Carcinogenesis vol.31 no.10 pp.1813–1821, 2010
doi:10.1093/carcin/bgq157
Advance Access publication August 10, 2010

Lycopene induces cell growth inhibition by altering mevalonate pathway and Ras signaling in cancer cell lines

Paola Palozza*, Maria Colangelo, Rossella Simone, Assunta Catalano, Alma Boninsegna, Paola Lanza[1], Giovanni Monego[2] and Franco O.Ranelletti[1]

Institute of General Pathology, Catholic University School of Medicine, L. Go F. Vito, 00168 Rome, Italy, [1]Institute of Histology, Catholic University School of Medicine, Largo Francesco. Vito, 00168 Rome, Italy and [2]Institute of Anatomy, Catholic University School of Medicine, Largo Francesco. Vito, 00168 Rome, Italy

*To whom correspondence should be addressed. Tel: +39 06 3016619; Fax: +39 06 3386446;
Email: p.palozza@rm.unicatt.it

Several evidences suggest that cancer cells have abnormal cholesterol biosynthetic pathways and prenylation of small guanosine triphosphatase proteins. Tomato lycopene has been suggested to have beneficial effects against certain types of cancer, including that of prostate, although the exact molecular mechanism(s) is unknown. We tested the hypothesis that lycopene may exert its antitumor effects through changes in mevalonate pathway and in Ras activation. Incubation of the *Ras*-activated prostatic carcinoma LNCaP cells with a 24 h lycopene treatment (2.5–10 μM) dose dependently reduced intracellular total cholesterol by decreasing 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase expression and by inactivating Ras, as evidenced by its translocation from cell membranes to cytosol. Concomitantly, lycopene reduced the Ras-dependent activation of nuclear factor-kappaB (NF-κB). Such a reduction was parallel to an inhibition of reactive oxygen species production and to a decrease in the phosphorylation ofc-jun N-terminal kinase, extracellular signal-regulated kinase 1/2 and p38. These effects were also accompanied by an arrest of cell cycle progression and by apoptosis induction, as evidenced by a decrease in cyclin D1 and phospho-AKT levels and by an increase in p21, p27 and p53 levels and in Bax:Bcl-2 ratio. The addition of mevalonate prevented the growth-inhibitory effects of lycopene as well as its increase in Ras cytoplasmatic accumulation and the subsequent changes in NF-κB. The ability of lycopene in inhibiting HMG-CoA reductase expression and cell growth and in inactivating Ras was also found in prostate PC-3, colon HCT-116 and HT-29 and lung BEN cancer cells. These findings provide a novel mechanistic insight into the growth-inhibitory effects of lycopene in cancer.

Introduction

Increased ingestion of tomatoes and tomato products containing lycopene has been shown to be associated with decreased risk of chronic diseases, including cancer. In particular, epidemiological studies reported a consistent inverse relationship between intakes of tomatoes/plasma lycopene levels and prostate (1,2), lung (3) and stomach cancers (3,4). Moreover, recent data evidence that lycopene supplementation may inhibit the progression of benign prostate hyperplasia, a risk factor for prostate cancer (5).

In addition, animal studies have shown antineoplastic effects of both tomato powder and purified lycopene supplementation. Boileau *et al.* (6) reported a significant inhibition of *N*-methy-*N*-nitrosourea-testosterone-induced carcinogenesis in rats following consumption of tomato powder. In a rat prostate cancer model, a supplementation with lycopene beadlets caused a significant reduction of tumor tissue (7). Tomato lycopene supplementation has been also shown to prevent the change in p53 target gene induced by cigarette smoke exposure in the gastric mucosa of ferrets (8), suggesting a protective effect of lycopene against development of gastric cancer.

Lycopene has been shown also to inhibit the growth of non-neoplastic human prostate epithelial cells, through cell cycle arrest (9) and tomato extracts have been reported to reduce prostate cancer cell survival in a dose-dependent manner (10). Physiologically, concentrations of lycopene have been also shown to induce mitochondrial apoptosis in LNCaP cells (11). Inhibitory effects on lycopene in mammary (12–14) as well as in endometrial (12), lung (12), colon (15,16) leukemia (17) and liver (18) cancer cell growth have been also reported.

Although there is significant evidence supporting the actions of lycopene as a potent antioxidant (19,20), there are a number of other potential mechanisms through which tomato products providing lycopene may reduce the risk for cancer. They include upregulation of connexin-43 and consequent enhancement of gap junction communication (21,22); modulation of growth factors and growth factor receptors, including insulin-like growth factor-1 (13,23,24) and platelet-derived growth factor-BB (25) immunoenhancement and decrease in inflammation (26); modulation of cyclo-oxygenase pathways (27) and xenobiotic metabolism (27) and gene methylation (28).

It is known that cancer cells have abnormal cholesterol biosynthetic pathways that are resistant to downregulation by cholesterol. The committed step in the biosynthesis of cholesterol and isoprenoids is catalyzed by 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which promotes the reductive deacylation of HMG-CoA to mevalonate (29). This pathway produces numerous bioactive signaling molecules including farnesyl pyrophosphate and geranylgeranyl pyrophosphate, which regulate transcriptional and posttranscriptional events that affect various biological processes, including cell growth (30).

Several reports indicate that prenylation, a lipid posttranslational modification, is involved in malignancy (31). In particular, many low-molecular-weight guanosine triphosphatases (GTPases) require prenylation to promote tumorigenesis (31). Among these, the Ras superfamily proteins usually require farnesylation. Subcellular localization of these proteins to intracellular membranes is essential for their function and it is mediated by posttranslational modifications with hydrophobic lipids such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate. These isoprenoids are transferred to cysteine residues (31).

In this study, we investigated the possibility that lycopene may modify cholesterol metabolism in cancer cells, secondary to its inhibitory effect on HMG-CoA reductase, the rate-limiting enzyme in cholesterol synthesis. We hypothesized that, through this inhibition, it can modify a key process in oncogene activation such as the prenylation of Ras and, thus, may control tumor cell growth. Our hypothesis seems sustained by the observation that both β-carotene and lycopene share similar initial synthetic pathways with cholesterol, which is synthesized in animal but not in plant cells. Moreover, recent studies suggest that lycopene is able to inhibit cholesterol synthesis in a macrophage cell line (32) and to reduce plasma low-density lipoproteins cholesterol concentrations in humans (33). On this basis, we have treated cell lines possessed a *ras* mutation (34), including the prostatic carcinoma LNCaP and PC-3 cells and the colon carcinoma HC-116 cells, and cell lines lacked a *ras* mutation (34), including the colon carcinoma HT-29 cells and the squamous carcinoma BEN cells, with lycopene, and we have studied the expression of HMG-CoA reductase as well as Ras activation in relation to cell growth. It has been reported that mutations in *ras* genes determined chronic activation of Ras (34).

Abbreviations: EDTA, ethylenediaminetetraacetic acid; GTPase, guanosine triphosphatase; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-kappaB; pAKT, phospho-AKT; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; THF, tetrahydrofuran; ROS, reactive oxygen species.

