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ORIGINAL CONTRIBUTION

Genistein and β-carotene enhance the growth-inhibitory effect of trichostatin A in A549 cells

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Abstract
Background The combination of anti-cancer drugs with nutritional factors is a potential strategy for improving the efficacy and decreasing the toxicity of chemotherapy. However, whether nutritional factors enhance the effect of trichostatin A (TSA), a novel anti-cancer drug, is unclear.
Aim We investigated the individual enhancing effect and its possible mechanisms of genistein, daidzein, β-carotene, retinoic acid, and α-tocopherol on the cell-growth-inhibitory effect of TSA in a human lung carcinoma cell line, A549.
Methods A549 cells were incubated with TSA (50 ng/mL) alone or in combination with the various nutritional factors for various times, and cell growth was measured. IMR90 cells, human lung fibroblasts, were also incubated with TSA alone or in combination with genistein or β-carotene to determine the selectivity of these treatments. In addition, we studied effects on the cell cycle, caspase-3 activity, and DNA damage (by comet assay) in A549 cells.
Results After treatment for 72 h, 10-μM genistein or β-carotene significantly enhanced the growth-inhibitory effect of TSA in A549 cells. Daidzein, retinoic acid, and α-tocopherol at the same concentration had no significant effect. However, genistein and β-carotene failed to enhance the cell-growth-arrest effect of TSA in IMR90 cells. Flow cytometric analysis showed that both genistein and β-carotene significantly increased the TSA-induced apoptosis in A549 cells. Genistein significantly enhanced TSA-induced caspase-3 activity in A549 cells by 34% at 24 h, and the caspase-3 inhibitor partly inhibited the enhancing effect of genistein on TSA-induced apoptosis. β-Carotene did not significantly affect TSA-induced caspase-3 activity. However, β-carotene rather than genistein enhanced TSA-induced DNA damage.
Conclusions Genistein and β-carotene enhance the cell-growth-arrest effect of TSA on A549 cells. Genistein exerts its effect, at least partly, by increasing caspase-3 activity; whereas β-carotene may enhance TSA-induced cell death mainly through a caspase-3-independent pathway.

Keywords Trichostatin A · Chemotherapy · Genistein · β-Carotene · Cell-growth-arrest

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Introduction

Trichostatin A (TSA) is a histone deacetylase inhibitor, which is a promising class of anti-cancer drugs, because it selectively induce the differentiation and apoptosis of various transformed cells [10]. The accumulation of acetylated forms of histones and nonhistone proteins is an important mechanism by which TSA affects the transcriptional patterns of many genes, including those associated with cell-growth-arrest and apoptosis [5]. Lung

cancer is the leading cause of cancer death in many countries, and the successful cure rate of this disease remains low. Recent studies have suggested that TSA may be a potential therapy for lung cancer [12, 24], because it induces apoptosis of lung cancer cells by activating the death receptor-mediated and mitochondrial pathways [12].

Various dietary factors, such as isoflavones, β-carotene, retinoic acid, and α-tocopherol, have been suggested to prevent the development of cancer [4, 13, 17, 20, 31, 33]. These dietary factors may exert anti-cancer effects by acting as anti-oxidants, by inducing apoptosis, by acting as anti-inflammatory agents, and by inducing differentiation [4, 22]. In addition, nutritional factors may cooperatively or synergistically enhance the effect of anti-cancer drugs to inhibit the proliferation of cancer cells through various mechanisms [7, 16]. For example, genistein synergistically enhances the preventive effect of tamoxifen on the growth of estrogen-dependent human breast carcinoma in mice by decreasing estrogen levels and activity [16]. Furthermore, combination therapy may reduce the toxicity of chemotherapy [7].

The aim of this study is to investigate whether the nutritional factors genistein, β-carotene, retinoic acid, and α-tocopherol, which are potential chemopreventive agents, enhance the effect of TSA on cell-growth-arrest/apoptosis and the possible mechanisms of such action in A549 cells. A549 cells are a cell line derived from human nonsmall-cell lung carcinoma [18]. To examine the selectivity of the combination treatment, we also evaluated the effect of TSA alone or in combination with nutritional factors in human embryo lung fibroblasts, IMR90 cells, a nontumor cell line.

