Apoptosis 2005; **10**: 1445–1456

DOI: 10.1007/s10495-005-1393-2

Lycopene induces apoptosis in immortalized fibroblasts exposed to tobacco smoke condensate through arresting cell cycle and down-regulating cyclin D1, pAKT and pBad

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Published online: 3 October 2005

There is a lot of interest in the health benefits of dietary carotenoids and on the relationship of these compounds with smoke. In particular, it is unknown if the enhanced cancer risk observed in smokers following β-carotene supplementation can be also found using other carotenoids. Here, we studied the effects of the tomato carotenoid lycopene on molecular pathways involved in cell cycle progression, apoptosis and survival in immortalized RAT-1 fibroblasts exposed to cigarette smoke condensate (TAR). Lycopene (0.5–2.0 μM) inhibited cell growth in a dose-and time-dependent manner, by arresting cell cycle progression and by promoting apoptosis in cells exposed to TAR. The arrest of cell cycle was independent of p53 and of 8-OH-dG DNA damage and related to a decreased expression of cyclin D1. Moreover, the carotenoid up-regulated apoptosis and down-regulated the phosphorylation of AKT and Bad in cells exposed to TAR. Such an effect was associated to an inhibition of TAR-induced expression of Cox-2 and hsp90, which is known to maintain AKT activity. This study suggests that lycopene, differently from β-carotene, can exert protective effects against cigarette smoke condensate.

Keywords: apoptosis; cell cycle; cigarette smoke condensate; immortalized cells; lycopene.

Introduction

The role of carotenoids in protecting tissues from cancer risk caused by chronic smoke is still under debate. Although many epidemiological studies suggest a protective role for carotenoids against cancer,[1] β-carotene supplements in humans have been shown to increase the risk of lung cancer among smokers in two out of three intervention trials. In the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study Group (ATBC trial), the carotenoid and/or vitamin E were given daily to smokers. After a period of six years, there was an increase by 18% in lung cancer.[2] In the Beta-carotene and Retinol Efficacy Trial (CARET trial), β-carotene along with preformed retinal were given for a period of four years to smokers and/or asbestos exposed workers.[3,4] In this period there was a 28% increase in lung cancer among those receiving β-carotene and vitamin A. In contrast to these studies, the Phisicians' Health Study was conducted among mainly nonsmokers: the carotenoid was given every other day and no effect was found on lung cancer risk in either the smokers or nonsmokers.[5]

Controversial results have been also found in animal models as well as in cultured cells. β-Carotene, at pharmacological,[6] but not at physiological,[7] concentrations increased cell proliferation and induced detrimental histopathological changes in the lungs of cigarette smoke-exposed ferrets. On the other hand, β-carotene failed to modulate lung tumor development induced by smoke in mice.[8] In a cell culture bioassay of carcinogenesis with BALC/c3T3 cells, Perocco *et al.*[9] reported an enhancement of benzo[a]pyrene-induced transformation when cells were treated with β-carotene. Interestingly, in the same model, the authors demonstrated an inhibitory activity of vitamin E and alpha-naphthoflavone on β-carotene enhanced transformation by benzo[a]pyrene and cigarette-smoke condensate. Recently, we have observed that β-carotene can exert detrimental effects in lung, mammary, larynx and colon cancer cells as well as in immortalized fibroblasts exposed to tobacco smoke condensate (TAR), by oxidizing DNA and modifying molecular pathways involved in cell proliferation and apoptosis.[10]

These controversial findings have prompted a more cautious program for the development of carotenoids as promising chemopreventive agents. In particular, it is still unclear the mechanism by which carotenoid molecules

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may induce procarcinogenic effects in smokers and it is unknown the role of carotenoids other than β-carotene, as modulators of cell growth following smoke exposure.

In the present work, we evaluated the role of lycopene on the growth of RAT-1 immortalized fibroblasts exposed to cigarette smoke condensate and we studied possible mechanisms by which lycopene and smoke may interact in regulating cell growth. In particular, we analysed the effects of the carotenoid and/or cigarette smoke condensate on cell cycle progression, apoptosis and survival and on molecular pathways involved in the control of these processes. Interest in lycopene, the major carotenoid present in tomatoes, is based on the fact that this carotenoid is present in plasma and breast milk of Western population at levels as high or higher than β-carotene.[11] In addition, tomato consumption has been shown to be associated with decreased risk of several cancers, including lung, gastrointestinal and prostatic cancers.[12–15] Supporting a role for lycopene as an anticancer agent are also a variety of animal model studies (for a review, see[16]) and in vitro cell culture studies.[17–23] In particular, it has been recently reported that lycopene supplementation inhibited lung squamous metaplasia in cigarette smoke-exposed ferrets.[24] Moreover, because reactive oxygen species (ROS) have been shown to be associated with carcinogenesis[25] and ROS are components from cigarette smoke,[26–29] there is a biologically plausible mechanism for lycopene action based on the fact that lycopene quenches singlet oxygen radicals to a greater degree than any other carotenoid.[30–32]

Materials and methods

Cell culture

RAT-1 immortalized fibroblasts (American Type Culture Collection, Rockville, MD, USA) were grown in MEM medium without antibiotics 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. TAR was delivered to the cells as dimethyl sulfoxide (DMSO) solutions. The amount of DMSO given to the cells was not greater than 0.1% (v/v). The final concentration given to the cells was 25 μg/ml. Such a concentration was the maximum one which did not induce necrotic effects in all the cells analyzed. Lycopene (kindly provided by Dr. Regina Goralczyk, DSM Nutritional Products, Basel, Switzerland) was delivered to the cells using tetrahydrofuran (THF) as a solvent, containing 0.025% butylated hydroxytoluene (BHT) to avoid the formation of peroxides.[33] The purity of lycopene was verified by HPLC. 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 greater than 0.1% (v/v). Control cultures received an amount of solvent (DMSO and THF) equal to that present in TAR and lycopene-treated ones. No differences were found between cells treated with DMSO plus THF and untreated cells in terms of cell number, viability and 8-hydroxyl-2′-deoxyguanosine (8-OHdG) levels. 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 haemocytometer counts were performed. The trypan blue dye exclusion method was used to evaluate the percentage of viable cells.

