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Ezetimibe reverses the inhibitory effects of dietary cholesterol on mammary tumorigenesis in rats

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There are concerns regarding increased cancer incidence in patients treated with ezetimibe, an inhibitor of the absorption of dietary cholesterol. Here we tested the hypothesis that ezetimibe will accelerate mammary tumorigenesis in rats. The drug was administered at a dose of 1 ppm in an AIN-93G diet that contained 0.3% cholesterol. This experimental diet and control diets that contained either no additions or cholesterol or ezetimibe only, were fed to groups of 30 Sprague-Dawley rats 3 days after they were treated with 50 mg/kg methylnitrosourea (MNU). All rats were euthanized 22 weeks after MNU administration. Tumor multiplicity was significantly smaller in rats fed cholesterol than those fed no cholesterol (1.84 ± 0.42 vs. 3.86 ± 0.86 respectively, $P < 0.05$), but was significantly greater in the cholesterol/ezetimibe group than the group fed only cholesterol (3.48 ± 0.59 vs. 1.84 ± 0.42 respectively, $P < 0.04$). The average weight of tumors/rat was also significantly larger in the cholesterol/ezetimibe group than those fed cholesterol alone (5.67 ± 1.15 vs. 2.56 ± 0.71 respectively, $P < 0.04$). As expected, ezetimibe prevented the cholesterol raising effect of the dietary cholesterol. These results show that ezetimibe reverses the inhibitory effect of dietary cholesterol on the development of rat mammary tumors.

A link between low serum cholesterol levels and increased risk of cancer has been recognized for some time,[1,2] but no clear pattern of risk has emerged with respect to breast cancer in particular. Studies have generally found no association[3] or an inverse association, particularly in women under 50 years of age.[4–6] Statins lower circulating cholesterol by inhibiting HMG-CoA reductase, the rate limiting enzyme in cholesterol biosynthesis. The evidence that statins play a role in breast cancer development is also equivocal, with some studies reporting a decrease in risk, others an increase.[7,8] The drug ezetimibe, an inhibitor of the absorption of dietary cholesterol, is being used as an alternative to statins to lower circulating cholesterol levels. Recent results from the Simvastatin and Ezetimibe in Aortic Stenosis (SEAS) trial,[9] have renewed concerns about the possible association between low cholesterol levels and cancer. In this trial, overall cancer incidence was increased significantly in the patients treated with simvastatin plus ezetimibe, with 105/943 cases compared to 70/929 in those receiving placebo ($P = 0.01$). There was an increased incidence of various cancers though none of the specific sites achieved statistical significance. Deaths from cancer were also more frequent in the treatment versus control groups (39 *vs.* 23 respectively, $P = 0.05$). A subsequent meta-analysis of cancer data from three ezetimibe trials including the SEAS trial, however, did not provide evidence of any effects of the drug on cancer rates.[10]

Human studies relating dietary cholesterol intake to breast cancer development have also been inconclusive. Three large prospective studies, the Nurses Health Study,[11] the Netherlands Cohort[12] and the National Health and Nutrition Examination Survey I Epidemiologic Follow-up Study (NHANES I)[13] did not detect any significant relationship between dietary cholesterol and breast cancer risk. In a pooled estimate from seven prospective studies, a small increase in breast cancer among women consuming higher amounts of dietary cholesterol was observed, but because the differences were small, the authors attributed this observation to chance.[14] It is clearly difficult to determine the independent effects of dietary cholesterol because of the strong correlations between cholesterol intake and other dietary variables such as animal fat. An additional complication is that individuals vary in their response to increases in dietary cholesterol, some showing an increase, others no response or, in some cases, even a reduction in serum levels.[15–17]

Studies in rodents have provided a more definitive relationship between dietary and circulating cholesterol and mammary carcinogenesis. A diet containing cholesterol that was shown to raise serum cholesterol levels decreased mammary gland HMG-CoA reductase activity and inhibited the development of chemically induced mammary tumors in rats.[18,19] Conversely, the bile acid binding resin

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cholestyramine, given to rats during the promotion phase of mammary carcinogenesis, reduced serum cholesterol levels, and increased the incidence of mammary tumors.[20] Feedback regulation of HMG-CoA reductase has been suggested to explain these observations.[18,21] In support of this notion, mevalonate, the product of HMG-CoA reductase, has been shown to promote the growth in mice of tumors derived from human breast cancer cells.[22]

