Lycopene Inhibits the Growth of Normal Human Prostate Epithelial Cells in Vitro[1,2]

Ute C. Obermüller-Jevic, Estibaliz Olano-Martin, Ana M. Corbacho, Jason P. Eiserich,* Albert van der Vliet, Giuseppe Valacchi, Carroll E. Cross and Lester Packer[†3]

*Department of Internal Medicine, Division of Pulmonary and Critical Care Medicine and *Department of Human Physiology, Division of Nephrology, University of California, Davis, CA 95616; †School of Pharmacy, Department of Molecular Pharmacology and Toxicology, University of Southern California, Los Angeles, CA 90089-9121*

ABSTRACT Lycopene has repeatedly been shown to inhibit the growth of human prostate cells in vitro. However, previous studies with lycopene have focused on cancer specimens, and it is still unclear whether this carotenoid affects the growth of normal human prostate cells as well. Therefore, we investigated the effects of lycopene on normal human prostate epithelial cells (PrEC) by treating them with synthetic all-E-lycopene (up to 5 μmol/L) and assessing proliferation via [^{3}H]thymidine incorporation. The effects of lycopene on cell cycle progression were investigated via flow cytometry. To elucidate whether lycopene modulates cyclins involved in cell cycle progression, protein expressions of cyclins D1 and E were analyzed. The results show that lycopene significantly inhibited the growth of PrEC in a dose-dependent fashion. Flow cytometry revealed a significant cell cycle arrest in the G0/G1 phase. This effect was confirmed by inhibition of cyclin D_1 protein expression, whereas cyclin E levels remained unchanged. The results demonstrate that lycopene inhibits growth of nonneoplastic PrEC in vitro. We hypothesize that lycopene might likewise inhibit the growth of prostatic epithelial cells in vivo. This might have an effect on prostate development and/or on enlargement of prostate tissue as found in benign prostate hyperplasia, a potential precursor of prostate cancer. J. Nutr. 133: 3356–3360, 2003.

KEY WORDS: • *lycopene* • *prostate* • *prostate hyperplasia* • *cell cycle* • *cell proliferation*

Prostate health is related to lifestyle and diet (1). Lycopene, a red carotenoid consumed primarily from tomatoes and a few other plant-based foods, is one of the most promising chemopreventive agents found in Western diets (2). Regular consumption of lycopene-rich foods has been reported to be associated with a 30 to 40% lower risk of prostate cancer (3,4), and lycopene rather than other carotenoids is inversely correlated to risk of prostate cancer (3,5–7). In pilot studies with cancer patients, daily ingestion of lycopene from tomato sauce or tomato extracts inhibited tumor growth and invasiveness (8,9). It is not yet proven, however, whether lycopene is the compound responsible for the beneficial effects associated with a high intake of tomatoes. Lycopene may just be a marker for a diet rich in lycopene-containing foods. Nevertheless, lycopene effectively inhibits growth of prostate cancer cells in cell cultures (10,11).

It is largely unknown whether lycopene also affects the growth of normal prostate cells. This may be important because hyperproliferation of epithelial cells in prostate tissue is a major reason for enlargement of the prostate in elderly men, a condition known as benign prostate hyperplasia (BPH). With increasing age BPH is more common; it is found in 20% of men aged 40 to 50 y and in 80% of men aged 70 to 80 y (12). It causes urinary tract obstruction and infection, with accompanying activation of inflammatory processes. Dietary factors likely play a role in the development of BPH, because epidemiological data suggest that the incidence of BPH in Asian men increases after adopting a Western lifestyle. Proliferation of normal prostate cells also occurs in prostatitis, a chronic inflammatory disease of the prostate. Increased proliferation and inflammatory processes in the prostate appear to be associated with the development of preneoplastic lesions. Both BPH and prostatitis are potential precursors of prostate cancer (13). It may be hypothesized that the beneficial effect of lycopene on the risk of prostate cancer, which has been observed in epide-

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[3] To whom correspondence should be addressed. E-mail: packer@usc.edu.

[4] Abbreviations used: BPH, benign prostate hyperplasia; ID_{25}, inhibitory dose with 25% effectiveness; ID_{50}, inhibitory dose with 50% effectiveness; PrEC, human normal prostate epithelial cells; PrEGM, prostate epithelial cell medium; THF, tetrahydrofuran.