TAR preparation

The particulate phase of cigarette smoke condensate (TAR) was provided by the British American Tobacco Italia (Rome, Italy). It was obtained by mechanically smoking cigarettes, using a smoking machine (Cerulean, ASM 516 model). Cigarettes were smoked using the puff profile (one 35-ml puff/min) to a butt length of 2.3 cm, as indicated by BAT protocol. The experimental conditions during smoking were: 22°C temperature, 60% humidity. The TAR from 20 cigarettes was trapped on filters and then extracted with deionised water. The aqueous solutions were filtered through a Whatman (0.2 μm) filter and dried in vacuo at room temperature. The dried cigarette total particulate matter was re-dissolved in DMSO and stored at −20°C.

Cell cycle analysis

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

Apoptosis detection

The percentage of apoptotic cells was determined by in situ nick end labelling (TUNEL).[34] Briefly, cells were centrifuged, fixed with acetone, and incubated for 5 min with the hybridisation buffer (Boehringer-Mannheim, Germany). Then 2.5 units of terminal deoxynucleotidyltransferase (Tdt) and 100 pmol biotin-dUTP

in hybridisation buffer were added and incubated for 1 h at 37°C. Thereafter, the cells were incubated with streptavidine-biotine-peroxidase complex for 30 min at room temperature. The sites of peroxidase binding were detected with diaminobenzidine. The percentage of TUNEL-positive apoptotic cells (labelling index, LI%) was counted at ×400 magnification. In the absence of Tdt, no unspecific staining was observed. For each slide, 3 randomly selected microscopic fields were observed and at least 100 cells per field were evaluated.

The activity of caspase-3 was measured by the fluorimetric assay as described.[34] Briefly, cells were incubated for the indicated times and then harvested. 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-AMC (Alexis Biochemicals), in a reaction buffer (10 mM HEPES, pH 7.5, containing 50 mM NaCl and 2.5 mM DTT) for 120 min at 37°C. The release of AMC was measured with excitation at 380 nm and emission at 460 nm using a fluorescence spectrophotometer.

Assay for 8-OHdG

Cytospin samples were prepared as follows: cells were diluted in sucrose buffer (0.25 M sucrose, 1.8 mM $CaCl_2$, 25 mM KCl, 50 mM Tris, pH 7.5) at a density of about 3.5×10^6 cells/ml. A total of 50 μl was added to carbowax-ethanol buffer (carbowax stock: 77 ml of PEG-1000 in 50 ml of water, 1 ml of stock in 74 ml of 70% ethanol) (Sigma, Italia, Milan, Italy) and mixed. Aliquots of 150 μl were placed into cytospin funnels and centrifuged at 300 rpm for 5 min on slides coated with aminopropyltriethoxysilane (Kindler, Freiburg, Germany). Samples were air-dried for 10–30 min, fixed in 95% cold ethanol (−20°C) for 10 min and stored at −20°C.

Detection of 8-OHdG by immunohistochemistry coupled with DAB (Vector, Burlingham) was carried out essentially as described by Yarborough *et al.*[35] 1F7 monoclonal antibody for 8-OHdG was kindly provided by Dr. R. M. Santella, Columbia School of Public Health, N.Y. Semi-quantitative evaluation of the staining was carried out by an optical microscope (ECLIPSE E600, Nikon, at 400×) connected to an Image-Pro plus Version 4.1 (Media Cybernetics, USA). Nuclear staining was evaluated in approximately 100 cells of randomly chosen images by operators who were blind to the status of cell treatment, as recommended in.[35] Negative and positive controls (untreated and 0.5 mM H_2O_2-treated cells, respectively) were included within each batch of slides. Detection of 8-OHdG by an HPLC-ECD method[36] in preliminary experiments validated the results obtained by immunohistochemical analysis.

Western blot analysis of cyclin D1, p53, Bax, total Bad, phospho-Bad-Ser,[136] Bcl-2, Bcl-xL, pAKT, AKT, hsp90, Cox-2 expression

Cells (10×10^6) were harvested, washed once with ice-cold phosphate buffered saline and gently lised for 30 min in ice-cold lysis buffer (1 mM $MgCl_2$, 350 mM NaCl, 20 mM HEPES, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM $Na_4P_2O_7$, 1 mM PMSF, 1 mM aprotinin, 1.5 mM leupeptin, 1 mM Na_3VO_4, 20% glycerol, 1% NP40). Cell lysates were centrifuged for 10 min at 4°C (10,000 × *g*) to obtain the supernatants, which were used for Western blot analysis. The anti-Cyclin D1 (clone 72-13G, catalog no. SC-450, anti-Bax (clone P-19, catalog no. SC-526), anti-pBad (Ser136) (catalog no. sc-7999), anti-Bad (catalog no. SSC-7899), anti-Bcl-xL S/L (clone L-19, catalog no. SC-1041), anti-AKT (clone B-1, catalog no. 5298) and anti-pAKT (clone Thr 308R, catalog no. 166646-R), anti-hsp90 (catalog no. SC-8262), anti-Cox-2 (clone C-20, catalog no. 1745) monoclonal antibodies were purchased from Santa Cruz Biotecnology, 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-labelled 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.

