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### 13. ABSTRACT (Maximum 200 Words)
HMG-CoA reductase is the rate-limiting enzyme in cholesterol biosynthesis that catalyzes the production of mevalonate. In addition to being a precursor of cholesterol, mevalonate is required by cells for DNA synthesis and cell cycle progression. We have investigated the hypothesis that the mevalonate pathway may be a useful target for cancer prevention and therapy. We have shown that the dietary fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) as well as the dietary isoprenoid geraniol that inhibit cell proliferation, are inhibitors of HMG-CoA reductase activity in breast cancer cells. The inhibitory effects of EPA and geraniol on cell proliferation, however, are independent of mevalonate. DHA, on the other hand, inhibits proliferation at least in part, by inhibiting mevalonate synthesis. We showed that exogenous mevalonate promotes growth of breast cancer cells in nude mice as well as proliferation of the cells in culture. This was associated with an increase in the passage of cells through the G1 restriction point of the cell cycle. Mevalonate caused increased cyclin A- and E-associated CDK2 activity. This was mediated by increased phosphorylation of CDK2 and decreased binding of CDK2 to the CDK1 p21cip. These findings may be important since common treatments to lower serum cholesterol increase mevalonate synthesis.

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INTRODUCTION

Disregulation of the mevalonate pathway is believed to contribute to uncontrolled tumor cell growth \(^1,2\), as products of mevalonate metabolism are required for DNA synthesis and cell proliferation \(^3\), energy metabolism \(^4\) and activation of oncogenes \(^5\). Malignant cells from diverse tumor types \(^6,8\) including mammary tumors \(^2\) have elevated mevalonate synthesis due to increased levels and activity of 3-hydroxy-3-methyl glutaryl Coenzyme A reductase (HMG-CoA reductase). Inhibition of the mevalonate pathway may therefore be a useful target for anti-cancer therapies. Statin drugs that inhibit mevalonate synthesis by competitively inhibiting HMG-CoA reductase cause a G1 phase arrest and eventual apoptosis in both normal \(^9,10,11\) and neoplastic cells \(^10,12-14\). In rodent models, statins have been shown to inhibit both mammary \(^15,16\) and colon cancer \(^17\). However, the use of statins as anti-cancer agents in humans is limited by highly efficient hepatic clearance \(^18\), and the occurrence of myotoxicity at doses required to inhibit tumor growth \(^19\). This has lead us to investigate dietary compounds with known anti-cancer effects that may act through the mevalonate pathway. We are also interested in the role of excess mevalonate in human breast cancer cell cycle control. Mammary mevalonate synthesis rates are regulated inversely with serum cholesterol levels. Understanding the role of mevalonate in mammary tumor growth therefore has great clinical significance as treatments for the prevention of heart disease often cause a fall in serum cholesterol. This may actually increase mammary mevalonate synthesis, and hence the risk of developing breast cancer in some individuals. Here we include Tasks 1 and 2 as previously reported in Annual Reports 2000 and 2001. Task 3 is reported below, along with additional work performed on the role of mevalonate in human breast cancer cell cycle control reported as Task 4.

BODY

Task 1. To determine the effects of dietary n-3 PUFAs on mammary tumorigenesis in LDL-R \(^+/-\) and LDL-R \(^-/-\) mice.

The first objective of Task 1 was to produce mammary tumors in LDL-R \(^+/-\) and LDL-R \(^-/-\) mice fed diets containing n-3 or n-6 PUFAs. Since writing the grant application, we completed a series of experiments in which we investigated the regulation of mevalonate synthesis in LDL-R \(^-/-\) mice fed n-3 or n-6 PUFAs \(^20\). Our results showed that dietary PUFAs and deletion of the LDL-R had independent effects on hepatic and mammary gland HMG-CoA
reductase and serum lipids and we observed a significant diet-gene interaction. The effects of PUFAs on HMG-CoA reductase in the mammary gland, but not the liver, were mediated by the LDL-R. We also observed that differences in HMG-CoA reductase and serum LDL-cholesterol, high density lipoprotein cholesterol and triglycerides between -/- and +/+ mice were dependent on whether mice were fed n-3 or n-6 PUFA.

In view of the differences in lipid metabolism that we observed in -/- and +/+ mice fed the different diets, we decided to make a few changes in the design of the tumorigenesis experiment of Task 1. We had proposed to use dimethylbenz(a)anthracene (DMBA) as the initiating agent. DMBA is typically given i.g. dissolved in corn oil, and its absorption is mediated by bile acids. As a lipid soluble compound, DMBA is transported from the liver to target tissues as a component of blood lipoproteins. We reasoned, therefore, that the difference in serum lipids between LDL-R +/+ and -/- mice would likely lead to differences in the pharmacokinetics of DMBA between the 2 types of mice. Thus, we decided that we must use the water-soluble initiating agent methylnitrosourea (MNU) instead of DMBA to prevent the potentially confounding effect that a difference in carcinogen uptake and delivery between groups might have on initiation.

