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Lycopene inhibition of IGF-induced cancer cell growth depends on the level of cyclin D1

■ **Summary** *Background* Insulin-like growth factors (IGFs) play an important role in normal and cancerous cell proliferation. Moreover, in recent studies IGF-I has been implicated as a major cancer risk factor. The tomato carotenoid lycopene and all-trans

retinoic acid (atRA) have been shown to inhibit growth factor-induced proliferation of different types of cancer cells. This action is associated with inhibition of cell cycle progression in G₀/G₁ phase. Cyclin D1 acts as a growth factor sensor in G₁ phase and is overexpressed in many breast cancer tumors. We have previously demonstrated that slow-down of serum-stimulated cell cycle progression from G₁ to S phase by lycopene correlates with reduction in cyclin D1 levels, suggesting that the expression of this protein is a main target for lycopene's action. *Aim of the study* To determine whether the reported reduction in cyclin D1 level is the key mechanism for lycopene and atRA inhibitory action on IGF-I-induced cell cycle progression. *Results* Human breast (MCF-7) and endometrial (ECC-1) cancer cells were synchronized in G₀/G₁ phase by serum deprivation followed by stimulation with IGF-I. Cell treatment with lycopene and atRA inhibited IGF-I-stimulated cell cycle progression from G₁ to S

phase and decreased retinoblastoma protein (pRb) phosphorylation. These events were associated with a reduction in cyclin D1 and p21^{CIP1/WAF1} level, but not that of p27^{KIP1}. To test the hypothesis that the decrease in cyclin D1 has a major role in the inhibitory effects of lycopene and atRA, we examined the ability of these two agents to suppress cell cycle progression in MCF-7/D1.13 cells which are capable of expressing cyclin D1 under the control of the Zn-inducible metallothionein promoter. Our results showed that ectopic expression of cyclin D1 can overcome cell cycle inhibition caused by lycopene and atRA. *Conclusions* Our findings suggest that attenuation of cyclin D1 levels by lycopene and atRA is an important mechanism for the reduction of the mitogenic action of IGF-I.

■ **Key words** lycopene – retinoic acid – breast cancer – cell cycle – cyclin D1

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Introduction

Insulin-like growth factors (IGFs) and their binding proteins (IGFBPs) play an important role in con-

trolling cell proliferation and differentiation as well as apoptosis and transformation [1]. Moreover, IGF-I has been implicated as a major cancer risk factor. It was reported that high blood levels of IGF-I

existing years before malignancy detection can predict increased risk for breast, prostate, colorectal and lung cancer [2–5]. We have previously demonstrated that lycopene inhibits the growth of breast, endometrial, lung cancer [6] and leukemic [7] cells. Furthermore, growth stimulation of MCF-7 human breast cancer cells by IGF-I was markedly reduced by low concentrations of lycopene [6, 8]. Interestingly, these effects were not accompanied by either necrotic or apoptotic cell death but were associated with a marked inhibition of serum- and IGF-I-stimulated cell cycle transition from G₁ to S phase [7–9].

Deregulated cell cycle is one of the major hallmark traits of cancer cells. Thus, elucidation of the mode by which carotenoids inhibit cell cycle progression would provide a mechanistic basis for the anti-cancer effect of these micronutrients. Cell cycle progression is activated by growth factors primarily during G₁ phase. The main components of the cell cycle machinery which act as growth factor sensors are the D type cyclins [10]. Cyclin D1 is an oncogene that is overexpressed in many breast cancer cell lines as well as in primary tumors [11]. Interestingly, many anticancer agents, including those used for breast cancer therapy, convey their inhibitory effect in G₁ phase primarily by reducing cyclin D1 levels (for example, pure antiestrogens [12, 13], tamoxifen [14], retinoids [15, 16], as well as progestins [17], and 1,25-dihydroxyvitamin D₃ [18]). In a recent study [9], we have demonstrated that the inhibition of serum-stimulated cell cycle traverse from G₁ to S phase by lycopene correlates with reduction in cyclin D1 levels, resulting in inhibition of both cdk4 and cdk2 kinase activity. Inhibition of cdk4 was directly related to lower amount of cyclin D1-cdk4 complexes while inhibition of cdk2 action was related to retention of p27 molecules in cyclin E-cdk2 complexes due to the reduction in cyclin D1 level. These results together with the fact that neither cyclin E nor cdk2 or cdk4 levels changed by lycopene treatment, suggest that the inhibitory effect of this carotenoid on cell cycle progression is mediated primarily by down-regulation of cyclin D1.