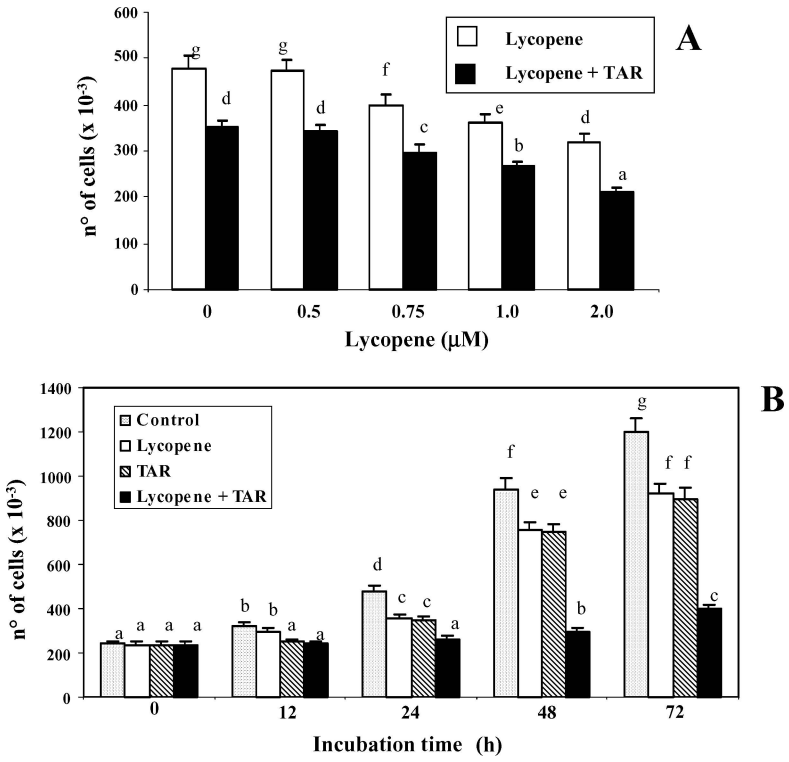
Extraction and analysis of lycopene

Lycopene was extracted with 1 vol methanol and 3 vol hexane from 10×10^6 cells after 24 h treatment with 1 μM lycopene in the absence or in the presence of TAR and analyzed by HPLC, as described in.[37]

Statistical analysis

Three separate cultures per treatment were utilized for analysis in each experiment. Values were presented as means ± SEM. Multifactorial two-way analysis of variance (ANOVA) was adopted to assess any differences among the treatments, the times and/or the concentrations (Figures 1A and B, Tables 1 and 2). 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 ANOVA was used to determine differences between treatment with TAR alone and in combination with lycopene in Figure 1. When significant values were found ($P < 0.05$), post hoc comparisons of means were made using Fisher's test. Differences were

Figure 1. Effects of TAR and lycopene, alone and in combination, on the growth of RAT-1 fibroblasts. Panel A shows the growth of cells treated with TAR at the concentration of 25 μg/ml and various lycopene concentrations for 24 h. Panel B shows the growth of cells treated with TAR (25 μg/ml) and with 1 μM lycopene for different periods of time. The values were the means ± SEM, $n = 7$. The significance was expressed by the presence of superscript letters. In Panel A, values not sharing the same letter were significantly different ($P < 0.005$) (Tukey's test). In Panel B, the treatment/time interaction was significant ($P < 0.05$). Values not sharing a letter were significantly different ($P < 0.001$) (Tukey's test).



analyzed using Minitab Software (Minitab, Inc., State College, PA, USA).

Results

To determine whether the association of smoke and lycopene resulted in changes of cell growth, we treated RAT-1 fibroblasts with TAR at the concentration of 25 μg/ml, alone and in combination with lycopene at various concentrations (Figure 1A) and for different incubation times (Figure 1B). As previously observed, the treatment with TAR alone was able to inhibit the growth of RAT-1 fibroblasts with respect to vehicle-control-treated cells. An inhibition of cell growth was also observed when lycopene was added to the cells at micromolar concentrations. The combined addition of TAR and lycopene remarkably decreased the growth of RAT-1 fibroblasts. Such an effect was dose- and time-dependent.

To elucidate the mechanism(s) responsible for the decrease in cell number by the treatment with TAR and lycopene, alone and in combination, we first examined the effects of these two compounds on cell cycle progression. As shown in Table 1, in vehicle-control cells, most RAT-1 fibroblasts were in G0/G1 phase. The treatment with lycopene (1 μM) and TAR (25 μg/ml) in combination resulted in a significant inhibition of cell cycle progression manifested by the accumulation of cells in the G0/G1 phase and by a decrease in percentage of cells in the S phase, which were maintained throughout the treatment (72 h). Such effects were more remarkable than those observed when the compounds were given alone to the cells.

In an attempt to explore the effects of lycopene and TAR on cell cycle-regulating proteins, we measured the expression of p53 (Figure 2A), following a 12 h-exposure to TAR (25 μg/ml) and lycopene (0.5 and 1 μM) in RAT-1 fibroblasts. In fact, it is well known that the tumor suppressor protein p53 has been shown to down-regulate cell proliferation following treatment with DNA damaging agents[38] and in a recent report we demonstrated that TAR was able to strongly increase DNA oxidative damage.[10] Lycopene alone did not significantly modify p53 expression with respect to control cells, whereas TAR alone remarkably increased it. However, such an effect was remarkably reduced by the combined addition of lycopene to the cells. To assess oxidative changes on DNA induced

Table 1. Effects of lycopene* and TAR# on cell cycle distribution of RAT-1 fibroblasts.