Materials and methods

Cell culture

Human prostate prostatic carcinoma LNCaP cells (American Type Culture Collection, Rockville, MD) was grown in RPMI 1640 (Laboratoires Eurobio, Les Ulis, France) medium; HCT-116 cell line (American Type Culture Collection) was grown in McCoy's 5a (Laboratoires Eurobio) medium; human prostate cancer PC-3 cell line (American Type Culture Collection) was grown in Dulbecco's modified Eagle's medium (Laboratoires Eurobio) medium; HT-29 human colon adenocarcinoma cells (American Type Culture Collection) were grown in modified Eagle's medium; squamous carcinoma BEN cells (American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium. The medium, without antibiotics, was supplemented with 10% fetal calf serum and 2 mM glutamine. Cells were maintained in log phase by seeding twice a week at density of 3×10^5 cells/ml at 37°C under 5% CO_2/air atmosphere. Lycopene (kindly provided by LycoRedNatural Products Industries Ltd, Beer Sheva, Israel) was delivered to the cells using tetrahydrofuran (THF) as a solvent, containing 0.025% butylated hydroxytoluene to avoid the formation of peroxides. The purity of lycopene was verified by high-peformance liquid chromatography. The stock solutions of lycopene were prepared immediately before each experiment. From the stock solutions, aliquots of lycopene were rapidly added to the culture medium to give the final concentrations indicated. The amount of THF added to the cells was not >0.1% (vol/vol). Control cultures received an amount of solvent (THF) equal to that present in lycopene-treated ones. No differences were found between cells treated with THF and untreated cells in terms of cell number and viability. After the addition of lycopene, the medium was not further replaced throughout the experiments. Experiments were routinely carried out on triplicate cultures. At the times indicated, cells were harvested and quadruplicate hemocytometer counts were performed. The trypan blue dye exclusion method was used to evaluate the percentage of viable cells.

Intracellular cholesterol assay

The analysis of intracellular cholesterol was performed as indicated (35). Cholesterol was extracted by disrupting 10×10^6 cells with an ultra-turrax homogenizer (on ice, 1 min, 12 000 r.p.m.) in 1 ml methanol (containing 0.1% butylated hydroxytoluene) and 3 ml hexane and centrifuged at 4000*g* for 2 min. After centrifugation, the hexane layer was transferred into another tube and was evaporated (N_2, 30 ± 1°C) to dryness. The extraction procedure was performed three times on each sample to ensure total removal of cholesterol. The hexane layers were evaporated in consecutive steps in the same tube and, then, dissolved in 200 µl hexane. A 20 µl aliquot was analyzed by high-peformance liquid chromatography equipped with a spectrophotometer (295-LC; PerkinElmer, Norwalk, CT) at 234 nm. A cyanide-bonded column, 25 × 0.46 cm cartridge format (Discovery-Cyano; Supelco, Bellefonte, PA), and 5 µm particle size were used. A 2 cm cartridge precolumn (Discovery-Cyano; Supelco) packed with the same material of the column was used. The mobile phase was hexane-2-propanol (95:5, vol:vol) at a flow rate of 1 ml.

Western blot analysis of HMG-CoA reductase, cyclin D1, p53, p21, p27, Bax, Bcl-2, Bcl-xL, pAKT, AKT, p38, p-p38, JNK, p-JNK, ERK1/2 and p-ERK1/2 expression

Cells (10×10^6) were harvested, washed once with ice-cold phosphate-buffered saline (PBS) and added for 30 min in an ice-cold lysis buffer as indicated (36). Cell lysates were centrifuged for 10 min at 4°C (10 000*g*) to obtain the supernatants, which were used for the analysis. The anti-HMG-CoA reductase (clone C-18, catalog no. SC-27580), anti-cyclin D1 (clone M-20, catalog no. SC-718), anti-p21 (clone C-19, catalog no. SC-397), anti-p27 (clone C-19, catalog no. SC-528) anti-Bax (clone P-19, catalog no. SC-526), anti-Bcl-xL S/L (clone L-19, catalog no. SC-1041), anti-AKT (clone C-20, catalog no. SC-5298), anti-phospho-AKT (pAKT) (clone N-19, P-19, catalog no. 166646-R), anti-p38 (clone C-20, catalog no. SC-535), anti-p-p38 (clone D-8, catalog no. SC-7973), anti-c-jun N-terminal kinase (JNK) (clone C-7, catalog no. SC-46006), anti-p-JNK (clone C-17, catalog no. SC-6254), anti-extracellular signal-regulated kinase (ERK)1/2 (clone K-23, catalog no. SC-135900) and anti-p-ERK1/2 (clone E-4, catalog no. SC-94) monoclonal antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. The anti-p53 (clone DO-1, catalog no. SC-126) and the anti-Bcl-2 (clone: Bcl-2/100/D5) monoclonal antibodies were purchased from YLEM, Rome, Italy. The blots were washed and exposed to horseradish peroxidase-labeled secondary antibodies (Amersham Pharmacia Biotech, Arlington Heights, IL) for 45 min at room temperature. The immunocomplexes were visualized by the enhanced chemiluminescence detection system and quantified by densitometric scanning.

Analysis of Ras cellular localization

Cells were scraped into 500 µl of lysis buffer [10 mM Tris–HCl, 10 mM NaCl, 3 mM MgCl2, 0.5% (octylphenoxy)polyethoxyethanol] containing aprotinin (100 U/ml), the calpain inhibitor *N*-acetyl-leucyl-leucyl-norleucinal (2 µg/ml), leupeptin (0.1 mM), phenylmethylsulfonyl fluoride (2 mM), pepstatin (5 µg/ml) and benzamidine (250 µg/ml) and homogenized by 15 passes through a 25 ga needle. The postnuclear supernatant was obtained by centrifugation (500*g*, 4°C, 10 min). The postnuclear fraction was further spun at 100 000*g* for 30 min (4°C, Beckman TLA 120.2 rotor) to obtain a pellet of the cell membrane fraction and cytosol fraction in the supernatant. The pellet was suspended in membrane resuspension buffer [10 mM Tris–HCl, 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethyleneglycol-bis(aminoethylether)-tetraacetic acid and 1% sodium dodecyl sulfate (SDS)] containing the protease inhibitors. Proteins from the cytosol and membrane (20–100*g*) fractions were mixed with SDS loading buffer and subjected to SDS–polyacrylamide gel electrophoresis on a 12% gel. Proteins were transferred electrophoretically to nitrocellulose membranes, which were blocked (16 h, 4°C) with 5% (wt/vol) non-fat dried milk in PBS. Membranes were incubated with a monoclonal antibody for pan Ras (clone F-132, catalog no. SC-32; Santa Cruz Biotechnology), followed by incubation with a peroxidase-conjugated anti-mouse IgG antibody (Santa Cruz Biotechnology). The immunocomplexes as indicated in western blotting assay.

Nuclear extracts and electrophoretic mobility-shift assay

Frozen cell pellets were processed to obtain nuclear extracts. Briefly, 12×10^6 cells were collected, washed twice and pelletted by 200*g* centrifugation for 10 min. The pellet was treated as indicated (37). Binding reactions containing 5 µg nuclear extracts, 10 mmol/l Tris–HCl (pH 7.6), 5% glycerol, 1 mmol/l EDTA, 1 mmol/l dithiothreitol, 50 mmol/l NaCl and 3 mg poly(dI-dC) were incubated for 30 min with 5000 c.p.m. of α-^{32}P-end-labeled double-stranded oligonucleotide in a total volume of 20 µl. The probe was 5′-AGTTGAGGGGACTTTCCCAGGC-3′. Labeling of the probe was obtained by incubating 5 pmol of oligonucleotide with 10 pmol [α-^{32}P]adenosine triphosphate and 3# UT4 polynucleotide kinase for 30 min at 37°C. The probe was then purified with MicroBIO-Spin P-30 columns. Complexes were separated on 60 g/l polyacrylamide gels with 45 mmol/l Tris-borate and 1 mmol/l EDTA, pH 8, buffer. After fixation and drying, gels were exposed on phosphor screens, which were then analyzed by phosphor/fluorescence imager STORM 840 (Molecular and Dynamics, Sunnyvale, CA). The intensity of the revealed bands was directly quantified by Image QuaNT software (Molecular Dynamics).

Analysis of p65 protein

Nuclear extracts, 25–30 µg of protein, were separated by SDS–polyacrylamide gel electrophoresis with 40–120 g/l Bis-Tris gels (NOVEX, San Diego, CA) and transferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA) with the use of a semidry system. Immunoblots were blocked overnight at 4°C in 50 g/l dried milk in PBS, pH 7.4, plus 0.05% Tween 20. Blots were incubated with polyclonal primary antibodies to p65 (clone 49.Ser 311, catalog no. SC-135769; Santa Cruz Biotechnology) in PBS plus 0.05% Tween 20 for 1–2 h at room temperature. The blots were visualized as described in western blotting assay.