Materials and methods

Materials

All chemicals used were of reagent grade or higher. Genistein, daidzein, β-carotene, α-tocopherol, retinoic acid, and TSA were from Sigma Chemical Co. (St. Louis, MO, USA). Tetrahydrofuran (THF) and dimethyl sulfoxide (DMSO) were from Wako Co. (Tokyo, Japan). Basal Medium Eagle, fetal bovine serum, trypsin, penicillin, streptomycin, sodium pyruvate, and nonessential amino acids were from GIBCO/BRL (Rockville, MD, USA).

Cell culture and cell growth test

A549 and IMR90 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and were cultured in Basal Medium Eagle containing 10% (v/v) fetal bovine serum, 0.37% (w/v) $NaHCO_3$, penicillin (100 units/mL), and streptomycin (100 µg/mL) at 37 °C in a humidified incubator under 5% CO_2 and 95% air. An equal number (2.5×10^4/mL) of cells was incubated for 24 h before the various treatments. At this point (0 h), the cell number was taken as 100%, and the cell growth of each treatment at the indicated times was expressed as a percentage (%) of that at 0 h. After being washed twice with phosphate-buffered saline (PBS; pH 7.4, containing 137-mM NaCl, 2.7-mM KCl, 1.5-mM KH_2PO_4, and 8.1-mM Na_2HPO_4), the cells were incubated in fresh culture medium containing TSA alone or in combination with the various nutritional factors. The medium was replaced every day. Cell growth was measured mainly by 3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyl-tetrazolium bromide (MTT) colorimetric assay [19], and the trends in cell growth inhibition induced by the various treatments were confirmed by Trypan blue assay [23]. Stock solutions of DMSO–genistein, DMSO–daidzein, THF–β-carotene, THF–retinoic acid, and methanol–α-tocopherol at a concentration of 10 mM were prepared freshly before each experiment. Control cells were incubated with solvent alone.

Flow cytometric assay

Flow cytometric analysis was performed with a FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) with WinMDI 2.8 software. To measure DNA, the level of propidium iodide (Sigma, St. Louis, MO, USA) incorporated into DNA was analyzed. Cells were harvested after the indicated time, washed 2 times with ice-cold PBS, and fixed in 3 mL of 70% ice-cold alcohol at −20 °C for at least 30 min [9, 28]. After centrifugation at 300 × g for 5 min, the cells were resuspended in PBS-containing 4 µg/mL propidium iodide and 0.5-mg/mL RNAase A and were incubated in the dark at 37 °C for 30 min. Fifteen thousand cells per sample were analyzed; the data were gated to exclude debris. Sub-G1, G1, and G2-M phase cells were determined from the DNA content histograms.

Caspase-3 activity

Caspase-3 activity was measured using a caspase-3 colorimetric protease assay kit (Chemicon, Billerica, MA, USA) according to the manufacturer's instructions. Protein concentrations of lysates were determined by the Lowry method [15]. An aliquot of cell lysates (80 µL) was incubated with the caspase-3 substrate at 37 °C for 2 h. Samples were analyzed at 405 nm in a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The relative caspase-3 activity of the control group was taken as 100. To confirm the importance of caspase-3 activity in apoptosis, in some experiments, A549 cells were pre-incubated with 1-µM Ac-DEVD-CHO (Chemicon, Billerica, MA,

USA) for 10 min, and then TSA or TSA + genistein were added to the medium for a further 48 h.

Comet assay (single-cell gel electrophoresis)

According to the method described by Collins et al. [2], single-cell gel electrophoresis was used to determine the DNA damage to A549 cells induced by various treatments. The DNA images were analyzed by computer with the Interactive Image Analysis Comet Assay III software (Perceptive Instrument, Haverhill, UK) as tail intensity (%DNA in tail).

