Effect of Dietary Lycopene on N-Methylnitrosourea-Induced Mammary Tumorigenesis

Leonard A. Cohen, Zhonglin Zhao, Brian Pittman, and Frederick Khachik

Abstract: Epidemiological studies suggest protective effects of lycopene-rich foods on several types of cancer, including prostate and gastrointestinal tract. Moreover, an inverse association between serum lycopene concentrations and several types of cancer has been reported. However, few studies have focused on breast cancer, and they have shown little association between lycopene consumption and cancer risk. In this report, we used the N-methylnitrosourea (NMU)-induced rat mammary tumor model to compare the effects of pure lycopene with a lycopene-rich tomato carotenoid oleoresin (TCO) on NMU-induced mammary tumorigenesis. Rats were fed diets supplemented with 250 and 500 ppm crystalline lycopene or TCO beginning seven days before initiation with NMU (55 days of age) to termination (18 wk after NMU). Neither pure lycopene nor lycopene in the form of a mixed carotenoid oleoresin exerted an inhibitory effect on tumor incidence, latency, multiplicity, volume, or total tumors per group compared with unsupplemented controls. Weight gains in all groups were similar. Assay of serum lycopene concentrations in lycopene-supplemented groups indicated that median levels of 7.12, 60, and 87 ng/ml were attained in blood of groups supplemented with 250 and 500 ppm lycopene and 250 and 500 ppm TCO, respectively. The results of this animal study are consistent with epidemiological reports indicating that lycopene does not protect against breast cancer.

Introduction

Largely on the basis of epidemiological studies showing that green and yellow vegetables protect against a variety of cancers (1,2), there has been growing interest in the ability of carotenoid pigments, and especially the vitamin A precursor β-carotene, to act as anticancer agents (3). However, the failure of two large-scale prospective intervention trials involving β-carotene (4,5) has refocused interest on a number of other carotenoids, particularly those that do not act as vitamin A precursors. Among these is the hydrocarbon carotenoid lycopene, which is found at high levels in tomatoes, pink grapefruit, and watermelon (6–8). Interest in lycopene as a potential chemopreventive agent is based on the following considerations: 1) lycopene is present at high levels in the serum and milk of most Western populations, often as high as or higher than β-carotene (9); 2) in epidemiological studies, an inverse correlation between dietary and blood levels of lycopene and the risk of cancer in various organs, including lung, colon, pancreas, bladder, gastrointestinal tract, and prostate (10–22), has been reported, although not all achieved statistical significance; 3) lycopene, in various forms, has been shown to inhibit the development of various experimental tumors, including liver, colon, breast, bladder, and lung (23–29); 4) lycopene has been shown to be the most efficient singlet oxygen quencher among common carotenoids (30,31) and has recently been shown to suppress oxidative changes in low-density lipoprotein particles in a human clinical intervention study (32); 5) like several other carotenoids, lycopene was found to upregulate intracellular gap junction communication by inducing connexin 43-mRNA expression (33,34); and 6) lycopene exhibited a dose-related inhibition of the mutagenicity of the heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine in the Salmonella typhimurium TA18 strain (35).

There have been relatively few animal model studies dealing with the effects of lycopene on breast cancer. Lycopene was shown to inhibit the proliferation of the human breast cancer cell line MCF7 (36) and to suppress tumor development in the spontaneous mammary tumor model (37). In the latter study a single extremely low dose of lycopene (5 x 10⁻⁴ %, wt/wt) was administered in the diet.

As noted by Khachik and co-workers (38), epidemiological evidence suggests that specific foods or food groups rich in lycopene, rather than lycopene per se, appear to confer protection against several cancers. They suggested, therefore, that experimental studies should test the entire carotenoid profile present in food such as tomatoes. In a previous study, Sharoni and colleagues (29), using the 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary tumor model,
reported that intraperitoneal administration of a lycopene-enriched tomato oleoresin suppressed mammary tumor development. In the present study, to more closely mimic human conditions, we have assessed the comparative inhibitory effects of a tomato carotenoid concentrate and pure lycopene, administered in the diet, in a well-established breast cancer model, namely, the N-methylnitrosourea (NMU)-induced mammary tumor system.

Materials and Methods

Animal Care and Adherence to Guidelines

The experimental protocol (see below) was approved by the American Health Foundation Institutional Animal Care and Use Committee. Animal care was conducted with strict adherence to institutional guidelines and to guidelines specified in the Guide for Care and Use of Laboratory Animals [DHHS Publ. No. (NIH) 85-23, revised 1985, Office of Science and Health Reports, Bethesda, MD 20892]. Three rats were housed together in a polystyrene cage that contained hardwood shavings and was covered with a filter top. The animal room was controlled for temperature (24 ± 2°C), light (12:12-hour light-dark cycle), and humidity (50%). Diets were provided in powdered form, and tap water was provided ad libitum. Stainless-steel "F"-type powder feeders were used to prevent scattering of food.

