Lycopene Increases Urokinase Receptor and Fails to Inhibit Growth or Connexin Expression in a Metastatically Passaged Prostate Cancer Cell Line: A Brief Communication

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The carotenoid lycopene, found in tomatoes, has been associated with decreasing prostate cancer risk. Potential mechanisms for this risk reduction include lycopene's status as a potent antioxidant, its inhibitory effect on cell proliferation, and its ability to increase intercellular gap junctional communication. Presently, in the United States, almost 200,000 men are diagnosed with prostate cancer and approximately 30,000 succumb to its metastatic effects. Therefore, novel treatment strategies are needed for patients who currently have the disease, especially those in advanced, i.e., metastatic status. In this study, we sought to determine if lycopene's inhibitory properties on premalignancy could be extended to advanced prostate cancer by assessing effects on a cell line derived through metastatic passage, the PC-3MM2. We report that in this cell line, lycopene has a potentially unwanted effect of upregulating expression of the urokinase plasminogen activator receptor and facilitating invasion while failing to significantly inhibit proliferation or to induce detectable levels of the gap junctional protein connexin 43 expression. Our results indicate that some caution should be taken with regard to use of lycopene to treat potentially advanced and metastatic prostate cancers. Exp Biol Med 228:967–971, 2003

Key words: urokinase plasminogen activator receptor; prostate cancer; lycopene; connexin 43

Lycopene, the carotenoid that gives tomatoes their red color, has generated much recent excitement as a potential chemopreventative for prostate cancer. This excitement is founded upon a number of epidemiological and prospective studies that associated lycopene consumption with reduced prostate cancer risk (1, 2). In addition, oral lycopene before prostatectomy was associated with reduced incidence of high grade prostatic intraepithelial neoplasia (HGPIN), increased apoptotic index, smaller tumors, and reduced prostate specific antigen (3), suggesting a potential use for lycopene in patients with preexisting prostate cancer in an early state of development. Although not all studies support the association of lycopene with reduced prostate cancer risk (2, 4, 5), much attention has been given to those studies that do show risk reduction. This attention has stimulated public interest in lycopene consumption, precipitating a proliferation of commercially available lycopene supplements (1, 2). Mechanistically, lycopene's antioxidant properties likely play a major role in tumor prevention; however, other reported effects could potentially be efficacious against established cancer cells. These effects would include lycopene's antiproliferative properties and its ability to increase connexin 43 expression, which enhances gap junction intracellular communication between preneoplastic and normal epithelial cells (3, 6).

To date, most lycopene studies involve its effects on inhibiting early stages of prostate carcinogenesis—stages in which chemoprevention is effective (1–4, 7–9). However, there are few investigations on effects of lycopene on advanced prostate cancer cells. Such investigations seem warranted because lycopene has shown a beneficial effect in reducing the grade in patients with organ-confined cancer and because of its effects beyond protection from oxidative damage (3, 6). In addition, patients with existing advanced disease that hear or read the advertising may be tempted to use lycopene as a complementary treatment.

Advanced prostate cancer cells, particularly those that successfully metastasize, are unique from developing cells within the primary tumor. Metastatic cells have acquired abilities to invade, intravasate, extravasate, and proliferate at secondary sites such as bone and therefore, because of their altered phenotype, could respond differently to lycopene than neoplastic, nonmetastatic cells.

One critical characteristic that metastatic cancer cells have acquired is the ability to dissolve basement membranes

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and the extracellular matrix (ECM). This degradative process is mediated largely by matrix metalloproteinases (MMPs) and the urokinase plasminogen activator (uPA) system consisting of uPA, its receptor, uPAR, and the inhibitor PAI-1 (10). uPA activates several MMPs (11, 12) in addition to activating plasmin, which degrades several matrix proteins. In prostate cancer, uPAR signaling enhances invasion and tumor cell growth (10, 13, 14) and has become an attractive target for inhibition in cell lines (10) and animal models (10, 15, 16).

