Lycopene Interferes With Cell Cycle Progression and Insulin-Like Growth Factor I Signaling in Mammary Cancer Cells

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Abstract: Recent studies have shown that high insulin-like growth factor I (IGF-I) blood level is a risk factor in breast and prostate cancer. The aim of this study was to determine whether the mitogenic activity of IGF-I in mammary cancer cells can be reduced by the dietary carotenoid lycopene. The anticancer activity of lycopene, the major tomato carotenoid, has been suggested by in vitro, in vivo, and epidemiological studies. Growth stimulation of MCF7 mammary cancer cells by IGF-I was markedly reduced by physiological concentrations of lycopene. The inhibitory effects of lycopene on MCF7 cell growth were not accompanied by apoptotic or necrotic cell death, as determined by annexin V binding to plasma membrane and propidium iodide staining of nuclei in unfixed cells. Lycopene treatment markedly reduced the IGF-I stimulation of tyrosine phosphorylation of insulin receptor substrate 1 and binding capacity of the AP-1 transcription complex. These effects were not associated with changes in the number or affinity of IGF-I receptors, but with an increase in membrane-associated IGF-binding proteins, which were previously shown in different cancer cells to negatively regulate IGF-I receptor activation. The inhibitory effect of lycopene on IGF signaling was associated with suppression of IGF-stimulated cell cycle progression of serum-starved, synchronized cells. Moreover, in cells synchronized by mimosine treatment, lycopene delayed cell cycle progression after release from the mimosine block. Collectively, the above data suggest that the inhibitory effects of lycopene on MCF7 cell growth are not due to the toxicity of the carotenoid but, rather, to interference in IGF-I receptor signaling and cell cycle progression.

Introduction

Insulin-like growth factors (IGFs) are important in the process of tumor formation and tumor cell proliferation (1,2). Recently, Chan and co-workers (3) have found a strong positive association between IGF-I levels and prostate cancer risk in participants in the Physicians’ Health Study. An equally strong association between this growth factor level and breast cancer risk was also reported in a case-control study within the Nurses’ Health Study cohort (4). Thus plasma IGF-I levels may be useful for identifying individuals at high risk for breast and prostate cancer and for developing risk reduction strategies, by lowering IGF-I levels or interference with its action.

The IGF autocrine/paracrine system is composed of several components. IGF-I and IGF-II are related peptides that are among the most active growth factors in various types of cancer, including breast cancer (1). The interaction of both ligands with IGF-I receptor results in tyrosine autophosphorylation of the receptor. This, in turn, leads to activation of downstream signaling cascades (5). The system also includes several IGF-binding proteins (IGFBPs), which mostly have a negative effect on IGF action (6). It has been demonstrated that tumor formation and progression directly correlate with the function/activity of IGF-I receptor in the tumor-forming cells (2,7,8). Therefore, intervention of IGF-I receptor signaling is becoming one of the goals of anticancer and preventive therapy.

A nutritional approach to the prevention of cancer, which has included assessment and evaluation of the anticancer effect of various micronutrients in the diet, has been prevalent for many years. Numerous epidemiological studies have demonstrated that consumption of vegetables and fruits reduces the risk of breast and other types of human cancers. Among plant constituents, carotenoids, mainly β-carotene, have been extensively studied and implicated as cancer-preventive agents (9). However, recent intervention studies with this carotenoid have revealed no beneficial effects (10,11) or have even shown a negative effect (12,13). These results seem to indicate that other potential anticancer factors are present in diets rich in vegetables and fruits.