Statistical analysis and calculation of synergy

Values are expressed as the mean ± SD of three replicates and were analyzed by using Student's t test for two-group comparisons or one-way ANOVA followed by Duncan's multiple-range test for comparisons of group means. p Values <0.05 were considered statistically significant. The synergy of treatments on the inhibition of cell growth was calculated using cell growth percentage as [control − combined treatment]/[(control − TSA treatment) + (control − nutrient treatment)], that is, [observed % inhibition/expected % inhibition] [1, 29]. According to this formula, a value greater than 1.0 is synergistic [1].

Results

Effects of genistein and β-carotene on TSA-induced cell-growth-arrest in A549 cells and in IMR90 cells

TSA at 25, 50 and 100 ng/mL inhibited the growth of A549 cells in a dose- and time-dependent manner; TSA at 100 ng/mL strongly induced cell death as early as 24 h, whereas at 25 ng/mL, TSA had no effect until 72 h (data not shown). We chose 50 ng/mL of TSA (TSA50), which significantly inhibited cell growth at 48 and 72 h by about 35 and 42%, respectively, to examine the combined effect of TSA with the test compounds. Co-incubation with 10 μM of genistein, an isoflavone, and β-carotene, a carotenoid, for 72 h significantly and synergistically enhanced the suppressing effect of TSA50 on the growth of A549 cells by about 32 and 34% ($p < 0.05$), respectively; whereas daidzein, α-tocopherol, and retinoic acid at the same dose had no significant effect (Table 1). Genistein and β-carotene themselves inhibited slightly but not significantly cell growth compared with the control group at 72 h ($p > 0.05$). In further time-course and dose-dependent studies, we found that genistein and β-carotene enhanced the growth-inhibitory effects of TSA in A549 cells in a dose-dependent manner (Fig. 1a, b).

We also evaluated the effect of TSA alone or in combination with genistein or β-carotene in human lung

Table 1 Effects of TSA or nutritional factors alone or combined on the growth of A549 cells

Treatment	Cell growth (%)	Observed inhibition (%)	Expected inhibition[1] (%)	Synergistic effect[2]
Control	467 ± 25[a]			
G	430 ± 25[a]	7		
BC	437 ± 19[a]	6		
D	476 ± 23[a]	−2		
RA	445 ± 21[a]	5		
E	469 ± 10[a]	0		
TSA	272 ± 28[b†]	42		
TSA + G	183 ± 13[a]	61*	49	1.24
TSA + BC	179 ± 21[a]	62*	48	1.29
TSA + D	225 ± 20[ab]	53	42	ND
TSA + RA	256 ± 5[b]	45	47	ND
TSA + E	293 ± 5[b]	37	42	ND

A549 cells were incubated with 50-ng/mL TSA alone or in combination with 10-μM genistein (G), β-carotene (BC), daidzein (D), retinoic acid (RA), or vitamin E (E) for 72 h. Values are means ± SD ($n = 3$). Values for the TSA treatment groups with different superscript letters are significantly different ($p < 0.05$)

ND not detected

* Denotes the value was significantly different from expected inhibition with the same treatment; † denotes the value was significantly different from that of the control

[1] The expected % inhibition is the sum of observed % inhibition of TSA and each nutrient factor alone

[2] The synergistic effect is calculated by using percentage cell growth as follows: [control − combined treatment]/[(control − TSA treatment) + (control − nutrient treatment)], i.e., [observed % inhibition/expected % inhibition]