The aim of the current study is to ascertain the importance of cyclin D1 as a target for lycopene anti-cancer activity. We validate and generalize our results by using two different cell lines and perform all experiments in parallel with a second anti-cancer agent, all-trans retinoic acid (atRA). This retinoid which is a metabolite of another carotenoid, β -carotene is also known to inhibit cell cycle progression at G₁ [15, 16]. Our hypothesis is that if cyclin D1 is indeed a prime target of lycopene, ectopic expression of this protein will overcome the growth inhibitory

effect of the carotenoid. This hypothesis is tested in a clone of MCF-7 cells capable of exogenously controlling cyclin D1 expression.

Materials and methods

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 Sigma-Aldrich (Israel). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), bovine insulin and Ca²⁺/Mg²⁺-free phosphate buffered saline (PBS) were purchased from Biological Industries (Beth Haemek, Israel). Lycopene was dissolved in THF at a concentration of 2 mM and stored at –20 °C. Immediately before the experiment, the stock solution was added to the cell culture medium, as described previously [6]. The final concentration of lycopene in the medium was measured spectrophotometrically after extraction in 2-propanol and *n*-hexane: dichloromethane. All procedures were performed under dim lighting.

Cell culture and treatment protocol

MCF-7, a human breast cancer cell line, was purchased from American Type Culture Collection (Rockwell, MD). ECC-1 human endometrial cancer cells were established and kindly provided by Dr. Pondichery Satyaswaroop (Hershey Medical Center, Pennsylvania State University) [19]. MCF-7.7 cells transfected with either p Δ MT (MCF-7.7p Δ MT) or p Δ MTcyclD1 (MCF-7.7D1.13) were established as described previously [20]. Cells were grown in DMEM containing penicillin (100 U/ml), streptomycin (0.1 mg/ml), nystatin (12.5 μ g/ml), insulin 0.6 μ g/ml, and 5% FCS. Cells were seeded into 75 cm² flasks (1.5 \times 10⁶ cells) in medium containing 5% FCS. Two days later, the medium was changed to one containing 0.5% FCS and solubilized lycopene or THF alone (see above). After 48 h of serum starvation (medium was replaced daily), cells were incubated in a medium containing IGF-I 30 nM with solubilized lycopene or vehicle. MCF-7.7 transfected cells were exposed to medium containing Zn (as ZnSO₄). Unless otherwise indicated, Zn concentration was 50 μ M. THF had no effect on cell growth or on the parameters measured in this study (data not shown). Cells harvested from 75 cm² flasks at different time points were used for both cell cycle and protein analyses by flow cytometry and western blotting, respectively.

■ Cell cycle analysis

Cells were trypsinized, collected and washed twice with PBS. Cell pellets were resuspended in 200 μ l PBS, fixed in 1 ml of 70% ethanol/30% saline and stored at -20°C . Cells were then washed twice with PBS followed by incubation for 40 min in 1 ml PBS containing 0.1% Triton X-100 and 30 μ g of RNase (DNase free) at room temperature. Ten μ g of propidium iodide were added and the suspension was incubated in the dark at room temperature for an additional 15 min. The suspension was then filtered through a 35 μ m filter and analyzed for DNA content by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA). The percent of cells in different phases of the cell cycle was determined using the ModFit (Verity Software House) program.

■ Western blot analysis

Cells were lysed as described previously [21] with modifications. Cell monolayers were washed twice in ice-cold PBS and then scraped into ice-cold lysis buffer A (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1.5 mM MgCl_2 , 1 mM EGTA, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 μ M phenylmethylsulfonyl fluoride, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 50 mM NaF, and 0.2 mM dithiothreitol). The lysates were incubated for 10 min on ice, and the cellular debris were cleared by centrifugation (13,000 rpm, 5 min, 4°C). Protein content of the samples was determined by the Bradford method using a protein assay kit (Bio-Rad). Equal amounts of protein (30–50 μ g) were separated by SDS-PAGE and then transferred to a PVDF membrane (Gelman, Inc.). Proteins were visualized using the ECL detection system (Amersham) after incubation overnight at 4°C with the following primary antibodies: cyclin D1 (HD-11), cyclin D3 (H-292), cyclin E (HE12), p27 (C-19), Cdk2(M2), Cdk4(C-22), p21 (C-19), p130 (C-20), p107 (C-18) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) or pRb (G3-245) from PharMingen (San Diego, CA). Protein abundance was quantitated by densitometry analysis. Although this is a semi-quantitative method for evaluating changes in protein levels, it is an accepted and widely-used procedure.