Recently, there has been a growing interest in the role of the tomato carotenoid lycopene in the prevention of cancer. Many epidemiological studies have suggested that lycopene...
decreases the risk for cancer of the breast (14), bladder (15), pancreas (16), lung (17), and prostate (18), as well as cervical intraepithelial neoplasia (19). Giovannucci (20) recently reviewed data from these and many other studies and found most of them to show inverse associations between tomato intake or blood lycopene level and the risk of cancer at a defined anatomic site. These epidemiological data were reinforced by studies showing the inhibitory effect of lycopene on tumor cell growth in vitro (21,22) and in vivo (23–28). We previously demonstrated (29) that lycopene inhibits mammary, endometrial, and lung cancer cell growth in a dose-dependent manner (concentration causing 50% inhibition approx 2 μM). This inhibition was detected after 24 hours of incubation and was maintained for at least three days. In contrast to cancer cells, human fibroblasts were less sensitive to lycopene and gradually escaped inhibition over time. The inhibitory effect of lycopene was greater in IGF-I-stimulated endometrial cancer cells than in those growing under basal conditions (29, 30). The latter finding suggests that a possible mechanism of lycopene action may be its interference with the mitogenic pathway of IGFs. Hence, in the present study, we investigated the mechanism of lycopene inhibitory effects on basal and IGF-I-stimulated growth of MCF7 breast cancer cells.

Materials and Methods

Materials

Human recombinant IGF-I and des(1–3)IGF-I were purchased from GroPep (Adelaide, Australia). Porcine insulin was from Eli Lilly (Indianapolis, IN). Lycopene purified from tomato extracts (>97%) was a gift from LycoRed Natural Products Industries (Beer Sheva, Israel). Tetrahydrofuran (THF), containing 0.025% butylated hydroxytoluene as an antioxidant, was purchased from Aldrich (Milwaukee, WI). Dulbecco’s modified Eagle’s medium (DMEM), fetal calf serum (FCS), and Ca2+/Mg2+-free phosphate-buffered saline (PBS) were obtained from Biological Industries (Beer Sheva, Israel). Annexin-V-FITC was a gift from Sigma Israeil Chemicals (Jerusalem, Israel).

Carotenoid Solutions

Lycopene was dissolved in THF at a concentration of 2 mM and stored at −20°C under nitrogen. Immediately before the experiment, this stock solution was added to the cell culture medium under nitrogen with vigorous stirring. Some precipitate formed during this procedure and was removed by filtration (Millex-HV, 0.45 μm, Millipore, Bedford, MA). The final concentration of lycopene in the medium was measured by spectrophotometry after extraction in 2-propanol and n-hexane-dichloromethane (31). The exact carotenoid concentration, which varies with each preparation of medium, was systematically recorded. The final THF concentration of 0.5% did not have any significant effect on all the measured parameters. All procedures were performed under reduced lighting.

Cell Culture and Cell Proliferation Assay

MCF7 human mammary cancer cells were grown in DMEM containing penicillin (100 U/ml), streptomycin (0.1 mg/ml), nystatin (12.5 μg/ml), 0.6 μg/ml insulin, and 10% FCS. Cells were seeded into 24-multiwell plates (35,000 cells/well) or 96-multiwell plates (5,000 cells/well) in medium containing 3% FCS. One day later the medium was changed to one containing solubilized lycopene or THF alone (see above). A relatively short half-life of the carotenoid (10–20 h) was found under similar conditions (29); thus the media were replaced daily to ensure continuous presence of the carotenoid during prolonged incubations. At the end of the incubation, 1.25 μCi/well of [3H]thymidine (specific radioactivity 5 mCi/mmol) was added for one hour. The nucleotide incorporation was stopped by addition of unlabeled thymidine (0.5 μmol). The cells were then trypsinized and collected on a glass-fiber filter with use of a cell harvester (Inotech). Radioactivity was determined by a radioactive image analyzer (BAS 1000, Fuji). Cell growth was also measured by direct cell counting with a Coulter counter after trypsinization and dilution in Isotone-II (Coulter Electronics, Luton, UK).

