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Chemopreventive effect of squalene on colon cancer

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Epidemiologic and laboratory studies suggest a cancer protective effect and/or lack of a tumor promoting effect by dietary olive oil as compared with other types of non-marine oils. Squalene, a constituent of olive oil, and a key intermediate in cholesterol synthesis may be regarded as partially responsible for the beneficial effects of olive oil, which include decreased mortality rates among populations with high olive oil consumption. Thus, in this study we have assessed the chemopreventive efficacy of squalene on azoxymethane (AOM)-induced colonic aberrant crypt foci (ACF). In addition, we measured the effect of squalene on serum cholesterol levels in the rats. Male F34 rats (5 weeks old) were fed the control diet (modified AIN-76A) or experimental diets containing 1% squalene or 320 p.p.m. sulindac. Two weeks later, all animals except those in vehicle (normal saline)-treated groups were s.c. injected with AOM (15 mg/kg body wt, once weekly for 2 weeks). At 16 weeks of age, all rats were killed, colons were evaluated for ACF and serum was assayed for the cholesterol levels. As expected, dietary administration of sulindac suppressed ACF development and reduced crypt multiplicity, i.e. number of aberrant crypts/focus. Administration of dietary squalene inhibited total ACF induction and crypt multiplicity by ~>46% ($P < 0.001$). Further, squalene at a level of 1% did not show any significant effect on serum cholesterol levels. Our finding that squalene significantly suppresses colonic ACF formation and crypt multiplicity strengthens the hypothesis that squalene possesses chemopreventive activity against colon carcinogenesis.

Introduction

Large bowel cancer is one of the leading causes of cancer deaths in both men and women in Western countries, including the United States (1). Evidence from epidemiological studies and laboratory animal assays suggests a relationship between colon cancer risk and dietary factors (2–4). Identification of naturally occurring carcinogens and anticarcinogens should lead not only to an understanding of carcinogenesis but should also provide new strategies for cancer prevention. Cancer risk effects of dietary olive oil have been established in several epidemiological studies by comparing people consuming this fat with those eating other non-marine oils (5–9). Most of the Mediterranean dietary case-control studies have also shown that people who eat high levels of olive oil had lower incidence of several cancers including cancer of the colon (6,7). Laboratory animal model studies have generally shown that olive oil has either no effect, or a protective effect on the prevention of a variety of chemically induced tumors (10–12). Our laboratory findings suggest that, compared with corn and sunflower oil, olive oil does not increase tumor incidence or growth in colon cancer models (13,14). Similar observations were made in mammary cancer models (10,11).

Most of the recent studies clearly suggest that the cancer promoting or protective effects of fat depend not only on the amount but also on the type of fat in terms of its fatty acid constituents (13–15). The 'protective' or 'non-promoting' activity of olive oil is often ascribed to its high content of oleic acid (C18:1, ω-9), a mono unsaturated fatty acid. But most of the studies carried out with animal fats that are rich in oleic acid, such as beef and poultry (35–45%), and with the vegetable fats, such as corn oil (25–35%), palm oil (43%), peanut oil (45–52%), soybean oil (25%) and sunflower seed oil (30–35%), have been largely associated with an increased risk of colon and breast cancer in humans. Also, these types of fats were generally shown to be promoters of chemically induced tumors in animals (10–13). Therefore it was important to consider whether other constituents may be unique to olive oil, and to examine qualitative or quantitative differences as compared with other fats and oils, that may account for the protective and/or non-promoting effects of tumorigenesis by olive oil.

Squalene is a triterpene that contains six isoprene units (Figure 1). It is present in olive oil at concentrations between 0.2–0.7%. In other common human dietary fats and oils it constitutes only 0.002–0.03% (16). It is a key intermediate in the biosynthetic pathway to steroids in plants and animals. Limited data are available on the cancer preventive properties of squalene in rodent models. Van Duuren and Goldschmidt (17) have shown that topical application of squalene to mouse skin inhibited benzo[*a*]pyrene (B[*a*]P*)-induced skin carcinogenicity. Murakoshi *et al.* (18) reported that topically applied squalene markedly suppressed the tumor promoting effect of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) on 7,12-dimethylbenz[*a*]anthracene (DMBA)-initiated mouse skin. Anti-tumorigenic activity of squalene has also been described by several Japanese investigators (19–21). To our knowledge, there are no studies indicating that the dietary administration of squalene has been tested in a colon cancer model or in any other carcinogenesis model other than skin.