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miological studies, may occur at early preneoplastic stages of the natural history of the disease.

Lycopene is a major carotenoid detected in the human prostate gland, and the concentrations of lycopene found in the human prostate are higher than in several other tissues (14). In human plasma the predominant form of lycopene is all-E-lycopene; however, in prostate tissue only 12 to 21% of total lycopene occurs as all-E-lycopene, the remainder occurring as cis isomers (14,15). Although lycopene appears to have beneficial effects on prostate cancer, the molecular mechanisms involved are still unclear. Lycopene is not a provitamin A carotenoid, but it possesses powerful antioxidant activity (16). Moreover, lycopene has been reported to inhibit the proliferation of several types of cancer cell lines (10,11,17) via modulation of cell signaling of growth factors and interference with cell cycle progression (17–20).

In the present study we investigated whether lycopene modulates growth and cell cycle of normal human prostate epithelial cells (PrEC), a nonimmortalized and primary cell strain.

MATERIALS AND METHODS

Chemical products. Crystalline lycopene (consisting of 95.5% all-E-lycopene, 4.0% 5-*cis*-lycopene and 0.5% other cis isomers) was a gift of Roche Vitamins (Basel, Switzerland). Basic aluminum oxide (grade I) and tetrahydrofuran (THF, UV-spectroscopy grade) were obtained from Fluka (Sigma-Aldrich, St. Louis, MO). All other solvents were HPLC-grade from Fisher Scientific (Los Angeles, CA). The THF, methanol (for sample extraction only) and *n*-hexane were further purified over a basic aluminum oxide open column (21) and stored for no longer than 24 h in amber glass vials under a nitrogen atmosphere at −20°C. Cell culture supplies were purchased from Costar (Corning, Cypress, CA).

Cell culture. Human prostate epithelial cells (PrEC) clone 6448 were obtained from Clonetics (San Diego, CA). Cells were maintained according to the manufacturer's instructions using Clonetics Prostate Epithelial Cell Medium (PrEGM) with an additional 1% antibiotics (penicillin/streptomycin). Cells were used at passages 3 to 6.

Lycopene preparation. Lycopene was freshly prepared as 1 mmol/L stock solution in THF before addition to the cell culture medium. Concentrations of lycopene in THF were determined by photometric measurement at $\lambda = 471$ nm ($E^{1\%}_{1cm} = 3450$) and purity was routinely confirmed by HPLC. The stock solutions were stored in amber vials under a nitrogen atmosphere at −20°C and used within 1 d. Lycopene was diluted into the culture medium at doses of up to 5 μmol/L and vigorously stirred before the addition to PrEC cells. All cell cultures except untreated control cells were treated with the same amount of 5 mL THF/L (20). Lycopene and THF were prepared with a minimum of exposure to air and light.

Lycopene extraction and HPLC analysis. Cell pellets were resuspended in 6 mol/L guanidine hydrochloride in 20 mmol/L potassium phosphate, pH 2.3, and then extracted twice with *n*-hexane (0.5 mg BHT/L). Hexane layers were combined and evaporated to dryness under a stream of nitrogen. The residue was redissolved in 500 μL methanol:THF (90:10 v/v, 0.5 mg BHT/L) and 50 μL was injected into the HPLC system. Reverse-phase HPLC was carried out on a Waters Breeze System consisting of a 1525 binary pump and a 717+ autosampler with a chiller connected to a 2487 dual wavelength absorbance detector (Waters, Milford, MA). A Waters C30 YMC Cartenoid column (5 μmol/L, 250 × 4.6 mm) was used, coupled to a YMC Cartenoid S guard column (5 μmol/L, 20 × 4.0 mm). A gradient system was performed with methanol:*tert*-butylmethylether: water (81:15:4 v/v/v) as eluent A and methanol:*tert*-butylmethylether:water (6:90:4 v/v/v) as eluent B according to the manufacturer's recommendations. The flow rate was 1 mL/min and lycopene was detected at 471 nm. Lycopene was quantified by reference to standard curves.