Hydroxymethylglutaryl-Coenzyme A (HMG-CoA) reductase, is regulated by a multivalent feedback mechanism controlled, in part, by intracellular cholesterol levels.[23] The cholesterol requirements of tissues are met by uptake from the circulation of low density lipoprotein (LDL) or by *de novo* synthesis. A fall in circulating cholesterol causes a decrease in its uptake via the LDL receptor, the resultant drop in intracellular cholesterol level leading to a compensatory stimulation of mevalonate synthesis through up-regulation of HMG-CoA reductase.[23,24] Rates of cholesterol biosynthesis are strongly correlated to rates of cell proliferation and inhibition of cholesterol synthesis leads to an inhibition of proliferation and apoptosis.[25] Alterations in the flux through the cholesterol biosynthetic pathway in pre-neoplastic or neoplastic cells may, therefore, affect tumor development and growth.

The studies reporting an inverse association between circulating cholesterol levels and cancer development including recent concerns regarding increased cancer incidence in patients treated with ezetimibe, led us to test the hypothesis that inhibiting the absorption of dietary cholesterol might accelerate tumor development. Here we show that ezetimibe given to rats after an initiating dose of the mammary carcinogen methylnitrosourea (MNU), does, indeed, reverse the inhibitory effects of dietary cholesterol on mammary tumorigenesis.

Material and Methods

Animals

One hundred and twenty pathogen free female Sprague-Dawley rats, 43 days of age (Charles River Laboratories, St Constant, Quebec), were housed at 24 ± 2° C and 50% humidity with a 12 h light-dark cycle and acclimatized for one week with food (AIN-93G diet) and water provided *ad libitum*.

Carcinogen treatment

MNU was obtained from Sigma-Aldrich (St Louis, MO) and stored at −20° C in the dark. Immediately before use, MNU was dissolved in physiological saline containing 0.05% acetic acid. At 50 days of age, a single dose of 50 mg/kg body weight MNU was administered i.p. to all the rats.

Diets

Diets were obtained from Dyets Inc (Bethlehem, PA). The experimental diets were formulated by adding 0.3% cholesterol (Maypro Industries Inc., Harrison, NY), 1ppm ezetimibe (Ezetrol, Merk Frosst, Kirkland, Quebec), or 0.3% cholesterol plus 1 ppm ezetimibe to AIN-93G diets at the expense of sucrose. Diets were added fresh to cages once per week.

Experimental protocol

Three days after carcinogen treatment, the rats were randomized into 4 groups (30/group) and were fed the control or one of the experimental diets for 22 weeks. They were weighed and palpated for mammary lesions weekly. Moribund animals, those with tumors larger than 2 cm or those remaining after 22 weeks were anaesthetized, blood samples taken by cardiac puncture, and then they were euthanized by cervical dislocation. Serum was stored at −20° C prior to analyis of total cholesterol using a colorimetric assay as described by the manufacturer (Teco Diagnostics, Anaheim, CA). Tumors were fixed in 10% formalin, embedded in paraffin, and sectioned and processed for histological evaluation by hematoxylin and eosin staining. Rats were cared for throughout in accordance with recommendations of the Canadian Council on Animal Care and the University of Toronto Animal Care Policies and Guidelines. The tumorigenesis protocol was reviewed by and received ethical approval of the University of Toronto Animal Care Committee. Data were analyzed by unpaired Student's t-tests. All experimental values are expressed as means ± SEM.

Results

Dose determination

In preliminary experiments to determine the dose of ezetimibe to use in our carcinogenesis experiment, five groups of 6 Sprague-Dawley rats at 8 weeks of age were fed for 10 days an AIN-93G diet containing 1% cholesterol at the expense of sucrose and supplemented with 0, 1, 2, 5, or 10 ppm ezetimibe. The 1 ppm dose reduced circulating cholesterol levels from 173.8 ± 16.8 to 74.0 ± 12.0 mg/dl ($p < 0.0007$), with no further reductions at higher doses. There were no effects of ezetimibe on body weights at any of the doses tested in this short term experiment (data not shown). In view of these results, we chose to use 1 ppm dietary ezetimibe for the carcinogenesis experiment and we reduced the dietary cholesterol level to 0.3% that we had used previously.[18]

Mammary tumorigenesis

Body weights and food intake did not differ between groups throughout the experiment (data not shown). At the termination of the experiment, 80–90% of the rats in the 4 groups had developed at least one mammary tumor. The final tumor incidences in each of the experimental groups did not differ from that of the control group and there were no differences in tumor latency between groups. Mammary tumors induced in rats by MNU have consistently been shown by us[18,26] and by others[27–29] to be adenocarcinomas. Histopathological examination of 40 tumors selected at random from rats fed cholesterol or cholesterol plus ezetimibe showed they were all, indeed, adenocarcinomas.

