonstrated by others

using hepatomas and leukemias (43,44) and may represent an adaptive mechanism by tumor cells to prevent a decrease in the mevalonate pool.

It is not clear why a previous study, also using Sprague–Dawley rats with a similar experimental design to ours, did not observe a decrease in DMBA-induced mammary tumorigenesis by dietary cholesterol (45). It is possible that the cholesterol used in that study was extensively oxidized, since serum levels were not elevated (45). Oxidized cholesterol is known to inhibit the absorption of cholesterol (46) and we have previously shown that mammary tumorigenesis is not inhibited by cholesterol that has been oxidized (4).

Although cholesterol has long been implicated in the development of cardiovascular disease (47), its role in human cancer development has not been thoroughly investigated. A review of epidemiological studies examining the relationship between dietary cholesterol and breast cancer concluded that cholesterol probably has no relationship with risk for the disease (48). Since, however, the intake of animal fat and protein, which may increase risk (48), correlate with the intake of cholesterol (49), it is unlikely that any protective effect of cholesterol could have been detected in these epidemiological studies. The oxidation state of cholesterol is also an important confounding variable that is not controlled in most studies. Furthermore, there is great variability in the absorption and metabolism of cholesterol among individuals (50). Although it is clearly inappropriate to recommend increasing dietary cholesterol to reduce the prevalence of breast cancer, the results of the present study underscore the importance of the mevalonate pathway in mammary tumorigenesis and suggest that other means of reducing mevalonate synthesis may be an effective approach to preventing and/or treating this disease. Indeed, we and others have recently proposed that the mevalonate pathway may mediate the inhibitory effects of fish oils on both mammary and colon tumorigenesis (51,52).

In summary, we have shown that dietary cholesterol inhibits mammary tumorigenesis induced by either MNU or DMBA. This inhibition is independent of the type or extent of mutations in the Ha-*ras* gene.

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