■ Statistical analysis

All experiments were repeated at least three times. The data represent the means \pm SEM unless stated

otherwise. The significance of the differences between the means of the various subgroups was assessed by the two-tailed Student's *t*-test. $P < 0.05$ was considered statistically significant. The calculations were performed using the GraphPad Prism 3.0 program (GraphPad Software, San Diego, CA).

Results

■ Lycopene and retinoic acid inhibition of IGF-I induced cell cycle progression is associated with decrease pRb and p130 phosphorylation and cyclin D expression

To assess the effect of lycopene and atRA on IGF-induced changes in cell cycle regulatory proteins, human breast (MCF-7) and endometrial (ECC-1) cancer cells were partially synchronized in G_1 phase by serum deprivation (0.5% FCS) for 48 h with or without lycopene (2–3 μ M) or atRA (1 μ M). This was followed by stimulation with 30 nM IGF-I, which drove the cells from G_1 into S phase (Fig. 1A). Similar to serum treatment of cells [9], IGF-I stimulation of MCF-7 and ECC-1 control cells for 24 h resulted in a substantial increase in the S phase population, whereas both lycopene and atRA treatment almost completely inhibited progression from G_1 to S phase.

Addition of IGF-I to serum-deprived control ECC-1 cells resulted in a time-dependent phosphorylation of pRb, i.e., accumulation of its slower migrating hyperphosphorylated (inactive) form beginning at 4 h (Fig. 1B). Similarly, an increase in the amount of two higher phosphorylated forms of p130 was observed. Pocket protein phosphorylation was markedly reduced and delayed in cells treated with either lycopene (Fig. 1B) or atRA (not shown). In MCF-7 cells, as previously demonstrated by us in serum-treated cells, the effects of these two agents on the phosphorylation state of pRb, and p130 (not shown) were similar to those observed in ECC-1 cells.

IGF-I stimulation of untreated MCF-7 cells, resulted in about a 6-fold increase in cyclin D1 level starting after 4 h and reaching a maximal level at 12 h (Fig. 1C, upper panel). Lycopene treatment inhibited the IGF-I induced increase in cyclin D1, and its maximal level achieved was only $35 \pm 5\%$ of that measured in the control cells. In control ECC-1 cells, IGF-I caused about a 5-fold increase in cyclin D1 levels at 24 h, whereas in the lycopene-treated cells, a much smaller increase was observed (Fig. 1C, middle panel), reaching $20 \pm 5\%$ of the maximal level measured in the control cells. A similar inhibition of cyclin D1 induction was observed also in atRA-treated