MNU initiates mammary tumorigenesis in mice, but a potential problem can be the development of lymphomas and ovarian tumors before mammary tumors have had the time to develop. For this reason, mice are usually treated with hormones in mammary tumorigenesis experiments to reduce the latency and promote the growth of mammary tumors. Indeed, the background strain of the LDL-R -/- mice, C57Bl, is poorly susceptible to chemically-induced carcinogenesis, and we felt it would be necessary to treat these animals in some way to promote mammary tumor development. We chose, therefore, to treat all of the mice with the synthetic hormone medroxyprogesterone acetate (MPA) that has previously been shown to promote mammary tumorigenesis in the mouse.

Work we performed to investigate the effects of n-3 and n-6 PUFAs feeding on mammary HMG-CoA reductase activity in LDL-R -/- versus wildtype mice was done using a 7% fat diet. However, as we indicated in the grant application, Ip et al. have shown that diets must contain at least 4% linoleic acid to ensure adequate levels of this essential fatty acid for maximal mammary tumor growth. While the 7% safflower oil diet we used previously contains adequate linoleic acid, the 7% fat menhaden oil diet would be deficient, and may, therefore,
artificially limit mammary tumor growth independent of any specific inhibitory effects of the individual PUFAs. To ensure adequate levels of linoleic acid in any future mammary tumorigenesis studies conducted, we have reformulated the test (n-3) and control (n-6) diets as follows: the 20% fat n-3 diet – 12% menhaden oil, 6% safflower oil, 2% soybean oil; the 20% fat n-6 diet – 18% safflower oil, 2% soybean oil.

In view of these changes from the diets we previously used, we decided it was necessary to perform a preliminary experiment to ensure that the decrease in mammary HMG-CoA reductase activity seen in LDL-R +/- mice compared to wildtype mice fed the 7% n-6 PUFA diet persisted when animals were fed the 20% n-6 PUFA diet. Briefly, 4 LDL-R +/- and 4 LDL-R +/+ mice were fed the 20% fat n-6 diet for one week. Animals were sacrificed by CO₂ asphyxiation and microsomes were isolated from mammary and liver tissues. Both mammary and hepatic HMG-CoA reductase activity levels were significantly lower in LDL-R +/- animals versus wildtype (see Figs 1a and 1b), and were, indeed, comparable to those we observed in mice fed the 7% n-6 diet 20.

In view of the issues discussed above, we decided it would be prudent to carry out a preliminary experiment to determine the feasibility of the full experiment described in Task 1. We decided to focus on the hypothesis that the lower HMG-CoA reductase activity in LDL-R +/- mice compared to +/+ mice (see Fig 1b) will lead to a lower mammary tumor incidence when the mice are fed diets rich in n-6 PUFAs.

Thirty female LDL-R knockout mice and 30 C57Bl wildtype controls were purchased from Jackson Labs at 5 weeks of age and acclimatized on an AIN-93G standard diet for one week. At 6 weeks of age animals were injected s.c. with 40 mg of slow-release medroxyprogesterone acetate (MPA) (Depo-Provera®) in the interscapular area. At 8 weeks of age, animals received a single dose of 50mg/kg MNU. One week post-MNU all animals were switched to the 20% n-6 diet for the duration of the experiment. Animals were weighed biweekly and palpated for the presence of mammary tumors weekly. Animals were given fresh food biweekly, and consumption per cage was recorded.

Throughout the study there was no significant difference in food consumption or weights between the two groups. Approximately one month post-initiation, some animals unexpectedly began “barbering”, a process of excessive grooming that leads at first to superficial hair loss, but if continued aggressively, to eventual skin damage and tissue destruction in the area affected. By
~2.5 months post-MNU, we sacrificed some animals that had significant damage to the skin and underlying connective tissue, or that had lost weight. Steps were taken to minimize barbering activity & damage – barbered animals were separated to prevent possible barbering by the dominant female in the cage, and animals with skin lesions were treated with varitene to prevent infection, and to lessen damage by reducing irritation in the area. Sacrificed animals were necropsied, and samples of mammary, liver, spleen, kidney, and ovaries were taken for histopathology. Several mice had grossly enlarged spleens, and all had marked anaemia. Because of the barbering activity, we decided to terminate the experiment at 6 months post-initiation. At this time, all mice were killed and samples of mammary gland, ovaries, liver, kidney, and spleen were placed in 10% buffered formalin for paraffin embedding. No palpable, or grossly visible mammary tumors were evident in either group. Slides prepared from mammary glands of mice indicated the growth of malignant lymphomas in mammary fat pads in most animals, but no mammary tumors were apparent. No differences in lymphoma incidence between LDL-R +/- and wildtype mice were found. The inherent poor susceptibility of C57Bl mice to chemically induced mammary carcinogenesis indicates caution in proceeding with further work on task 1, or in studies utilizing similar models.

