Lycopene and Lutein Inhibit Proliferation in Rat Prostate Carcinoma Cells

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Abstract: Consumption of lycopene, a carotenoid without provitamin A activity, has been associated with a lower risk of prostate and breast cancer. Lutein is another carotenoid that may be associated with a reduced risk of age-related macular degeneration, the leading cause of blindness in adults 65 years of age and older. Bioactive compounds such as lycopene and lutein, derived from natural plant sources, have been shown to act at low substrate levels through the action of intrinsic cytokines and growth factors and their receptors within tissues, particularly those of the fibroblast growth factor and transforming growth factor \( \beta \) families. The effects of grapefruit-derived and commercial lycopene and lutein preparations on androgen independent cultured malignant type II tumor cells [Dunning R3327AT3 or AT3 cells (androgen-responsive, slow-growing tumor cells with well developed epithelium and stroma)] were compared to their benign parent type I tumor epithelial cells (DTE). Results demonstrated that both lycopene, in an \( \alpha \)-cyclodextrin water soluble carrier, and lutein inhibited malignant AT3 cells in a concentration and time-dependent manner. No such effect was observed when benign DTE cells were examined, demonstrating selective inhibition of extremely malignant AT3 prostate cancer cells relative to their benign parent. Lutein demonstrated a similar but slightly diminished response as lycopene. When cells were treated with cocktails of lycopene and lutein, no synergistic or additive effect occurred. These studies are consistent with epidemiological studies that show inverse relationships of these carotenoids with prostate cancer.

Introduction

Prostate cancer has become the second leading cause of cancer-related death among men in the United States (1). Incidence and mortality data, age adjusted to the 2000 population standard of the United States, indicate that there were slightly over 230,000 new cases in 2004, with 29,900 deaths occurring. American men have a 1 in 6 lifetime probability of developing prostate cancer. It was estimated that prostate cancer would account for 33% of cancer cases in men in 2006.

Epidemiologic studies suggest that consumption of vegetables and fruits rich in carotenoids reduces the risk of prostate cancer (2,3). Lycopene is the predominant carotenoid in human plasma, contributing between 21% and 43% of total plasma carotenoid level, i.e., \( \sim 0.5 \) \( \mu \)mol/l of plasma (4). Tissue levels vary from 1 nmol/g wet wt in adipose tissue up to 20 nmol/g wet wt in adrenal and testes. Lycopene is an acyclic polyene with 11 conjugated double bonds and 2 nonconjugated double bonds. In nature, it is mainly found as an all-trans isomer. Hydrogen and carbon atoms in lycopene are seen arranged in the linear and all-trans form (Fig. 1A). Tomato is the main source of lycopene in the human diet, although other fruits such as guava, watermelon, and citrus fruits, e.g., the Rio Red grapefruit, are among those that contain moderately high levels of the carotenoid. In all cases, the lycopene levels in crops vary and may be altered by several preharvest and postharvest factors (5,6).

Lutein is another carotenoid of biological significance. Lutein, a xanthophyll, consists of chains with 8 conjugated double bounds containing closed rings on each end of the chain (Fig. 1B). This carotenoid is found in fruits and vegetables, especially dark green leafy vegetables such as kale and spinach. Emerging scientific evidence suggests that lutein may help maintain the health of human eyes. Studies have shown that diets high in lutein may be associated with a reduced risk of age-related macular degeneration, the leading cause of blindness in adults 65 years of age and older (7,8).
Bioactive compounds such as lycopene and lutein have been shown to produce biological effects at low substrate levels. It has been hypothesized that some phytochemicals, including carotenoids, act through intrinsic cytokines, growth factors, and their receptors, particularly those of the fibroblast growth factor (FGF) and transforming growth factor β (TGF-β) families (9–11).