Measurement of reactive oxygen species

Cells were harvested to evaluate reactive oxygen species (ROS) production using the di(acetoxymethyl ester) analog (C-2938) of 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (Molecular Probes, Eugene, OR) as described (37). Before the addition of the fluorescent probes, 2×10^6 cells were washed to eliminate the amount of lycopene not cell-associated. Fluorescent units were measured in each well after 30 min incubation with 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (10 µM) by use of a Cytofluor 2300/2350 Fluorescence Measurement System (Millipore Corporation). Lycopene did not alter the basal fluorescence of 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate.

Cell cycle analysis

Cell cycle stage was analyzed by flow cytometry. Aliquots of 10^6 cells were harvested by centrifugation, washed in PBS, fixed with ice-cold 70% ethanol and treated with 1 mg/ml RNAse for 30 min. Propidium iodide was added to a final concentration of 50 µg/ml. Data were collected, stored and analyzed using Multicycle software.

Apoptosis detection

The activity of caspase-3 was measured by the fluorimetric assay as described (36). Briefly, cells (2×10^6) were lysed in 50 mM Tris–HCl buffer, pH 7.5, containing 0.5 mM EDTA, 0.5% IGEPAL and 150 mM NaCl. Cell lysates were incubated with 50 µM caspase-3 fluorogenic substrate, Ac-DEVD-7-amino-4-methylcoumarin (Alexis Biochemicals, San Diego, CA), in a reaction buffer (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.5, containing 50 mM NaCl and 2.5 mM Diethyldithiocarbamate) for 120 min at 37°C. The release of 7-amino-4-methylcoumarin was measured with excitation at 380 nm and emission at 460 nm using a fluorescence spectrophotometer.

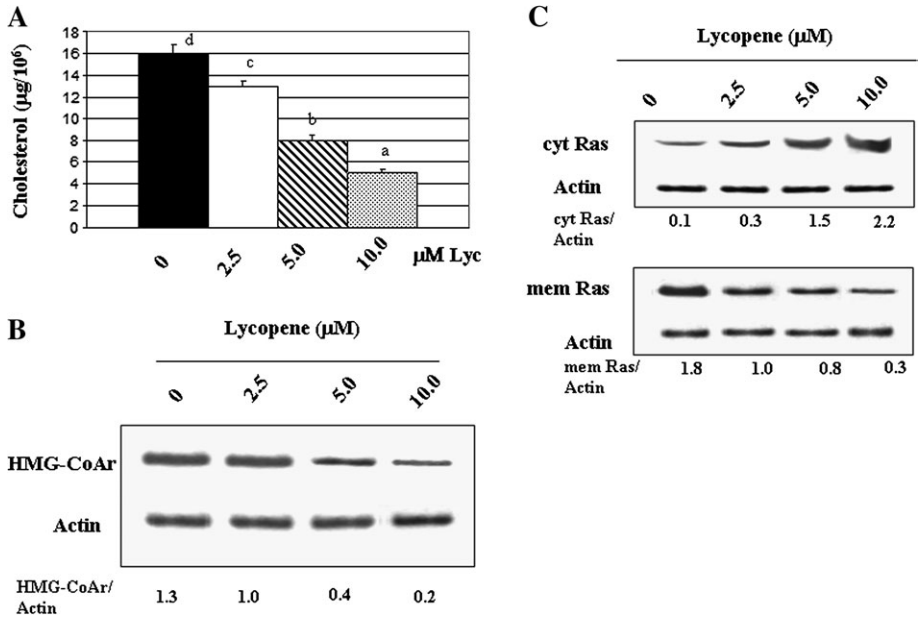

Fig. 1. Effects of varying lycopene concentration on the expression of cholesterol levels, HMG-CoA reductase and Ras translocation (cyt, cytoplasmatic; mem, membrane) in LNCaP cells treated for 24 h. In panel (**A**), the values were the means ± SEMs, $n = 5$. The values not sharing the same letter were significantly different ($P < 0.05$, Fisher's test). Panels (**B** and **C**) represent western blot analyses. The values in the western blot panels represented the ratio of the indicated protein and actin.

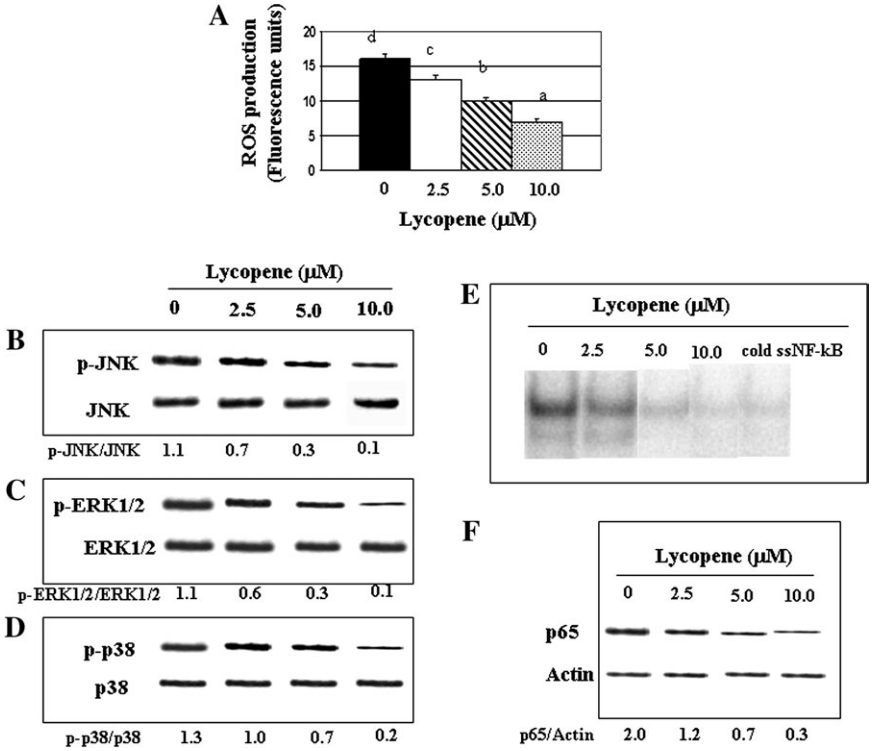

Fig. 2. Effects of varying lycopene concentrations on ROS production [panel (**A**)], MAPK expression [panels (**B**–**D**)], NF-κB-binding activity [panel (**E**)] and p65 [panel (**F**)] expression in LNCaP cells. In panel (E), the specificity was demonstrated by using excess unlabeled NF-κB oligonucleotides (=cold ssNF-κB), which competed away binding. In panel (A), the values were the means ± SEMs, $n = 3$. The values not sharing the same letter were significantly different ($P < 0.05$, Fisher's test). Panels (B–D and F) represent western blot analyses. The values in the western blot panels represented the ratio of the indicated protein and actin.

Statistical analysis

Three separate cultures per treatment were utilized for analysis in each experiment. Values were presented as means ± SEMs. Multifactorial two-way analysis of variance was adopted to assess any differences among the treatments (Figures 5A and 6A–C). When significant values were found ($P < 0.05$), post hoc comparisons of means were made using the Tukey's Honestly Significant Differences test. One-way analysis of variance was used to determine differences between the different lycopene concentrations and the different treatments (Figures 1A, 2A and 4A and C). When significant values were found ($P < 0.05$), post hoc comparisons of means were made using Fisher's test. Differences were analyzed using Minitab Software (Minitab, State College, PA).

