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Vitamin C Inhibits Benzo[a]pyrene-Induced Cell Cycle Changes Partly via Cyclin D1/ E2F Pathway in Human Embryo Lung Fibroblasts[1]

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Objective To study the molecular mechanism of the inhibitory effects of vitamin C on benzo[a]pyrene (B[a]P)-induced changes of cell cycle in human embryo lung fibroblast (HELF) cells. **Methods** The stable transfectants, HELF transfected with antisense cyclin D1 and antisense CDK4, were established. Cells were cultured and pretreated with vitamin C before stimulation with B[a]P for 24 h. The expression levels of cyclin D1, CDK4, E2F1, and E2F4 were determined by Western blot. Flow cytometric analysis was employed to detect the distributions of cell cycle. **Results** B[a]P significantly elevated the expression levels of cyclin D1, E2F1, and E2F4 in HELF cells. Vitamin C decreased the expression levels of cyclin D1, E2F1, and E2F4 in B[a]P-stimulated HELF cells. Dose-dependent relationships were not found between the different concentrations of vitamin C (10, 100, 500, 1000, and 5000 μmol/L) and the expression levels of cyclin D1, E2F1, and E2F4 in HELF cells. The expression levels of cyclin D1, E2F1, and E2F4 in B[a]P-treated transfectants were lower than those in B[a]P-treated HELF cells. The expression levels of cyclin D1 and E2F4 treated with vitamin C and antisense cyclin D1 were decreased compared with those treated with antisense cyclin D1 alone. The effects of vitamin C combined with antisense CDK4 on the expression levels of cyclin D1 and E2F1/E2F4 were similar to those of antisense CDK4 alone. B[a]P progressed HELF cells from G1 to S phase. Both vitamin C and antisense cyclin D1 suppressed the changes of cell cycle progressed by B[a]P. However, antisense CDK4 did not attenuate the above changes. Vitamin C combined with antisense CDK4 markedly suppressed B[a]P-induced changes of cell cycle as compared with antisense CDK4. But the inhibitory effects of vitamin C combined with antisense cyclin D1 on B[a]P-induced changes of cell cycle were similar to those of vitamin C alone or antisense cyclin D1 alone. **Conclusions** B[a]P progressed HELF cells from G1 to S phase *via* intracellular signaling pathway of cyclin D1/E2F. Vitamin C may modulate this signaling pathway to protect cells from injury caused by B[a]P.

Key words: B[a]P; Ascorbic acid; Cyclin D1; E2F; Cell cycle; Antisense

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) such as B[a]P are widespread environmental contaminants formed as byproducts of combustion[1]. PAHs have been found to be potent mammary carcinogens in rodents[2-3]. It is believed that B[a]P requires biological activation through oxidative metabolism to be carcinogenic. The ultimate carcinogenic metabolite has been considered to be the cytochrome P450 isozyme- and microsomal epoxide hydrolase-derived metabolite, BPDE, which forms stable adducts with DNA[1,4].

In mammalian cells, proliferation is controlled by a series of positive and negative regulatory factors. The functions and regulation of the cyclin

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Abbreviations: PAH, polycyclic aromatic hydrocarbon; B[a]P, Benzo[a]pyrene; BPDE, (±)-7β, 8α-dihydroxy-9 α,10 α-epoxy-7,8,9,10,-tetrahydrobenzo(a)pyrene; CDK4, cyclin-dependent kinase 4; pRb, retinoblastoma protein; HELF, human embryo lung fibroblast; FBS, fetal bovine serum; DTT, 1,4-dithiothreitol; SDS, sodium dodecyl sulphate, DMSO, dimethyl sulfoxide.