Treatment	Cell Cycle								
	12 h			24 h			72 h		
	G0/G1	S (%)	G2/M	G0/G1	S (%)	G2/M	G0/G1	S (%)	G2/M
Control	51.8 ± 2.0^{a}	33.0 ± 1.4^{D}	$15.2 \pm 1.5^{*}$	52.0 ± 2.0^{a}	31.0 ± 1.2^{D}	$17.0 \pm 1.0^{*}$	52.3 ± 2.1^{a}	29.0 ± 2.5^{D}	$18.7 \pm 2.3^{*}$
Lycopene	59.6 ± 2.5^{b}	25.5 ± 1.3^{C}	$14.9 \pm 2.3^{*}$	59.1 ± 2.8^{b}	26.9 ± 1.3^{C}	$14.0 \pm 1.9^{*}$	60.2 ± 3.0^{b}	26.0 ± 1.5^{C}	$13.8 \pm 2.7^{*}$
TAR	60.1 ± 2.3^{b}	25.7 ± 1.2^{C}	$14.2 \pm 2.0^{*}$	59.0 ± 2.5^{b}	24.5 ± 1.1^{C}	$15.5 \pm 1.8^{*}$	59.7 ± 2.6^{b}	24.1 ± 1.5^{C}	$16.2 \pm 1.6^{*}$
TAR + Lycopene	67.7 ± 2.1^{c}	21.7 ± 1.1^{B}	$16.6 \pm 1.5^{*}$	68.1 ± 2.9^{c}	17.0 ± 2.1^{A}	$14.9 \pm 1.9^{*}$	70.3 ± 3.2^{c}	16.0 ± 2.0^{A}	$13.7 \pm 2.7^{*}$

*Lycopene was added to the cells at the concentration of 1 μM. #TAR was added at 25 μg/ml. The values were the means $\pm$ SEM of 5 experiments. Treatment/time interaction was significant ($P < 0.05$). The significance was expressed by the presence of different superscript letters and symbols. Within the same cell cycle phase, values not sharing the same superscript letter or symbol were significantly different (G0/G1: $P < 0.001$; S: $P < 0.001$) (Tukey's test).

Table 2. Effects of lycopene* and TAR#, alone or in combination, on Apoptosis induction in RAT-1 fibroblasts.

Treatment	Apoptosis					
	12 h		24 h		72 h	
	TUNEL (% positive cells)	Caspase-3 assay (% vs Control)†	TUNEL (% positive cells)	Caspase-3 assay (% vs Control)	TUNEL (% positive cells)	Caspase-3 assay (% vs Control)
Control	2.0 ± 0.2^{a}		2.0 ± 0.2^{a}		2.3 ± 0.2^{a}	
Lycopene	2.1 ± 0.3^{a}	98 ± 9^{A}	2.4 ± 0.2^{a}	105 ± 11^{A}	2.6 ± 0.3^{a}	110 ± 12^{A}
TAR#	1.9 ± 0.3^{a}	102 ± 10^{A}	4.0 ± 0.5^{b}	135 ± 14^{B}	22.6 ± 1.9^{d}	500 ± 20^{D}
Lycopene* + TAR#	7.0 ± 1.0^{c}	220 ± 19^{C}	13.5 ± 1.5^{c}	463 ± 42^{D}	30.7 ± 2.6^{e}	705 ± 70^{E}

*Lycopene was added to the cells at the concentration of 1 μM.#TAR was added at 25 μg/ml. The values were the means $\pm$ SEM of three experiments. Treatment/time interaction was significant ($P < 0.05$). The significance was expressed by the presence of different superscript letters. Values not sharing the same superscript letter were significantly different (TUNEL, 12 h: $P<0.005$, 24 h: $P<0.001$, 72 h: $P < 0.002$) (Caspase-3 assay, 12 h: $P < 0.001$, 24 h: $P < 0.001$, 72 h: $P < 0.002$) (Tukey's test)
†Caspase-3 activation in control cells was 2000 $\pm$ 180 Fluorescence units.

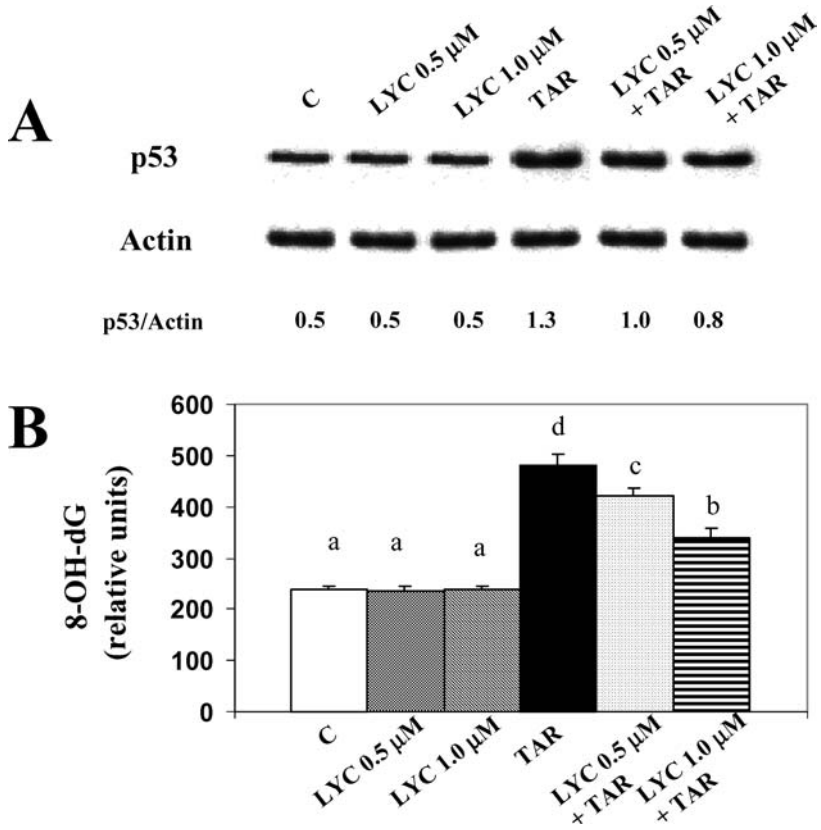
by TAR and lycopene, we measured the levels of 8-OH-dG in RAT-1 fibroblasts treated with TAR (25 μg/ml) and lycopene at the concentration of 0.5 and 1 μM for 12 h (Figure 2B). Accordingly with the changes in p53 expression, a significant increase in 8-OHdG-specific immunoreactivity was detected in fibroblasts treated with TAR. In contrast, the combination of TAR and lycopene significantly reduced the formation of 8-OH-dG with respect to TAR alone. On the other hand, no significant changes in the levels of this oxidative marker were found in cells treated with lycopene alone with respect to control cells.