In this study, we assessed the potential effects of lycopene on expression of uPAR and proliferation in a prostate cancer bone metastatic cell line derived from repeated metastatic selection (17, 18). We report that in this PC-3MM2 cell line, lycopene has the potentially unwanted effects of upregulating expression of the uPAR and facilitating invasion while failing to significantly inhibit proliferation or to induce detectable levels of connexin 43 expression. These results suggest caution should be taken with regard to use of lycopene to "treat" potentially advanced and metastatic prostate cancers.

The human prostate cell line, PC3, was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and stocks were propagated in Ham's F12-K medium (Sigma Chemicals, St. Louis, MO) plus 10% fetal bovine serum (FBS; Gibco-Life Technologies, Grand Island, NY). The PC-3MM2 cell line, a mandibular metastasis (18), was a generous gift from Dr. Isaiah Fidler (Anderson Cancer Center, Houston, TX). PC-3MM2 stock cultures were maintained in modified Eagle's medium (MEM; HyClone Laboratories, Logan, UT), supplemented with 10% FBS, 1 m*M* sodium pyruvate (Sigma Chemicals), 1× non-essential amino acids solution (HyClone Laboratories), 1× MEM vitamins solution (Cellgro, Herndon, VA), and 2 m*M* L-glutamine (Sigma Chemicals). MCF-7 breast carcinoma cells were obtained from the ATCC, and stock cultures were grown in DMEM (Hyclone) with high glucose and 10% FBS.

For conditioned medium preparations used in immunoblots, cell lines were cultured to 70% confluence in 150-mm diameter dishes then growth with serum-free stock medium for 24 hrs. Conditioned medium (20 ml) was collected from each dish of cultures and was concentrated approximately 40-fold (Centricon Plus-20 5,000 MWCO). Cells numbers from the plates of cultures used to condition the medium were counted and media were loaded into lanes as equal cell number equivalents—100,000 cells of conditioned medium per lane.

For growth studies, cells were seeded at 2000 cells/cm^2 in 24-well plates. After 24 hr, medium from each well of cells was aspirated, washed with phosphate-buffered saline (PBS), and refed with 1 ml of assay medium. This time point was designated Day 0. For PC-3MM2, PC-3, and MCF-7 cells, the growth assay medium consisted of the cell's stock media without supplements except 1.0% FBS ± lycopene. Media were refreshed after 4 days and cells were trypsinized and counted every other day using a Coulter Counter (Hialeah, FL).

Lysates of cells for immunoblotting were prepared by removing the cells from dishes with a cell scraper (Corning, Corning, NY), pelleting the cells in PBS, and lysing them using EBC buffer (50 m*M* Tris-HCl, pH 7.5, 120 m*M* NaCl, and 0.5% Nonidet P-40) (19). Protein samples (100 μg/lane) and conditioned medium samples corresponding to 200,000 cells per lane were electrophoresed under nonreducing conditions through a 7.5% SDS-polyacrylamide gel. Samples for connexin 43 detection were reduced before electrophoresis. After electrophoresis, conditioned media samples or cell lysates were transferred to polyvinylidene difluoride membranes. Membranes were blocked for 1 hr in 4.0% nonfat dry milk/1.0% bovine serum albumin (BSA) in 140 m*M* NaCl and Tris-HCl, pH 7.5, at room temperature, then incubated with primary antibodies overnight at 4°C. Immunoblots were developed using enhanced chemiluminescence (Western Lightning; Perkin-Elmer Life Sciences, Boston, MA).

In vitro invasion was assayed essentially as described (14, 20) using Transwell polycarbonate membrane inserts with 8.0-μm pores (Falcon Corporation, Cowley, UK) coated with 100 μl of a 1.0 mg/ml dilution of growth factor-reduced Matrigel basement membrane matrix containing 5 n*M* uPA. Twenty thousand cells plated into the upper chamber were allowed to cross through the matrix toward a chemoattractant (NIH 3T3 cell-conditioned medium) and attach to the under surface of the membrane over a 6-hr period after which 10 high-power fields per insert were scored for invading cells (two inserts per condition). The cells per high-power field were averaged to obtain the data shown in Table I.