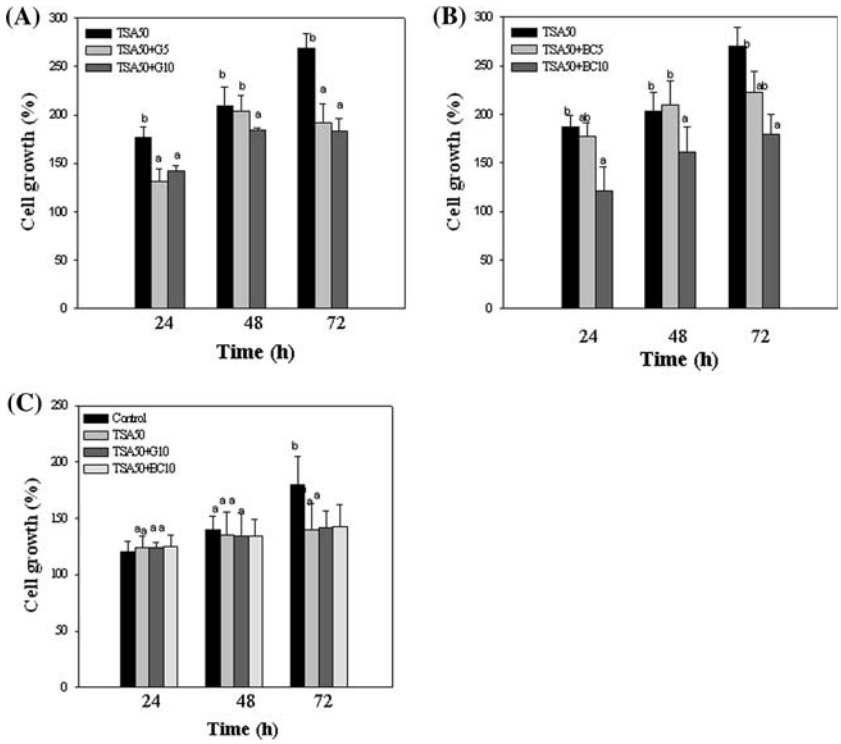

Fig. 1 Time- and dose-dependent effects of TSA alone or in combination with genistein or β-carotene on the growth of A549 cells (**a**, **b**, respectively) or on the growth of IMR90 cells (**c**). The cells were incubated with TSA (at 0 or 50 ng/mL; control and TSA50, respectively) alone or in combination with 5- or 10-μM genistein (G5, G10) or β-carotene (BC5, BC10). Values (means ± SD, $n = 3$) at the same treatment time with different *superscript letters* are significantly different ($p < 0.05$)

fibroblasts, IMR90 cells. The results showed that the inhibitory effects of TSA alone on IMR90 cell growth were markedly lower than those on A549 cells (Fig. 1c). In addition, genistein and β-carotene failed to enhance the cell-growth-arrest effect of TSA in IMR90 cells.

Effect of genistein or β-carotene on TSA-induced apoptosis

The amount of apoptotic cells was calculated on the basis of the appearance of cells in the sub-G1 phase [14]. As shown in Fig. 2, TSA50 induced apoptosis and G2/M phase arrest in A549 cells from 24 h onward. Ten micromolar of β-carotene and genistein significantly increased the percentage of apoptosis rather than G2/M arrest in a time-dependent manner. The extent of the increase in apoptotic cells induced by TSA alone or combined with genistein or β-carotene seemed to peak at 48 h (about 2–3-fold of the level at 24 h of each treatment).

Activation of caspase-3

As shown in Fig. 3A, TSA50 significantly increased caspase-3 activity to a peak at 24 h. Genistein significantly enhanced the effect of TSA on increasing caspase-3 activity by 34% at 24 h, but the enhancing effect of genistein declined at 48 h. In contrast, the effect of β-carotene was not significant. To confirm the role of caspase-3 in the enhancing effect of genistein on apoptosis induced by TSA, A549 cells were pre-incubated with 1-μM Ac-DEVD-CHO, a caspase-3 inhibitor, and were then incubated with TSA or TSA + genistein for a further 48 h. The results of flow cytometry showed that the presence of Ac-DEVD-CHO significantly suppressed the enhancing effect of genistein on TSA-induced apoptosis by about 55% (Fig. 3b).