Task 2. To determine whether transgenic mice that overexpress the LDL-R in the mammary gland develop fewer tumors than wild-type controls.

Transgenic mice are currently created in two predominant background strains – FVB and C57Bl. Both of these strains are poorly susceptible to the induction of chemically-induced mammary tumors. Given our experience with chemical carcinogenesis in C57Bl mice (see Task 1), and that other, more susceptible strains of mice are unavailable for generation of transgenic animals, we realized that it would be prudent to pursue another approach to test our central research hypothesis. The principle aim of this work has been to establish definitively whether mevalonate plays a role in mammary carcinogenesis. To this end we have examined two possible alternative routes of investigation. The first involved the use of the well-established rat chemical carcinogenesis model, which reproducibly yields a high incidence of mammary tumors (>80% in our hands) with short latency (<26 weeks). We proposed to investigate the effects of exogenous mevalonate on the growth of chemically-induced rat mammary tumors. An abundant exogenous mevalonate supply available to growing transformed cells of the mammary gland would down-
regulate HMG-CoA reductase activity, while still allowing cells to produce all of the downstream mevalonate-derived metabolites. This model would be equally as useful for us as the transgenic LDL-R mouse and would avoid the problems associated with the mouse being resistant to chemical carcinogenesis. However, it was first necessary to address the feasibility issue of delivery of mevalonate to target tissues. To determine a method of mevalonate delivery to rat mammary glands, several small preliminary studies were conducted, as follows:

Study 1: Ten female Sprague Dawley rats were fed a diets containing either 0 or 1% mevalonate (wt/wt) for a period of 1 week. Animals were then sacrificed and liver and mammary gland tissues were assayed for HMG-CoA reductase activity. Enzyme activity down-regulation was taken as evidence of product-feedback inhibition, indicating delivery of mevalonate to target cells. While hepatic HMG-CoA reductase activity decreased substantially (Fig 2a), mammary gland reductase activity did not change significantly (Fig 2b). Taken together, this likely indicates rapid hepatic clearance of mevalonate during first pass through the liver following intestinal absorption, and therefore inadequate delivery of mevalonate to the mammary gland.

Study 2: As an alternative route to oral administration of mevalonate, we assessed the ability of mini-osmotic pumps to deliver a physiological dose of mevalonate to the rat mammary gland. As in study 1, effective delivery of mevalonate was assessed by measuring feedback inhibition of HMG-CoA reductase. Briefly, 10 female SD rats, age 12 weeks, were randomized to two groups, and surgically implanted s.c. with a 200 uL mini-osmotic pump (flow rate of 0.25uL/hour, and pumping ‘life’ 28 days) containing either 1mg/ul mevalonate, or isotonic saline. Rats were sacrificed two weeks after pump implants, and HMG-CoA reductase activity was assessed. Results again indicated no significant difference in mammary reductase activity between groups (Fig. 3), indicating insufficient delivery of mevalonate to mammary cells by 200 uL pumps.

Study 3: To assess whether a greater dose of mevalonate to rats would reach target cells of the mammary gland, 10 rats were implanted s.c. with 2 mL mini-osmotic pumps capable of releasing a ten-fold greater dose of mevalonate than that used in study 2 (flow rate 2.5uL/hour). However, even at this dose, mevalonate released s.c. failed to reach cells of the mammary gland (Fig 4).

At this time, we began to explore a second alternative avenue of investigation to address the role of the mevalonate pathway in mammary carcinogenesis. Athymic nude mice are able to accept xenografts, including an injection of human breast cancer cells, which subsequently grow to form a palpable tumor. Tumor growth in nude mice has been shown to be affected by
treatments administered to the host mouse, including drug treatments and alterations in diets. We proposed to perform an experiment wherein the ability of exogenous mevalonate to modulate the growth of human breast cancer cells inoculated into the mammary fat pad of nude mice would be assessed. A feasibility study was performed in Balb/c mice, the background strain for the nude mouse, to determine a mode of delivery of exogenous mevalonate to the mammary gland. S.c. implantation of mini-osmotic pumps (flow rate 0.25uL/h) containing mevalonate significantly down-regulated mammary HMG-CoA reductase activity (Fig 5) indicating effective delivery of mevalonate to mammary glands of mice.