Aberrant crypt foci (ACF), which are early preneoplastic lesions, have consistently been observed in experimentally induced colon carcinogenesis in laboratory animals (22–25). Pretlow *et al.* (26) have also shown that these lesions are present in the colonic mucosa of patients with colon cancer

***Abbreviations:** ACF, aberrant crypt foci; AOM, azoxymethane; B[*a*]P, benzo[*a*]pyrene; DMBA, 7,12-dimethylbenz[*a*]anthracene; HMG-CoA reductase, hydroxymethyl glutaryl-coenzyme A reductase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

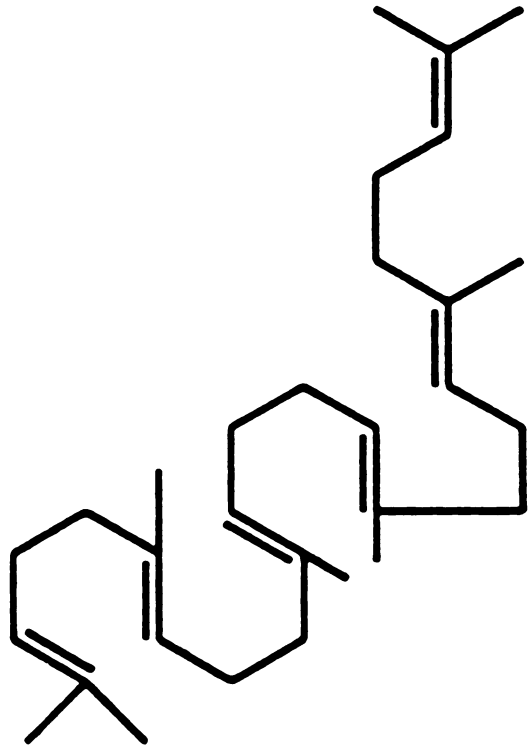

Fig. 1. Structure of squalene.

and have suggested that such aberrant crypts are putative precursor lesions from which adenomas and carcinomas may develop. ACF may express mutations in the *APC* gene and *ras* oncogene that appear to be biomarkers of colon cancer development (26,27). There is some debate concerning the carcinogenic potential of ACF in humans. Jen *et al.* (28) have suggested that hyperplastic foci, often harboring *ras* mutations, are of low neoplastic potential, and that dysplastic foci harboring *APC* mutations are early neoplastic lesions, but current data are not conclusive and may differ in the rat. There is evidence that several inhibitors of ACF formation reduce the incidence of colon tumors in laboratory animals (23–25) suggesting that ACF induction can be used to evaluate novel agents for their potential chemopreventive properties against colon cancer.

The present study was designed to evaluate the inhibitory activity of squalene on AOM-induced ACF formation in the colon of male F344 rats. The major goal of this study was to determine whether this natural agent is conceivably an effective chemopreventive agent in pre-clinical efficacy studies and, eventually in human clinical trials.

Materials and methods

Animals, diets, carcinogen and squalene

AOM (CAS: 25843–45–2) was purchased from Ash Stevens (Detroit, MI). Squalene was bought from Aldrich Chemical Company Inc. (Milwaukee, WI) and sulindac was purchased from Sigma Chemicals (St Louis, MO). Sulindac, a known inhibitor of colon carcinogenesis, was included in the current study as a positive control (29). Weanling male F344 rats were purchased from Charles River Breeding Laboratories (Kingston, NY). All ingredients of the semipurified diet were bought from Dyets Inc. (Bethlehem, PA) and were stored at 4°C until the experimental diets were prepared. The rats were held in quarantine for 1 week and had access to modified AIN-76A semipurified control diet [casein, 20%; D,L-methionine, 0.3%; corn starch, 52%; dextrose, 13%; corn oil, 5%; alphacel, 5%; mineral mix (AIN), 3.5%; vitamin mix (AIN), 1%; choline bitartrate, 0.2%]. They were randomly distributed by wt into various dietary groups and were transferred to an animal holding room

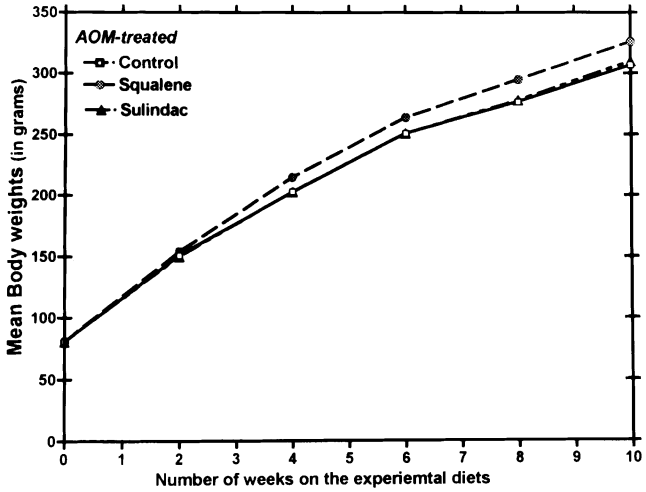

Fig. 2. Effect of squalene and sulindac on male F344 rat body wt gain.

where they were housed in plastic cages, three rats/cage, under controlled conditions of a 12-h light/12-h dark cycle, 50% relative humidity and 21°C room temperature. Experimental diets were prepared by mixing squalene (1% w/w) or 320 p.p.m. sulindac with modified AIN-76A control diet. Squalene and sulindac were incorporated into the experimental diets at the expense of corn starch.