Cellular uptake of lycopene. Cells were seeded at a density of 100,000 cells per 35-mm culture dish and covered with 2 mL of PrEGM. The medium was renewed after 24 h. Cells were grown to a density of ~60% and then given fresh medium containing lycopene or THF alone. After 24 or 72 h cells were washed twice, harvested in PBS and then frozen at −80°C until HPLC analysis.

[³H]Thymidine incorporation assay. Cells were seeded at a density of 1.2×10^4 cells per 35-mm culture dish and covered with 2 mL of PrEGM. After 24 h, the medium was replaced with fresh medium containing 90% lower amounts of growth factors. The medium also contained lycopene or THF alone. Neither lycopene nor THF was added to untreated control cells. After 48 h of growth factor starvation the synchronized cells were added to 0.6 μCi [^{3}H]thymidine (Amersham Biosciences, Piscataway, NJ) for 12 h. The [^{3}H]thymidine incorporation assay was performed as previously described (22). Radioactivity was measured using a Beckman LS5000TD liquid-scintillation counter (Beckman Coulter, Fullerton, CA).

Flow cytometry. Cells were seeded at a density of 2×10^5 cells per 100-mm culture dish and covered with 5 mL of PrEGM. After 24 h, the medium was replaced by 10 mL fresh medium containing 90% lower amounts of growth factors. The medium also contained lycopene or THF alone. Untreated control cells contained neither lycopene nor THF. After 48 h the synchronized cells were restimulated with complete PrEGM containing fresh amounts of lycopene or THF (or neither) for 12 h. Cells were harvested by trypsinization and centrifuged. The cell pellet was redissolved in 1 mL of PBS and 100 μL of the cell suspension was used for cell counts by the trypan blue exclusion assay. The remaining cells were washed with 4 mL of PBS, centrifuged again and redissolved in 0.5 mL of PBS. After addition of the cell suspension to 4.5 mL of ice-cold ethanol, the samples were frozen at −20°C.

For flow cytometry analysis, samples were centrifuged for 5 min at 200 × *g*, washed with 5 mL of PBS and then resuspended in 1 mL of freshly prepared staining solution [propidium iodide (1 g/L) diluted 1:50 in 0.1% (v/v) Triton X-100 in PBS containing RNAse A (0.2 g/L)]. The analysis was performed after 30 min of incubation at room temperature. 10,000 cells were analyzed for cell cycle distribution using a Beckton Dickinson FACScan flow cytometer equipped with a 488-nm argon laser and CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA). Cell cycle distribution was analyzed by Mod-fit software (Verity Software House, Topsham, ME).

Western blot analysis. Cells were grown and treated with lycopene as described above for cell cycle progression analysis. After starvation for 48 h, synchronized cells were restimulated with complete PrEGM containing lycopene or THF alone (or neither) and maintained for 12 h until harvest for protein extraction. Cells were washed twice in PBS and frozen at −20°C. Cellular protein was extracted using RIPA buffer (Santa Cruz Biotechnology, Santa Cruz, CA). Protein was quantified with a Detergent Compatible Protein Assay (Bio-Rad Laboratories, Hercules, CA), and 20 μg total protein lysate was subjected to SDS-PAGE. Cyclin D1 and cyclin E proteins were incubated with antibodies and visualized by means of the ECL+ detection system (Amersham Biosciences). The following antibodies were used: mouse monoclonal IgG2b anticyclin D1 (A-12) (sc-8396, Santa Cruz); mouse monoclonal anticyclin E2 (HE-12) (sc-9568, Santa Cruz); antimouse HRP-conjugate (Bio-Rad Laboratories).

Statistical analysis. Data were analyzed in two-group comparisons by means of Student's *t* test; $P < 0.05$ was considered statistically significant.

RESULTS

Cellular uptake of lycopene. Following incubation of cell cultures with 5 μmol/L all-E-lycopene, uptake into PrEC cells was 0.885 ± 0.291 and 0.704 ± 0.196 nmol/mg protein after 24 and 72 h, respectively ($n = 3$). No medium change was carried out during the incubation with lycopene. The observed levels of cellular uptake of all-E-lycopene were comparable to previously reported data, e.g., using LNCaP prostate cancer cells (23).