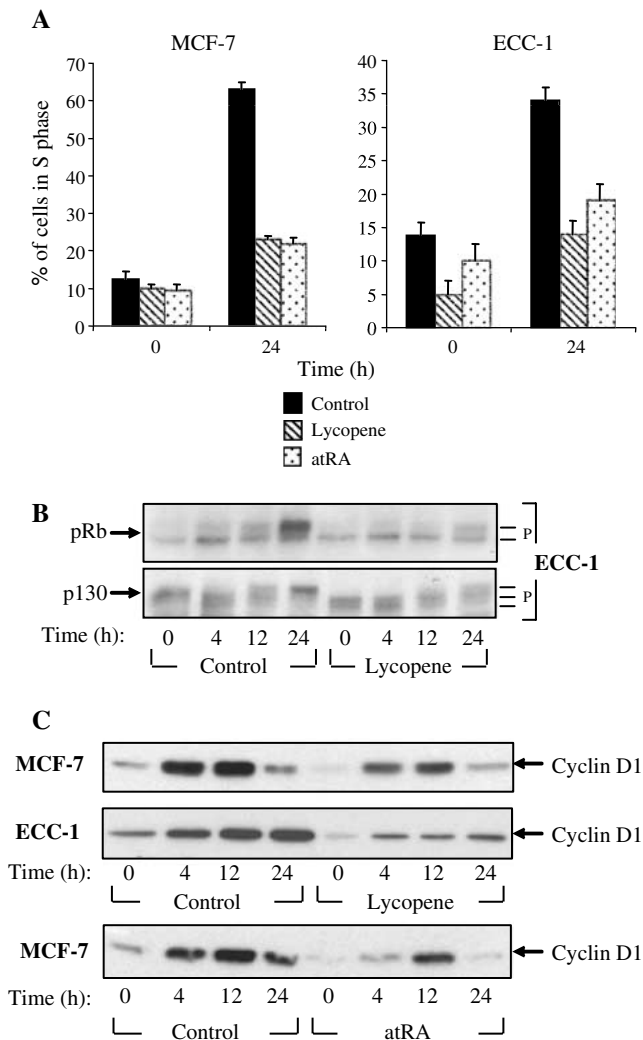


Fig. 1 Lycopene and atRA inhibit cell cycle progression and reduce pRb phosphorylation and cyclin D1 protein levels. Exponentially grown MCF-7 and ECC-1 cells were synchronized in G₁ phase (0 h) by serum deprivation (0.5% FCS) for 48 h in the presence of lycopene (2–3 μ M), atRA (1 μ M) or 0.5% THE (control) and then stimulated with 30 nM IGF-I for 24 h. **(B)** Percentage of MCF-7 and ECC-1 cells in S phase. Cells were harvested and DNA content was analyzed by flow cytometry. **(B, C)** Cells were harvested at the indicated times and whole cell lysates were subjected to SDS-PAGE and Western blot analysis using antibodies against pRb, p130 and cyclin D1. One representative experiment of three similar experiments is shown. **(B)** Expression of pocket proteins in ECC-1 cells. The slower mobility (hyperphosphorylated) and the faster mobility (hypophosphorylated) forms of pRb and three phosphorylated forms of p130 are indicated. **(C)** Expression of cyclin D1 in MCF-7 and ECC-1 cells

MCF-7 (Fig. 1C, lower panel) and ECC-1 cells (not shown). In agreement with previous results, cyclin E levels and CDK2 and CDK4 levels did not change during stimulation with IGF-I in the absence or presence of lycopene in MCF-7, and ECC-1 (data not shown) cells.

■ The effect of lycopene and atRA on MCF-7 cells capable of exogenously controlling cyclin D1 expression

In order to substantiate our assumption that reduction of cyclin D1 plays a crucial role in the inhibitory action of lycopene and atRA on IGF-I activity, we examined the effects of these compounds on the cell cycle in MCF-7.7D1.13 cells, which are capable of ectopic expression of cyclin D1 under control of the Zn-inducible metallothionein promoter [20]. To achieve the optimal Zn concentration needed to induce cell cycle progression from G₁ to S phase after serum starvation, a concentration dependence of the cellular response to ZnSO₄ (0 to 75 μ M) was determined 24 h after onset of stimulation. The percentage of S phase cells reached a plateau of about 50% at 50 μ M ZnSO₄ (Fig. 2B). Zn had no effect on cell cycle phase distribution in MCF-7.7p Δ MT cells transfected with the vector alone.

Lycopene or atRA treatment of cyclin D1-transfected cells did not alter cell cycle progression induced by Zn stimulation (Fig. 2B). In contrast, IGF-I stimulation of these cells was markedly inhibited (by about 50%) by both lycopene and atRA. It is important to note that the response of the cyclin D1-transfected cells to IGF-I, measured as progression of cells from G₁ to S phase, was much lower than the response observed in parental MCF-7 cells (compare Fig. 1B to 2B). Reduced sensitivity of the transfected cells to 17- β estradiol, another stimulator of cell cycle progression, has been observed previously [20].

When Zn was added to the cyclin D1-transfected cells, a prominent increase in cyclin D1 expression took place (Fig. 2C), which was not reduced by lycopene treatment. We also found that p21 levels increased in parallel to cyclin D1 levels with no significant difference between the lycopene- or vehicle-treated cells (Fig. 2C). This is in contrast to the lower level of p21 in the lycopene-treated parental cells [9]. The levels of p27 were unchanged throughout the experiment, similar to the findings in parental MCF-7 cells.