Tyrosine Phosphorylation of Insulin Receptor Substrate 1 in Intact Cells

Cells grown in six-well plates (500,000 cells/well) were incubated for 12 hours without serum, washed twice with PBS, then incubated with IGF-I in 2 ml of PBS containing 1 mg/ml of BSA (essentially globulin-free; Sigma Chemical, St. Louis, MO) at 37°C for three minutes. After stimulation, plates were placed on ice, and cells were washed rapidly with ice-cold PBS and frozen in liquid N2. Cells were then thawed on ice in 0.4 ml of lysis buffer [1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 50 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), 150 mM sucrose, 80 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 2 mM EDTA, 2 mM EGTA, 2 mM sodium orthovanadate, 50 mM sodium fluoride, 0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml trypsin inhibitor], and the lysates were cleared by centrifugation (12,000 g for 20 min at 4°C). The protein content was determined using bicinchoninic acid protein assay reagent (Pierce Chemical, Rockford, IL). One hundred-microgram protein samples were separated by 7.5% SDS-polyacrylamide gel under reducing conditions. The Rainbow protein molecular weight markers (Amersham, Buckinghamshire, UK) were used. Proteins were then transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA), and the tyrosine-phosphorylated insulin receptor substrate 1 (IRS-1) was detected using rabbit polyclonal anti-phosphotyrosine

102 Nutrition and Cancer 2000
antibodies (Upstate Biotechnology, Lake Placid, NY) at 1 µg/ml. Bands were detected by enhanced chemiluminescence kit (Amersham, Buckinghamshire, UK), according to the manufacturer’s suggested procedures. Quantitation was done by the Image Analysis System (GDS 5000, UVP). IRS-1 was identified by protein stripping and reprobing the polyvinylidene difluoride membranes with anti-IRS-1 antibody (IRS-1-CT, Upstate Biotechnology).

Iodination Procedures

IGF-I and des(1–3)IGF-I were iodinated by the chloramine-T procedure and separated from free radioactive iodide on a Sephadex G-25M (PD-10 column, Pharmacia, Uppsala, Sweden), as previously described (32). Specific radioactivity was about 9,000 cpm/fmol.

Determination of IGF-I Receptor and Cell Surface-Associated IGFBPs

IGF-I receptor assay was performed using [125I]des(1–3)IGF-I (an IGF analog that does not bind to IGFBPs), essentially as described by us previously (33). Cell surface-associated IGFBPs were determined as a portion of [125I]labeled IGF-I binding displaced by unlabeled IGF-I in the presence of 100 nM unlabeled des(1–3)IGF-I in every assay point to exclude [125I]labeled IGF-I binding to IGF receptors, as verified by us previously (32,33). Radioligand binding was measured in monolayers of cells (150,000 cells/well) in 24-well plates. Cells were washed twice with PBS and incubated for 2.5 hours at 4°C with 150,000 counts/min of [125I]labeled IGF-I or [125I]labeled des(1–3)IGF-I in 0.2 ml of PBS containing 1 mg/ml of bovine serum albumin (essentially globulin-free). After incubation, cells were washed three times with ice-cold PBS and dissolved in 0.5 M NaOH. Radioactivity was then measured in a gamma counter. The dissociation constants (Kd) and the number of binding sites were analyzed as described by Munson and Rodbard (34) using the LIGAND program for the final Scatchard analysis. IGF-I receptors were also analyzed by flow cytometry (see below). Anti-IGF-I receptor antibody (IGF-IR 3B7, Santa Cruz, Santa Cruz, CA) was used as the primary antibody, and phycoerythrine-conjugated F(ab')2 fragment, goat anti-mouse immunoglobulin G (Jackson Immunoresearch, West Grove, PA) was used as the secondary antibody.

Flow Cytometry

Flow cytometric studies were performed using the FACStar flow cytometer (Becton-Dickinson, Mountain View, CA). The obtained data were analyzed by PC-Lysis (Becton-Dickinson) and ModFit (Verity Software House) computer programs.

Cell Cycle Analysis

Cells were trypsinized, collected, and washed twice with PBS. Cell pellets were resuspended in 200 µl of PBS, fixed in 1 ml of 70% ethanol-30% PBS, and stored at −20°C. Cells were washed twice with PBS, then incubated for 40 minutes in 1 ml of PBS containing 0.1% Triton X-100 and 50 µg of ribonuclease (deoxyribonuclease free) at room temperature. Propidium iodide (10 µg) was added, and the suspension was incubated in the dark at room temperature for an additional 15 minutes. The suspension was then filtered through a 60-µm mesh filter and analyzed for DNA content. The percentage of cells in different phases of the cell cycle was determined by flow cytometry with a ModFit program. The sub-G1 (subdiploid) cell population was analyzed essentially as described above, except the ethanol-fixed cells were kept for at least one week at −20°C.