Lycopene (#L-9879; Sigma Chemicals; *all-trans* configuration) was reconstituted to 10 m*M* in chloroform and was stored in aliquots at −80C°. For treatment of cells, the dilution of chloroform was greater than or equal to 1:2000 and control culture plates were treated with a dilution of chloroform equal to the highest volume used in any experimental plate. Antibodies for uPAR (R&D Systems, Minneapolis, MN) and connexin 43 (Sigma Chemicals) were used at dilutions of 0.5 and 2.0 μg/ml, respectively.

We have previously demonstrated that the PC-3MM2 has at least two characteristics that could give it a selective advantage in metastasis—a greater level of the invasion-associated urokinase receptor (21) and a more rapid rate of proliferation than PC-3 or PC-3M prostate cancer lines (22), which are two prostate cancer cell lines from which the PC-3MM2 was ultimately derived (18). Because we had observed that uPAR levels increased with metastatic selection, we wished to determine if lycopene could be used to reduce uPAR levels and/or invasion/proliferation in these advanced cancer cells.

Lycopene was added to PC-3MM2 cultures at a dose of 1.0 μ*M* and lysates were assessed for uPAR expression. The use of the 1.0 μ*M* level was based on reported plasma and

prostatic concentrations of lycopene (23). To our surprise, lycopene treatment resulted in the increased expression of the uPAR (Fig. 1A, left panel). To examine the effects of lycopene on the other components of the uPA system, we next assessed expression of uPA and PAI-1. Our results showed that lycopene treatment had little effect on expression of uPA and PAI-1 in PC-3MM2 cell lysates (Fig. 1A, center and right panels), although activity for uPA and PAI-1 were not directly assayed. Because both uPA and PAI-1 are secreted, and uPAR can be cleaved into a soluble form (suPAR), we also analyzed conditioned media for suPAR, uPA, and PAI-1 levels and found little to no effect of lycopene on uPA or PAI-1 but an increase in suPAR (Fig. 1B). Therefore, our results indicated that lycopene selectively modulates the uPA system in this cell line by increasing cellular uPAR expression perhaps contributing more uPAR for constitutive cleavage and release.

To determine if this effect of lycopene stimulation on the uPAR was a general phenomenon or observed in this cell model derived from repeated metastatic passage, we checked uPAR levels in two other cancer cell lines—the PC-3 and the MCF-7. The PC-3 line was selected because it is the parental prostate cancer cell from which the PC-3MM2 was ultimately derived (18). Although MCF-7 are breast cancer cells, they have previously been shown to be growth inhibited by lycopene stimulation (6) and therefore we have used them as a positive control. Our results demonstrated that lycopene had no effect on expression of the

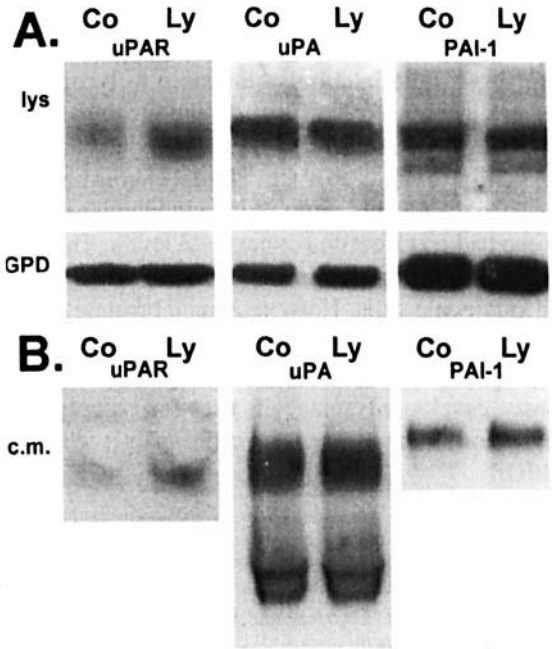

Figure 1. Lycopene induces urokinase receptor but not uPA or PAI-1 in PC-3MM2 cells. (A) Cell lysates (100 μg of total protein) of control (Co) and lycopene-treated (Ly) cells were immunoblotted for uPAR, uPA, and PAI-1 expression. Lycopene was used at a dose of 1.0 μ*M*. Protein loading was verified by glyceraldehyde-phosphate dehydrogenase (GPD) expression. (B) Conditioned medium collected and concentrated from control and lycopene-treated cells was analyzed for expression of soluble uPAR, uPA, and PAI-1. Medium equal to that secreted by 100,000 cells was loaded into each well.

