Lycopene Inhibits Experimental Metastasis of Human Hepatoma SK-Hep-1 Cells in Athymic Nude Mice\textsuperscript{1,2}

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Abstract

Lycopene has been shown to inhibit tumor metastasis in vitro, but it is unclear whether lycopene is antimitastatic in vivo. Here, nude mice were orally supplemented 2 times per week for 12 wk with a low or high dose of lycopene [1 or 20 mg/kg body weight (BW)] or with \textit{b}-carotene (20 mg/kg BW). Two weeks after the beginning of supplementation, mice were injected once with human hepatoma SK-Hep-1 cells via the tail vein. Plasma levels of matrix metalloproteinase (MMP)-2 and vascular endothelial growth factor (VEGF) increased gradually in tumor-injected mice (tumor controls) following tumor injection but were markedly lowered by lycopene or \textit{b}-carotene supplementation. Ten weeks after tumor injection, mice were killed and tumor metastasis was found to be confined to the lungs. Compared with the tumor controls, high-lycopene supplementation lowered the mean number of tumors from 14 ± 8 to 3 ± 5 (\textit{P} < 0.06) and decreased tumor cross-sectional areas by 62% (\textit{P} < 0.05). High-lycopene supplementation also decreased the positive rate of proliferating cellular nuclear antigen (PCNA), the level of VEGF, and protein expressions of PCNA, MMP-9, and VEGF in lung tissues. However, high-lycopene increased the protein expression of \textit{nm23}-H1 (an antimitastatic gene) by 133% (\textit{P} < 0.001). For most variables measured, effects of lycopene were dose dependent and the effect of \textit{b}-carotene was between those of high-dose and low-dose lycopene. These results show that lycopene supplementation reduces experimental tumor metastasis in vivo and suggest that such an action is associated with attenuation of tumor invasion, proliferation, and angiogenesis. J. Nutr. 138: 538–543, 2008.

Introduction

Tumor metastasis, a major cause of death for cancer patients, is a multistep process that includes acquisition of the capacity to separate from the primary tumor and invasion of the underlying basal lamina to gain access to the cardiovascular or lymphatic circulation (1). Circulating tumor cells must then exit the cardiovascular system and proliferate within the stroma of the lung to produce a secondary tumor. Altered expression of the putative metastasis suppressor gene \textit{nm23}-H1 (2,3) is considered to play an important role during the acquisition of metastatic ability. Furthermore, changes in matrix metalloproteinases (MMP)\textsuperscript{5} (4–6) and angiogenic factors, such as vascular endothelial growth factor (VEGF) (7–9) and interleukin (IL)-12 (10,11), may contribute to tumor metastasis. MMP-2 and MMP-9 have been implicated in malignant tumor progression, partly because they degrade collagen type IV, V, VII, and X, fibronectin, and gelatin of basement membranes (12–14).

Epidemiologic studies have shown that high-lycopene intakes are associated with lowered risks of several types of cancer (15,16). Recent cohort studies have also shown decreased risks for liver cancer (17) and prostate cancer (18) in people with either high serum levels of lycopene or a regular and high intake of lycopene or tomatoes. In vitro, lycopene has been reported to inhibit the invasion of rat ascites hepatoma AH109A cells in a dose-dependent manner up to 5 \textmu mol/L (19) and to reduce the motility of brain tumor cells (20). We recently showed that lycopene inhibits the migration and invasion of human hepatoma SK-Hep-1 cells in vitro and that these effects are associated with the upregulation of \textit{nm23}-H1 (21). Hwang and Lee (22) also demonstrated that lycopene inhibits adhesion, invasion, and migration of SK-Hep-1 cells and that these actions are associated with decreased activity of MMP-2 and MMP-9. However, it is unclear whether lycopene is antimitastatic in vivo.

In this study, we employed an in vivo model using nude mice administered lycopene orally for 12 wk and injected with SK-Hep-1 cells once via tail vein 2 wk after lycopene supplementation. This tumor injection model does not duplicate all of the steps required for metastasis from a primary tumor, but it has the principle advantage of standardizing the onset of the

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\textsuperscript{5} Abbreviations used: BW, body weight; H&E, histological; IHC, immunohistochemical; IL, interleukin; MMP, matrix metalloproteinase; PCNA, proliferating cellular nuclear antigen; VEGF, vascular endothelial growth factor.

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invasion by injection tumor cells into the blood circulation and can be used to measure the ability of malignant cells to extravasate and form tumors in the lungs (23).