Analysis of Apoptotic and Necrotic Cell Populations

Cells were collected after incubation for 10 minutes with 0.2 mg/ml of crystalline trypsin solution (Beth Haemek) at 37°C and washed twice in buffer containing 10 mM HEPES, 140 mM NaCl, and 2.5 mM CaCl2 at room temperature. Cells were resuspended in 0.5 ml of the above-described buffer supplemented with 0.2 µg of annexin-V-FITC and incubated for 30 minutes at room temperature in the dark (35). Propidium iodide (10 µg) was added, and the suspension was incubated for an additional 15 minutes. The cell suspension was then filtered through a 60-µm mesh filter and analyzed by flow cytometry.

Electrophoretic Mobility Shift Assay of AP-1 Binding Capacity

The assay was performed using an oligonucleotide containing four tandem repeats of the TPA-responsive element (TRE) consensus sequence (5'-TGACTCATGACTCATGACTCATGACTCATCA-3'), as previously described (36). Cells were washed twice with ice-cold PBS, scraped by a rubber policeman, collected by centrifugation, and resuspended in buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.5 mM AEBSF, 10 µg/ml of aprotinin, and 10 µg/ml of leupeptin. The cells were lysed by passage through a 21-gauge needle, and nuclei were collected by centrifugation at 12,000 g for 15 minutes at 4°C. The nuclear pellet was resuspended in buffer containing 20 mM HEPES (pH 7.9), 1.5 mM MgCl2, 0.5 mM DTT, 25% (vol/vol) glycerol, 0.2 mM EDTA, 0.42 M NaCl, 0.5 mM AEBSF, 10 µg/ml of aprotinin, and 10 µg/ml of leupeptin and lysed by passage through a 21-gauge needle. Lysates were clarified by centrifugation at 12,000 g for 15 minutes at 4°C and dialyzed against 50 volumes of buffer containing 20 mM HEPES (pH 7.9), 20% (vol/vol) glycerol, 1.5 mM MgCl2, 0.1 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM AEBSF, 10 µg/ml of aprotinin, and 10 µg/ml of leupeptin.
for six hours. The TRE consensus sequence oligonucleotide was end-labeled with \([\gamma^{32}\text{P}]\text{ATP}\) by T4-polynucleotide kinase. Protein content of the sample was determined by the method of Bradford using a protein assay kit (Bio-Rad), 10 \(\mu\text{g}\) of nuclear lysate were incubated at room temperature with the labeled TRE oligonucleotide for 30 minutes, and DNA-protein complexes were separated from the unbound probe on 5% TBE-polyacrylamide gel. The gel was dried and analyzed by a radioactive image analyzer (BAS 1000, Fuji Photo Film, Tokyo, Japan).

**Statistical Analysis**

Data were analyzed by Student’s \(t\)-test; \(p < 0.05\) was considered statistically significant.