Carotenoids

The lycopene-rich tomato carotenoid oleoresin (TCO) was a 5.7% suspension of tomato carotenoids in medium-chain triglycerides (MCT) (a gift from Dr. J. Clark, Henkel, LaGrange, IL). With use of a high-performance liquid chromatography (HPLC) equipped with a photodiode array detector, it was determined that the suspension contained 66% lycopene, 22% ß-carotene, 6% phytofluene, 5% phytene, and 0.07% ζ-carotene (Table 1). Crystalline lycopene (mol wt 536; a gift from LycoRed, National Product Industries, BeerSheva, Israel, via Chemoprevention Branch, National Cancer Institute, Bethesda, MD) was suspended at 4% in MCT (“Neobee M-S.” lot 797890; a gift from F. Dowell, Stephan, Maywood, NJ). The crystalline lycopene was added to MCT, and the suspension was prepared by use of a Branson sonicator (Branson Instruments, Concord, CT) for 15 minutes.

Dose Selection

Previous studies conducted in this laboratory (39) indicated that TCO fed in the AIN-76A diet over the range 50, 124, 248, 496, and 1,240 ppm exerted no untoward effects on body weight gain or overall animal health. Tissue and blood distribution studies indicated that significant levels of lycopene could be detected in animals fed as low as 50 ppm. In general, the highest levels were found in the range 496–

<table>
<thead>
<tr>
<th>Carotenoid</th>
<th>Amount</th>
<th>%</th>
<th>%Total carotenoid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lycopene</td>
<td>37,504</td>
<td>3.7</td>
<td>66</td>
</tr>
<tr>
<td>ß-Carotene</td>
<td>12,443</td>
<td>1.2</td>
<td>22</td>
</tr>
<tr>
<td>Phytofluene</td>
<td>3,349</td>
<td>0.3</td>
<td>6</td>
</tr>
<tr>
<td>Phytene</td>
<td>2,794</td>
<td>0.28</td>
<td>5</td>
</tr>
<tr>
<td>ζ-Carotene</td>
<td>440</td>
<td>0.04</td>
<td>0.7</td>
</tr>
<tr>
<td>2,6-Cycloxyccopene-1,5-diol</td>
<td>471</td>
<td>0.04</td>
<td>0.7</td>
</tr>
<tr>
<td>Total carotenoids</td>
<td>57,001</td>
<td>5.7</td>
<td>100</td>
</tr>
</tbody>
</table>

a: TCO, tomato carotenoid oleoresin.
b: Data are from Ref. 39.

1,240 ppm; for this reason, two doses were selected for the present study, 250 and 500 ppm.

Protocol for Experimental Mammary Tumor Induction

One hundred virgin female Sprague-Dawley rats (starting age 28 days, Charles River, Kingston, NY) were maintained on the standard Open Formula Rat and Mouse Ration (NIH-07) diet (4.5% fat, 23.5% protein, 50% carbohydrate, and 4.5% fiber) (Zeigler Brothers, Gardners, PA) until they were placed on experimental diets. All rats were then assigned to one of five experimental groups of 20 animals each by recognized randomization procedures (25) to equalize initial weight: Group 1, lycopene (250 ppm); Group 2, lycopene (500 ppm); Group 3, TCO (250 ppm); Group 4, TCO (500 ppm); Group 5, control (Table 2). At 50 days of age, all rats received a single dose (40 mg/kg body wt) of NMU (Ash Stevens, Detroit, MI) by tail vein injection. NMU was dissolved in a few drops of 3% acetic acid and diluted with distilled water to give a stock solution of 10 mg of NMU per milliliter, which was administered within two hours of preparation (40).