The first observations on biological properties of lycopene were reported in 1959, which demonstrated that intraperitoneally injected lycopene increased the survival rate of irradiated mice and mediated their resistance toward bacterial infections (12). More recently, Giovannucci et al. (2) reported that ingestion of tomato-based foods and increased plasma lycopene level was significantly associated with a lower risk of prostate cancer. Pastori et al. (13) reported that lycopene, at 1 μmol/l in association with 50 μmol/l α-tocopherol, inhibited the proliferation of PC-3 cells and DU-145 human prostate cancer cells. Although it does not have provitamin A activity, lycopene exhibits at least 3 anticarcinogenic mechanisms: first, lycopene is the most efficient singlet O2 quencher of the carotenoids (14); second, lycopene enhances gap-junction intercellular communication (15); and third, lycopene acts to inhibit potent autocrine mitogenic activity induced by insulin-like growth factors (16,17). Results from in vitro studies show that lycopene exhibits inhibitory effects on endometrial (18), mammary tumor cell lines (MCF-7) (19), and HL-60 leukemia cell lines (20) and is more potent than α- or β-carotene in inhibiting human lung cancer cell (NCI-H226) growth (21).

Based on these inhibitory effects of lycopene on other tumor cell lines, we sought to compare the effects of this carotenoid on androgen-independent, malignant type II prostate tumor cells with their benign parent type I tumor epithelial cells (DTE). The influence of lutein on cell growth of both tumor lines was also examined as well as a cocktail of both carotenoids.

Materials and Methods

Materials

Standard lycopene (≥90%) and lutein (90–94%) were purchased from Sigma Chemical, St. Louis, MO. Tetrahydrofuran (THF) containing 0.025% butylated hydroxytoluene antioxidant and α-cyclodextrin hydrate (98%) was purchased from Fisher Chemical (Suwanee, GA). Dulbecco’s modified Eagle’s medium (DMEM, 1 × liquid containing L-glutamine), Roswell Park Memorial Institute (RPMI 1640 1 × liquid containing L-glutamine), fetal bovine serum (FBS), Hanks’ Balanced Salt Solution (HBSS) and pronase were purchased from Gibco Invitrogen Corporation (Carlsbad, CA). The culture media RD is a mixture of RPMI and DMEM in equal parts. Lycopene was extracted and purified from the Texas Rio Red grapefruit according to the following procedures.

Grapefruit Lycopene Extraction

Three grapefruits were peeled and homogenized in a blender. The juice sample (100 ml) was mixed with an equal amount of petroleum ether by vigorous stirring for 2 h. The extract was centrifuged at 13,000 g, and the supernatant was passed through a NaSO₄ column (20 cm × 13 mm). The extract was then evaporated to about 3 to 5 ml using a rotary evaporator under vacuum at 30°C, transferred to a 15 ml vial, and evaporated further with liquid nitrogen to 1.0 ml. Lycopene was precipitated by adding 10 ml of 95% ethanol, and the precipitate was allowed to settle at room temperature for 5 min before being filtered. The clear filtrate was discarded, and the residue was washed with 10 ml of petroleum ether. The residue was air dried and loaded to a silica gel (10 g) column (40 cm × 13 mm), which was preconditioned with petroleum ether. The lycopene was eluted with increasing polarity with chloroform. Equivalent eluting fractions...
were pooled and evaporated with liquid nitrogen and weighed.

**HPLC Analysis and Grapefruit Lycopene Purity**

Lycopene was analyzed and quantitated with a Spectra System Model P-4000 high-performance liquid chromatography (HPLC; Thermo Separation Products, Waltham, MA, equipped with a quaternary HPLC pump and fitted with a C18 analytical column (15 cm × 4.6 mm inside diameter, 5 microns particle size). Alltech, Deerfield, IL). The auto injection system (Spectra System AS 3000, Providence, RI) employed a 20 µl sample loop. Detection was achieved with a UV 6000 LP wavelength detector set at wavelength of 474 nm. The isocratic mobile phase used was methanol:THF:water (67:27:6) at a flow rate of 1.0 ml/min. Lycophene was quantified using ChromQuest (Waltham, MA) software. Samples were dissolved in 1.0 ml of THF and then diluted to 2 ml with a solution of 3 parts methanol and 1 part THF. Samples were filtered through a 0.2 µm nylon filter, and 20 µl of the sample was injected into the HPLC. The concentration of lycopene in a 12 g sample of the homogenized grapefruit juice from the extraction described above was measured using HPLC. Based on the standards, the lycopene content was calculated to be 4 mg per 100 g of juice. The above extraction yielded 1.6 mg of dried grapefruit lycopene product. Purity of grapefruit lycopene, based on HPLC elution profiles of lycopene standards, was estimated to be ≥ 90.