Effect of genistein or β-carotene on TSA-induced DNA damage

Because the uncoiled DNA induced by TSA may increase the DNA damage caused by the attack of many active compounds, we investigated the effect of nutritional factors on DNA damage induced by TSA. Using the comet assay, we found that TSA50 markedly increased the level of DNA damage after incubation for 24 h (Fig. 4). Ten micromolar of β-carotene significantly enhanced the TSA-induced DNA damage by 64% at 24 h, whereas genistein had no effect.

Discussion

The combination of nutritional factors with anti-cancer drugs has been suggested to be a potential strategy for cancer therapy [6, 25]. In this study, we first showed that genistein or β-carotene at 5 or 10 μM, the physiological concentrations after supplementation of these compounds

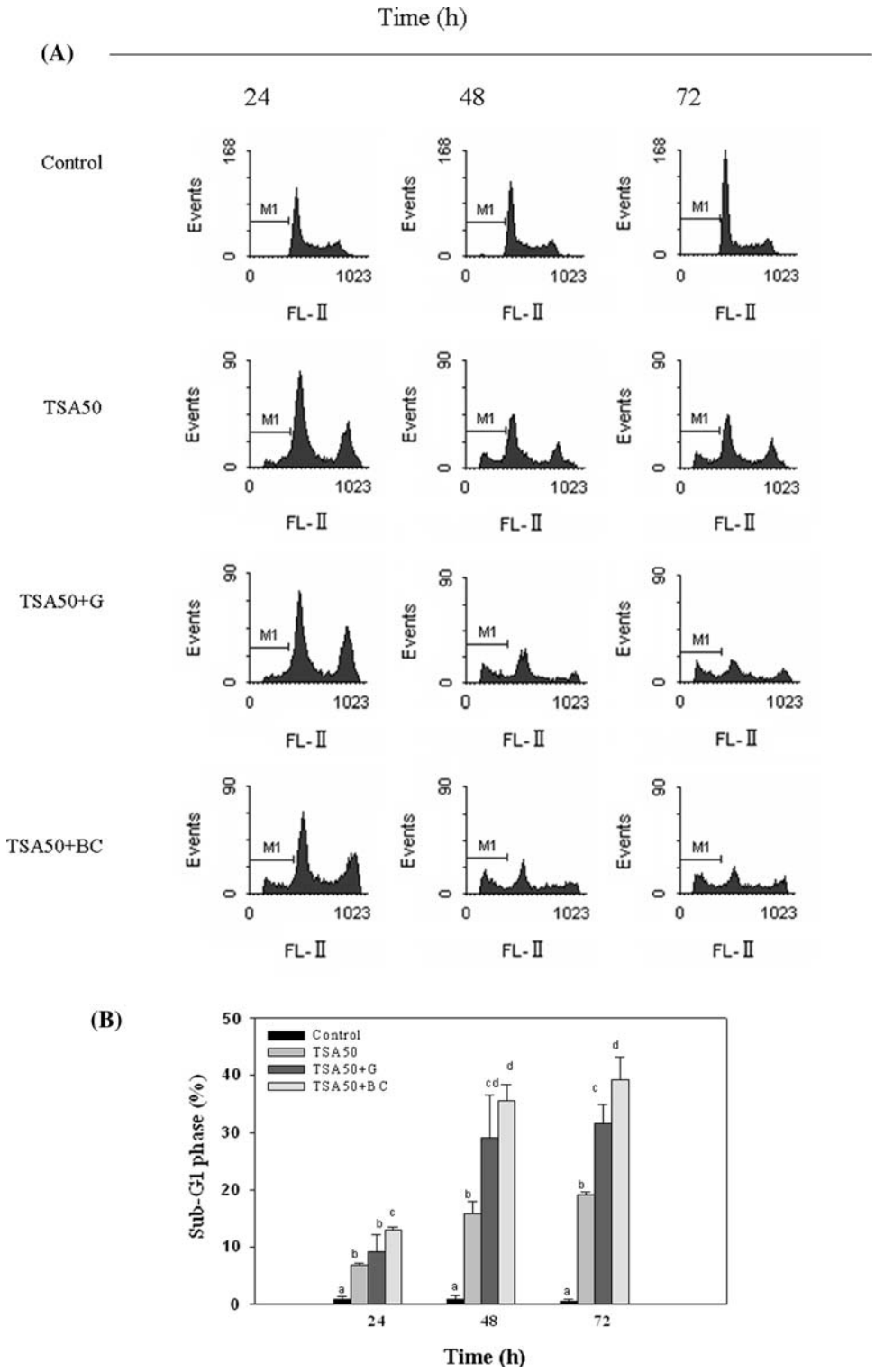

Fig. 2 Effects of various treatments on cell cycle progression and apoptosis in A549 cells. Cells were incubated with 50-ng/mL TSA (TSA50) alone or in combination with 10-μM genistein (G) or β-carotene (BC) for the indicated times. **a** DNA content histogram, **b** the percentage of cells in the sub-G1 phase. Values (means ± SD, $n = 3$) at the same treatment time with different *superscript letters* are significantly different ($p < 0.05$)