It has been reported that cyclin D1 plays a regulatory key role during the G0/G1 phase of the cell cycle.[39] Therefore, we measured the expression of this protein in our model and in our experimental conditions (Figure 3). Fibroblasts treated with the combination of lycopene and TAR for 12 h showed a strong decrease in cyclin D1 expression, which was much greater than the inhibitions induced individually by TAR and lycopene.

To investigate whether cell apoptosis can be affected by smoke and/or lycopene treatments, we measured both the percentage of TUNEL positive cells and the cleaved caspase-3 relative to controls, in RAT-1 fibroblasts following the different treatments for 12, 24 and 72 h (Table 2). Neither lycopene (1 μM) nor TAR (25 μg/ml) significantly modified the percentage of apoptosis with respect to control cells at 12 h, as evidenced by TUNEL method. However, a weak increase in the percentage of apoptotic cells was observed following TAR treatment for 24 h, which became remarkable at 72 h, whereas lycopene treatment was still uneffective. On the other hand, the combination of lycopene and TAR induced apoptosis precociously (at 12 h) and in a greater extent (at 72 h) than that observed in the presence of TAR alone.

Because caspases play a central role in the commitment to apoptosis, we have also analysed the effects of lycopene and TAR on the activation of caspase-3, one of the most important cell death executioner for apoptosis.

Figure 2. Effect of TAR, alone and in combination with lycopene, on the expression of p53 (panel A) and on the levels of 8-OHdG (panel B) in RAT-1 fibroblasts treated for 12 h. TAR was added to the cells at the concentration of 25 μg/ml and the carotenoid at the concentration of 0.5 and 1 μM. Panel A: representative Western blot analysis. Panel B: The values were the means ± SEM, $n = 5$. The significance was expressed by the presence of superscript letters. Values not sharing the same letter were significantly different ($P < 0.05$, Fisher's test)



According with the results obtained by TUNEL method, we found that the treatment with a combination of lycopene and TAR resulted in a strong increase in 7 amido-4 methylcoumarin fluorescence, indicative of the activation of caspase-3 in RAT-1 fibroblasts. Such an increase was more precocious and greater than that observed in the presence of TAR alone (Table 2).

In an effort to investigate the molecular pathway involved in apoptosis induction by the combination of TAR and lycopene, we also examined the effects of these two compounds, alone and in combination, on the expression of the apoptosis promoter proteins Bax and Bad and on the expression of the apoptosis blocking proteins Bcl-2 and Bcl-XL (Figure 4). The phosphorylated form of Bad was not modified by lycopene alone, but it was increased by TAR alone after 12 h of incubation. Such an increase was counteracted by lycopene addition, which strongly decreased Bad phosphorylation in fibroblasts exposed to TAR. No changes in the expression of total Bad were found during the treatments. Moreover, no remarkable changes in Bax expression were observed at 12 h in cells following the addition of TAR and/or lycopene to RAT-1 fibroblasts. However, it should be noted that a 72-h treatment increased Bax expression and reduced Bad phosphorylation in fibroblasts treated with TAR, whereas it was still ineffective on these two proteins in cells treated with lycopene alone (data not shown). In contrast, neither Bcl-2 nor Bcl-XL expression were significantly modified by the treatments with TAR and/or lycopene for both 12 h (Figure 3) and 72 h (data not shown).

Several observations indicate that the cellular response to various chemotherapeutic agents is dependent on the AKT-dependent survival pathway.[40] Moreover, AKT has been reported to protect cells from apoptosis.[40] Therefore, we investigated the levels of phosphorylated and total AKT in cells following treatment with TAR and lycopene (Figure 5A). TAR alone significantly increased pAKT expression, while the carotenoid alone was uneffective in modifying this protein. On the other hand, the combination of TAR and lycopene strongly decreased it.

It is known that hsp90 maintains the AKT activity by binding to AKT and by preventing PP2A-dependent phosphorylation of AKT.[41] Therefore, we measured the levels of this heat shock protein in RAT-1 fibroblasts

Figure 3. Representative Western blot analysis of the expression of cyclin D1 in RAT-1 fibroblasts treated with TAR, alone and in combination with lycopene, for 12 h. TAR was added to the cells at the concentration of 25 μg/ml and the carotenoid at the concentration of 0.5 and 1 μM.