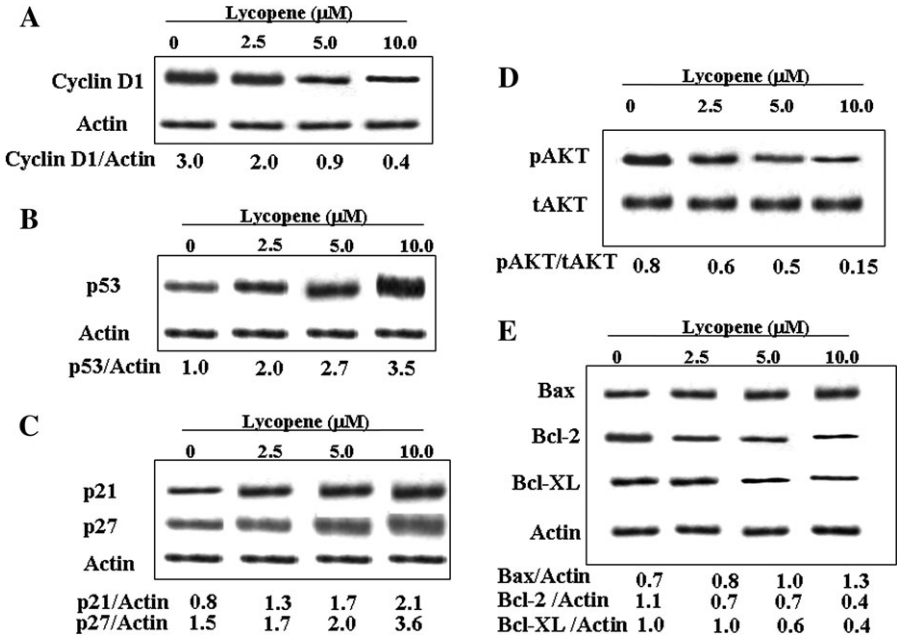

Fig. 3. Effects of varying lycopene concentrations on the expression of cell-cycle related proteins [cyclin D1, panels (**A**); p53, panel (**B**); $p21^{WAF1/CIP1}$ and p27, panel (**C**)], Akt (t, total; p, phosphorylated) [panel (**D**)] and apoptosis-related proteins [Bcl-2, Bcl-XL and Bax, panel (**E**)] in LNCaP cells treated for 24 h. Representative western blot analyses. The values in the western blot panels represented the ratio of the indicated protein and actin.

Results

Effects of lycopene on HMG-CoA reductase expression, intracellular cholesterol content and Ras localization

We treated the prostatic carcinoma LNCaP cells with lycopene (2.5–10 μM) and we measured the intracellular cholesterol content (panel A) and HMG-CoA reductase expression (panel B) after 24 h of incubation (Figure 1). Our results show that the levels of total cholesterol were progressively decreased by the addition of increasing concentrations of lycopene (Figure 1, panel A). The carotenoid was also able to reduce the expression of the enzyme in a dose-dependent manner (Figure 1, panel B). Concentrations of lycopene <2.5 μM had no effect in reducing cholesterol levels and HMG-CoA reductase expression (data not shown).

One determinant of Ras activity is its translocation from the cytosol to cell membranes, including the plasma membrane. To determine whether lycopene reduced Ras membrane translocation, we measured the relative amount of Ras protein in isolated cell membranes and in cytosol (Figure 1, panel C). A 24 h treatment of LNCaP cells with lycopene markedly decreased the translocation of Ras to the cell membrane, whereas the expression of this protein in the cytosol was increased.

Effects of lycopene on Ras-dependent pathways involved in redox regulation

Numerous observations have suggested a role for the Ras superfamily of small GTPase in redox regulation, and ROS have been reported as important downstream effectors for Ras protein. To quantify the level of intracellular ROS generated in tumor cells, we used a fluorimetric assay. ROS production was measured after 3 h of incubation in LNCaP cells (Figure 2, panel A). Lycopene significantly reduced ROS production in a dose-dependent manner, starting the inhibition at the concentration of 2.5 μM (data not shown).

Since the increase in ROS levels and the modulation of Ras expression have been reported to affect the phosphorylation of mitogen-activated protein kinases (MAPKs), we also examined the expression of the JNK (Figure 2, panel B), ERK1/2 (Figure 2, panel C) and p38 (Figure 2, panel D) in LNCaP cells treated for 24 h. Lycopene induced a dose-dependent decrease in the level of the phosphorylated forms of JNK (p-JNK), ERK1/2 (p-ERK1/2) and p38 (p-p38).

ROS and MAPKs have been shown to activate NF-κB, able to modulate several proteins involved in the regulation of cell cycle and apoptosis. Therefore, we measured the activation of NF-κB in LNCaP cells treated with different concentrations of lycopene for 24 h (Figure 2, panel E). In the absence of lycopene, tumor cells evidence NF-κB DNA binding. The addition of the carotenoid inhibited such a binding in a dose-dependent manner. Similar results were found when nuclear extracts were prepared from LNCaP cells in the absence and in the presence of lycopene and nuclear translocation of the NF-κB p65 subunit was detected by western blotting after 24 h (Figure 2, panel F). In the absence of lycopene, nuclear translocation of the NF-κB subunit p65 was observed in tumor cells. Such an effect was inhibited by a 24 h treatment with lycopene. Interestingly, the inhibition of NF-κB DNA-binding activity by lycopene paralleled that of both MAPK phosphorylation and ROS production, starting from the same carotenoid concentration (2.5 μM) (data not shown).

Effects of lycopene on proteins involved in cell cycle progression, survival and apoptosis

It is known that NF-κB controls cell growth in several ways, affecting several cell cycle- and apoptosis-related proteins, including cyclin D1, cyclin kinase inhibitors and Bcl-2 family proteins. In an attempt to explore the effects of lycopene on cell cycle progression, we measured the content of the G_0/G_1 phase-related cyclin D1 (Figure 3, panel A) and those of the cyclin kinase inhibitors p53 (Figure. 3, panel B), $p21^{WAF1/CIP1}$ and p27 (Figure 3, panel C) in LNCaP cells incubated for 24 h. Lycopene-treated cells exhibited a dose-dependent decrease of cyclin D1 and a dose-dependent increase in p53 and $p21^{WAF1/CIP1}$ and p27 as compared with the respective vehicle-control-treated cells. We also examined AKT expression and its phosphorylation in the tumor cells (Figure 3, panel D). Treatment of LNCaP cells with lycopene for 24 h decreased AKT phosphorylation in a dose-dependent manner.

Moreover, we evaluated the effect of lycopene on apoptosis-regulating proteins (Figure 3, panel E), examining the expression of Bax which acts as apoptosis promoter and that of Bcl-2 and Bcl-XL,