Experimental procedure

At 5 weeks of age, groups of rats were fed the modified AIN-76A (control) or experimental diets containing 1% squalene or 320 p.p.m. sulindac. At 7 weeks of age, all animals except the vehicle-treated rats received AOM s.c. once weekly for 2 weeks at a dose rate of 15 mg/kg body wt per week. Animals intended for vehicle treatment were given an equal volume of normal saline. The rats were continued on control and experimental diets until the termination of the study when they were 16 weeks of age. All animals were killed by CO_2 euthanasia. Blood samples were collected from individual rats to analyze for the cholesterol. The colons were removed, flushed with Krebs Ringer solution, opened from cecum to anus, and fixed flat between two pieces of filter paper in 10% buffered formalin. Microscope slides were placed on top of the filter paper to ensure that the tissue remained flat during fixation. After a minimum of 24 h in buffered formalin, the colons were cut into 2-cm segments, starting at the anus; for the next 5–10 min they were placed in a Petri dish containing 0.2% methylene blue in Krebs Ringer solution. They were then placed, mucosal side up, on a microscope slide and observed through a light microscope. ACF were recorded according to standard procedures that are being used routinely in our laboratory. Aberrant crypts were distinguished from the surrounding normal crypts by their increased size, significantly increased distance from lamina to basal surface of cells, and the easily discernible pericryptal zone. The parameters used to assess the aberrant crypts were occurrence and multiplicity. Crypt multiplicity was determined as the number of crypts in each focus and categorized as containing up to three, four or more aberrant crypts/focus. One observer without knowing the identity of agents under study scored all colons; scores were checked at random by a second observer. Cholesterol was assayed according to a standard procedure (30).

Statistical analysis

All results were expressed as the means ± SEM and were analyzed by one-tailed Student's *t*-test. Differences were considered statistically significant at $P < 0.05$.

Results

General observation

The body wts of rats treated with vehicle or AOM and fed the control or experimental diets containing 320 p.p.m. sulindac were comparable throughout the study period (Figure 2). However, rats fed the squalene diet showed slightly higher body wts than rats in the control diet group ($P > 0.05$), indicating absorption of squalene. In vehicle-treated animals, administration of experimental diets containing squalene or

Table I. Effect of dietary squalene on AOM-induced colonic ACF formation in male F344 rats

Experimental groups	No. of rats at risk	Number of aberrant crypts per focus				Total ACF/Colon
		1 crypt	2 crypts	3 crypts	4 or more crypts	
AOM-treated						
Control diet	12	15 ± 2.5[a]	41 ± 6.2	30 ± 4.4	29 ± 3.2	114 ± 15
1% Squalene	12	5 ± 1.2[b,***]	22 ± 4.1[b,***]	17 ± 2.7[b,*]	17 ± 2.5[b,**]	62 ± 8.8[b,***]
320 p.p.m. Sulindac	12	4.5 ± 0.8[b,***]	18 ± 2.4[b,***]	15 ± 1.8[b,***]	16 ± 2.1[b,***]	54 ± 5.6[b,***]
Saline-treated						
Control diet	6	0	0	0	0	0
1% Squalene	6	0	0	0	0	0
320 p.p.m. Sulindac	6	0	0	0	0	0

[a]Mean ± SEM.
[b]Values in the vertical column are significantly different form the control group by Student's *t*-test, *$P < 0.01$; **$P < 0.001$; ***$P < 0.0001$.

sulindac did not produce any gross changes in the liver, kidney, intestine and lungs.

Aberrant crypts

Rats receiving saline and fed the control or experimental diets showed no evidence of ACF formation in the colon (Table I). In rats fed the control diet, AOM treatment induced, on the average, ~114 ACF/colon and 29 foci containing multiple (four or more) aberrant crypts/focus (Table I). As expected, ACF were predominantly observed in the distal colons. Efficacy endpoints used in this study were inhibition of total occurrences of ACF as well as reduction of number of multicrypts (four or more) of aberrant crypts. In the present study, sulindac, which has been shown to be a strong inhibitor of colon carcinogenesis in animal assays and has reduced polyps in patients with familial polyposis, was also found to be an effective inhibitor of total occurrence of ACF/colon (52%) and of multicrypt clusters containing two, three or even four or more crypts/focus (45–56%). Administration of 1% squalene significantly suppressed the total number of ACF/colon (>45% inhibition, $P < 0.001$) as compared with the control diet. Aberrant crypt multiplicities per focus were significantly decreased (40–50%, $P < 0.01$ to 0.0001). Administration of 1% squalene for ~10 weeks did not alter the serum cholesterol levels when compared with the control diet group (80.3 ± 2.3 mg/dl in the control diet group versus 83.2 ± 2.1 mg/dl in squalene diet group).