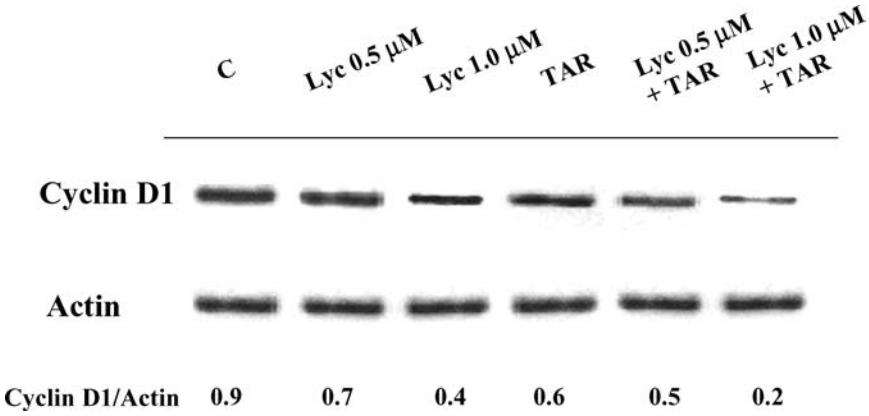
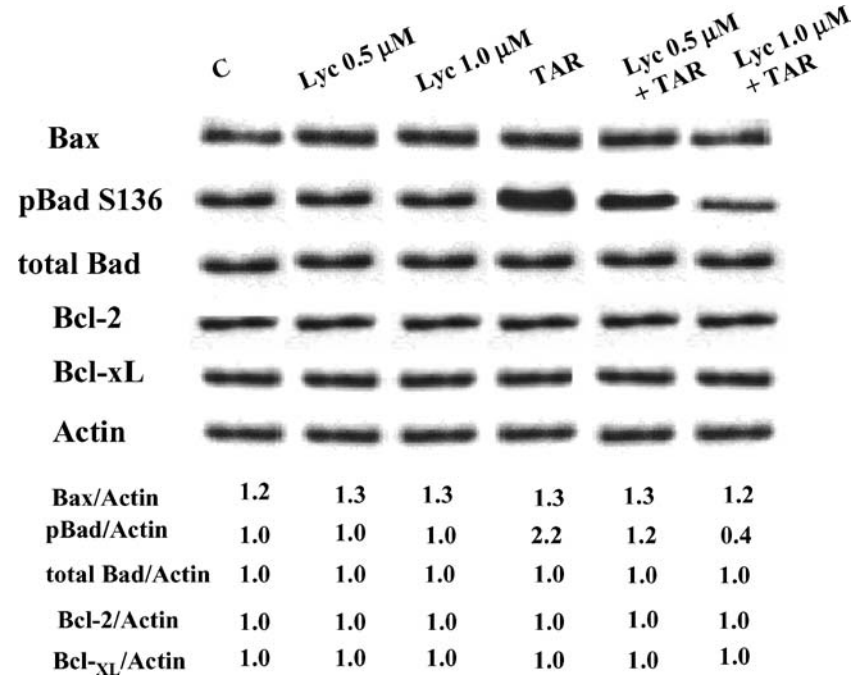


Figure 4. Representative Western blot analysis of the expression of Bax, pBad, total Bad, Bcl-2 and Bcl-xL in RAT-1 fibroblasts treated with TAR, alone and in combination with lycopene, for 12 h. TAR was added to the cells at the concentration of 25 μg/ml and the carotenoid at the concentration of 0.5 and 1 μM.



following a 12-h treatment with TAR and lycopene (Figure 5B). TAR markedly increased hsp90 expression, whereas lycopene alone was ineffective. On the other hand, lycopene stongly inhibited TAR-induced hsp90 expression.

Since it has been reported that cigarette smoke condensate induces the expression of Cox-2[10,42] and that this protein confers resistance to apoptosis[43] and promotes tumor survival, we examined Cox-2 expression following treatment with lycopene (0.5 and 1 μM) and TAR (25 μg/ml) in RAT-1 fibroblasts (Figure 6). TAR alone increased the expression of Cox-2. On the other hand, lycopene alone weakly decreased it. However, the combination of TAR and lycopene strongly inhibited Cox-2 expression.

Lycopene was incorporated and/or associated to RAT-1 fibroblasts. This effect was observed at 12 h of treatment and became maximum at 24 h. At this time, the

Figure 5. Representative Western blot analysis of the expression of AKT (panel A) and heat shock protein (hsp)-90 (panel B) in RAT-1 fibroblasts treated with TAR, alone and in combination with lycopene, for 12 h. TAR was added to the cells at the concentration of 25 μg/ml and the carotenoid at the concentration of 0.5 and 1 μM.