The results in Fig. 2 indicate that lycopene and atRA can decrease cyclin D1 levels only when it is induced by the physiological stimulant, IGF-I, because, as expected, when cyclin D1 was induced ectopically the two compounds could no longer decrease its level and, more importantly, they did not inhibit cell cycle progression. However, the lack of inhibition of Zn-stimulated cell cycle progression might result not from the difference in the mechanisms of IGF-I- and Zn-stimulated cyclin D1 induction but merely from the high levels of this protein expressed in the presence of 50 μ M Zn. To

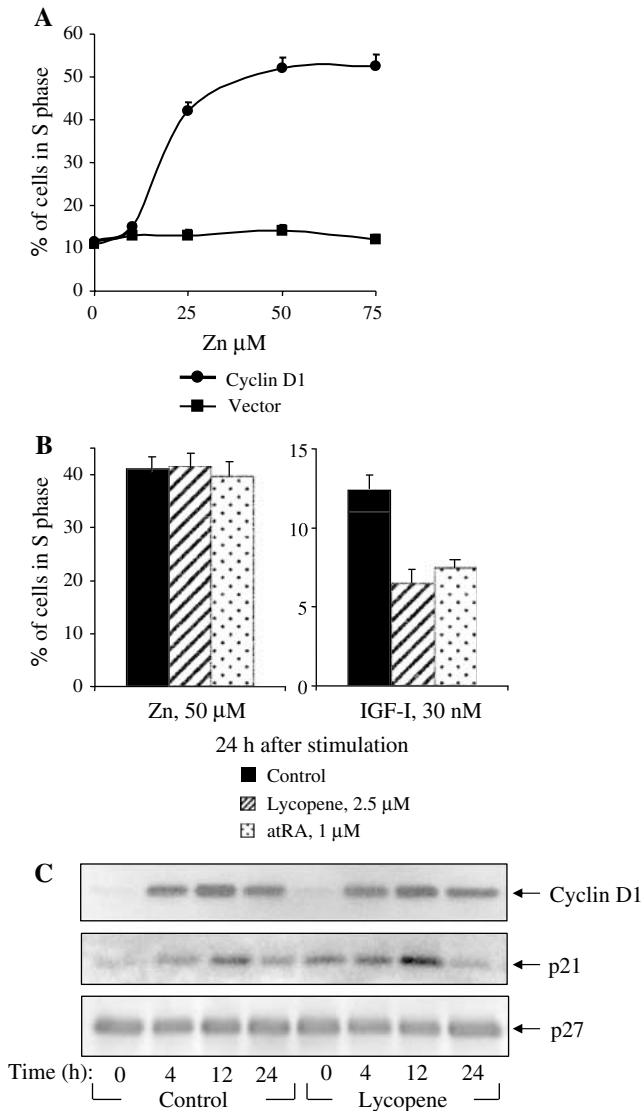


Fig. 2 Effects of lycopene and atRA on cell cycle and regulatory protein expression induced by Zn and IGF-I in cyclin D1-transfected cells. **(A)** MCF-7.7D 1.13 cells transfected with p Δ MTcycD1 (cyclin D1) and MCF-7.7p Δ MT cells transfected with p Δ MT (vector) were synchronized in G₁ phase by serum deprivation for 48 h. Increasing concentrations (0–75 μ M) of ZnSO₄ were added to the arrested cells for 24 h. Cells were harvested and percentage of cells in S-phase was analyzed by flow cytometry. **(B)** MCF-7.7D 1.13 cells were treated with lycopene and atRA and stimulated with 50 μ M ZnSO₄ or 30 nM IGF-I, as described in Fig. 1. The results shown are the percentage of the S-phase cell population measured 24 h after stimulation. Before stimulation, 5.2 \pm 0.4% of the cells were in S phase (not shown). Data of three separate experiments are presented as the mean \pm SEM. **(C)** Western blot analysis of cyclin D1, p21 and p27 expression in whole cell lysates obtained from lycopene-treated and untreated (control) MCF-7.7D 1.13 cells at the indicated times after stimulation with 50 μ M ZnSO₄. A representative experiment of three similar experiments is shown.

exclude this latter possibility we repeated the experiments but with lower concentrations of Zn to achieve a level of induction of cyclin D1 similar to that observed in IGF-I stimulated cells in the absence

of lycopene. The results demonstrated that even though the levels of cyclin D1 were similar in 30 nM IGF-I and 20 μ M Zn-stimulated cells (Fig. 3A, B), lycopene was capable of inhibiting only IGF-I stimulation of cyclin D1 expression and cell cycle progression (Fig. 3B, C). Thus, it appears that ectopic induction of cyclin D1 can rescue cells from the inhibitory effect of lycopene.