To determine the effects of increased exogenous mevalonate on the growth of human breast cancer cells in nude mice, the following experiment was performed. 60 female athymic nude mice, age 8 weeks, were inoculated in the right thoracic mammary fat pad with $10^6$ highly malignant, metastatic MDA-MB-435 human breast cancer cells. One week following inoculation, animals were surgically implanted s.c. in the inter-scapular area with a mini-osmotic pump containing either 1g/L mevalonate or isotonic saline. Animals were fed an AIN 93G control diet throughtout the experiment. Beginning 2 weeks after pump implantation, when wounds had healed, animals were weighed and palpated weekly for tumors. Palpable tumors were measured using Vernier calipers. At monthly intervals, spent pumps were removed surgically, and replaced with fresh pumps. At week 13, animals were sacrificed and tumors were weighed and measured. Sections of mammary gland, tumor, and perfused lungs were preserved in 10% buffered formalin.

Tumors in both groups grew at similar rates until the time period following the third pump replacement. From approximately 9 weeks onward, tumors in mice treated with mevalonate grew at a significantly greater rate than controls. By week 13, tumors in mevalonate treated animals had a greater surface area (Fig 6a) and weight (Fig 6b) versus controls. We are currently performing immunohistochemistry to determine whether increases in tumor size resulting from mevalonate treatment are associated with evidence of enhanced angiogenesis in tumor sections. Our results would indicate that growth of mammary tumors in this model is limited by the concentration of mevalonate, a result that supports our hypothesis that this metabolite plays a key role in controlling the rate of cell proliferation.
Task 3. To determine the extent to which growth inhibition of MCF-7 cells by the n-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and the isoprenoids geraniol and β-ionone, is mediated by the mevalonate pathway.

In initial experiments we have optimized cell culture conditions. The issue of ensuring that the lipophilic fatty acids and isoprenoids are adequately solubilized in aqueous media has prompted us to experiment with varying culture conditions, including different levels of ethanol (up to 1%) and albumin (up to 2g/L). Reported conditions in the literature vary considerably, even for commonly used cell lines, and so we wanted to ensure that culture conditions achieved optimal solubilization of test compounds while maintaining favorable growth conditions and minimizing toxicity of vehicles used. Our protocol involved extensive mixing and pre-incubation of test compounds with fetal bovine serum (FBS) to allow for maximal binding to the protein carrier. Final ethanol concentrations were minimized, with a maximum concentration in medium of 0.1%. Low-serum medium (2 to 5% FBS) was used to support MCF-7 growth during treatments with fatty acids, while complete medium (10% FBS) was used in all isoprenoid experiments.

The effects of the n-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on MCF-7 cell proliferation were assessed. Cells grown in 96 well plates were treated for 11 days with increasing levels of the test fatty acids (10, 30, 50, and 70μM). Cell proliferation rates were determined by BrdU incorporation as measured by ELISA. Both fatty acids inhibited cell proliferation in a dose-dependent manner (Figs. 7a & 7b). Similar effects were seen on HMG-CoA reductase activity. Both EPA and DHA inhibited HMG-CoA reductase activity in MCF-7 cells grown for 8 days in 75 dL flasks (Figs. 8a & 8b). The maximum effect was seen at 30μM for both fatty acids, and there was no greater inhibition of HMG-CoA reductase activity above this level. Inhibition of HMG-CoA reductase activity by fish oils was highly correlated with inhibition of cell proliferation (Figs. 9a & 9b). We next assessed the ability of mevalonate to restore proliferation in cells treated with EPA or DHA. Cells grown in the presence of 30μM EPA or DHA (the level at which maximum effects on HMG-CoA reductase were observed) were treated with increasing levels of mevalonate, up to 1mM. Mevalonate increased proliferation in DHA treated cells by up to ~60% compared to cells grown in the absence of exogenous mevalonate (Fig.10b). This indicates that inhibition of mevalonate synthesis mediates, at least in part, the effects of DHA on MCF-7 cell proliferation. EPA treated cells, however, failed to
respond to treatment with mevalonate, even at the highest (1mM) treatment level (Fig. 10a). Treatment of cells with 1mM mevalonate alone (in the absence of n-3 PUFAs) did not significantly promote proliferation.

The effects of the dietary isoprenoids geraniol and β-ionone on MCF-7 cell proliferation and in vitro HMG-CoA reductase activity were assessed. Cells were grown in HG-DME:F12 medium with 10% FBS and 1% antibiotics for 7 days in the presence of increasing concentrations of test compounds (100, 200, 300, 400, 500 μM β-ionone or 100, 300, 500, 700 μM geraniol). Final concentrations of ethanol were 0.1% in all treatment and control groups. After 7 to 8 days, cell proliferation was determined by BrdU incorporation as measured by ELISA, and mevalonate synthesis was measured by radiochemical assay of HMG-CoA reductase activity in cell extracts. Both isoprenoids significantly inhibited MCF-7 cell proliferation (geraniol IC50~500μM, β-ionone IC50~200μM). Only geraniol, however, also inhibited HMG-CoA reductase activity (Figs. 11a & 11b). This finding was both novel and unexpected as β-ionone is known to inhibit hepatic HMG-CoA reductase activity, and regulation of HMG-CoA reductase activity by non-sterol isoprenoids is reported to be intact in tumor cells. To determine causality, mevalonate rescue experiments were subsequently performed in which cells were grown coordinately in the presence of growth inhibitory levels of isoprenoids with up to 2mM supplemental mevalonate. Despite a highly significant correlation between inhibition of HMG-CoA reductase activity and cell proliferation by geraniol ($r^2=0.89, p<0.01$) mevalonate failed to rescue proliferation inhibition by either isoprenoid tested (Figs 12a & 12b).