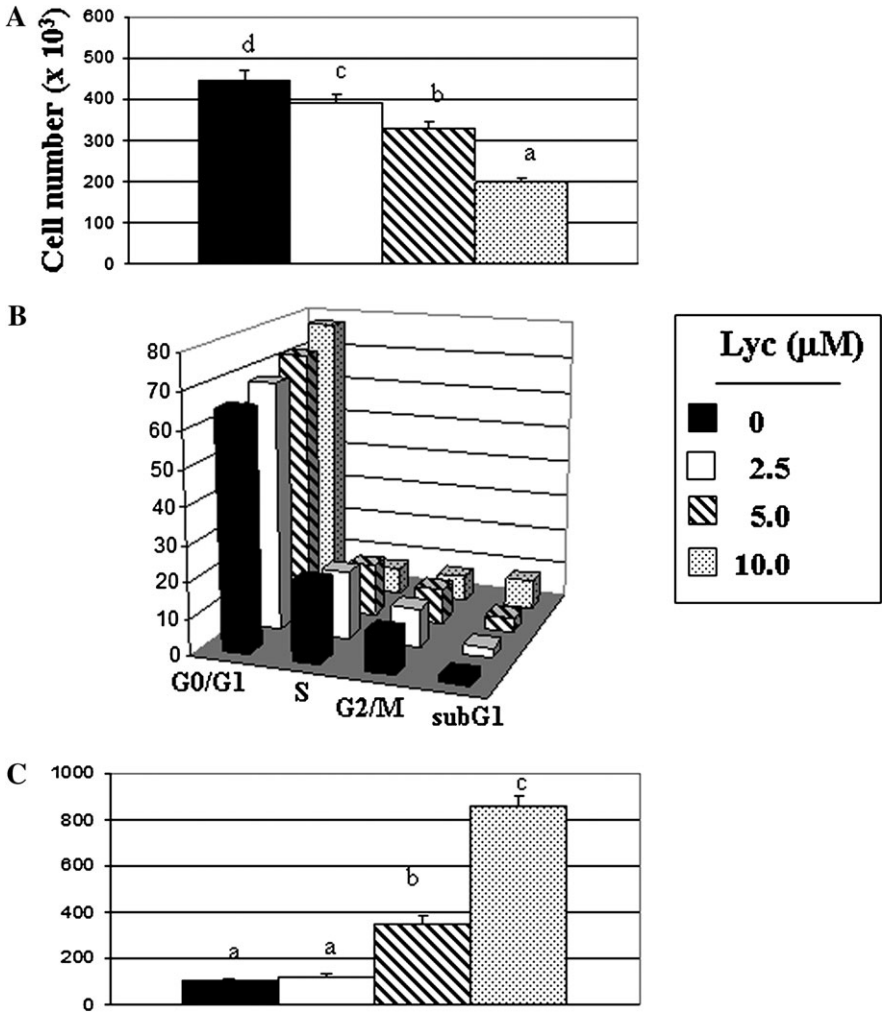

Fig. 4. Effects of varying lycopene concentrations on cell number [panel (**A**)], cell cycle progression [panel (**B**)] and apoptosis [panel (**C**)], measured as caspase-3 activation, in LNCaP cells treated for 24 h. The number of LNCaP cells at time zero was 300×10^{-3}. The values were the means ± SEMs, $n = 3$. In panels (A and C), values not sharing the same letter were significantly different ($P < 0.05$, Fisher's test).

which are known to suppress programmed cell death, in LNCaP cells treated for 24 h. The treatment with lycopene significantly reduced the expression of both Bcl-2 and Bcl-XL and increased that of Bax.

Effects of lycopene on cancer cell growth, cell cycle progression and apoptosis

We, then, measured cell growth after 24 h of incubation (Figure 4, panel A). The number of seeded cells at time zero (24 h before the measurement) was 300×10^{-3}. Lycopene was able to inhibit the growth of LNCaP cells in a dose-dependent manner. A similar inhibition was evident also at 72 h (data not shown). A significant inhibition of cell growth was found at lycopene concentrations starting from 2.5 μM because at lower concentrations, the carotenoid was unable in inducing growth inhibition (data not shown). It is interesting to note that the reduction in cell number by lycopene, at least at the highest concentration, partially resulted from cell death. This was clearly evident at 10 μM lycopene where the number of cells (200×10^{-3}) after 24 h of treatment was smaller than the number of seeded cells (300×10^{-3}).

To elucidate possible mechanism(s) responsible for the reduction of cell number by lycopene in LNCaP cells, we first examined whether such a reduction was associated with cytostatic effects due to changes in cell cycle progression. The cell cycle distribution of LNCaP cells incubated in the absence or in the presence of varying concentrations of lycopene for 24 h is shown in Figure 4, panel B. In the absence of lycopene, ∼20% of cancer cells were in S phase and >60% of them in G_0/G_1 phase. However, after a 24 h addition of lycopene, we observed a net dose-dependent increase in the percentage of cells in G_0/G_1 phase, which was maintained throughout the treatment (72 h) (data not shown). The G_0/G_1 accumulation was accompanied by a corresponding reduction in the percentage of cells in S phase. In addition, the presence of a distinct sub-G_1 peak (subdiploid DNA content), suggestive of the presence of apoptotic cells, was found following lycopene treatment.

Apoptosis induction by lycopene in tumor cells was further studied by evaluating the activation of caspase-3 (Figure 4, panel C). In LNCaP cells, we found that a 24 h treatment with lycopene resulted in a dose-dependent increase in 7-amido-4-methylcoumarin fluorescence, indicative of the activation of caspase-3. The pro-apoptotic effects paralleled those obtained by cell cycle analysis. These findings were consistent with the changes in cell cycle- and apoptosis-related proteins observed in LNCaP cells.

Effects of mevalonate on lycopene-induced changes in cell growth, RAS translocation and NF-κB activation

The important role of mevalonate pathway in modifying the growth-inhibitory effects of lycopene is clearly demonstrated by our observation that the carotenoid at the concentration of 10 μM was ineffective in inhibiting cancer cell growth in the presence of 100 μM mevalonate, as observed in LNCaP cells (Figure 5, panel A) treated

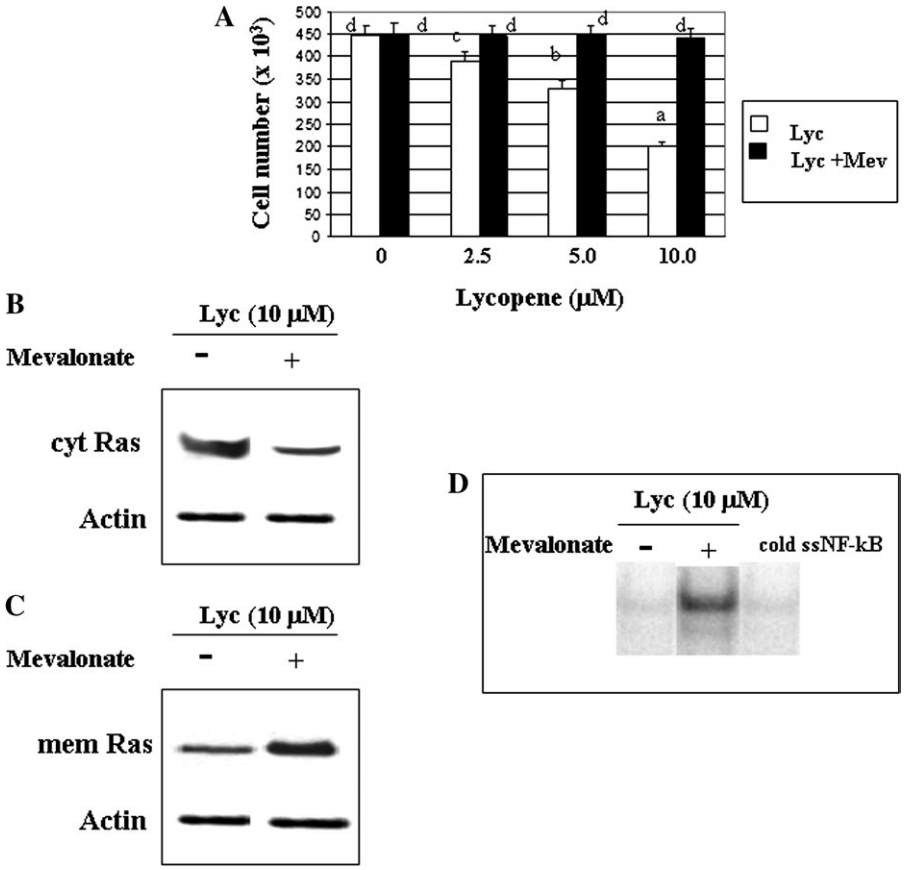

Fig. 5. Effects of mevalonate (mev) addition, in the presence and in the absence of lycopene, on cell growth [panel (**A**)], Ras translocation (cyt, cytoplasmatic; mem, membrane) [panels (**B** and **C**)] and NF-κB DNA-binding activity [panel (**D**)] in LNCaP cells. In all the panels, lycopene was shown at the concentrations of 10 μM, the incubation time was 24 h and mev was added at the concentration of 100 μM. In panel (D), the specificity was demonstrated by using excess unlabeled NF-κB oligonucleotides (=cold ssNF-κB), which competed away binding. In panel (A), the values were the means ± SEMs, $n = 3$. Values not sharing the same letter were significantly different ($P < 0.005$, Tukey's test). Panels (B and C) represent western blot analyses. The values in the western blot panels represented the ratio of the indicated protein and actin.

for 24 h. Moreover, mevalonate addition completely prevented the effects of the carotenoid on Ras translocation in LNCaP cells treated for 24 h (Figure 5, panels B and C). Finally, a 24 h treatment with mevalonate prevented the effects of lycopene in reducing NF-κB activation (Figure 5, panel D) in LNCaP cells.