Materials and Methods

Chemicals. Lycopene was purchased from Wako. Anti-nm23 mouse monoclonal antibody and anti-MMP-9 monoclonal antibody were purchased from BD Co. and US-Biological, respectively. Antiproliferating cellular nuclear antigen (PCNA), anti-VEGF, and anti-mouse IgG-horseradish peroxidase polyclonal antibody were purchased from Santa Cruz Bio-technology. VEGF and IL-12 ELISA kit were purchased from R&D. All chemicals used are of reagent or higher grade.

Hepatocarcinoma cells for injection to nude mice. SK-Hep-1 human hepatoma cells were grown in DMEM containing 10% (v/v) fetal bovine serum, 0.37% (wt%) NaHCO₃, penicillin (100 kU/L), streptomycin (100 kU/L) in a humidified incubator under 5% CO₂, and 95% air at 37°C. The survival rate of cells was always higher than 95% by Trypan blue exclusion. Before injection into the mice, the cells were harvested by trypsinization and washed 3 times with cold serum-free medium and then injected in a total volume of 0.1 mL using a 1-mL 27G2 latex-free syringe (BD) within 30 min of harvest.

Animals, diet, and treatment. Adult (6- to 8-wk-old) male athymic nude mice (17–22 g) were purchased from the Animal Center of the National Science Council. We recently showed that athymic nude mice are better accumulators of lycopene than F344 rats and BALB/c mice and thus should be more useful for studying the in vivo effects of lycopene (24). The study protocol was approved by the Animal Research Committee of National Chung Hsing University.

The mice were housed individually in hanging wire mesh cages with controlled temperature (25 ± 2°C), humidity (65 ± 5%), and alternating 12-h-light/dark cycles. Upon arrival, the mice were acclimated for 2 wk. During the entire experimental period (including acclimation), the mice consumed a standard rodent diet (Lab 5001, Purina Mills) and water ad libitum. The standard diet contained 59.8% carbohydrate, 23.4% protein, 4.5% crude fat, and 4.3 mg β-carotene/kg and had no detectable amounts of lycopene, as indicated by the supplier. The mice (7–8 mice per group) were orally administered with lycopene [1 and 20 mg/kg body weight (BW)] or β-carotene (20 mg/kg BW) in corn oil twice per week (25) for 12 wk. The doses and the feeding regimen used here were based on those of our recent study (24) with slight modification. Corn oil alone (10 mL/kg BW) was given to the noninjected control mice. Two weeks into the supplementation, the mice were injected once with SK-Hep-1 cells [5 × 10⁶ cells] into the lateral tail vein (26,27) and blood samples were drawn once per week from the retro-orbital plexus for the measurement of MMP and VEGF. BW were also measured once weekly.

At the end of experiment (10 wk after tumor injection), the mice were killed by CO₂ asphyxiation. Blood samples were collected from both the retro-orbital plexus and heart in a 10-mL vacutainer tube containing K₂EDTA and were centrifuged (400 × g; 10 min) to obtain plasma. Lungs were grossly examined and we determined the number of pulmonary tumors by counting visible tumor foci (28). A portion of the organ was removed and fixed in 10% buffered formalin. The left lobe of the lung (the largest areas from each lobe) was processed for histological (H&E) staining. Plasma and the remaining tissues were stored at −80°C until analyses.

Lung metastasis. Macroscopic in all 5 lung lobes of each mouse was pictured at the time of killing. Quantitative analysis of lung metastasis and the cross-sectional area of tumors in randomly selected fields were measured using the image Pro Plus software (Media Cybernetics).

Gelatin zymography. Plasma activities of MMP-2 and MMP-9 were determined by gelatin zymography according to a protocol developed by Kleiner and Stertler-Stevenson (29). The plasma samples were pooled because of the limited plasma volume per mouse. Before the assay, plasma samples were diluted 1:10 with PBS. The relative MMP activities were quantitated by Matrox Inspector 2.1 software.