**Task 4.** To investigate the mechanism by which mevalonate promotes mammary tumor cell growth.

We reported in Task 2 that we determined that mevalonate promotes the growth in nude mice of tumors derived from an inoculation of MDA-MB-435 human breast cancer cells (Figs. 6a and 6b). The finding that excess mevalonate promotes mammary tumorigenesis is both novel and exciting as mevalonate is a common cellular metabolite, and its synthesis can be increased by several factors including dietary compounds such as linoleic acid, and drugs that alter serum cholesterol levels, such as cholestyramine. Understanding how mevalonate promotes mammary tumor growth may provide considerably insight into the development of deregulated growth control in transformed cells.
In our 2002 Annual Report we showed that mevalonate added to cell culture medium for 60 hours promotes the growth of MDA-MB-435 cells (Fig. 13), associated with increased passage of cells through the G1-phase restriction point into S-phase (Table 1). Cell cycle progression is regulated in part by the activity of a group of kinases, known as cyclin-dependent kinases (CDKs) that are present at relatively constant levels during all phases of the cell cycle.\textsuperscript{29,30} Their activity is modulated by activating and deactivating phosphorylations and by interaction with two types of regulatory proteins.\textsuperscript{29} The first are the cyclins that bind and activate CDKs. Progression of cells through early G1 phase is governed by CDK4, which couples with cyclins D1, D2, and D3.\textsuperscript{29,30} CDK2-cyclin E controls passage of cells through the late-G1 restriction point and into S phase.\textsuperscript{29,30} After this commitment to replication occurs, CDK2 releases cyclin E, and instead couples with cyclin A.\textsuperscript{31} CDK2-cyclin A catalyzes reactions necessary for DNA synthesis and chromosomal replication.\textsuperscript{31} The second group of regulatory proteins are CDK-inhibiting proteins (CDKIs).\textsuperscript{32} These are small molecular weight proteins that bind to and inhibit CDKs.\textsuperscript{29,32} The G1 phase CDKIs p21\textsuperscript{cip1} and p27\textsuperscript{kip1} are powerful modulators of cell cycle progression, and relative binding levels of these proteins may actually play a greater role in determining CDK activity than cyclin abundance.\textsuperscript{33}

We have measured relative expression levels of protein regulators of the G1/S phase transition, including the kinases CDK4 and CDK2, their respective regulatory subunits cyclin D, and cyclins E and A, and the CDKIs p21\textsuperscript{cip1} and p27\textsuperscript{kip1}. We have also performed measures of CDK2 in vitro kinase activity as well as relative activation state, including the presence of an activating phosphorylation, and relative binding with cyclins E and A and CDKIs p21\textsuperscript{cip1} and p27\textsuperscript{kip1}. CDK2 is necessary both for entry into, and transit through S phase.\textsuperscript{31} In our experiments we have found an increase in the proportion of cells in S phase, at the expense of the proportion of cells in G1 (Table 1). While CDK4-cyclin D1 also catalyzes important events in the passage of cells through G1, we have hypothesized that CDK2 is a highly critical effector of mevalonate-induced cell cycle effects. This is supported by studies demonstrating that over expression of cyclin E (required for CDK2 activity)\textsuperscript{34}, but not cyclin D1 (required for CDK4 activity)\textsuperscript{35}, can overcome the blockade of cell cycling by mevalonate depletion.

Treatment of MDA-MB-435 cells with mevalonate (up to 5mM) resulted in a slight (~25%) increase in cyclin A expression at all concentrations (Fig 14). Cyclin E expression was not affected by mevalonate treatment (Fig 14). Cyclin D1 expression decreased with increasing
levels of mevalonate treatment, to ~65% of untreated control at the 1mM and 5mM treatment levels (Fig. 14). This likely reflects the expected decreased percentage of cells in early G1 phase as mevalonate increases entry of cells into late G1 and S phase (Table 1) where cyclin A and cyclin E expression would be preferentially stimulated. Expression of both p21cip1 and p27kip1 decreased with mevalonate treatment (Fig. 14) with maximal declines seen at 1mM and 0.5mM for each CDKI, respectively. Decreased expression of the CDKIs p21cip1 and p27kip1, as well as increased expression of cyclin A may help to drive the entry of mevalonate treated cells into active replication as was evident by flow cytometry (Table 1).