**Carotenoid Preparation for Cell Culture**

Carotenoids were dissolved in THF at 2 mM and mixed with an equal volume of α-cyclodextrin at 2 mM (in ethanol). This solution was shaken for 2 h to allow the α-cyclodextrin to incorporate the respective carotenoid inside its structure. All solvents were evaporated, and the remaining sample was added to RD cell culture media and incubated at 37°C for an additional 2 h. The solution was then passed through a sterile filter (pore size 0.2 microns). All sample preparations were performed under dim light.

**Cell Lines and Culture Conditions**

Androgen-independent, malignant rat prostate carcinoma AT3 cells from the Dunning R3327AT3 tumor and their benign parent, DTE, were maintained as monolayers in RD medium supplemented with 2% FBS. These cell lines were first established from 7 biologically distinct in vivo Dunning R3327 rat prostatic tumor sublines (22). The AT3 exhibits a high growth rate and maintains a very high metastatic ability. The DTE line exhibits a slow growth rate and low metastatic ability. Growth rates (cell doubling times in h) of the high metastatic lines, e.g., AT3, are 2-8-fold greater than those of cell lines exhibiting low metastatic ability. Cell stocks from both cell lines were grown in 75 cm² tissue culture flasks in a 5% CO₂ humidified atmosphere at 37°C and passaged using pronase in HBSS.

**Inhibition of Cell Growth**

Cells were seeded in 24-well plates at a density of 1 × 10⁴ cells per well and were incubated for 2 h at 37°C to attach to the well plates. The prepared solutions of RD cell culture media, THF, and α-cyclodextrin containing lycopene were then added to the wells. Carrier, THF, and α-cyclodextrin had no significant effect on cell growth of either cell line. In preliminary ranging studies, cells exposed to grapefruit lycopene concentrations of 0, 0.04, 0.4, 5, 10, and 20 µM were allowed to grow undisturbed for 4 days before being harvested. The study was repeated using equivalent tomato lycopene concentrations. As responses to the 2 sources of lycopene were similar, 0.02 µM to 10 µM concentrations of tomato lycopene were used in concentration dependency studies. In time-course studies, cells were exposed to 2.0 µM tomato lycopene and harvested after each day for 4 days. Similar studies, time-course and concentration dependency, were conducted for lutein and a cocktail of lycopene and lutein [phytochemical bioactive molecules (PBAMS)].

**Mitogenic Assay for Growth Factor Studies**

Growth factor studies essentially followed the methods employed by Liu et al. (23). Androgen-independent, malignant rat prostate carcinoma AT3 cells from the Dunning R3327AT3 tumor and their benign parent, DTE, were seeded in 24-well plates at a density of 1 × 10⁶ cells per well in a serum free, low calcium RSFAA media. Control cells were grown in normal RD cell culture media. After 2 h, the media was replaced with fresh media. Experimental cells were exposed to 10 µM tomato lycopene in serum free, low Ca media with FGF-1 (10 mg/ml). Control studies were conducted in the same manner with only the carrier and without lycopene. At the end of 24 h, [³H] thymidine was added at a concentration of 0.5 µCi/ml, and cells were incubated for 6 h. Nucleotide incorporation was halted by addition of unlabeled thymidine. Cells were harvested and radioactivity measured using a scintillation counter.