**Table 1** Activities of MMP-9 and MMP-2 and concentrations of VEGF and IL-12 in plasma of control and tumor-bearing male nude mice supplemented with 1 or 20 mg lycopene/kg BW (LP1, LP20) or 20 mg β-carotene/kg BW (BC20) for 12 wk.1,2

<table>
<thead>
<tr>
<th>Group</th>
<th>MMP-9</th>
<th>MMP-2</th>
<th>VEGF</th>
<th>IL-12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 24</td>
<td>100 ± 19</td>
<td>39 ± 8</td>
<td>7540 ± 1649</td>
</tr>
<tr>
<td>Control + tumor</td>
<td>217 ± 35</td>
<td>154 ± 19</td>
<td>214 ± 45</td>
<td>3834 ± 1253</td>
</tr>
<tr>
<td>LP1 + tumor</td>
<td>181 ± 51</td>
<td>126 ± 18</td>
<td>90 ± 14</td>
<td>7533 ± 1915</td>
</tr>
<tr>
<td>LP20 + tumor</td>
<td>139 ± 29</td>
<td>90 ± 21</td>
<td>51 ± 15</td>
<td>6547 ± 1364</td>
</tr>
<tr>
<td>BC20 + tumor</td>
<td>180 ± 51</td>
<td>113 ± 23</td>
<td>96 ± 20</td>
<td>5836 ± 1194</td>
</tr>
</tbody>
</table>

1 Values are means ± SD, n = 3–7. Means in a column with superscripts without a common letter differ, P < 0.05.
2 After 2 wk of supplementation, mice were injected once with SK-Hep-1 cells via the tail vein and were killed 10 wk after tumor injection.
1.09 ± 0.63%, 1.00 ± 0.31%, and 1.07 ± 0.13%, respectively, with no significant differences among the treatments.

Time-course changes in plasma MMP-2 activities and VEGF levels during the experimental period. We determined only the activity of MMP-2 in the plasma during the experimental period because of the limited amounts of plasma. Plasma MMP-2 activities and VEGF levels were significantly induced in tumor controls during the 12-wk experimental period; lycopene supplementation decreased MMP-2 and VEGF in a dose-dependent manner and the effect was evident immediately after tumor injection (Fig. 1). The effect of β-carotene (20 mg/kg BW) was smaller than that of low-lycopene supplementation. Interestingly, plasma MMP-2 activity in the high-lycopene group was lower than that in the nontumor-injected controls over the entire experimental period.

Plasma levels of MMP-9, MMP-2, VEGF, and IL-12 at the time of killing. The plasma activities of MMP-9 and MMP-2 in the tumor controls were significantly elevated by tumor injection (Table 1). High-lycopene treatment significantly decreased the activities of MMP-9 and MMP-2, which did not differ from those of the nontumor-injected controls. Low lycopene and β-carotene significantly decreased only MMP-2 activity. Tumor injection markedly increased the level of VEGF and this increase was essentially abolished by high-lycopene treatment, whereas low lycopene and β-carotene also significantly decreased the VEGF level compared with tumor controls. By contrast, plasma levels of IL-12 significantly decreased with tumor injection and were restored by lycopene (both low and high doses) and β-carotene supplementation.

Inhibition of experimental metastasis by lycopene. At the time the mice were killed, metastatic nodules were clearly noted microscopically in the lungs of mice that received the tail vein injection with SK-Hep-1 cells (data not shown), whereas only slight tumor formation was observed in the livers of some of the mice and no nodules were found in any other organs examined. Only 1 of the 8 mice in the tumor control group had not developed a lung tumor, whereas 2 of 8 and 4 of 7 mice in the low- and high-lycopene groups, respectively, had no tumor formation (Table 2). The number of tumors was significantly decreased by both high-lycopene and β-carotene treatments and the effects of 2 treatments did not differ.

Supplementation with low and high lycopene decreased the tumor cross-sectional area by 19 and 62% (P < 0.05), respectively (Table 2). β-Carotene supplementation also decreased the tumor cross-sectional area 49% to 1.22 ± 0.61 mm² (P < 0.05).

Correlation of MMP-9, MMP-2, VEGF, and IL-12 to tumor number. Tumor number in the lungs of nude mice was positively correlated with the activities of MMP-9 (r = 0.98; P = 0.004), MMP-2 (r = 0.93, P = 0.021), and VEGF (r = 0.88, P = 0.041) and negatively correlated with the level of IL-12 (r = −0.75; P = 0.148).

IHC assays. In vivo cell proliferation and angiogenesis were evaluated using anti-PCNA and anti-VEGF antibodies, respectively (Fig. 2). The percentage of positive PCNA tumor cells in the tumor control group was 6.5-fold greater than in the noninjected control group (P < 0.05) (Table 3). Low- and high-lycopene supplementation decreased percent positive PCNA by ~32 (P = 0.069) and 74% (P < 0.01), respectively, compared with the tumor controls. High-lycopene supplementation was more effective than β-carotene in decreasing percent positive PCNA (74% vs. 43% for low-lycopene and 61% for β-carotene).