Diets

The Teklad 7001 (4% fat) rodent diet (Teklad, Madison, WI) was used. Experimental diets were prepared in our diet kitchen in 15-kg lots by mixing the basal diet with different carotenoids in a Hobart planetary mixer under low light conditions. Diets were stored at 4°C in the dark until use. Rats were fed three times per week, and feeders were removed and washed after each feeding. The rats were fed the experimental diets seven days before NMU administration and remained on these diets for the duration of the experiment (18 wk). The stability of lycopene in the diet mix was tested previously (39). The homogeneity and concentration in the diet mix were determined before the start of bioassay, and random samples were analyzed periodically. Approximately 25% of lycopene in the diet is lost after six days at ambient temperature (39).
Table 2. Effect of Carotenoid on Mammary Tumor Incidence, Multiplicity, Volume, and Latency

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Agent</th>
<th>Rats at Risk</th>
<th>Mean Latency, days</th>
<th>Mean Multiplicity</th>
<th>Mean Tumor Volume, cm³</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control (MCT)⁵</td>
<td>18/20</td>
<td>90</td>
<td>94 ± 30</td>
<td>2.2 ± 1.3</td>
</tr>
<tr>
<td>2</td>
<td>Lycopene (250 ppm)</td>
<td>19/20</td>
<td>95</td>
<td>93 ± 30</td>
<td>2.2 ± 1.8</td>
</tr>
<tr>
<td>3</td>
<td>Lycopene (500 ppm)</td>
<td>18/20</td>
<td>90</td>
<td>91 ± 27</td>
<td>2.8 ± 2.2</td>
</tr>
<tr>
<td>5</td>
<td>TCO (250 ppm)</td>
<td>18/20</td>
<td>90</td>
<td>89 ± 28</td>
<td>2.8 ± 2.4</td>
</tr>
<tr>
<td>5</td>
<td>TCO (500 ppm)</td>
<td>19/20</td>
<td>95</td>
<td>84 ± 29</td>
<td>3.2 ± 1.9</td>
</tr>
</tbody>
</table>

a: Incidence was evaluated by Fisher’s exact test; all comparisons were not significant. Mean multiplicity was evaluated by analysis of variance followed by Dunnett’s test; all comparisons were not significant. Mean volume was evaluated by analysis of variance followed by Dunnett’s test; all comparisons were not significant. Mean latency was evaluated by Kaplan-Meier test; all comparisons were not significant.

b: Values are means ± SD.

c: MCT, medium-chain triglyceride.

Observation Schedule

At weekly intervals, beginning four weeks after NMU injection, each rat was weighed, and the location of palpable tumor(s) and the date they were found were recorded.

Serum Collection

Blood was obtained by heart puncture under ketamine anesthesia. Blood was collected in evacuated, sterile Autosep tubes (Terumo Medical, Elton, MD). Serum and formed elements were separated by centrifugation at 200 g for 20 minutes at 4°C. Serum was stored at −20°C in the dark until assayed.

Necropsy and Histopathology

Approximately 18 weeks after NMU administration, the experiment was terminated. Rats were sacrificed by carbon dioxide inhalation, mammary tumors (classified as palpable or nonpalpable but grossly visible) were excised, and three diameters (length × height × width) were measured by vernier caliper. The tumors were then fixed in 10% buffered Formalin, embedded in paraffin blocks, and stained with hematoxylin-eosin for histological examination. Histological diagnosis of mammary tumors was based on criteria outlined by Young and Hallowes (41) and Russo and associates (42).

Statistical Analysis

Tumor-free survival was estimated separately for each group by the Kaplan-Meier product-limit estimate for censored data (43). The survival distributions for each treatment group were then compared with controls by the log-rank test (44,45). The purpose of the analysis was to test the null hypothesis that the survival distributions of all those groups were equal. In addition to the overall test of significance, pairwise comparisons between groups were made.

Tumor incidence (expressed as the percentage of tumor-bearing animals) was compared among the groups by Fisher’s exact test. Overall dose-related associations between tumor incidence and type of oil were tested by use of Armitage’s test for linear trends in proportions (46,47). Tumor multiplicity among the groups (categorized as 0, 1–2, 3–4, and 5 tumors/rat) was compared by use of the Mantel-Haenszel (48) χ² test. In addition, overall dose-related associations for tumor multiplicity among treatment groups were tested by linear regression analysis. In addition, tumor volume and multiplicity were compared for each treatment group using one-way analysis of variance followed by Dunnett’s multiple comparisons test (49).

Tumor volumes were assessed by applying the following formula: volume (cm³) = D₁² × D₂, with D₁ representing the largest tumor diameter and D₂ the next-largest tumor diameter in centimeters.

The overall weight gains in the animals of all groups were compared by use of single-classification analysis of variance with repeated measures followed by Dunnett’s test (49,50). The test of interest was the interaction between weight and time to evaluate the null hypothesis of no difference in weight gain over time among the groups. Pairwise comparisons among the groups were also conducted.

All statistical tests were two tailed and were considered statistically significant at p < 0.05. Significance tests for all pairwise comparisons were adjusted for multiple comparisons by multiplying the actual P value by the number of comparisons made (in this case, 5) for the evaluation of statistical significance.