in humans [8, 30], had the potential to enhance the growth-inhibitory effect of TSA50 in A549 cells. A previous study, using higher concentrations (25 and 50 μM), also demonstrated that genistein enhances the anti-tumor effect of TSA in osteosarcoma cells [26]. Furthermore, this study showed that genistein and β-carotene significantly increased the TSA-induced apoptosis of A549 cells. On the other hand, genistein and β-carotene failed to enhance the cell-growth-arrest effect of TSA in IMR90 cells. These results imply that the combination of genistein or β-carotene with TSA has the potential for specifically inhibiting cancer cell growth.

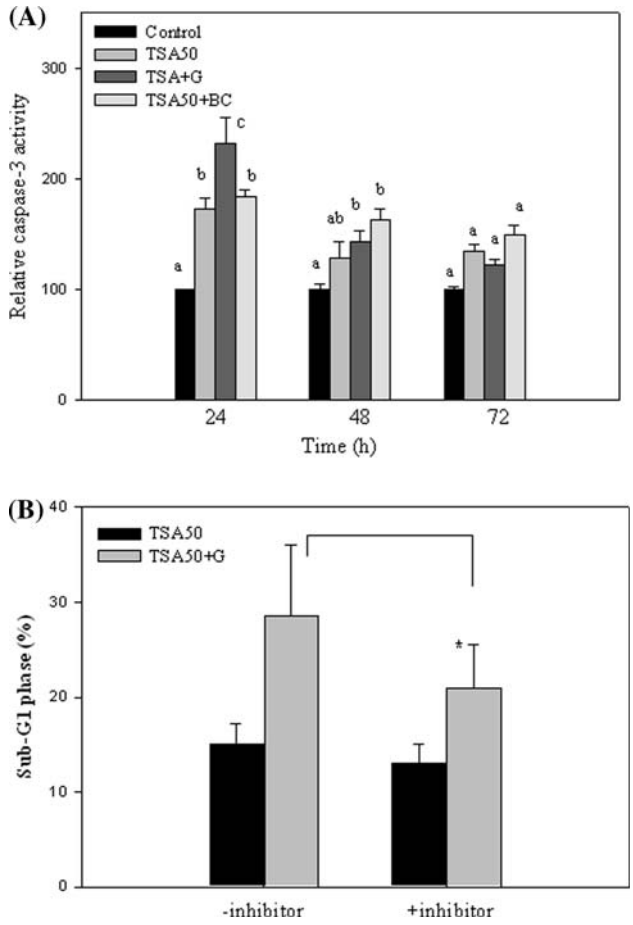

Fig. 3 **a** Effects of various treatments on caspase-3 activity in A549 cells. Cells were incubated with 50-ng/mL TSA (TSA50) alone or in combination with 10 μM of genistein (G) or β-carotene (BC) for the indicated times. Values (means ± SD, $n = 3$) at the same treatment time with different *superscript letters* are significantly different ($p < 0.05$). **b** Effects of a caspase-3 inhibitor, Ac-DEVD-CHO, on the apoptosis in A549 cells. The *asterisk* indicates an effect significantly different from the same treatment but without caspase-3 inhibitor