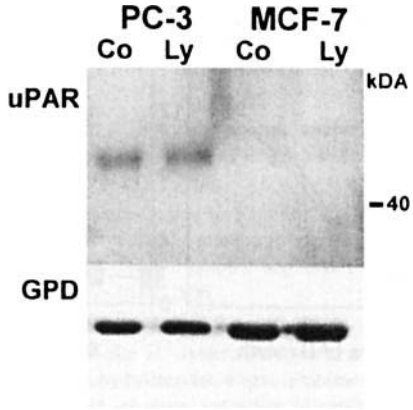

Figure 2. Lycopene treatment does not alter urokinase receptor expression in lysates from PC-3 and MCF-7 cell lines. One hundred micrograms of total protein was immunoblotted for uPAR (upper panel) and glyceraldehyde-phosphate dehydrogenase (GPD, lower panel).

uPAR in the PC-3 or MCF-7 lines, although uPAR levels in MCF-7 cells were barely detectable (Fig. 2). The lack of response in these two cell lines suggests that the PC-3MM2 line has acquired a unique phenotype that can paradoxically use lycopene to enhance uPAR expression.

uPAR facilitates matrix degradation through binding of uPA and subsequently localizes plasmin activation. Therefore, it is possible that increased expression of uPAR by lycopene could lead to increased invasiveness. We tested this hypothesis using a standard *in vitro* invasion assay and found that indeed, lycopene-treated cells were almost twice as invasive as untreated cells (Table I).

Lycopene has been widely reported to inhibit cell proliferation and this effect has provided justification for its use as a prostate cancer chemopreventative (1–4, 7–9). We tested lycopene in proliferation assays and compared its effect with effects on PC-3 and MCF-7 cell lines (Fig. 3). These proliferation assays revealed that although lycopene inhibited growth rates of the PC-3 and MCF-7 lines over an 8-day period, it had minimal effect on growth of the PC-3MM2 cells. Therefore, it appears that the PC-3MM2 line has lost growth inhibitory response to lycopene.

The PC-3 line was previously reported to be inhibited by lycopene only with simultaneous addition of α-tocopherol (23); however, this study used a much greater quantity of serum (10.0% vs 1.0%), a higher initial cell concentration and it was conducted over a shorter time period (1 day). The experimental conditions we used allowed for a more sensitive assessment of lycopene-induced growth inhibition, yet there was no effect on proliferation of the PC-3MM2 line.

Table I. Effect of Lycopene Treatment on *in vitro* Invasion

	Number of cells per HPF
Control	18.2 ± 3.0
Lycopene treated	34.6 ± 6.2

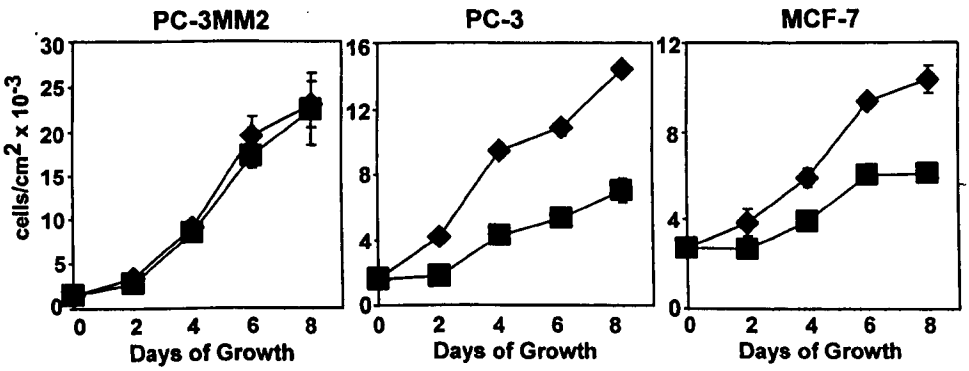

Figure 3. Lycopene effect on proliferation of tumor cell lines. Lycopene (1.0 μ*M*) was added to cultures of PC-3MM2, PC-3, and MCF-7 cells for an 8-day period. Triplicate cell counts were taken every other day from treated (■) and untreated wells (filled diamond). Error bars represent standard deviations.