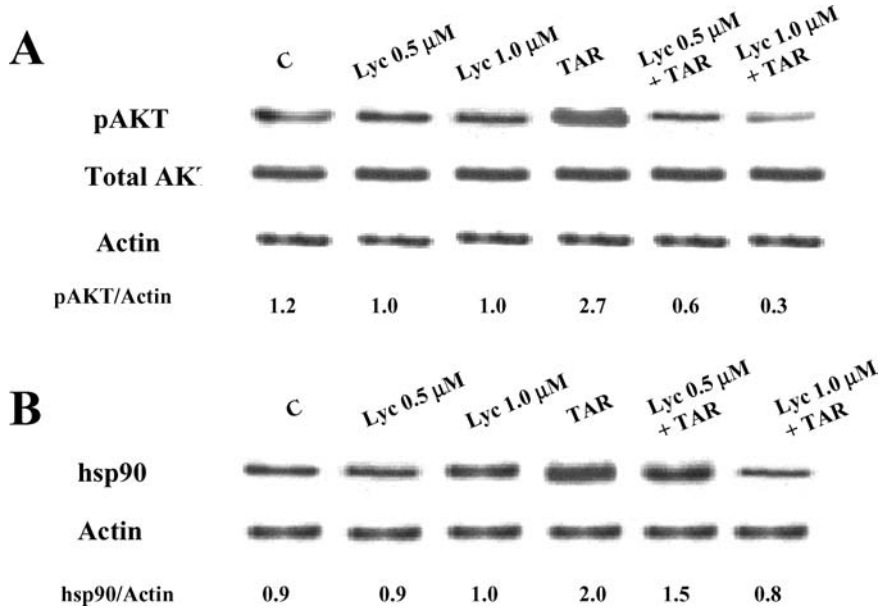
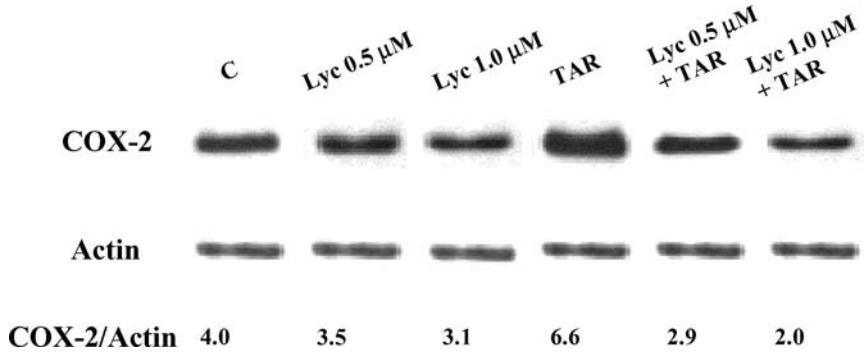


Figure 6. Representative Western blot analysis of the expression of Cox-2 in RAT-1 fibroblasts treated with TAR, alone and in combination with lycopene, for 12 h. TAR was added to the cells at the concentration of 25 μg/ml and the carotenoid at the concentration of 0.5 and 1 μM.



carotenoid, added to the cells at the concentration of 1 μM, reached the concentration of 80 pmol/10^6 cells. The concomitant presence of TAR caused a significant reduction of the carotenoid content into the cells. At 24 h of incubation, such a reduction was about 50%.

Discussion

It is still unclear the mechanism by which carotenoid molecules may induce procarcinogenic effects in smokers and it is unknown the role of carotenoids other than β-carotene, as modulators of cell growth following smoke exposure. Cigarette smoke exposure has been considered a strong risk factor for cancer since it promotes genomic instability and the development of neoplasia by affecting molecular pathways involved in cell proliferation and apoptosis. In this study, we have demonstrated that the tomato carotenoid lycopene strongly inhibited cell growth in immortalized fibroblasts, as well as in lung and prostate cancer cells, exposed to cigarette smoke condensate, by arresting cell cycle progression and by promoting apoptosis. The growth-inhibitory effects of the carotenoid were dose- and time-dependent and occurred at lycopene concentrations (0.5–2.0 μM) which are in the range of lycopene levels found in the serum of supplemented subjects. In fact such low lycopene concentrations are found in the blood of individuals consuming average-to high amounts of tomato products and, thus, may serve to inhibit cancer process. The results of this

study are consistent with the findings from other experimental studies that have shown growth-inhibitory effects by lycopene alone in different tumor cell lines, including prostate,[23] mammary,[17,18,21,22] endometrial[22] cancer cells and promyelocytic leukaemia cells.[20] On the other hand, they completely differed from the results reported in our previous study, showing that a combination of TAR and β-carotene was able to increase cell growth with respect to TAR alone.[10]

We clearly reported that lycopene was able to enhance the arrest of cell cycle progression induced by TAR alone in RAT-1 fibroblasts. TAR-exposed fibroblasts treated with lycopene showed a delay in cell cycle at the G0/G1 phase and a concomitant reduction in S phase. On the other hand, fibroblasts treated with lycopene alone showed the same effects, although in a lower extent. The regulation of cell cycle progression by lycopene is under active study and seems to involve a modulation of cyclins, cyclin-dependent kinases and cyclin-dependent kinase inhibitors. This report confirms prior work that demonstrates that these two growth-regulatory programmes, the cell cycle machinery and lycopene signalling, are coupled through regulation of cyclin D1, the G0/G1-phase cyclin.[22] It is well known that cyclin D1 is a known oncogene and it is over-expressed in several cancer cell lines.[39] Thus, reduction in cyclin D1 by lycopene treatment may contribute to its proposed action in prevention of prostate and breast cancer. Interestingly, fibroblasts treated with TAR also exhibited low levels of cyclin D1, as recently reported,[10] but such an effect became very remarkable in the presence of the carotenoid.

Moreover, in contrast to β-carotene, the arrest of cell cycle progression by lycopene in RAT-1 fibroblasts exposed to TAR did not involve p53 pathway.[10] The carotenoid counteracted the effects of TAR on p53 expression by significantly decreasing it. Such a finding is not surprising in view of the fact that p53 responds to stress signals that can cause oncogenic alterations, such as DNA damage.[38] Cells increased their content of 8-OH-dG as a consequence of smoke exposure and this results in an increased expression of p53. Given its potent antioxidant function,[30–32] lycopene may protect cells against TAR-induced DNA oxidation. Lycopene has been shown to reduce the amount of oxidative DNA damage in cell culture as well as in animal models (for a review, see[44]). Moreover, several studies demonstrated that tomato consumption protected human leukocytes against oxidative DNA damage in vitro.[45] Bowen *et al.*[46] provided the first in vivo evidence that a lycopene-rich diet reduced oxidative DNA damage also in prostate. According with these findings, in our study, cells exposed to a combination of TAR and lycopene significantly decreased their 8-OH-dG levels and, in turn, their p53 content.