Discussion

The present study was undertaken to evaluate the chemopreventive activity of dietary squalene against ACF formation in the rat colon. ACF are putative preneoplastic lesions. Since multiplicity of four or more aberrant crypts/focus has been a fairly consistent predictor of colon tumor outcome (25,26,31), we used this criterion to evaluate squalene for its potential chemopreventive properties. The results of this study confirm our earlier efficacy study with sulindac (29) and provide additional evidence that crypt multiplicity and ACF are predicative of colon tumor incidence. Sulindac was found to be a strong inhibitor of chemically-induced colon carcinogenesis in animal models: it is currently under evaluation in human clinical chemoprevention trials (29,32). Dietary squalene clearly inhibits experimentally induced-ACF in male F344 rats. This is the first study to demonstrate that dietary administration of squalene suppresses a model of chemically-induced carcinogenesis. It supports at least in part, the hypothesis that some of the observed anti-tumor promoter activity of olive oil is due to squalene. Topically applied squalene has previously been shown to be inhibit B[*a*]P-induced skin carcinogenesis in mice (17). Murakoshi *et al.* (18) have shown that topically applied squalene significantly suppressed the tumor promoting effect of TPA on mouse skin tumors after initiation with DMBA. Further, some studies have revealed that squalene potentiates the effects of several anticancer agents (19–21). One of the advantages of squalene is that, unlike synthetic chemopreventive agents, it is a naturally occurring compound that is both produced endogenously and present in many human foods. Also, it is interesting to note that sharks, which have high tissue levels of squalene, have been claimed to be resistant to cancer (33)

The exact mechanism(s) involved in the inhibitory effect of AOM-induced colon carcinogenesis by dietary squalene is not fully known. Squalene is a key intermediate in the biosynthesis of cholesterol, bile acids and sterols. It is possible that the observed inhibitory effect of dietary squalene in the present study may be due to the modulation of the cholesterol biosynthetic pathway. Strandberg *et al.* (34) have reported that rats given 1% dietary squalene for 5 days strongly suppressed (~80%) HMG-CoA reductase activity in liver microsomes. Inhibition of HMG-CoA reductase, a rate-limiting control step in the cholesterol biosynthetic pathway, may lead to the reduction of a series of intermediates such as mevalonate, geranyl pyrophosphate and farnesyl pyrophosphate. Farnesyl pyrophosphate is a source for the prenylation of oncogenes such as *ras-P*[21.] This prenylation (farnesylation) process is a post-translational modification of oncogenes that enables them to acquire full oncogenic activity (35,36). Prevention of farnesylation suppresses the activation of oncogene proteins as signal transducing agents in the regulation of cell transforming activity (37). It is possible that dietary squalene may inhibit HMG-CoA reductase activity in colonic mucosal cells leading to AOM-induced colonic ACF suppression. Alternatively, it is possible that dietary squalene could modulate the biosynthesis of the colon tumor promoting bile acids. Previous studies from our laboratory have shown that certain bile acids are potent colon tumor promoters (13).

We also find that dietary administration of 1% squalene over a 10-week period did not increase serum cholesterol levels. This observation is very important, because, theoretically, dietary supplementation of squalene could augment cholesterol and bile acid production, resulting in enhanced atherosclerotic disease or possibly, colon tumor promoting effects. Kritchevsky *et al.* (38) have described that dietary administration of 3% squalene in a high-cholesterol diet to

rabbits for 14 weeks, failed to elicit more atheromas than similar cholesterol-fed controls. Strandberg *et al.* (39) fed 900 mg squalene daily to humans for a period of 7–10 days, and produced a 17-fold increase in serum squalene, but no significant increase in serum cholesterol levels. Since short-term studies are insufficient to fully answer questions of long-term effects of higher than normal dietary squalene intake with regard to the metabolism of cholesterol and other metabolites, long-term studies are warranted to examine such effects.

In conclusion, this study demonstrates that dietary administration of squalene inhibits the formation of preneoplastic lesions in the colon, with no significant effect on serum cholesterol levels. Further experiments, including pre-clinical efficacy and mechanistic studies are warranted to fully evaluate this natural compound for its cancer preventive properties and to understand its mode of action.

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