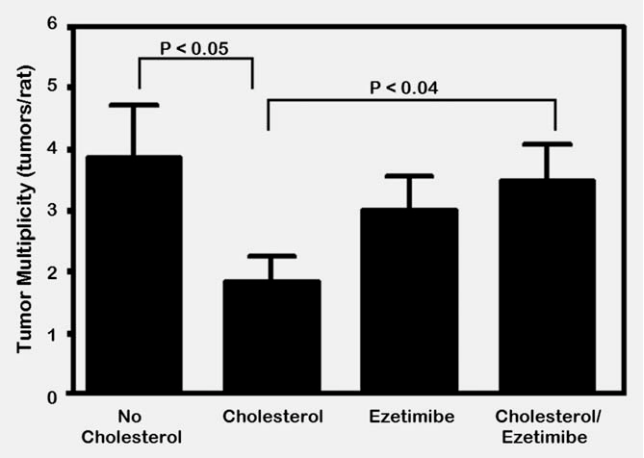

Figure 1. Effect of AIN-93G diets containing no cholesterol, 0.3% cholesterol, 1ppm ezetimibe, or 0.3% cholesterol plus 1 ppm ezetimibe on mammary tumor multiplicity in Sprague-Dawley rats initiated with MNU. The control diet was AIN-93G. Means ± SEM, $n = 30$/group.

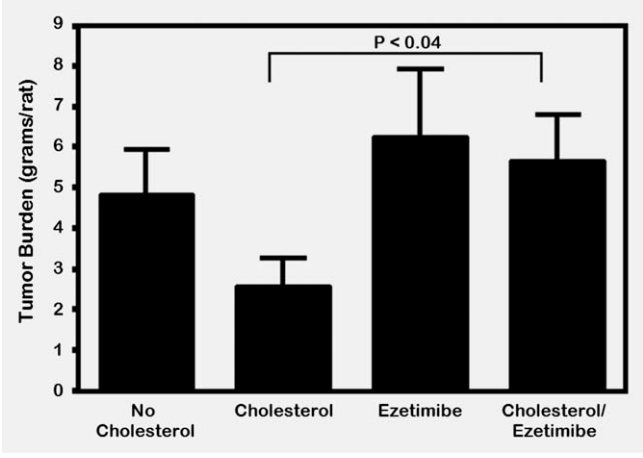

Figure 2. Effect of AIN-93G diets containing no cholesterol, 0.3% cholesterol, 1ppm ezetimibe, or 0.3% cholesterol plus 1 ppm ezetimibe on mammary tumor burden in Sprague-Dawley rats initiated with MNU. The control diet was AIN-93G. Means ± SEM, $n = 30$/group.

Figure 1 illustrates that the tumor multiplicity (average number of tumors per rat) was significantly smaller in the rats fed cholesterol than those with no cholesterol in their diet. However, the tumor multiplicity was significantly greater in the cholesterol/ezetimibe group than the group fed the diet containing cholesterol but no ezetimibe. The tumor burden (average weight of tumors per animal) was also significantly larger in the rats fed cholesterol plus ezetimibe than in those fed cholesterol alone (Fig. 2). The animals in the group fed cholesterol seemed to develop smaller tumors than those with no cholesterol in their diet, although this difference did not achieve significance ($p = 0.11$).

Figure 3 shows the serum cholesterol values for the 4 groups at the time they were euthanized. As expected, the total serum cholesterol was significantly greater in the cholesterol fed group than either the group fed no cholesterol or the groups that consumed ezetimibe.

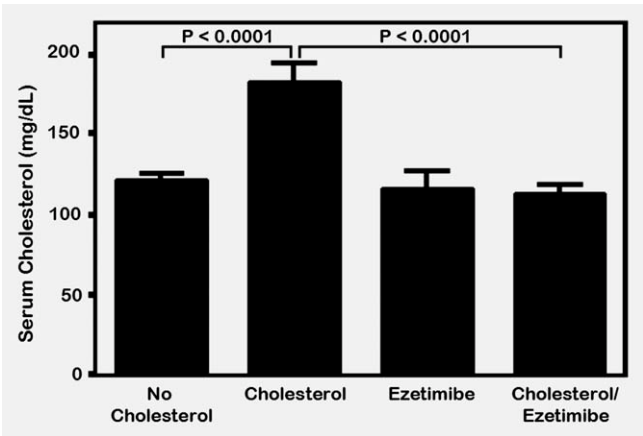

Figure 3. Effect of AIN-93G iets containing no cholesterol, 0.3% cholesterol, 1ppm ezetimibe, or 0.3% cholesterol plus 1 ppm ezetimibe on total serum cholesterol in Sprague-Dawley rats initiated with MNU. The control diet was AIN-93G. Means ± SEM, $n = 30$/group.