**Results**

**Lycopene Inhibits Basal and IGF-Induced Thymidine Incorporation**

We first evaluated whether lycopene inhibits basal and IGF-stimulated proliferation of MCF7 mammary cancer cells. To this purpose, cells were preincubated for one day in the presence of increasing concentrations of lycopene, then stimulated with IGF-I for one to three days in the presence of the same concentrations of lycopene. The results obtained after two days of IGF-I stimulation are shown in Figure 1. To reduce the interference of serum-derived growth factors, these experiments were performed in the presence of 0.5% FCS. Under these conditions, lycopene did not affect \([\text{H}]\text{thymidine incorporation during the one-day preincubation period, before the addition of IGF-I. In cells incubated without lycopene, IGF-I stimulated \([\text{H}]\text{thymidine incorporation by about twofold. Lycopene at } 3 \text{\(\mu\text{M}\) inhibited basal and IGF-induced cell growth (\(p < 0.05\)), but the IGF effect was inhibited to a greater extent. At lower concentrations (0.75 or 1.5 \(\mu\text{M}\)), \([\text{H}]\text{thymidine incorporation was significantly inhibited only in cells growing in the presence of IGF-I, whereas basal growth was not affected. Because \([\text{H}]\text{thymidine incorporation is an indirect method for measuring cell growth, the above results were confirmed in several experiments by direct cell counting (Figure 1B). The increase in cell number by IGF-I and the decrease by 3 \(\mu\text{M}\) lycopene under basal and IGF-I-stimulated conditions (Figure 1B) were similar to the changes in \([\text{H}]\text{thymidine incorporation (Figure 1A). The validity of \([\text{H}]\text{thymidine incorporation for analysis of cell proliferation was similarly confirmed in a previous study (29). The reduction in cell number by lycopene, as shown above, can result from inhibition of cell cycle progression and/or induction of cell death. Thus we next examined these two possibilities.**

**Lycopene Slows Cell Cycle Progression and Does Not Cause Cell Death**

In unsynchronized cells, under conditions in which lycopene inhibits cancer cell growth (29), there was no change in the cell cycle phase distribution (see Figure 4, A and B). Therefore, cell cycle progression was studied in cells synchronized by two methods: serum starvation and mimosine block. It has been shown that, after growth arrest of breast cancer cells by serum deprivation, restimulation with insulin, IGF-I, and several other growth promoters resulted in cell cycle progression through the G\(_1\) phase and subsequent entry into the S phase (37). Accordingly, control and lycopene-treated cells were partially synchronized by serum starvation (0.5% FCS) followed by IGF-I stimulation for 26 hours (Figure 2). Serum starvation of lycopene-treated and untreated cells resulted in accumulation of cells in the G\(_0\)-G\(_1\) phase. The number of cells in this phase increased from 52% in unstarved cells to 70% in starved cells. A concomitant decrease from 33% to 20% and from 15% to 10% was noted in the S and G\(_2\)/M phases, respectively. IGF-I treatment of the

![Figure 1](image-url)

*Figure 1. Effect of lycopene (lyco) treatment on \([\text{H}]\text{thymidine incorporation and cell number in control and insulin-like growth factor I (IGF-I)-stimulated MCF7 mammary cancer cells. Cells were preincubated for 1 day in 0.5% fetal calf serum (FCS) containing indicated concentrations of lycopene, then 30 nM IGF-I was added for 2 days where indicated. Values at end of preincubation were the same for all experimental conditions and were subtracted from all data. A: \([\text{H}]\text{thymidine incorporation. Values are means } \pm \text{ SEM of } 3 \text{ different experiments, with } 4 \text{ replicate wells in each experiment. B: cell counting. Values are means } \pm \text{ SEM of } 2 \text{ different experiments, with duplicate counts under each experimental condition. THF, tetrahydrofuran; cpm, counts/min.**

104 Nutrition and Cancer 2000
partially synchronized control cells induced a twofold increase in the number of cells to 40% in the S phase and to 17% in the G2/M phase. This increase was completely abrogated by lycopene pretreatment.

The slowing of cell cycle progression by lycopene was demonstrated also in cells synchronized by treatment with the plant amino acid mimosine. Mimosine reversibly inhibits DNA synthesis in mammalian cells, arresting them in the G1-S boundary after the restriction point (38). The cells were synchronized by 36 hours of incubation with mimosine (500 μM) with or without lycopene (Figure 3). Mimosine caused accumulation of cells in the G0/G1 phase (85% vs. 52% in nonsynchronized cells). Control or lycopene-treated cells were then released from the block by mimosine removal, and cell cycle progression was monitored after 7 and 20 hours. Removal of mimosine caused rapid cell cycle progression in control cells, and after seven hours, 40% of control cells progressed to the middle-late S phase. In contrast, only 16%...
of lycopene-treated cells were present in the early S phase seven hours after mimosine removal. After 20 hours, 40% were present in the early S phase and only 22% were found in the middle-late S phase (similar to that seen in control cells 7 h after release).