Our study also shows that fibroblasts exposed to TAR in the presence of lycopene enhanced their capability to undergo apoptosis, as evidenced by TUNEL method and caspase-3 assay. In these cells, apoptosis induction was very remarkable and evident after 12 h. On the other hand, cells treated with TAR alone underwent apoptosis only after 24 h of incubation and, in this case, the percentage of apoptotic cells was much lower than that observed in the presence of TAR and the carotenoid. Cells treated with lycopene alone, at least at the doses and at the times indicated in this study, did not show significant differences in the percentages of apoptotic cells with respect to control cells. According with this, Hall[47] and Kotake-Nara *et al.*[48] did not find apoptosis induction by lycopene in different human prostate cancer cells even at very high lycopene concentrations, although recent studies seem to suggest that apoptosis can be detected using 5 μM lycopene[49] or even lower concentrations of the carotenoid (<3 μM)[50] in LNCaP human prostate cancer cells within 12 h[50] or 48 h[49] of treatment. Lycopene was also apparently unable to induce apoptosis in HL-60 leukemic cells, although the oxidative metabolites of lycopene acted as apoptosis inducers.[51,52] It has been recently reported that tomato sauce, rich in lycopene, increased the percentage of apoptotic cells in prostate carcinoma in much higher extent than in benign prostatic hyperplasia.[53] All these findings seem to suggest that lycopene preferentially acts as an apoptosis inducer when it is administrated at high concentrations and in particular cell types. Hyperplastic cells are less sensitive than carcinoma cells to lycopene-induced apoptosis. In this context, the lack of apoptotic effects by lycopene alone observed in our study may be explained by the low dose of lycopene used as well as by the fact that RAT-1 fibroblasts are an immortalized but not a transformed cell line.

Accordingly with the results on apoptosis, we found that a 12 h-treatment with TAR alone suppressed Bad-mediated apoptosis by inducing the phosphorylation of Bad at Ser136. Such an effect was maintained at 72 h (data not shown). At this time, an increased expression of Bax was observed in cells treated with TAR alone (data not shown), which became relevant at 36 h, as previously reported by us. The enhancement of Bax expression was presumably due to the increased levels of p53, which is known to promote apoptosis through this protein in conditions of persistent DNA damage.[38] On the other hand, when lycopene was added to fibroblasts in combination with TAR, the phosphorylation of Bad was completely blocked by the carotenoid. Such an effect was observed at 12 h but was also maintained for 36 h (data not shown). The discovery of a substantial and sustained decrease in Bad phosphorylation by lycopene under smoke conditions has been also recently reported by Liu *et al.* in lung of ferrets.[24]

It is known that phosphorylation of Bad and, consequently, the capability of cells to undergo apoptosis, is controlled by multiple pathways, including AKT.[40] Bad

phosphorylation by AKT (at serine 136) inactivates Bad by inducing its interaction with 14-3-3 proteins. This binding induces a conformational change in Bad, which allows binding and phosphorylation of Bad by PKA. Our study suggests that AKT regulation may have a key role in the proapoptotic effects of lycopene under smoke conditions. In fact, while TAR-treated cells exhibited high levels of pAKT, cells treated with a combination of TAR and lycopene strongly decreased it. The heat shock protein hsp90 was increased in fibroblasts treated with TAR alone, but decreased in cells treated with a combination of TAR and lycopene. These data are particularly interesting since it has been reported that Hsp90 maintains the AKT activity by binding to AKT and by preventing PP2A-dependent dephosphorylation of AKT.[41] Moreover, hsp90 also prevents proteasome-dependent degradation of PDK1, which is known to activate AKT.[54] On the other hand, our observation that lycopene was able to minimize the effects of TAR on hsp90 is not surprising in view of the fact that heat shock proteins increase as a consequence of oxidative stresses, including smoke,[55,56] and that lycopene acts as a potent cellular antioxidant.[30–32,57] The modulation of hsp90 by lycopene under smoke conditions could be a further suggestive intracellular mechanism to explain the modulatory activity of lycopene on Bad. In fact, it has been recently proposed that lycopene is able to down-regulate Bad phosphorylation through the IGF pathway and the up-regulation of IGFBP-3,[24] which acts as a potent inhibitor of PI3K/AKT/PKB pathway.[58]

It has been recently reported that cigarette smoke condensate induced the expression of Cox-2[10,42,59] and that the levels of this protein may be a suitable marker for following cancer progression.[60] We have also demonstrated that lycopene treatment decreased the expression of Cox-2, which we recently reported to act as an anti-apoptotic protein.[43]

Conclusions

Further work is needed to better clarify the different behaviour of β-carotene and lycopene under smoke conditions. It is possible that the different outcome between the lycopene and β-carotene studies in our cell models may be attributable to the differences in the levels of carotenoids that accumulate in the cells. Moreover, it is unclear if the activity of lycopene may reside in the carotenoid molecule or in metabolic products that may be formed from it. Although such gaps, this study has important implications for future studies regarding chemopreventive effects of lycopene against smoke-induced cancer. In particular, we have demonstrated that the tomato carotenoid lycopene strongly inhibited cell growth in immortalized fibroblasts exposed to cigarette smoke condensate. The carotenoid may mediate its protective effects against smoke through a decrease in the expression of cyclin D1, which arrests cell cycle progression and a down-regulation of the phosphorylation of AKT, and Bad, which promote apoptosis.

Acknowledgments

The authors thank Dr. Regina Goralczyk, DSM Nutritional Products, Human Nutrition and Health, Carotenoid Section, Basel, Switzerland for the generous provision of purified lycopene and Dr. Alfredo Nunziata of BAT Italia for kindly providing cigarette TAR. This work was supported by MIUR-D1

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