CDK2 activity is essential for initiation of DNA synthesis and completion of chromosome replication. We assessed whether changes in known regulators of CDK2 activation state were evident with mevalonate treatment of cells, and whether in vitro CDK2 activity was increased. CDK2 enzyme levels were not significantly increased as determined by Western blot analysis (Fig 15a). However, as determine by migration shift assay during SDS-PAGE 36, there was an ~25% increase in the 33 kDa CDK2 form that occurs when CDK2 has been activated by phosphorylation on threonine 160. This indicates that mevalonate increases activation of CDK2 (Fig. 15b). We also found that mevalonate treatment decreases the amount of p21cip1 bound to CDK2 (Fig. 15c). CDK2 immunoprecipitates from extracts of cells treated with mevalonate were subjected to SDS-PAGE, and immunoblotted for the CDKI p21cip1. Figure 15c shows that mevalonate decreases the inactivating binding of p21cip1 to CDK2, which should also increase the activation state of cellular CDK2. Finally, we performed a histone H1 phosphorylation assay to assess CDK2-specific activity. Immunoprecipitates of cyclin A or cyclin E from mevalonate treated cells were incubated with histone H1 and [γ32P]-ATP. Phosphorylation of histone H1 by CDK2 was determined by radiography of gels following SDS-PAGE. Equal loading was confirmed by Coomassie blue staining of gels. Specific kinase activity of CDK2 associated with cyclin E and cyclin A both increased dramatically with increasing mevalonate treatment (Fig. 15d), in parallel with concentration dependent effects of mevalonate on cell proliferation rates (Fig 13).

Our results therefore indicate that mevalonate promotes the growth of human breast cancer cell through modulation of cell cycle progression, specifically through changes that result in increased CDK2 activity.
KEY RESEARCH ACCOMPLISHMENTS

- The n-3 PUFAs DHA and EPA, and the dietary isoprenoid geraniol, but not β-ionone, were shown to inhibit HMG-CoA reductase activity in human breast cancer cells (MCF7).
- The inhibitory effects of the n-3 PUFA EPA, and the isoprenoids geraniol and β-ionone on MCF-7 cell proliferation were demonstrated to be independent of effects on mevalonate synthesis. However, inhibition of mevalonate synthesis mediates, at least in part, the inhibitory effects of DHA on MCF-7 proliferation.
- Exogenous mevalonate was demonstrated to promote the growth of tumors derived from MDA-MB-435 human breast cancer cells in athymic nude mice.
- Exogenous mevalonate was demonstrated to promote proliferation of MDA-MB-435 human breast cancer cells in culture, associated with an increase in passage of cells through the G1 restriction point into S phase.
- Effects of mevalonate were found to be mediated, at least in part, by changes in cell cycle regulatory proteins. This included increased expression of the positive cell cycle regulator cyclin A, and decreased expression of the inhibitory CDKIs p21cipl and p27kip1.
- Mevalonate caused increased cyclin A and cyclin E associated CDK2 activity. This was mediated by increased activating phosphorylation of CDK2, and decreased binding of CDK2 to the CDKI p21cipl.

REPORTABLE OUTCOMES

1. Mammary cancer cell HMG-CoA reductase is susceptible to regulation by the n-3 PUFAs EPA and DHA, and by the dietary isoprenoid geraniol, but not β-ionone. Effects of EPA, geraniol, and β-ionone on mevalonate synthesis by mammary cancer cells are correlative, but not causative, indicating that inhibitory effects of these compounds on cell proliferation are not mediated through the mevalonate pathway. However, inhibition of mevalonate synthesis by DHA does appear to partially mediate inhibition of proliferation. Two manuscripts are being prepared from this work.
2. Mevalonate promotes the growth of tumors in vivo, and proliferation of mammary cancer cells in vitro. These effects are associated with alteration in molecular regulators of cell cycle control that promote passage of cells beyond the G1 restriction point and, therefore,
elicit the onset of DNA synthesis and cell proliferation. A manuscript reporting outcomes from this work is currently in preparation.


Personnel receiving pay from the research effort: Robin E. Duncan, Ph.D. candidate.