Collectively, the above results clearly indicate that lycopene slows cell cycle progression through the G₁ and S phases. This effect was not accompanied by apoptotic or necrotic cell death, as observed in the following experiments.

The presence of cells with sub-G₁ DNA content was previously used as an indicator of apoptosis in MCF7 cells (39). Analysis of DNA histograms after two days of incubation of control and lycopene-treated cells (Figure 4, A and B) did not reveal any sub-G₁ peak. On the other hand, treatment with 20 μM cisplatin, known to induce apoptosis (40) and thus serving as a positive control, resulted in G₂ arrest and massive apoptosis, as revealed by the appearance of a large sub-G₁ peak (Figure 4C).

Phosphatidylserine “flip-flop” is one of the early-middle markers of apoptosis (41) and was detected also in MCF7 cells undergoing apoptosis in response to all-trans-retinoic acid (42). Titration of phosphatidylserine in the outer plasma membrane leaflet of cells, by annexin-V-FITC binding and flow cytometry, did not show any increase in annexin-V-positive cells after lycopene treatment of cells growing in

![Figure 4](image4.png)

**Figure 4.** Lack of lycopene effect on cell apoptosis. DNA content was analyzed in cells incubated for 48 h in medium containing 3% FCS in absence (A) or presence (B) of lycopene or treated for 20 h with 20 μM cisplatin (C). Cell cycle analysis was performed as described in Materials and Methods. C: designation of various cell cycle phases and sub-G₁ peak containing apoptotic bodies. A representative example of 3 similar experiments is shown.

3% serum (Figure 5, A and B). Again, treatment with 20 μM cisplatin resulted in the appearance of an apoptotic cell population, as evidenced by the presence of cells displaying phosphatidylserine on the outer leaflet of the plasma membrane (Figure 5C).

We previously proposed that lycopene does not cause necrotic cell death on the basis that lactate dehydrogenase accumulation was not detected in conditioned medium of lycopene-treated cells (29). In the present study we confirmed this result by analyzing DNA staining with propidium iodide as a second fluorescent probe in the experiment with annexin-V-FITC (Figure 5). This DNA-binding dye cannot penetrate the intact cell membrane; thus the absence of an increase in a propidium iodide-positive population in lycopene-treated cells indicates that the carotenoid does not induce necrotic cell death (Figure 5, A and B). In this double-staining experiment, the propidium iodide-positive cells were also annexin-V positive, since the protein can probably penetrate the necrotic cells and bind to phosphatidylserine at their inner plasma membrane leaflet. The necrotic cell population was more pronounced in the cisplatin-treated cells (Figure 5C).
The effects of lycopene on apoptosis were tested primarily after two days of treatment (Figures 4 and 5), because at this time a significant inhibitory effect on cell number was shown by the carotenoid. To exclude the possibility that apoptosis occurred at a different time, measurements were performed also after one day and after four days and revealed similar results (data not shown).

**Lycopene Inhibits Signal Transduction of IGF-I**

To study the mechanism of inhibition of IGF-I-induced cell growth, we first analyzed the function of IGF-I receptors by measuring IGF-I-induced IRS-1 tyrosine phosphorylation in control and lycopene-treated MCF7 cells. This 185-kDa protein is one of the major tyrosine-phosphorylated proteins observed on IGF stimulation (1). Cells were stimulated with IGF-I, and lysates were analyzed by Western immunoblotting with an anti-phosphotyrosine antibody (Figure 6A). Lycopene did not change the basal level of IRS-1 tyrosine phosphorylation (cf. Lanes 1 and 2), but IGF-I-induced tyrosine phosphorylation (set at 100%) was inhibited by 60 ± 12% (cf. Lanes 3 and 4). This reduction in IRS-1 phosphorylation was not accompanied by a change in the level of the protein, as estimated by Western blot with anti-IRS-1-specific antibody (Figure 6B).