Lycopene effects on cell proliferation. Lycopene significantly inhibited the growth of PrEC cells as determined by [^{3}H]thymidine incorporation assay (**Fig. 1**). There was no

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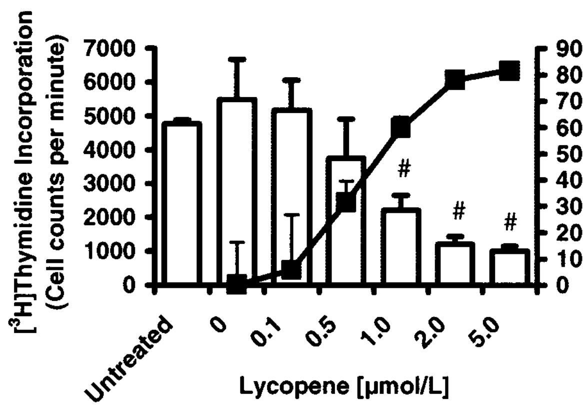

FIGURE 1 Inhibition of proliferation of normal prostate epithelial cells by lycopene in vitro. Data represent the extent of [^{3}H]thymidine incorporation (cell counts per minute; bars) and the percentage of growth inhibition (%; line). One representative result from three independent experiments is shown. Values are means ± SEM, $n = 3$ ($^{\#}P < 0.05$, lycopene-treated vs. vehicle-treated cells).

significant difference in cell growth observed between cells treated with the vehicle alone and untreated control cells. Inhibition of cell proliferation by lycopene was found to be dose dependent. A significant inhibition was observed at a dose of 1 μmol/L lycopene or higher ($P < 0.05$). The calculated inhibitory doses with 25 and 50% effectiveness (ID_{25} and ID_{50}) for lycopene were 0.4 and 0.8 μmol/L, respectively. The maximum inhibition of ~80% was achieved at a dose of 2 μmol/L lycopene. A higher amount of lycopene did not alter cell proliferation significantly. To assess the validity of the results obtained from the [^{3}H]thymidine incorporation assay, cell proliferation was also determined in a different set of four independent experiments via cell counts (trypan blue exclusion test). Cell count results revealed a similar growth inhibition by lycopene (data not shown).

Effects of lycopene on cell cycle progression. To determine whether the observed inhibition of cell proliferation was due to effects on cell cycle progression, a flow cytometry analysis was performed (**Fig. 2**). Treatment of cells with 0.5 μmol/L lycopene did not result in a significant change of the cell cycle compared with vehicle-treated cells. However, a dose of 5 μmol/L lycopene led to a significant accumulation of cells in the G0/G1 phase (71% vs. 56%; $P < 0.05$) and significantly fewer cells in the S phase (22% vs. 31%; $P < 0.05$) compared with vehicle-treated cells. The difference in the G2/M phase was statistically not significant (9% vs. 14%; $P = 0.05$).

It is noteworthy that when the cells treated with vehicle alone were compared with untreated control cells, it became obvious that the vehicle itself had already affected cell cycle progression to some extent, because a significant accumulation of cells in the G0/G1 phase was observed (56% vs. 48%; $P < 0.05$). Thus, all studies on the effects of lycopene were compared with vehicle-treated cells.

Cyclin protein expression. To determine whether the modulation of cell cycle progression by lycopene was accompanied by cellular changes in cyclin D1 and cyclin E protein expression, Western blot analyses were performed. After stimulation of synchronized cells with growth factors, cyclin D1 protein expression increased in untreated cells, vehicle-treated cells and, to a lesser extent, in cells treated with 0.5 μmol/L lycopene (**Fig. 3**). However, in cells treated with 5 μmol/L lycopene, no cyclin D1 expression was detected. Cyclin E levels remained constant throughout the experiments and were unaffected by the treatment with lycopene or vehicle alone.

DISCUSSION

Highly proliferative prostate epithelium as found in BPH is a risk factor for prostate cancer (24), and inhibition of epithelial cell proliferation is a common strategy to lower the risk of cancer. The primary PrEC cell line appears to represent a suitable model for in vitro investigation of the chemopreventive effects of lycopene. The PrEC cells have been previously used to study the effects of dietary factors on the growth and cell cycle of normal prostate epithelial cells (25,26).