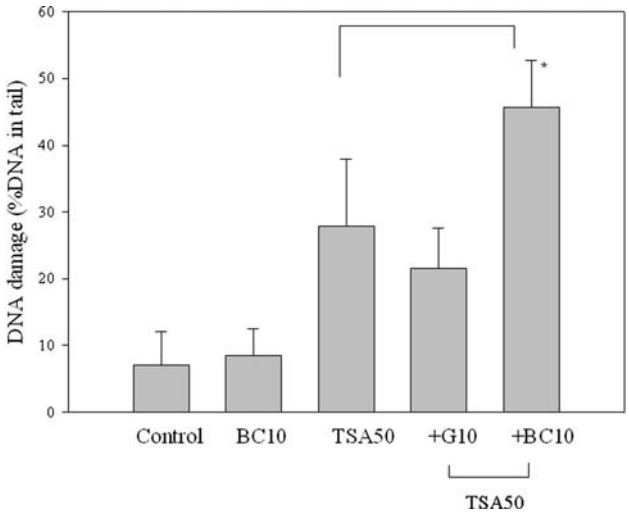

Fig. 4 Effects of various treatments on DNA damage in A549 cells. Cells were incubated with 50-ng/mL TSA (TSA50) alone or in combination with 10-μM genistein (G) or β-carotene (BC) for 24 h. Values (means ± SD, $n = 3$) among TSA50-treated groups marked with *asterisks* indicate an effect significantly different from that for TSA50 alone ($p < 0.05$)

Caspase-3 is an important executioner of apoptosis and has been associated with the mechanism by which TSA induces apoptosis of A549 cells [12]. Our results showed that genistein significantly increased TSA50-induced caspase-3 activation at 24 h. Although the increase of caspase-3 activity induced by TSA + genistein declined at 48 h, the increase of apoptosis in A549 cells induced by the combined treatment seemed to peak at 48 h. It may be that caspase-3 activation occurs earlier than apoptosis. Furthermore, the enhancing effect of genistein on TSA-induced apoptosis was partly but significantly suppressed by a caspase-3 inhibitor, Ac-DEVD-CHO, implying that the increase in caspase-3 activity plays an important role in the enhancing effect of genistein. Our results are consistent with a recent study showing that the combination of genistein and tumor necrosis factor-related apoptosis–inducing ligand (TRAIL) synergistically induce the activation of several caspases, including caspase-3, through the activation of death receptors as well as mitochondrial dysfunction in human gastric adenocarcinoma AGS cells [11]. However, we cannot rule out the possibility that other caspases or apoptotic proteins are also involved in the effects of genistein.

Although the enhancing effect of β-carotene on TSA-induced cell death was similar to that of genistein, β-carotene did not significantly affect caspase-3 activity, implying that the enhancing effect of β-carotene was mainly through a caspase-3-independent pathway. Using the comet assay, we found that β-carotene rather than genistein can enhance TSA-induced DNA damage, which is signaled to the mitochondria and causes apoptosis through multiple independent pathways [21]. The comet assay is a sensitive method for determining single- or double-strand breaks in DNA induced by genotoxic agents [3]. However, this assay cannot detect fragmentation in DNA during apoptosis, because the apoptotic fragments are too small to be detected [3]. Thus, the mechanisms by which β-carotene increases TSA-induced apoptosis may be associated with the increase in DNA damage. Growing evidence has shown that β-carotene is sensitive to oxidative stress and that it's oxidized products increase DNA damage [27, 32]. We also hypothesized that the increase in DNA damage induced by β-carotene was associated with the oxidative products of β-carotene, because in our preliminary study we found that TSA50 alone increased the generation of reactive oxygen species (determined by fluorescence probe) in A549 cells (data not shown).

In conclusion, this study demonstrates the potential of genistein and β-carotene at physiological concentrations on enhancing the growth inhibitory of TSA in A549 cells. However, further studies are necessary to determine the relevance of these findings in vivo.

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