To examine whether lycopene inhibition of IGF-I receptor signaling can also be observed downstream to IRS-1, we studied the IGF-induced binding capacity of the AP-1 transcriptional regulatory complex. The activation of AP-1 transcriptional activity is a middle-term event in the mitogenic signaling pathway of many growth factors (43). As we anticipated, lycopene completely blocked IGF-I-induced upregulation of AP-1 binding (Figure 7). In contrast to the inhibition of IGF-I-induced AP-1 binding capacity, lycopene increased its basal level (Figure 7, cf. Lanes 5 and 3).

Inhibition of IGF-I receptor signaling might have resulted because lycopene modulated the number and affinity of the IGF-I receptors. However, neither affinity ($K_d$ approx 1 nM) nor number of IGF-I receptors (28,000 ± 5,600 and 27,600 ± 4,900 receptors/cell in control and lycopene-treated cells, respectively) was altered after two days of lycopene treatment, as measured by $^{125}$I-labeled des(1–3)IGF-I binding and calculated by Scatchard analysis. The lack of an effect on IGF-I receptor number was confirmed by the results of flow cytometric analysis with use of a monoclonal anti-IGF-I receptor antibody (Figure 8). We recently reported that cell surface-associated IGFBP-3 inhibits IGF-I receptor function (32,44); thus an increase in their number could mediate the inhibitory effect of lycopene on IGF-I signaling. Because of cross-reactivity of anti-IGFBP antibodies with many membrane proteins, we were unable to identify the type of the membrane-associated IGFBPs in MCF7 cells. However, by measuring $^{125}$I-labeled IGF-I binding (Figure 9), we showed that lycopene treatment resulted in about a 60% increase in the number of cell surface-associated IGFBPs (93,400 ± 8,800 and 147,300 ± 10,100 IGFBPs/cell in control and lycopene-treated cells, respectively). No significant change in the affinity of these proteins to IGF-I ($K_d$ approx 2 nM) was observed (Figure 9).

**Discussion**

In the present study we show that the mechanism of lycopene inhibition of mammary cancer cell proliferation involves interference in cell cycle progression and in the signaling pathway of IGF-I. We found that IGF-I-stimulated cell growth was inhibited by lycopene concentrations that were lower than those needed for inhibition in unstimulated cells. Similar results were obtained in Ishikawa endometrial cancer cells (29). Such low lycopene concentrations (<1 μM) are found in the blood of individuals consuming average-to-high amounts of tomato products (45) and thus may serve to inhibit cancer progression.

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**Figure 6.** Effect of lycopene treatment on IGF-I-stimulated tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1). Cells were treated for 2 days with 0.5% THF (control, Lanes 1 and 3) or 5 μM lycopene (Lanes 2 and 4) in presence of 0.5% FCS. During last 12 h, serum was replaced by 1 mg/ml of BSA (essentially globulin-free). Cells were washed and incubated for 3 min with 30 nM IGF-I (Lanes 3 and 4) or buffer alone (Lanes 1 and 2). Proteins containing phosphotyrosine were detected in cell lysates by Western immunoblotting with anti-phosphotyrosine antibody (A). Membranes were then stripped and reblotted with anti-IRS-1 antibody (B). Bar graph shows means ± SEM of densitometric data of 4 experiments.
The suppression of IGF-I-induced cell growth was associated with inhibition of IGF signaling, as evidenced by a decrease in IGF-I stimulation of tyrosine phosphorylation of IRS-1 and binding capacity of AP-1. This inhibition of IGF-I receptor activity was not due to reduction of IRS-1 level, receptor number, or receptor affinity. We recently reported that cell surface-associated IGFBP-3 could negatively modulate IGF-I receptor function (32,44). Thus the increase in the number of cell surface-associated IGFBPs, as seen in the present study, may explain, at least partially, the inhibitory effect of lycopene on IGF-I receptor function. However, because of antibody cross-reactivity, it was not possible to identify the type of the IGFBP. In another study (46), the metabolite of β-carotene, retinoic acid, which inhibits MCF7 cell growth, was observed to stimulate autocrine production and release of various IGFBPs, but no information was presented for cell surface-associated IGFBPs. We previously observed a similar role of cell surface-associated IGFBPs in the effect of tamoxifen on cell growth (47). This antiestrogen inhibits proliferation of mammary cancer cells while, paradoxically, stimulating endometrial cancer cell growth. These opposite effects were inversely correlated with changes in membrane-associated IGFBPs but not with changes in the number or affinity of IGF-I receptors. More studies are needed to clarify whether lycopene inhibition of IGF-stimulated mammary cancer cell growth is solely due to these IGF-signaling events or also to additional cellular effects.
Although this is the first report showing that lycopene interferes with a growth factor signal transduction mechanism, an effect of another carotenoid on the epidermal growth factor receptor has recently been demonstrated (48). In that study, prevention of cervical carcinogenesis by beta-carotene was due to induction of apoptosis in cervical dysplastic cells via downregulation of the epidermal growth factor receptor protein.