Effects of lycopene on HMG-CoA expression, Ras translocation and cell growth in other cancer cell lines

The effects of lycopene on HMG-CoA reductase expression, Ras translocation and cell growth were also studied in other cancer cell lines, including cells possessed a *ras* mutation (32), such as the prostatic carcinoma PC-3 cells and the colon carcinoma HC-116 cells, and cells lacked a *ras* mutation (32), including the colon carcinoma HT-29 cells and the squamous carcinoma BEN cells (Figure 6). In all these cells, lycopene was given at the concentration of 10 μM for 24 h. The carotenoid markedly reduced HMG-CoA expression (Figure 6, panel A), enhanced cytoplasmatic Ras translocation from cell membrane (Figure 6, panel B) and inhibited cell growth (Figure 6, panel C) in all the cell lines analyzed. Interestingly, lycopene was more potent as a cell growth-inhibitory agent in cells expressing a *ras* mutation than in cells lacking it. In fact, the percentages of cell growth inhibition by lycopene ranged from 47 to 56% in cells expressing the mutation, whereas they ranged from 20 to 29% in cells lacking it. The addition of 100 μM mevalonate (panel C) prevented the growth-inhibitory effects of lycopene, suggesting that mevalonate pathway has a key role in the anticancer activity of the carotenoid.

Discussion

Although it seems to be evident from many studies in mammary (12–14), endometrial, lung (12) colon (15,16) leukemia (17) and liver (18) cancer cells that lycopene is able to act as an antitumor agent by arresting cell proliferation and/or by inducing apoptosis, the exact mechanism(s) of its beneficial action is still under debate.

In this study, we reported a novel molecular mechanism of lycopene in cancer control. For the first time, we showed that the carotenoid may arrest cell cycle progression and induce apoptosis in cancer cells, by directly inhibiting the mevalonate pathway and by impairing Ras membrane localization and cell signaling.

The activity of HMG-CoA reductase in animal cells has been shown to be sensitive to negative regulation by both sterols and non-sterol products of the mevalonate pathway (36). Lycopene is a polyisoprenoid synthesized in plants from mevalonate via HMG-CoA reductase pathway. In plants, as well as in animal cells, HMG-CoA reductase is regulated by an end-product repression. Our results show that lycopene was able to reduce the expression of HMG-CoA reductase in a dose-dependent manner in both prostate and lung cancer cells. Such a control probably involves a posttranscriptional mechanism, as suggested for isoprenoids and other carotenoids, including β-carotene (38). The inhibition of HMG-CoA reductase by lycopene was also accompanied by a reduction in intracellular cholesterol levels. These data are in agreement with other observations showing that lycopene is able to inhibit cholesterol synthesis in cultured macrophages (32) and in human subjects (33).

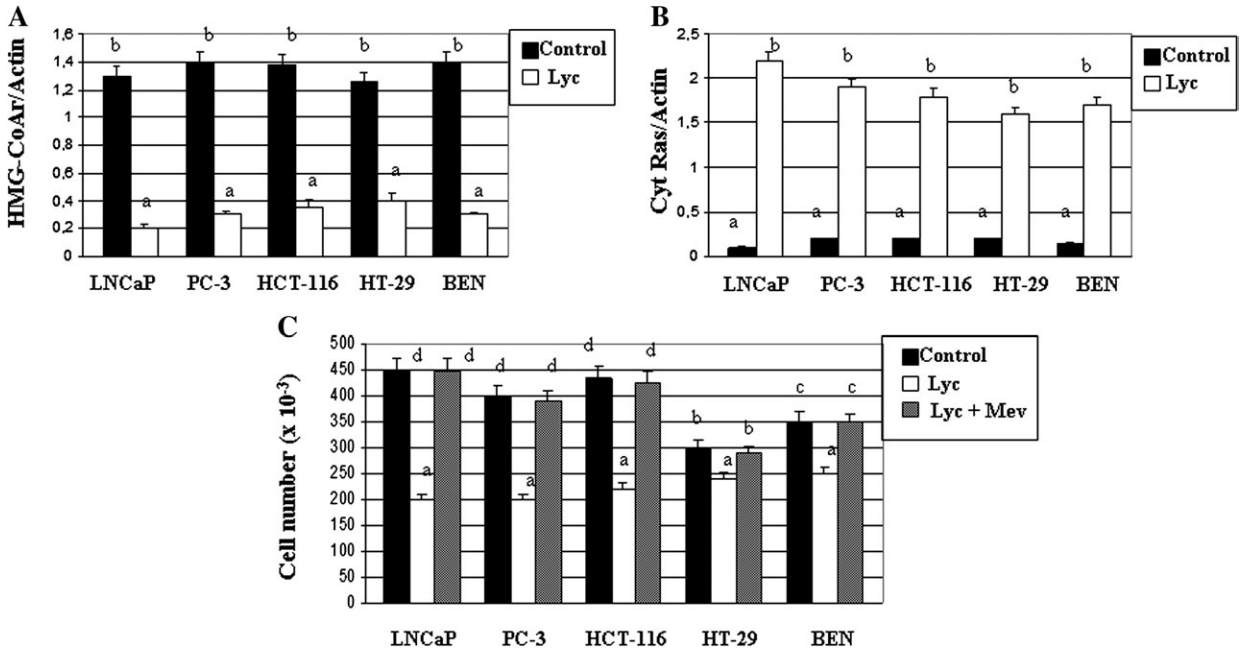

Fig. 6. Effects of lycopene on HMG-CoA expression [panel (**A**)], cytoplasmatic (cyt) Ras levels [panel (**B**)] and cell growth [panel (**C**)] in different cancer cell lines. In all the panels, the carotenoid was added at the concentration of 10 μM for 24 h. In panel (C), mev (100 μM) was added in combination with lycopene. The values were the means ± SEMs, $n = 3$. In the panels, values not sharing the same letter were significantly different [panel (A): $P < 0.005$; panel (B): $P < 0.002$; panel (C): $P < 0.001$,Tukey's test].

It has been recently shown that membrane association appears to be an important function in mevalonate-derivative modifications of several important proteins such as many low molecular GTPase. Members of the Ras GTPase family are major substrates for post-translational modification by farnesylation (39), a process essential for their proper membrane localization and activation (40). GTPases function as molecular switches, cycling between an active guanosine triphosphate-bound and an inactive guanosine diphosphate-bound state thereby mediating cellular responses through their association with numerous effector molecules including kinases (41). Since it has been reported that one determinant of Ras activity is its translocation from the cytosol to cell membranes, we measured the relative amount of Ras protein in isolated cell membranes and in the cytosol. Our results show that lycopene impaired the localization of Ras in cell membranes in cancer cells, which lead to cytoplasmatic accumulation and subsequent inactivation of this protein.

Numerous observations have suggested a role for the Ras superfamily of small GTPase in redox regulation, and ROS have been reported as important downstream effectors for both Ras and Rac proteins. According to these observations, in our study, lycopene was able to inactivate Ras protein and concomitantly to inhibit intracellular ROS production in a dose-dependent manner, confirming its role as an intracellular redox agent. The antioxidant properties of lycopene are well known. Lycopene was found to inactivate several kinds of free radicals, including hydrogen peroxide, nitrogen dioxide (42,43) thiyl (RSz) and sulphonyl (RSO2z) radicals (44). Moreover, lycopene and tomato products have been reported to inhibit lipid and DNA oxidation (10) previously reported in normal as well as in tumor cells.