![FIGURE 1](https://academic.oup.com/jn/article-abstract/138/3/538/4670246)

**FIGURE 1** Time-course changes in plasma activities of MMP-2 (A) and VEGF (B). Male athymic nude mice were supplemented with 1 or 20 mg lycopene/kg BW (LP1, LP20) or with 20 mg β-carotene/kg BW (BC20) for 12 wk. Two weeks into the supplementation, the mice were injected once with SK-Hep-1 cells (5 × 10⁵ cells). Each data point represents a single replicate obtained from pooled plasma from 7–8 mice per group.

<table>
<thead>
<tr>
<th>Group</th>
<th>Tumor incidence, %</th>
<th>Tumor cross-sectional area (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8 (0)</td>
<td>9 ± 4 a</td>
</tr>
<tr>
<td>Control + cancer</td>
<td>7 (88)</td>
<td>3 ± 5 b</td>
</tr>
<tr>
<td>LP1 + cancer</td>
<td>6 (75)</td>
<td>1.22 ± 0.26 b</td>
</tr>
<tr>
<td>LP20 + cancer</td>
<td>6 (86)</td>
<td>1.22 ± 0.26 b</td>
</tr>
<tr>
<td>BC20 + cancer</td>
<td>7 (66)</td>
<td>1.22 ± 0.26 b</td>
</tr>
</tbody>
</table>

1 Values are means ± SD, n = 7–8 unless otherwise noted. Means in a column with superscripts without a common letter differ, P < 0.05.
2 After 2 wk of supplementation, mice were injected once with SK-Hep-1 cells via the tail vein and were killed 10 wk after tumor injection.
3 The tumor incidence in the treated groups did not differ from that in the tumor control group (chi-square test).
The percentage of positive VEGF cells was markedly higher in the tumor controls than in the noninjected controls and low- and high-lycopene supplementation decreased the percent positive VEGF by 21 (\(P = 0.124\)) and 54% (\(P < 0.05\)), respectively (Table 3). β-Carotene also tended to decrease the percent positive VEGF 32% (\(P = 0.071\)).

**PCNA, VEGF, MMP-9, and nm23-H1 protein expressions in lung tissues.** Protein expressions of PCNA in the lungs were markedly elevated in the tumor controls and high-lycopene supplementation significantly decreased the level, whereas supplementation with either low lycopene or β-carotene had no effect (Fig. 3; Table 4). Similarly, high-lycopene supplementation significantly decreased the protein expressions of VEGF and MMP-9, whereas supplementation with either low lycopene or β-carotene had no effect. By contrast, tumor injection significantly decreased protein expression of nm23-H1 in lung tissues and low-lycopene supplementation partially restored the protein expression, whereas high-lycopene and β-carotene supplementation completely restored the expression.

**Concentrations of lycopene and β-carotene in tissues of nude mice after supplementation for 12 wk.** Lycopene was not detectable in the lung in the nude mice without lycopene supplementation (Table 5). Lycopene supplementation resulted in dose-dependent increases in lycopene concentrations in the lungs. β-Carotene was detectable in the lungs of nude mice before supplementation and was markedly increased in the lungs after supplementation.

**Discussion**

Although lycopene has been shown to inhibit tumor metastasis in vitro (19–22), little is known whether it is antimetastatic in vivo. We chose SK-Hep-1 cells to study the metastasis in nude mice, because we had investigated the antimetastatic actions and mechanisms of lycopene in vitro (21,32). Tumor metastasis in the nude mice was evidenced by markedly increased plasma levels of MMP-2 and VEGF almost immediately following tumor injection as well as increased MMP-9 and decreased IL-12 levels in the plasma at the end of experiment (10 wk after tumor injection). Tumor metastasis occurred essentially in the lungs where protein expressions of PCNA and VEGF were markedly increased in the tumor controls.

**TABLE 3** Positive rates of PCNA and VEGF in lung tissues of male nude mice supplemented with 1 or 20 mg lycopene/kg BW (LP1, LP20) or 20 mg β-carotene/kg BW (BC20) for 12 wk.