Uptregulation of the binding capacity of AP-1 transcriptional complex is a common downstream step in IGF-I and other growth factor signaling. Interestingly, although lycopene blocked IGF-I-induced upregulation of AP-1, it did increase the basal level of AP-1 binding activity. Experiments are in progress to clarify the involvement in these effects of various proteins of the Fos and Jun families, which are components of the AP-1 transcription complex. In a recent review, Sun and Oberley (49) focused on the regulation of transcription factors by oxidation/reduction status. Particularly, it was found that antioxidants activate AP-1 binding activity. Because lycopene is a potent antioxidant (50), these data agree well with the carotenoid-induced elevation of basal AP-1 binding activity shown in the present study. However, not all reports support the notion that the AP-1 induction is an antioxidant response (51).

Effects of carotenoids on AP-1 transcription complex have not been reported; however, a recent study has revealed the effect of another active food ingredient, curcumin (diferuloylmethane), on several transcriptional regulatory complexes (52). This compound, known to possess an anti-inflammatory and anticarcinogenic activity, was found to inhibit tumor necrosis factor- and hydrogen peroxide-mediated activation of nuclear factor-kB in human myeloid cells. AP-1 binding factors were also found to be downmodulated by curcumin, whereas the Sp1 binding factor was unaffected. It remains to be investigated whether lycopene regulates transcription systems other than AP-1.

The lycopene-induced delay in cell cycle progression after serum starvation or mimosine synchronization strongly indicates that the carotenoid slows the cell cycle. Mimosine is known to block the cell cycle at the G1-S boundary after the restriction point (38). Thus it appears that lycopene affects the phase that follows the mimosine point of action. In addition, lycopene inhibits the action of IGF-I on the cell cycle, which has been suggested to stimulate the cell cycle at the G0-G1 transition (53). We recently found that inhibition of HL-60 cell growth by lycopene is accompanied by inhibition of cell cycle progression in the G0-G1 phase (22). Another carotenoid, alpha-carotene, had a similar effect in neuroblastoma cells (54). We are investigating the effect of lycopene on molecular components of cell cycle machinery, where we previously observed a reduction in the level of cyclin D and the activity of cyclin-dependent kinase-4 and kinase-2 as well as a hypophosphorylated state of the retinoblastoma protein (unpublished observations).

Lycopene inhibition of the action of IGF-1, the level of which has been shown to strongly correlate with risk of breast and prostate cancer (3,4), may be one of the mechanisms for the preventive effect of lycopene on these (14,18) and other (20) cancers. Lycopene is the major carotenoid in human plasma in some Western countries where the diet is rich in tomato and tomato products. However, its levels may be low and vary considerably in individuals on low-carotenoid diets. It also should be noted that the activity of lycopene may reside in the carotenoid molecule or in a metabolic product that may be formed from it. Elucidation of the mechanism of lycopene action on cancer cell growth shouldendorse its inclusion as an important component of dietary regimens.

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