**Statistics**

Data are reported as the mean of 4 replicates ± SD. Student’s t-test was employed to compare control and experimental parameters with P ≤ 0.05 as a statistical endpoint.
Results

Lycopene inhibits the malignant type II prostate AT3 cells in a concentration dependent manner regardless of the source of the carotenoid (Fig. 2A). The effects of various concentrations of lycopene on the cells harvested after 4 days showed that lycopene, from 0.2–10.0 µM, significantly inhibited the malignant AT3 growth in comparison to a carrier vehicle control. Similar studies on the effect of increasing concentrations of lycopene on the benign tumor DTE cells did not show any significant inhibitory effects at any concentration (Fig. 2B).

Time course studies done over a period of 4 days with a constant concentration (2.0 µM) of lycopene showed significant inhibition, compared to control, beginning at Day 2. After 4 days, AT3 cell growth was inhibited by 49% compared to control (Fig. 3A). Similar time studies showed no significant effects on the benign DTE cells (Fig. 3B).

Time course studies with a constant concentration (2.0 µM) of lutein also showed significant inhibition of malignant AT3 cell growth beginning at Day 2 when compared to its vehicle control and was 42% of the control at Day 4 (Fig. 4). Studies with increasing concentrations (0.02–10.0 µM) of lutein did not show any significant effect on DTE cell growth (data not shown).

Similar time course studies of an equal molar cocktail of lycopene and lutein failed to demonstrate a synergistic or additive effect of the 2 carotenoids. A comparison of carotenoid and cocktail inhibition of malignant AT3 cell growth is shown in Fig. 5. The level of inhibition of the cocktail was not significantly different from that of either lycopene or lutein. The apparent specificity of the inhibitory effect of these carotenoids is clearly demonstrated by comparing the effects of the cocktail on malignant AT3 cells (Fig. 6A) and benign DTE cells (Fig. 6B).

Results (not shown) of experiments assessing the mitogenic activity of external FGF or TGF-β indicated that lycopene had no significant effect on FGF or TGF-β moderation of mitogenic activity in either of the cell types.

Figure 2. A: Concentration-dependent inhibition of type II prostate AT3 (androgen-responsive, slow-growing tumor cells with well developed epithelium and stroma) cells by lycopene over a constant time of 4 days (*, P < 0.0001). Data are mean ± SD; n = 4. B: Effects of increasing concentrations of lycopene (in α-cyclodextrin) on DTE (benign parent type I tumor epithelial) cells over a constant time of 4 days. Data are mean ± SD; n = 4; NS.
A: Time course studies (4 days) of a constant lycopene (2.0 µM) concentration (in α-cyclodextrin) on AT3 (Androgen-responsive, slow-growing tumor cells with well developed epithelium and stroma) cells (*, P < 0.05; **, P < 0.0001). Data are mean ± SD; n = 4. B: Time course studies (4 days) of a constant lycopene (2.0 µM) concentration (in α-cyclodextrin) on DTE (benign parent type I tumor epithelial) cells. Data are mean ± SD; n = 4; NS.

Discussion

McCarty (24) characterized malignant cells by their up-regulation, or constitutive activation, of multiple signaling pathways that promote proliferation and inhibit apoptosis, enabling the cells to invade and migrate through target tissues while evoking angiogenesis. Similarly, down-regulation, or loss of pathways that oppose the upregulating events, is often seen. Unfortunately, therapies that are initially effective in beneficially modulating these pathways often lead to selection for androgen-independent cells where subsequent amplification and/or mutations of the androgen receptor gene enables the receptor to be activated by nonandrogenic entities such as growth factors. Further, increased expression of various androgen receptor coactivators can promote adequate androgen receptor activity even in a low-androgen environment (25,26). This illustrates not only the complexity of the process of progression of transformed cells to the malignant state but also the seemingly insurmountable task of devising a single therapeutic strategy that is effective for multiple targets. It is most likely that therapies will be targeted to specific targets in the progression to malignancy. Thus, the current studies with lycopene and lutein have particular relevance, as both selectively target proliferation of highly malignant cells.

Lycopene has been shown to influence cell cycle progression and inhibit growth of a number of cancer cell types (18–21,27). Recently, it has been shown that lycopene inhibits the growth of normal human prostate epithelial (PrEC) cells, and it was suggested that the carotenoid might play a role in benign prostate hyperplasia—a potential precursor of prostate cancer (28).