CONCLUSIONS

In normal rodent mammary tissue, a diet rich in n-3 PUFAs inhibits HMG-CoA reductase activity, thereby limiting endogenous mevalonate synthesis compared to a diet rich in n-6 PUFAs. To investigate whether n-3 PUFAs inhibit breast tumor cell proliferation by inhibiting endogenous mevalonate synthesis, we performed cell culture work in which the ability of mevalonate to rescue inhibition of MCF-7 cell proliferation by n-3 PUFAs (EPA and DHA) was ascertained. Inhibition of HMG-CoA reductase activity by DHA, but not EPA, is causally linked to inhibition of cell proliferation. While both EPA and DHA also inhibit mammary cancer cell growth through alternate pathways, effects on mevalonate synthesis mediate at least some of the effects of DHA, demonstrating an exciting and novel mechanism. Like fish oils, dietary isoprenoids have been shown to inhibit hepatic HMG-CoA reductase activity in rodent and avian models 37-38, and to inhibit mammary carcinogenesis in rats 39-41. However, the effect of these compounds on mammary HMG-CoA reductase activity has not been investigated. We
determined that while both geraniol and β-ionone reportedly inhibit hepatic HMG-CoA reductase activity, only geraniol inhibited HMG-CoA reductase activity in MCF-7 human breast cancer cells. Inhibition of HMG-CoA reductase activity by geraniol was highly correlated with effects on cell proliferation. However, mevalonate rescue experiments determined that this relationship was not causal. Our research indicates that while EPA and geraniol inhibit breast cancer cell mevalonate synthesis, these effects are not causative, and therefore these compounds do not display “statin-like” effects. DHA, however, may be a candidate for the development of preventative therapies that target statin-inhibited pathways, but with lower toxicity. Other mechanisms of action should be explored to better understand the nature of the inhibitory effects of EPA, and the dietary isoprenoids geraniol and β-ionone on breast cancer.

While technical limitations precluded our use of transgenic and knockout mice in the chemical carcinogenesis studies we originally proposed, work involving the nude mouse-tumor cell inoculation model was productive. Tumors derived from highly malignant MDA-MB-435 human breast cancer cells grew more rapidly in nude mice implanted s.c. with mini-osmotic pumps containing mevalonate, compared to controls, and had greater weights and volume at the end of the experiment (13 weeks). This indicates that growth of mammary tumors in this model is limited by the concentration of mevalonate, a result that supports our hypothesis that this metabolite plays a key role in controlling the rate of cell proliferation. Investigations involving MDA-MB-435 cells in culture have shed light on the specific nature of that role. Mevalonate promotes the proliferation of cells by increasing levels of the CDK2-activating protein cyclin A, while decreasing levels of the CDK2-inhibiting proteins p21cip1 and p27kip1. CDK2 in vitro kinase activity associated with both cyclin A and cyclin E is increase in mevalonate treated cells. Decreased inhibitory binding of CDK2 by the CDK1 p21cip1, and increased activating phosphorylation of CDK2 on threonine 160 mediate this.

Mevalonate, a key metabolite in all living cells, is required for the synthesis of cholesterol and hence membranes, and for the production of the isoprenoids that have growth regulatory functions. Indeed, mevalonate is required for DNA synthesis and cell proliferation. Several phytochemicals as well as fish oils, can inhibit mevalonate synthesis in cells. However, our work has demonstrated that these compounds do not inhibit mammary cancer cell proliferation through effects on mevalonate synthesis, and therefore they are not statin-like in nature. We have demonstrated that mevalonate promotes the growth in nude mice of tumors
derived from human breast cancer cells. Mevalonate also increases cell proliferation in vivo, associated with increased entry of cells into S phase, and active cell cycling. Mammary mevalonate synthesis is increased by multiple factors, including increased dietary linoleic acid content and decreased serum cholesterol levels. Recommendations for the prevention of heart disease advise steps that may increase mammary mevalonate synthesis. These include replacing saturated fat with dietary PUFAs, which are predominantly of the n-6 type in the North American diet. Diets rich in n-6 PUFAs are well established as strong promoters of mammary cancer. Reducing serum cholesterol by increasing cholesterol and bile acid excretion through the use of bile acid sequesterants has also been shown to promote experimental mammary carcinogenesis. Our finding that mevalonate promotes mammary tumor growth may provide a mechanism to explain the promoting effects of linoleic acid and cholestyramine on mammary carcinogenesis. Our research may also have clinical significance. Both mammary cancer incidence and heart disease risk increase in women with advancing age. Our findings indicate that the use of bile-acid sequesterants in hypolipidemic therapy in women, especially those with established risk factors for breast cancer, may be contraindicated.

REFERENCES:


Appendix

Table and Figures
Table 1. The effects of mevalonate on cell cycle distribution.