In our study lycopene significantly inhibited PrEC cell growth in a dose-dependent manner. Although low doses of up

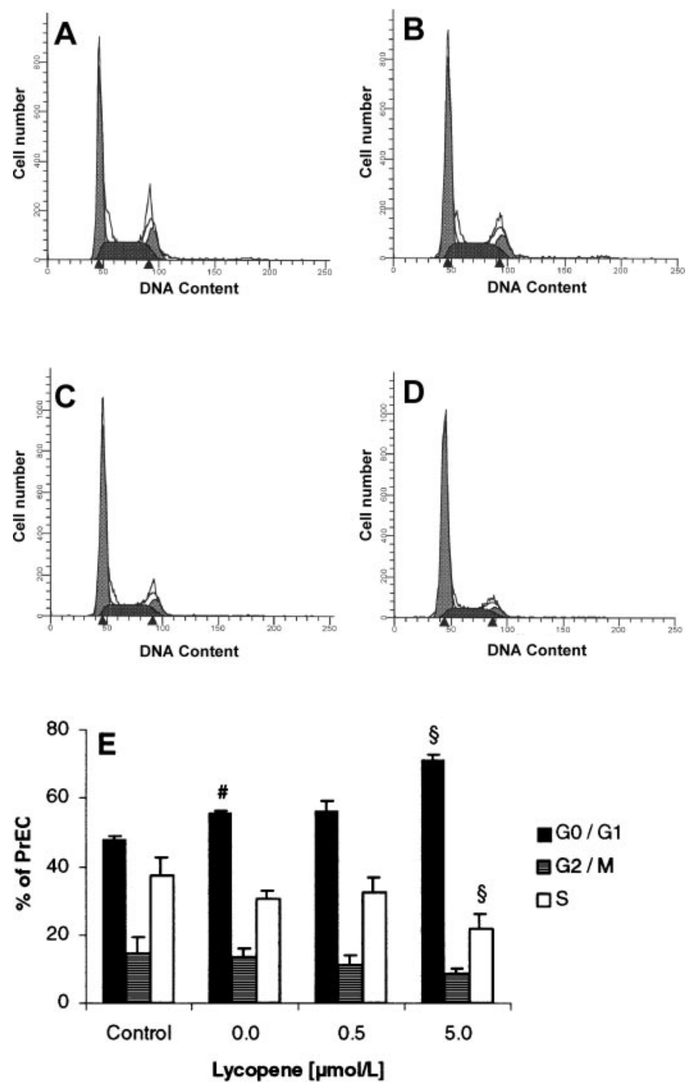

FIGURE 2 Inhibition of cell cycle progression in normal prostate epithelial cells by lycopene. Cell cycle progression was measured by flow cytometry. The distribution of cells in different stages of the cell cycle in untreated control cells (*A*) and cells treated with THF alone (*B*), 0.5 μmol/L lycopene (*C*) and 5 μmol/L lycopene (D) is shown, along with a summary of the percentage of cells in each phase of the cell cycle (*E*). One representative result from three independent experiments is shown. Values are means ± SEM, $n = 4$. ($^{\#}P < 0.05$ vehicle-treated vs. untreated cells; $^{\S}P < 0.05$ lycopene-treated vs. vehicle-treated cells).

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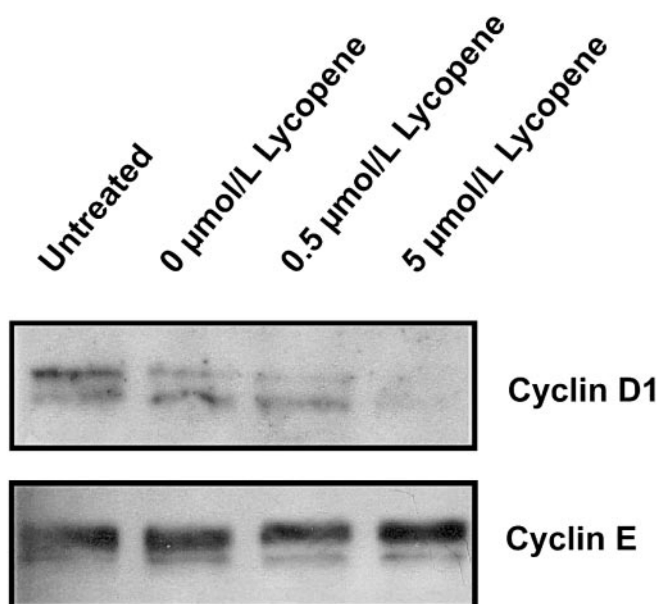