Carotenoid Extraction

For the qualitative and quantitative assessment of carotenoids, diet and serum were processed according to methods developed previously (9). Carotenoids were extracted from the diet by mechanical agitation with a mixture of acetonitrile (85%), hexane (2.5%), methanol (10%), and methylene chloride (2.5%) followed by centrifugation and removal of the organic fraction. The efficiency of extraction of carotenoids into the organic layer, with use of echinonene, an
oxygenated derivative of β-carotene not present in tomatoes as internal standard, varied from 36% to 60% for diet and from 75% to 100% for serum.

Extraction from 1 ml of serum involved addition to the sample of 1 ml of ethanol and internal standard (echinone) followed by centrifugation to remove precipitated protein. Two milliliters of hexane were then added to the ethanol fraction, the hexane layer was decanted twice, and the combined extract was evaporated to dryness under N₂ gas. The residue, containing carotenoids, was then brought up in the injection solvent before HPLC analysis.

Carotenoid Quantitation

Lycopene and secondary carotenoids were analyzed by reverse-phase HPLC, as previously described (39). Analyses were conducted on a model 510 HPLC (Waters, Milford, MA) equipped with a Microsorb 5-μm cis column (Rainin, Emeryville, CA) and an ultraviolet-visible detector (model SPD-10A, Shimadzu, Kyoto, Japan). Retention times using this system were 32 minutes for lycopene (470 nm), 3 minutes for ζ-carotene (400 nm), 41-42 minutes for phytofluene (350 nm), and 43-44 minutes for phytoene (290 nm).

Results

Mammary Tumor Yields

At either dose administered, TCO exerted a null effect on NMU mammary tumorigenesis compared with controls when expressed in terms of incidence, latency, multiplicity, volume, or total tumors per group (Table 2, Figures 1 and 2). Likewise crystalline lycopene had no measurable effect on any of the above parameters compared with controls regardless of dose (Table 2, Figures 1 and 2).

Nonadenocarcinomas were rare: two in Group 1 (controls), one in Group 2 (250 ppm lycopene), two in Group 3 (500 ppm lycopene), two in Group 4 (250 ppm TCO), and none in Group 5 (500 ppm TCO); hence, analysis of mammary adenocarcinoma-only data resulted in a similar null outcome as total mammary tumors. Adenocarcinoma-only tumor incidence was 85% in Group 1, 95% in Group 2, 90% in Group 3, 85% in Group 4, and 95% in Group 5. Unscheduled termination due to severely necrotizing tumors or other deleterious health effects were as follows: one in Group 1, two in Group 2, two in Group 3, three in Group 4, and four in Group 5. Histological examination revealed no pattern of decreased degree of differentiation in the tumors obtained from treated or control groups. Animal weight gains were similar in all groups (Figure 3), indicating that the presence of lycopene in the diet did not impair feeding behavior.

Carotenoid Analyses

Serum levels of lycopene in the treated groups varied widely (Table 3), with mean levels in the lycopene groups almost an order of magnitude lower than in the TCO groups.
A rough dose-response trend was observed, with the group supplemented at 250 ppm exhibiting approximately one-half the serum concentration of the group supplemented at 500 ppm.

Discussion

The present results indicate that neither pure crystalline lycopene nor a lycopene-rich tomato oleoresin inhibited the growth and development of NMU-induced mammary tumors. These results contradict several previous experimental studies dealing with the effects of lycopene on in vitro proliferation of human breast cancer cells and in vivo mammary tumor studies. Levy and co-workers (36) reported that lycopene inhibited [3H]thymidine uptake in MCF7 human breast cancer cells, with a half-maximal inhibitory concentration of 1-2 μM, and that α- and β-carotene were far less effective inhibitors. The effect of lycopene on cell proliferation (i.e., increase in cell numbers per unit time) was not reported. The authors also reported that lycopene suppressed insulin-like growth factor-I-stimulated growth and suggested that lycopene's inhibitory effects were mediated by an autocrine-paracrine system. Comparison of these studies with ours is difficult, since in the former case, lycopene was used to inhibit an established in vitro cancer cell line and, in the latter, as a chemopreventive agent in a whole animal feeding study.