It is known that Ras activation is the first step in activation of MAPK cascade. Therefore, we measured the effects of the carotenoid on the levels of the redox-sensitive p38, JNK and ERK1/2. It has been previously reported that lycopene is able to modulate MAPK signaling in retinal pigmental epithelial cells (45) as well as in breast cancer cells (46). According to these findings, lycopene was able to decrease the phosphorylation of the three redox-sensitive MAPK kinases in both the cancer cell lines.

Carotenoids, including β-carotene and lycopene, have been shown to regulate pathways converging at the NF-κB-binding sites (37,47). Here, we showed that lycopene inhibited the binding activity of NF-κB in LNCaP cells. The decrease in NF-κB-binding ability by lycopene may be mediated, at least in part, by its suppression of MAPK signaling and by its inhibition of ROS production.

It is known that NF-κB controls cell growth in several ways, affecting several cell cycle- and apoptosis-related proteins, including cyclin D1, p21, p27, p53, PI3K/Akt, Bax and Bcl-2. Our study clearly shows that lycopene blocked the G_1/S phase of cell cycle and suggests that possible targets of such a block could be cyclin D1 and some inhibitors of cell cycle. In fact, the carotenoid suppressed cyclin D1 levels and increased p21, p27 and p53 levels. These changes were accompanied by apoptosis induction, revealed by the presence of a distinct sub-G_1 peak (subdiploid DNA content) in the cell cycle analysis and by the activation of caspase-3. In agreement with these findings, at the concentration responsible for apoptosis induction, the carotenoid also reduced pAKT phoshorylation and increased the Bax:Bcl-2 ratio.

Previous reports evidence that lycopene (9,10,13,37,48) or lycopene extracts (49) induced a G_1/S cell cycle arrest, which is corroborated by the downregulation of cyclins, including cyclin E (50) and cyclin D1 (16,49) and/or by the upregulation of cyclin A and p27 (50). Hwang and Bowen (10) demonstrated an interference of tomato paste with the cell cycle of LNCaP cells in the G_2/M-phase, although Hantz *et al.* (11) found that purified lycopene, at concentrations lower than those used in this study, did not affect the proliferation but modified the apoptosis in LNCaP cells. These observations suggest that lycopene may alter cell cycle-regulatory proteins depending on the way and the dose of lycopene administration.

The cancer-preventive effect of lycopene mediated by its ability to induce apoptosis has been previously reported (25,27,46,48,49). It has been suggested that it may depend on the intracellular formation of auto-oxidant products of lycopene rather than to lycopene itself (51).

Interestingly, in our study, lycopene was more effective as a growth-inhibitory agent in cells possessing a K-*ras* mutation and resulting in constitutively activated Ras protein (34) than in cells

lacking the mutation. The impairment of membrane localization of the abnormal protein, abolishing its function, is probably responsible for the marked growth-inhibitory effects of lycopene observed in *ras*-mutated cells.

The inhibition of mevalonate pathway has a key role in the growth-inhibitory effects of lycopene as well as in carotenoid ability to induce cytoplasmatic Ras accumulation and to inhibit NF-κB activation in LNCaP cells. In fact, mevalonate, added in combination with lycopene, completely prevented such effects, as evidenced in Figure 5.

In conclusion, from our study, we can suggest that lycopene may alter mevalonate pathway through inhibition of HMG-CoA reductase. This may induce a decreased prenylation of Ras, which, in turn, may modulate redox-sensitive molecular pathways responsible for NF-κB activation and consequently, for cell cycle arrest and apoptosis induction, as evidenced by decreased cyclin D1 and pAKT levels, increased p21, p27 and p53 levels and changes in Bax:Bcl-2 ratio. The specificity of this mechanism is underlined by the observation that the addition of mevalonate completely prevented the growth-inhibitory effects of lycopene as well as its changes in Ras cytoplasmatic accumulation and the subsequent changes in NF-κB. These findings suggest that lycopene and/or tomato products could be potent anticancer compounds in chemopreventive and therapeutic applications and provide a novel mechanistic insight into the inhibitory effects of lycopene on the growth of human cancer cells.

Funding

LYCOCARD, European Integrated Project n° 016213.

Acknowledgements

We are very much grateful to Dr Yoav Sharoni and LycoRedNatural Products Industries Ltd for lycopene supply.

Conflict of Interest Statement: None declared.