Discussion

The extensive amount of data showing the beneficial role of active dietary components in cancer prevention and the growing evidence implicating IGF-I as a significant cancer risk factor imply that there might be a link between the actions of some micronutrients and this growth factor. We addressed this question by studying the effects of lycopene, the major tomato carotenoid, and atRA, the metabolite of another carotenoid, β -carotene, on cell cycle apparatus in IGF-I-stimulated human breast and endometrial cancer cells. We have recently found that lycopene-induced reduction in cyclin D1 levels in serum-stimulated MCF-7 cells results in a decrease in cdk2 and cdk4 activity [9]. In the current study we have extended the range of cell types by adding ECC-1 endometrial cancer cells, and focused on the effects of lycopene on a specific and important growth factor, IGF-I. Our results suggest that the mechanism by which lycopene and atRA inhibit IGF-I action in cancer cells is based on the suppression of IGF-I-stimulated cyclin D1 induction by these two compounds. Cyclin D1 is a known oncogene [22] and a key element in cell cycle progression. To directly test the question of whether reduction of cyclin D1 levels is the basis of the anti-proliferative action of lycopene, we compared changes in cyclin D1 levels and cell cycle progression in IGF-I- and Zn-treated MCF7.7D1.13 cells expressing cyclin D1 under control of a Zn-inducible promoter. We assumed that if cyclin D1 induction is a main target of lycopene action then its ectopic expression would rescue the cells from inhibition. Alternatively, if additional components essential to cell cycle progression that are downstream to cyclin D expression are affected by lycopene and atRA, then the cell cycle would still be inhibited in spite of the ectopic expression of cyclin D1. Indeed, our results showed that even when cyclin D1 was induced by IGF-I and by Zn to similar levels in untreated cells, the amount of cyclin D1 expressed under Zn control was sufficient to rescue the cells from lycopene inhibition of the cell cycle. These data support the suggestion that control over cyclin D1 levels, whether through synthesis or degradation, serves as a crucial element in lycopene