Nagashawa and others (37) reported that lycopene fed in the AIN-76A diet at 0.5 ppm suppressed the development of mammary tumors in a high mammary tumor strain of SHM virgin mice. In that study, small numbers of animals were used (9 controls and 14 experimental), and blood levels of lycopene were not measured. Hence, the possibility arises that the model as used did not have sufficient statistical power to determine statistical significance with any confidence. Also, at such low dietary levels, it appears doubtful that measurable levels of lycopene could be detected in blood. This is particularly true, since lycopene appears to be absorbed relatively poorly from the diet (51-54). Sharoni and associates (29) reported that injection of a lycopene-rich tomato oleoresin at 10 mg/kg body wt ip twice per week for 2 weeks before mammary tumor induction by DMBA and for an additional 16 weeks resulted in significantly fewer tumors per rat than in untreated rats. However, lycopene injections had no effect on tumor incidence, latency, or mean tumor area. Comparison of the study of Sharoni and associates with our study is difficult because of differences in the tumor model (they used the host-activated carcinogen DMBA, and we used the direct-acting carcinogen NMU) and route of administration (they used intraperitoneally injected lycopene, and we incorporated lycopene in the diet). Hence, the entire process of digestion and absorption into lymphatic chylomicrons was circumvented in the study of Sharoni and associates.

Diet itself may play a role as an intervening variable in studies on lycopene as a chemopreventive agent (51,52).

### Table 3. Lycopene Concentration in Serum of Rats Fed Lycopene-Containing Diets

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Agent</th>
<th>n</th>
<th>Mean ± SD</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>6</td>
<td></td>
<td>7</td>
<td>0-10</td>
</tr>
<tr>
<td>2</td>
<td>Lycopene (250 ppm)</td>
<td>6</td>
<td>6 ± 4</td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>Lycopene (500 ppm)</td>
<td>6</td>
<td>10 ± 3</td>
<td>12</td>
<td>6-13</td>
</tr>
<tr>
<td>4</td>
<td>TCO (250 ppm)</td>
<td>6</td>
<td>63 ± 14</td>
<td></td>
<td>60</td>
</tr>
<tr>
<td>5</td>
<td>TCO (500 ppm)</td>
<td>6</td>
<td>94 ± 31</td>
<td>87</td>
<td>58-148</td>
</tr>
</tbody>
</table>

* a: Statistical significance is as follows: Group 4 less than Group 5: p < 0.05 (t-test), p < 0.03 (t-test with log transformation), and p < 0.045 (nonparametric test). Groups 2 and 3 less than Groups 4 and 5: p < 0.0001 (t-test and t-test with log transformation) and p < 0.0005 (nonparametric test).

Comparing the results of our previous study (39) with the present study, it appears that lycopene is more readily extracted from the AIN-76A (75-100%) semipurified diet than the grain-based Teklad diet (30%). The reasons for this are unclear but may be related to differences in the matrix into which lycopene is incorporated. It also appears that blood levels of lycopene were lower in animals fed lycopene in the grain-based Teklad diet than in animals fed lycopene in the casein-based AIN-76A diet (39). For example, in TCO-fed rats in our previous study (39), blood levels of 174-366 and 109-266 ng/ml were measured in the groups supplemented at 250 and 500 ppm, respectively, an order of magnitude greater than that reported here. This may be due to a strain-specific effect; i.e., the outbred Sprague-Dawley rat was used in the present study, and the inbred Fischer 344 rat was used in the previous study. In addition, the fat content of the diet could be a determinant of lycopene bioavailability. The Teklad diet contains 4% fat (mainly soybean oil), whereas the AIN-76A diet contains 5% fat (corn oil). Because lycopene is extremely lipophilic, its absorption across the intestinal wall and incorporation into chylomicrons and ultimately low-density lipoprotein particles may be a function of the quality and quantity of lipid in the diet (51).

It has been reported that lycopene is inefficiently absorbed from the diet in the gastrointestinal tract of rats compared with other carotenoids and that the presence of one carotenoid may influence the absorption of a second carotenoid (53,54). In this study, we found an almost 10-fold difference in the absorption of lycopene from the diet into the bloodstream when administered in equal amounts as a lycopene-rich TCO or as a lipid suspension of crystalline lycopene. The physicochemical reasons for this remain to be determined. Nonetheless, despite the marked difference in serum lycopene concentrations, no effect was seen in terms of the inhibition of mammary tumorigenesis with either form of lycopene. As seen in Table 1, TCO contains several different carotenoids, including β-carotene. Although not assayed for, on the basis of our previous studies (39), it is most likely that these also are absorbed, carried in the circulation, and distributed to various organs, including the mammary gland. It appears, therefore, that in this
model lycopene lacks chemopreventive activity whether administered alone or in the presence of the entire tomato carotenoid profile.

Epidemiological studies tend to support the results reported here. Although lycopene (or lycopene-rich foods) may protect against cancer of the cervix (11,15), prostate (12,13,55,56), and gastrointestinal tract (2,14,57), it does not appear to protect against breast cancer (58–61). Future studies on the interaction between dietary fat and lycopene uptake may shed more light on lycopene’s role in cancer prevention.

Acknowledgments and Notes

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