The current studies, conducted on the effects of lycopene from tomato and grapefruit on the growth of the malignant rat prostate cancer cells (AT3), showed that lycopene inhibited growth of these malignant cells in a concentration-dependent manner. Similar studies conducted on the benign DTE cells showed no inhibitory effects. Time course studies, over a 4–5 day treatment period, confirmed that both grapefruit and tomato lycopene have inhibitory effects on the highly malignant AT3 cells, while having no effect on the benign DTE cells. Control studies conducted using the carrier α-cyclodextrin did not show any effects on either AT3 or DTE cell types. These results demonstrate that lycopene from both sources selectively inhibits the extremely malignant, highly proliferative AT3 prostate cancer cells relative to their benign parent. The differential results also indicate that the inhibitory effect on AT3 cells is not a general cytotoxic effect, although the possibility that these carotenoids may influence programmed cell death in malignant cells remains a strong possibility.

As lutein has also been identified in human prostate tissue, the effects of lutein and a cocktail (PBAM) of lutein and lycopene on AT3 and DTE rat prostate cancer cell lines were examined. Lutein was found to have an inhibitory effect on the malignant cell line (AT3) at 2, 3, and 4 days, with a 42% inhibition of lutein treated cells at Day 4. When comparing the effects of lutein on AT3 cells with that of lycopene, our results suggest that lycopene may be the more potent carotenoid, with a 49% inhibition of lycopene cells at day 4 (Fig. 5).

A number of recent reports indicate that a cocktail of PBAM better represents the physiological concentrations of bioactive molecules within cells (29,30). However, similar effects of PBAM to those of either lycopene or lutein, alone, on AT3 and DTE cells cell growth were found in both time curve and concentration dependency studies. The effects of
the cocktail, showing similar selective inhibition on AT3 cells in comparison to DTE cells and no synergistic or additive response, suggest that both carotenoids act on a common physiologic target (30).

In conclusion, we have observed that lycopene, lutein, or a combination of both differentially inhibit growth of a highly malignant line of prostate tumor cells (AT3) while exerting no effect on growth of the benign tumor parental cell line (DTE). This inhibitory effect is concentration dependent and does not represent a general cytotoxic response. Studies with PBAMS suggest that the carotenoids act on a common physiologic target. Regardless of mechanism(s), the importance of these

Figure 6. A: Time course studies (4 days) of a constant lutein (Lut; 2.0 µM) and lycopene (Lyc; 2.0 µM) concentration (in α-cyclodextrin) on AT3 cells (androgen-responsive, slow-growing tumor cells with well developed epithelium and stroma) (*, \( P < 0.05 \); **, \( P < 0.0001 \)). Data are mean ± SD; \( n = 4 \).

B: Time course studies (4 days) of a constant lutein (2.0 µM) and lycopene (2.0 µM) concentration on DTE (benign parent type I tumor epithelial) cells. Data are mean ± SD; \( n = 4 \); NS.
observations rests with the potential of this cell model to explore not only the mechanism(s) of carotenoid action in malignant progression but to explore the underlying steps in tumor progression from a benign state to frank malignancy.

Acknowledgments and Notes

We thank Dr. Mikio Kan for providing FGF-1 and TGF-β, Maki Kan and Thanh Tran for their expert technical assistance, and Ms. Natachia Olivo for assistance with manuscript preparation. BSP was supported by the USDA-CSREES IFAFS#2001-52102-02294 and USDA-CSREES#2005-34402-14401 “Designing Foods for Health” through the Vegetable & Fruit Improvement Center. RSG was partially supported by University of Houston-Victoria faculty research grants. Address correspondence to Dr. Richard S. Gunasekera, 3007 N. Ben Wilson Drive, Victoria, TX 77901. Phone: 361-570-4218. FAX: 281-275-3008. E-mail: GunasekeraR@uhv.edu.

Submitted 19 December 2006.

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