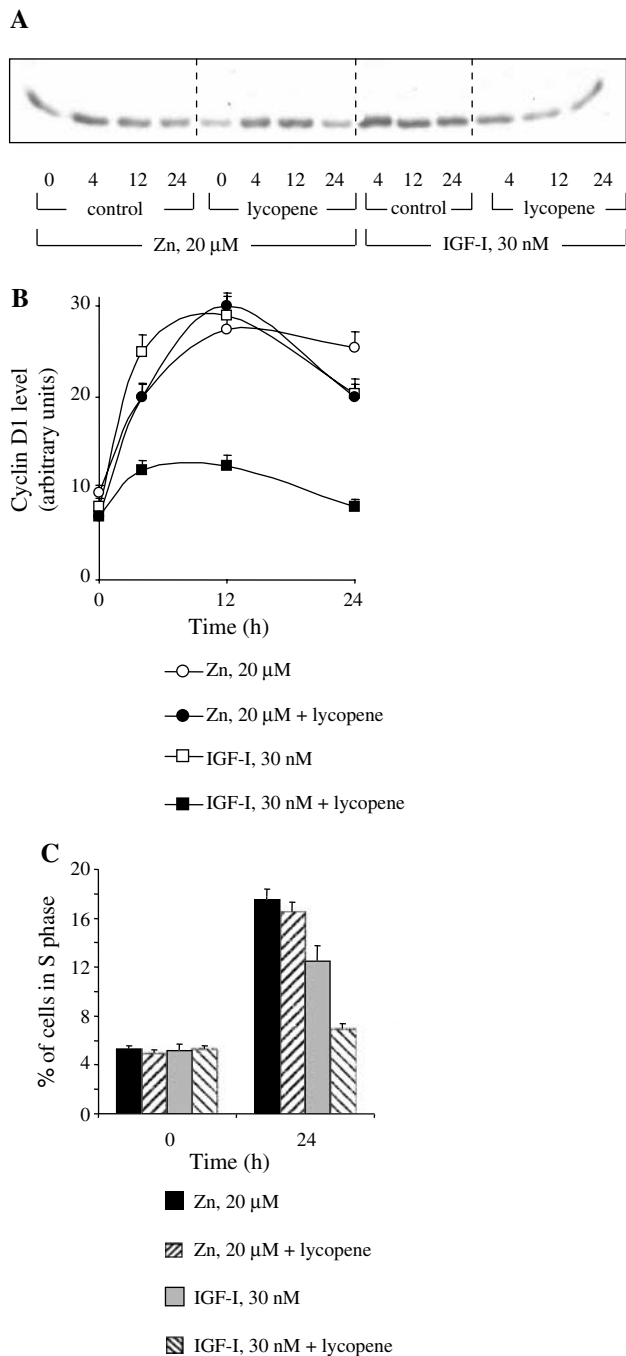


Fig. 3 Effects of lycopene on cell cycle and cyclin D1 expression induced by 20 μ M Zn in comparison to 30 nM IGF-I in cyclin D1-transfected cells. Serum-deprived MCF-7.7D 1.13 cells were treated with lycopene and stimulated with 20 μ M Zn or 30 nM IGF-I essentially as described in Fig. 4. **(A)** Western blot analysis of cyclin D1 expression in whole cell lysates obtained from lycopene-treated and untreated (control) cells at the indicated times after stimulation. A representative experiment of three similar experiments is shown. **(B)** Densitometric analysis of cyclin D1 levels shown in panel A. Results are the mean \pm SEM of three experiments. **(C)** Percentage of S-phase population of lycopene-treated and untreated (control) cells 24 h after addition of 20 μ M ZnSO₄ or 30 nM IGF-I. Results are presented as the mean \pm SEM of three experiments

and atRA inhibition of IGF-I mitogenic signals thus implicating this protein as a major target for the anticancer activity of these agents. However, these results do not exclude the possibility that other proteins such as various transcription factors, take part in the inhibition of cancer cell proliferation by lycopene and atRA. For example, we recently demonstrated that carotenoids stimulate the electrophile/antioxidant response element (EpRE/ARE) which is activated by the Nrf2 transcription factor [23]. Activation of this transcription system induces the production of phase II detoxification enzymes which remove carcinogens from the cells and thus are important for cancer prevention. The possibility that Nrf2 may have a role in inhibition of cancer cell proliferation is also under study.

IGF-I-mediated induction of p21 was markedly reduced by both lycopene and atRA treatment. A similar decrease in p21 abundance during inhibition of the cell cycle has been described previously in studies with retinoids [15, 16]. Several mechanisms could be responsible for this effect, such as inhibition of p53 pathways or disruption of growth factor signals known to induce p21 protein when cells re-enter the cell cycle [24, 25]. It was suggested that p21 promotes cyclin-CDK complex assembly and is essential for its kinase activity [26–28]. In a previous report [9], we have shown that p21 induction is linked with the induction of cyclin D1. The suggestion that cyclin D1 induction is an ultimate cause of p21 induction can be tested directly in cells that express cyclin D1 under a control that is independent of normal cellular mechanisms. Indeed, in the current study, similar to findings described by Hiyama et al. [29], ectopic expression of cyclin D1 resulted in a parallel increase in p21 levels. Since p21 may have a role in the functioning of cyclin D1-cdk complexes, our results suggest the possibility that lowering the levels of p21 may serve as an additional mechanism for the inhibitory action of lycopene on cancer cell proliferation.

We compared the effects of lycopene to those of atRA, since atRA is a metabolite of β -carotene that, like lycopene, is found abundantly in vegetables and fruits. Furthermore, we have previously shown that, like retinoids, lycopene or one of its derivatives can interact at low affinity with members of the nuclear receptor family [30]. Retinoids are known to affect the IGF system in several complementary ways, one of which is to increase IGFBP-3 expression thereby reducing availability of the ligand to the IGF receptor. This increased expression of IGFBP-3 results in reduction of the mitogenic signal of IGF-I [31]. Similarly, lycopene increases the levels of membrane-associated IGF binding proteins [32]. In addition, we recently found that supplementation with tomato lycopene can reduce IGF-I blood level

as (Walfisch et al., unpublished results). Retinoids also reduce cyclin D1 levels by augmenting the degradation of cyclin D1 protein through the ubiquitin-proteasome pathway [31, 33]. In the present study, in agreement with others, we demonstrated that atRA reduces cyclin D1 levels and inhibits cell cycle progression. More importantly, we showed that the inhibitory effect of atRA, similar to that of lycopene, is abrogated by ectopic expression of cyclin D1.

In conclusion, the present study demonstrates that the inhibition of the mitogenic activity of IGF-I on

breast and endometrial cancer cells by lycopene and atRA is mediated, at least in part, through reduction of cyclin D1 levels.

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