<table>
<thead>
<tr>
<th>Phase</th>
<th>% of total cells</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>G0-G1</td>
<td>71</td>
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<tr>
<td>S</td>
<td>16</td>
</tr>
<tr>
<td>G2-M</td>
<td>13</td>
</tr>
</tbody>
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Cells were grown for 60 hours at the indicated concentrations of mevalonate, then harvested by trypsinization and stained with PI for FACS analysis.
Liver HMG-CoA Reductase Activity

Figure 1a  Liver HMG-CoA reductase activity in LDL-R/- versus LDL-R +/- mice fed a 20% fat diet rich in n-6 PUFAs (measured in pmol mevalonate produced per minute per mg microsomal protein), p<0.05.
Mammary Gland HMG-CoA Reductase Activity

Figure 1b Mammary HMG-CoA reductase activity in LDL-R/- versus LDL-R +/- mice fed a 20% fat diet rich in n-6 PUFAs. *p<0.02.
Liver HMG-CoA Reductase Activity

Figure 2a  Liver HMG-CoA reductase activity in Sprague Dawley rats fed an AIN 93G control diet containing 0% or 1% mevalonate (wt/wt). p<0.02
Mammary Gland HMG-CoA Reductase Activity

Figure 2b  Mammary HMG-CoA reductase activity in Sprague Dawley rats fed an AIN 93G control diet containing either 0% or 1% mevalonate (wt/wt).
Mammary Gland HMG-CoA reductase activity

Figure 3  Mammary HMG-CoA reductase activity in rats implanted s.c. with a 200uL mini-osmotic pump delivering mevalonate or isotonic saline (0.25uL/h).
Figure 4  Mammary HMG-CoA reductase activity in rats implanted s.c. with a 2mL mini-osmotic pump delivering mevalonate or isotonic saline (2.5uL/hr).
Mammary Gland HMG-CoA reductase activity

![Graph showing HMG-CoA reductase activity](image)

Figure 5 Mammary HMG-CoA reductase activity in Balb/c mice implanted s.c. with 200uL mini-osmotic pumps containing mevalonate or isotonic saline (0.25uL/h). *p<0.005
Figure 6a  Surface area of tumors derived from MDA-MB-435 human breast cancer cells growing in athymic nude mice treated with either mevalonate or isotonic saline delivered via mini-osmotic pumps. *p<0.05
Tumor Weights

Figure 6b  Final weights of tumors derived from MDA-MB-435 human breast cancer cells grown in nude mice treated with mevalonate or saline via s.c. implaned mini-osmotic pumps. p<0.05
Figs. 7a & 7b. Effect of EPA and DHA on MCF-7 cell proliferation. Cells were treated with increasing levels of n-3 PUFAs as indicated for 11 days. Proliferation rates were assessed by ELISA assay of BrdU incorporation. Treatment of cells with fatty acids at levels of 30μM or above significantly inhibited proliferation compared to controls (*p<0.01).
Figs. 8a & 8b. Effects of EPA and DHA on HMG-CoA reductase activity in MCF-7 cells. Cells were treated for 8 days. Data represent mean activity assayed from 3 separate flasks (*p<0.01 versus control).
Figs. 9a & 9b. Correlation between cell proliferation rate and HMG-CoA reductase activity in MCF-7 cells treated with varying levels of EPA (a) or DHA (b).
Fig. 10a. Mevalonate does not restore cell proliferation in cells treated with 30μM EPA. 1mM mevalonate had no effect on cell proliferation.
Fig. 10b. Mevalonate partially rescued cell proliferation in cells treated with 30μM DHA. Treatment of cells inhibited by DHA with 0.5mM mevalonate caused a 41±9% increase in cell proliferation, and with 1mM mevalonate, a 60±5% increase in cell proliferation. Rescue of DHA inhibition of cell proliferation by mevalonate was highly significant, *p<0.001.
Figs. 11a & 11b. Geraniol inhibits both MCF-7 cell proliferation (○-) and HMG-CoA reductase (●-) activity compared to control, whereas β-ionone inhibits only cell proliferation (*p<0.01).
Figs. 12a & 12b. Mevalonate fails to rescue cell proliferation in cells treated with either geraniol or β-ionone, indicating that mechanisms other than inhibition of HMG-CoA reductase mediate the inhibitory effects of these isoprenoids on MCF-7 cell proliferation. (*p<0.01 vs control).
Fig. 13. Effects of mevalonate on growth of MDA-MB-435 cells in culture. Cells were grown for 60 hours in serum free media in the presence of increasing levels of mevalonate, and proliferation rates were assessed by nuclear incorporation of BrdU (*p<0.01 vs control).
Figure 14: Immunoblots of indicated proteins and relative densities as assessed by density scanning of images following chemiluminescence.
Figure 15: Measures of CDK2 activation state and activity. a) Immunoblot of CDK2 and density quantification following chemiluminescence. b) Immunoblot of CDK2 (34kDa) and CDK2 activated by phosphorylated at threonine 160 (33kDa) (density blot of 33kDa form). c) Immunoblot of p21cip1 detectable after SDS-PAGE of immunoprecipitated CDK2. d) Cyclin E and cyclin A associated histone H1 kinase activity in extracts of cells grown in increasing concentrations of mevalonate. Phosphorylation of histone H1 is a highly specific measure of CDK2 activity.