Lycopene has been reported to exert a stabilizing effect on premalignancies through a number of pathways, including a critical upregulation of cell-cell communication via the gap junction protein connexin 43 (3, 6). To determine if this effect was preserved in PC-3MM2 cells, we immunoblotted cell lysates for connexin 43 expression but found no detectable quantities before or after lycopene treatment (Fig. 4). Interestingly, however, lycopene addition did result in increased levels in the PC-3 line. There was no change in connexin 43 levels in MCF-7 cells (data not shown). These results suggest that the effect of lycopene on connexin 43 expression in prostate cancer lines may become lost with the selections in metastatic passage.

Alternative medications that are heavily advertised as cancer preventatives may be used by some patients for treatment; hence, men diagnosed with prostate cancer may consume lycopene products in an attempt to treat their disease. However, our results with the PC-3MM2 cell line do not support lycopene use to inhibit metastatic cancer cells. In fact, increased expression of uPAR in some cells by lycopene could actually enhance invasion while having little or no effect on inhibiting proliferation or cell-cell adhesion properties. Interestingly, another carotenoid supplement, β-carotene, was once promoted as a method to decrease lung cancer in smokers, but trials with β-carotene indicated an increase risk in some individuals (2). It must be noted that our results were obtained exclusively with one cell line isolated experimentally from a heterogeneous population of tumor cells and our findings in culture cannot be generalized to advanced stage disease. The tumor microenvironment is a complex milieu of tumor, stroma, normal epithelium, endothelium, and immunological representatives as well as growth factors, cytokines, matrix proteins, and proteases. These environmental components will modulate effects of lycopene on uPAR levels and invasion *in vivo*; therefore, animal studies will be required to further assess the effects reported here. Such studies can also effectively address the potential metabolic conversion of *trans* to *cis* lycopene, tissue distribution, and bioavailability.

An ideal cell culture comparison of lycopene effects would involve both cells representing advanced disease and patient-matched premalignant cells; however, with lines derived from human tumors, this is not possible. Therefore, we compared the effects of lycopene treatment on PC-3MM2 cells with effects on PC-3 cells and found that the PC-3 line still retained some inhibitory responses to lycopene treatment such as reduced proliferation and increased connexin 43 expression and was unaffected by lycopene treatment in expression of uPAR. The MCF-7 breast cancer line behaved similarly to the PC-3 in terms of growth inhibition, although lycopene had no effect on connexin 43 levels. These results suggest that lycopene can inhibit growth pathways in established carcinoma cells. However, some prostate carcinoma cells may develop lycopene growth resistance, perhaps through the selection pressures involved in metastasis, and also may develop advantageous uses for lycopene signals that promote cancer-stage specific matrix degradation potential.

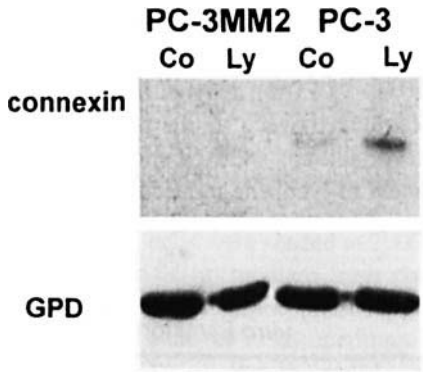

Figure 4. Lycopene treatment does not induce detectable levels of connexin 43 expression in PC-3MM2 but does in PC-3 cells. Equivalence of loading was verified by glyceraldehyde-phosphate dehydrogenase (GPD, lower panel).

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