Discussion

In this study, we first showed that ezetimibe given to rats at a dose of 1 ppm in the diet, significantly lowered serum cholesterol levels, comparable to the reductions reported by van Heek et al.[30] If we assume an average body weight of 250 g and an average food intake of 20 g/day, our rats were exposed to 0.08 mg/kg body weight per day. The human dose is typically 10 mg,[31] which is equivalent to 0.14 mg/kg body weight for a 70 kg person.

The carcinogenesis experiment confirmed our previous observation that rats fed 0.3% cholesterol in their diets developed fewer mammary tumors than those fed no cholesterol.[18,19] Our results show, however, that addition of ezetimibe to the diet reversed the inhibitory effect of the cholesterol. The addition to the diet of ezetimibe alone had no effect on tumor formation confirming a recent study in which ezetimibe was shown not to be genotoxic and, as expected, was not carcinogenic in standard 2-year assays in mice and rats.[32] The results support previous findings that the non-absorbable resin cholestyramine promoted the development of rat mammary tumors induced by dimethylbenzanthracene (DMBA).[21] Cholestyramine binds to bile acids in the GI tract preventing their re-absorption resulting in an increased conversion of cholesterol into bile acids, thereby lowering circulating cholesterol levels.[33,34] Ezetimibe binds to the cholesterol transport protein Niemann Pick C1-like 1 (NPC1L1) to inhibit intestinal absorption of dietary and biliary sterols.[35] In response to this inhibition, there is a reciprocal increase in hepatic cholesterol synthesis,[36] but this does

not fully compensate for the decreased absorption, leading to a reduction in serum total and LDL cholesterol.[37] Ezetimibe has recently been shown to act similarly in rats.[38] Since the drug undergoes enterohepatic circulation leading to negligible systemic exposure,[31] its promoting effects are likely to be due to the lowering of circulating cholesterol. El-Sohemy et al. showed that HMG-CoA reductase activity in the mammary gland is reduced by dietary cholesterol, suggesting that a decrease in mevalonate synthesis is one mechanism by which circulating cholesterol could inhibit tumorigenesis.[24] Conversely, the decrease in serum cholesterol caused by ezetimibe or cholestyramine may lead to a compensatory up-regulation of mammary gland HMG-CoA reductase activity with an increase in mevalonate synthesis that may increase the proliferation of preneoplastic or neoplastic cells.[22] Other mechanisms, however, are clearly possible and further work is needed in this area.

Our results suggest that inhibiting cholesterol absorption in people eating Western-type diets that contain high levels of cholesterol, may increase the risk of cancer. In addition to pharmacological approaches to treat hypercholesterolemia using drugs such as ezetimibe, a number of countries allow the addition of phytosterols to margarine because these compounds act within the small intestine to limit cholesterol absorption.[39] There are two general classes of phytosterols—stanols and sterols. Plant stanols are relatively unabsorbed, while the plant sterols can reach significant levels in the blood and exert effects independent of their role of limiting cholesterol absorption. A number of case-control studies, have shown that dietary phytosterol intake assessed by food frequency questionnaires is associated with reduced risk for cancers at a number of sites including breast.[22,40–42] These studies do not definitively establish a cause-effect relationship since there are many other potentially cancer protective components of plant-based foods. Data from animal studies relating phytosterol intake to cancer risk have been inconsistent. Raicht et al.[43] have shown that a diet containing a 2% phytosterol mixture (95% β-sitosterol, 4% campestreol, 1% stigmasterol) inhibited colon tumor development induced in rats by intra-colonic administration of MNU. Quilliot et al.,[44] on the other hand, observed no effect of 24 mg/rat/day of phytosterols (55% β-sitosterol, 41% campesterol, 4% stigmasterol) on colon carcinogenesis induced by intra-rectal instillation of MNU. Clearly, more research on phytosterols is required before we can be assured of the long term safety of these compounds with respect to cancer development.

In summary, the present results demonstrate that ezetimibe reverses the inhibitory effects of dietary cholesterol on mammary tumorigenesis in rats, likely by decreasing circulating cholesterol levels. Our data provide an experimental basis for the epidemiological findings that inhibiting cholesterol absorption may increase the risk of cancer.

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