FIGURE 3 Lycopene downregulates cyclin expression in normal prostate epithelial cells in vitro. Cells were subjected to Western blot analysis for cyclin D1 and cyclin E protein expression. One representative result from three independent experiments is shown.

to 0.5 μmol/L lycopene were only slightly effective, higher doses of lycopene significantly inhibited cell growth ($P < 0.05$). The calculated ID_{25} and ID_{50} values for PrEC cells of 0.4 and 0.8 μmol/L, respectively, are physiologically relevant concentrations of lycopene, because blood levels of lycopene are frequently found in this range in humans that consume a diet rich in tomatoes and/or tomato products or supplement their diet with carotenoids (9,27). This suggests that regular intake of lycopene may result in blood levels of lycopene likely to affect prostatic epithelial cell growth in men.

The observed inhibition of cell proliferation by lycopene of up to 82% in normal prostate epithelial cells was even stronger than previously reported for prostate cancer cells. Pastori et al. (1998) reported an ~20% inhibition of DU-145 prostate cancer cell proliferation by a dose of 5 μmol/L lycopene (11). Kotake-Nara et al. (10) reported an ~25% inhibition of three different prostate cancer cell lines (LNCaP, DU-145, PC-3) by 5 μmol/L lycopene (10). In our study, a 20% inhibition of PrEC cell proliferation was achieved at a dose of 0.3 μmol/L lycopene.

The observed effect of lycopene on inhibition of cyclin D1 expression in G0/G1 arrested cells, with no effect on cyclin E levels, confirms previous reports on cancer cells. Although no reports on prostate cancer cells exist, it has been reported that lycopene suppresses cell cycle progression in breast cancer cells primarily via inhibition of cyclin D1 expression (19). In the same study, lycopene inhibited downstream events in cell signaling, resulting in impaired transition to the S phase, e.g., inhibition of cdk2 kinase activity and decrease of pRb phosphorylation. The mechanism of lycopene interference with cell signaling in normal prostate epithelial cells, leading to downregulation of cyclin D1 and ultimately to cell cycle arrest in G0/G1, warrants further investigation.

This is the first report of lycopene effects on nonneoplastic prostate cells. It is unknown whether lycopene modulates the growth of other nonneoplastic cell types apart from prostate cells. The observed growth inhibition of lycopene in prostate cells, if confirmed in vivo, could affect both normal prostate development and enlargement of the prostate. During maturation of the prostate gland, enhanced cell proliferation is a physiological process. No reports exist, however, on potential negative effects of the consumption of lycopene-rich foods on prostate development and organ function. In contrast, the common pathologic hyperproliferation of prostate cells in adult men developing BPH may be positively affected by lycopene. The effects of lycopene on normal prostate development and organ function need further clarification. Furthermore, it is not known whether the observed effects on normal prostate epithelial cells are specific for lycopene or may also be observed with other carotenoids. However, a study of prostate cancer cells by Kotake-Nara et al. (10) revealed an inhibition of cancer viability (as assessed by MTT assay) by several carotenoids, with differing degrees of inhibition (10). The 5,6-monoepoxy carotenoids neoxanthin and fucoxanthin caused an even stronger inhibitory effect on prostate cancer cells than that of lycopene. However, lycopene is one of the carotenoids preferably accumulated in human prostate. This suggests that lycopene effects on prostate cells may play an important role in prostate health.

We conclude that lycopene effectively inhibits the growth of normal human prostate epithelial cells. If lycopene inhibits the growth of prostate cells in vivo, this may likely affect the growth of prostate tissue. Further studies of lycopene effects on BPH, prostate development and organ function are warranted. Inhibition of proliferation of normal prostate cells is one plausible way that lycopene may lower the risk of prostate cancer.

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