<table>
<thead>
<tr>
<th>Group</th>
<th>PCNA</th>
<th>VEGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9 ± 1a</td>
<td>14 ± 3c</td>
</tr>
<tr>
<td>Control + tumor</td>
<td>68 ± 6a</td>
<td>68 ± 14a</td>
</tr>
<tr>
<td>LP1 + tumor</td>
<td>46 ± 21ab</td>
<td>54 ± 18ab</td>
</tr>
<tr>
<td>LP20 + tumor</td>
<td>18 ± 14bc</td>
<td>31 ± 11bc</td>
</tr>
<tr>
<td>BC20 + tumor</td>
<td>35 ± 22de</td>
<td>46 ± 19de</td>
</tr>
</tbody>
</table>

1 Values are means ± SD, \(n = 3–7\). Means in a column with superscripts without a common letter differ, \(P < 0.05\).
2 After 2 wk of supplementation, mice were injected once with SK-Hep-1 cells via the tail vein and were killed 10 wk after tumor injection.
expressions of MMP-9, PCNA, and VEGF were significantly increased and the protein expression of nm23-H1 gene was markedly decreased. Importantly, we showed that oral supplementation with lycopene, especially at the higher dose (20 mg/kg BW, 2 times per week) in nude mice injected with tumor cells markedly decreased the tumor metastasis in the lungs and effectively reinstated the protein expression levels of MMP, VEGF, PCNA, and nm23-H1.

A possible mechanism underlying the antimetastatic actions of lycopene is the inhibition of MMP, especially MMP-2 and MMP-9, which degrades collagen type IV, a major component of basement membranes (12–14). Our findings are consistent with those from in vitro studies using SK-Hep-1 cells showing that lycopene inhibits the secretion of MMP-2 and MMP-9 (23) and the expression of MMP-9 protein and mRNA (32). Another possible antimetastatic mechanism of lycopene is by inhibition of angiogenesis, as we observed here that the levels of VEGF, an angiogenic marker, in plasma and tissue were decreased by lycopene supplementation in a dose-dependent manner. In addition, the increases of MMP-2 and MMP-9 are associated with angiogenesis (4–6). Indeed, inhibition of MMP-2 has shown great promise with synthetic inhibitors as antitumor agents (antiangiogenesis, antiproliferation, and antimetastasis) in preclinical models (33).

Another possible antimetastatic mechanism of lycopene is the inhibition of cell proliferation that leads to decreased tumor size (tumor cross-sectional area). PCNA, an auxiliary protein of the DNA polymerase δ, is a proliferation-associated marker used in different neoplasms in relation to clinical behavior (34). The PCNA expression, which peaks in late G1 and S phases of the cell cycle (35), is positive in tumor cells (36). In this study, we demonstrated that lycopene supplementation significantly decreased tumor cell proliferation (as shown by decreased percent positive PCNA cells). Our results are similar to those of Tang et al. (37), which showed that lycopene inhibits the growth of human prostate cancer cells in vitro and in BALB/c nude mice. Liu et al. (38) also showed that lycopene protects against smoke-induced lung carcinogenesis in ferrets, possibly through attenuation of PCNA protein expression in lung tissues. Interestingly, Lian et al. (39) showed that apo-10'-lycopenoic acid, a lycopene cleavage product metabolized in mammalian tissues, inhibits lung tumorigenesis in the A/J mouse model in vivo. Similarly, the mechanism of β-carotene on lung tumor prevention may be mediated through its metabolites products, such as β-apo-14'-carotenolic acid, or through their conversion to retinoic acid (40,41).

Still another possible mechanism by which lycopene inhibits metastasis in vivo is through increased expression of nm23-H1, a tumor metastasis suppressor gene (2,3). Decreased expression of nm23 gene was found in some primary lesions of human metastatic hepatoma (42,43). The present findings that lycopene supplementation (at 1 and 20 mg/kg BW) significantly restored nm23-H1 protein expression in lung tissues of tumor-injected nude mice agree with those of our recent report (22) that lycopene upregulates the expressions of nm23-H1 in SK-Hep-1 cells, which may lead to inhibition of cell migration and invasion.

In summary, this study demonstrates that lycopene supplementation markedly inhibits the growth of metastatic tumors developed in the lungs of athymic nude mice injected with human hepatoma cells. These effects of lycopene are likely associated with inhibition of tumor invasion (attenuation of MMP and enhancement of nm23-H1), proliferation (attenuation of PCNA), and angiogenesis (attenuation of MMP and VEGF but enhancement of IL-12) in the lungs of the nude mice. These results warrant further studies of lycopene as a potential chemopreventive or chemotherapeutic agent.
We thank C-H. Chuang, C-Y. Lin, and Y-E. Fan for technical assistance.

Literature Cited