References

1. Giovannucci,E. *et al*. (2002) A prospective study of tomato products, lycopene, and prostate cancer risk. *J. Natl Cancer Inst.*, **94**, 391–398.
2. Giovannucci,E. *et al*. (1995) Intake of carotenoids and retinol in relation to risk of prostate cancer. *J. Natl Cancer Inst.*, **87**, 1767–1776.
3. Giovannucci,E. (1999) Tomatoes, tomato-based products, lycopene, and cancer: review of the epidemiologic literature. *J. Natl Cancer Inst.*, **91**, 317–331. Review.
4. Liu,C. *et al*. (2008) Nutrition and gastric cancer risk: an update. *Nutr. Rev.*, **66**, 237–249. Review.
5. Schwarz,S. *et al*. (2008) Lycopene inhibits disease progression in patients with benign prostate hyperplasia. *J. Nutr.*, **138**, 49–53.
6. Boileau,T.W. *et al*. (2003) Prostate carcinogenesis in N-methyl-N-nitrosourea (NMU)-testosterone-treated rats fed tomato powder, lycopene, or energy-restricted diets. *J. Natl Cancer Inst.*, **95**, 1578–1586.
7. Siler,U. *et al*. (2004) Lycopene and vitamin E interfere with autocrine/paracrine loops in the Dunning prostate cancer model. *FASEB J.*, **18**, 1019–1021.
8. Liu,C. *et al*. (2006) Lycopene supplementation prevents smoke-induced changes in p53, p53 phosphorylation, cell proliferation, and apoptosis in the gastric mucosa of ferrets. *J. Nutr.*, **136**, 106–111.
9. Obermüller-Jevic,U.C. *et al*. (2003) Lycopene inhibits the growth of normal human prostate epithelial cells *in vitro*. *J. Nutr.*, **133**, 3356–3360.
10. Hwang,E.S. *et al*. (2005) Effects of tomato paste extracts on cell proliferation, cell-cycle arrest and apoptosis in LNCaP human prostate cancer cells. *Biofactors*, **23**, 75–84.
11. Hantz,H.L. *et al*. (2005) Physiologically attainable concentrations of lycopene induce mitochondrial apoptosis in LNCaP human prostate cancer cells. *Exp. Biol. Med. (Maywood)*, **230**, 171–179.
12. Levy,J. *et al*. (1995) Lycopene is a more potent inhibitor of human cancer cell proliferation than either alpha-carotene or beta-carotene. *Nutr. Cancer*, **24**, 257–266.
13. Karas,M. *et al*. (2000) Lycopene interferes with cell cycle progression and insulin-like growth factor I signaling in mammary cancer cells. *Nutr. Cancer*, **36**, 101–111.
14. Chalabi,N. *et al*. (2007) Immunohistochemical expression of RARalpha, RARbeta, and Cx43 in breast tumor cell lines after treatment with lycopene and correlation with RT-QPCR. *J. Histochem. Cytochem.*, **55**, 877–1683.
15. Salman,H. *et al*. (2007) Lycopene affects proliferation and apoptosis of four malignant cell lines. *Biomed. Pharmacother.*, **61**, 366–369.
16. Tang,F.Y. *et al*. (2008) Lycopene inhibits growth of human colon cancer cells via suppression of the Akt signaling pathway. *Mol. Nutr. Food Res.*, **52**, 646–654.
17. Amir,H. *et al*. (1999) Lycopene and 1,25-dihydroxyvitamin D3 cooperate in the inhibition of cell cycle progression and induction of differentiation in HL-60 leukemic cells. *Nutr. Cancer*, **33**, 105–112.
18. Hwang,E.S. *et al*. (2006) Inhibitory effects of lycopene on the adhesion, invasion, and migration of SK-Hep1 human hepatoma cells. *Exp. Biol. Med. (Maywood)*, **231**, 322–327.
19. Rao,A.V. (2004) Processed tomato products as a source of dietary lycopene: bioavailability and antioxidant properties. *Can. J. Diet Pract. Res.*, **65**, 161–165.
20. Das,S. *et al*. (2005) Lycopene, tomatoes, and coronary heart diseases. *Free Radic. Res.*, **39**, 449–455.
21. Stahl,W. *et al*. (2000) Stimulation of gap junctional communication: comparison of acyclo-retinoic acid and lycopene. *Arch. Biochem. Biophys.*, **373**, 271–274.
22. Vine,A.L. *et al*. (2005) Transcriptional regulation of connexin 43 expression by retinoids and carotenoids: similarities and differences. *Mol. Carcinog.*, **43**, 75–85.
23. Kanagaraj,P. *et al*. (2007) Effects of lycopene on insulin-like growth factor-1, IGF binding protein-3 and IGF type-1 receptor in prostate cancer cells. *J. Cancer Res. Clin. Oncol.*, **133**, 351–359.
24. Liu,C. *et al*. (2003) Lycopene supplementation inhibits lung squamous metaplasia and induces apoptosis via up-regulating insulin-like growth factor-binding protein 3 in cigarette smoke-exposed ferrets. *Cancer Res.*, **63**, 3138–3144.
25. Wu,W.B. *et al*. (2007) Inhibitory effect of lycopene on PDGF-BB-induced signalling and migration in human dermal fibroblasts: a possible target for cancer. *Biochem. Soc. Trans.*, **35**, 1377–1378. Review.
26. Riso,P. *et al*. (2006) Effect of a tomato-based drink on markers of inflammation, immunomodulation, and oxidative stress. *J. Agric. Food. Chem.*, **54**, 2563–2566.
27. Heber,D. *et al*. (2002) Overview of mechanisms of action of lycopene. *Exp. Biol. Med. (Maywood)*, **227**, 920–923.
28. King-Batoon,A. *et al*. (2008) Modulation of gene methylation by genistein or lycopene in breast cancer cells. *Environ. Mol. Mutagen.*, **49**, 36–45.
29. Brown,M.S. *et al*. (1980) Multivalent feedback regulation of HMG-CoA reductase, a control mechanism coordinating isoprenoid synthesis and cell growth. *J. Lipid Res.*, **21**, 505–517. Review.
30. Swanson,K.M. *et al*. (2006) Anti-cancer therapy: targeting the mevalonate pathway. *Curr. Cancer Drug Targets.*, **6**, 15–37. Review.
31. Saxena,N. *et al*. (2008) RAS: target for cancer therapy. *Cancer Invest.*, **26**, 948–955. Review.
32. Fuhrman,B. *et al*. (1997) Hypocholesterolemic effect of lycopene and β-carotene is related to suppression of cholesterol synthesis and augmentation of LDL receptor activity in macrophages. *Biochem. Biophys. Res. Commun.*, **233**, 658–662.
33. Elson,C.E. *et al*. (1999) Isoprenoid-mediated inhibition of mevalonate synthesis: potential application to cancer. *Proc. Soc. Exp. Biol. Med.*, **22**, 294–310.
34. Rajesh,D. *et al*. (1999) Ras mutation, irrespective of cell type and p53 status, determines a cell's destiny to undergo apoptosis by okadaic acid, an inhibitor of protein phosphatase 1 and 2A. *Mol. Pharmacol.*, **56**, 515–525.
35. Chen,B.H. *et al*. (1994) Evaluation of the analysis of cholesterol oxides by liquid chromatography. *J. Chromatogr. A*, **661**, 127–136.
36. Palozza,P. *et al*. (2008) The sensitivity to β-carotene growth-inhibitory and pro-apoptotic effects is regulated by caveolin-1 expression in human colon and prostate cancer cells. *Carcinogenesis*, **29**, 2153–2161.
37. Palozza,P. *et al*. (2003) Beta-carotene regulates NF-kappaB DNA-binding activity by a redox mechanism in human leukemia and colon adenocarcinoma cells. *J. Nutr.*, **133**, 381–388.
38. Peffley,D.M. *et al*. (2003) Plant -derived monoterpenes suppress hamster kidney cell 3-hydroxy-3-methylglutaryl coenzyme a reductase synthesis at the post-transcriptional level. *J. Nutr.*, **133**, 38–44.
39. Jackson,S.M. *et al*. (1997) Signaling molecules derived from the cholesterol biosynthetic pathway. *Subcell. Biochem*, **28**, 1–21.
40. Sinensky,M. (2000) Functional aspects of polyisoprenoid protein substituents: roles in protein-protein interaction and trafficking. *Biochim. Biophys. Acta*, **1529**, 203–209. Review.

41. Argmann,C.A. *et al*. (2005) Regulation of macrophage cholesterol efflux through hydroxymethylglutaryl-CoA reductase inhibition: a role for RhoA in ABCA1-mediated cholesterol efflux. *J. Biol. Chem.*, **280**, 22212–22221.
42. Böhm,F. *et al*. (1995) Carotenoids protect against cell membrane damage by the nitrogen dioxide radical. *Nat. Med.*, **1**, 98–99.
43. Böhm,F. *et al*. (2001) Dietary uptake of lycopene protects human cells from singlet oxygen and nitrogen dioxide—ROS components from cigarette smoke. *Photochem. Photobiol. B.*, **64**, 176–184.
44. Mortensen,A. *et al*. (1997) Comparative mechanisms and rates of free radical scavenging by carotenoid antioxidants. *FEBS Lett.*, **418**, 91–97.
45. Chan,C.M. *et al*. (2009) Lycopene inhibits PDGF-BB-induced retinal pigment epithelial cell migration by suppression of PI3K/Akt and MAPK pathways. *Biochem. Biophys. Res. Commun.*, **388**, 172–176.
46. Chalabi,N. *et al*. (2007) Expression profiling by whole-genome microarray hybridization reveals differential gene expression in breast cancer cell lines after lycopene exposure. *Biochim. Biophys. Acta*, **1769**, 124–130.
47. Huang,C.S. *et al*. (2007) Lycopene inhibits matrix metalloproteinase-9 expression and down-regulates the binding activity of nuclear factor-kappa B and stimulatory protein-1. *J. Nutr. Biochem.*, **18**, 449–456.
48. Ivanov,N.I. *et al*. (2007) Lycopene differentially induces quiescence and apoptosis in androgen-responsive and –independent prostste cancer cell lines. *Clin. Nutr.*, **26**, 252–263.
49. Palozza,P. *et al*. (2007) The growth-inhibitory effects of tomatoes digested in nitro in colon adenocarcinoma cells occur through down-regulation of cyclin D1, Bcl-2 and Bcl-XL. *Br. J. Nutr.*, **98**, 789–795.
50. Nahum,A. *et al*. (2001) Lycopene inhibition of cell cycle progression in breast and endometrial cancer cells is associated with reduction in cyclin D levels and retention of p27(Kip1) in the cyclin E-cdk2 complexes. *Oncogene*, **20**, 3428–3436.
51. Zhang,H. *et al*. (2003) A novel cleavage product formed by autoxidation of lycopene induces apoptosis in HL-60 cells. *Free Radic. Biol. Med.*, **35**, 1653–1663.

Received February 3, 2010; revised July 5, 2010; accepted July 8, 2010