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D1-CDK4(6)-pRB-E2F cascade have been reviewed thoroughly in recent years. There are many checkpoints in cell cycle. One of the important checkpoints is the G1-S checkpoint in late G1 phase. Cyclin and its partner Cdks, rate-limiting for cells to enter S phase, are important regulating genes in this checkpoint. It has been reported that cyclin D1-CDK4 complex plays an important role in many types of cancer. Amplification and overexpression of the cyclin D1 gene have been reported in many types of cancer, including breast, bladder, head and neck, lung, and larynx carcinomas[5-12]. E2F is a heterodimeric transcription factor composed of one of six E2F-family proteins bound to a DP family member. Through its interaction with pRb, E2F regulates the expression of growth-related genes by binding to E2F consensus sequences in their promoter. E2F can act as a positive and a negative element, in the free form or bound to pRb, respectively[13]. All the six E2F family members bind to the same DNA sequence, but additional levels of regulation have been observed as follows: the "activating" E2F1, E2F2, and E2F3 associate with pRb; E2F-pRb complexes are found primarily in G1; the "repressive" E2F4 and E2F5 prefer binding to p107 and p130; and E2F6 is the most recently identified[14-15].

Ascorbic acid, one of the important water soluble vitamins, is widely distributed in fresh fruit and vegetables. Many health benefits have been attributed to ascorbic acid such as antioxidant, anti-atherogenic, anti-carcinogenic and immunomodulatory effects. A number of clinical and epidemiological studies on anti-carcinogenic effects of ascorbic acid have not shown any conclusive beneficial effects on various types of cancer except for gastric cancer[16]. One of the most consistent epidemiological findings is that the reduced risk of cancer is associated with high intake of ascorbic acid or vitamin C rich foods. Biochemical and physiological evidence suggests that ascorbic acid functions as a free radical scavenger and inhibits the formation of potentially carcinogenic N-nitroso compounds, thus offering protection against stomach cancer[17-19]. Low intake of ascorbic acid and other vitamins is associated with an increased risk of cervical cancer[20-23].

Naidu *et al.*[24] have found that ascorbyl stearate-induced anti-proliferative and apoptotic effects in ovarian cancer are mediated through cell cycle arrest and modulation of the IGFIR and PI3K/AKT2 survival pathways. However, as the molecular mechanism underlying anti-carcinogenic activity of ascorbic acid is not elucidated, the relation between ascorbic acid and cancer is still debatable. Although ascorbic acid was discovered in 17th century, the exact role of this vitamin in human biology and health is still a mystery in view of many beneficial claims and controversies.

The present study investigated whether vitamin C pretreatment attenuated the expression of cell cycle regulators induced by B[a]P in HELF cells and changed the cell cycle. Results of this study indicate that vitamin C has antagonistic effects on cell growth and DNA synthesis. The inhibitory effects of vitamin C may be attributed to its blockage of signaling pathways involving cyclin D1/E2F.

MATERIALS AND METHODS

Reagents

RPMI 1640 medium was obtained from GIBCO. L-glutamine, propidium iodide (PI), ascorbic acid, B[a]P and RNaseA were obtained from Sigma. Gentamycin sulfate was from Amresco. Transfectam reagent was obtained from Promega. Antisense cyclin D1 plasmid and antisense CDK4 plasmid were established by Yan *et al.*[25]. The peroxidase-conjugated affinity antibodies IgG were purchased from Jackson. We used the following antibodies: goat polyclonal anti-actin (c-11), rabbit polyclonal anti-E2F1 (c-20), E2F4 (c-20), rabbit monoclonal anti-CDK4 (c-22), and mouse monoclonal cyclin D1 (A-12) (Santa Cruz Biotechnology). Nitrocellulose membranes were from Bio-Rad. The enhanced chemical luminescence detection system (ECL kit) was purchased from Santa Cruz Biotechnology.

Cell Preparation and Culture Condition

HELF cell line was purchased from Peking Union Medical College. Cells were cultured in RPMI-1640 with 2.0 g $NaHCO_3$ per liter, supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 50 μg/mL gentamycin sulfate, in a humidified incubator at 37℃ with 5% CO_2. The cultures were dissociated with trypsin and transferred to new 50 cm^2 culture flasks one to three times per week.

Generation of Stable Transfectants

HELF cells were cultured in a 6-well plate until they reached 85%-90% confluence. Ten micro litre of Transfectam reagent and 2 μg antisense cyclin D1 plasmid or antisense CDK4 plasmid were used to transfect each well in the absence of serum. After 2 h, the medium was replaced with RPMI-1640 containing 10% FBS. Approximately 48 h after the transfection, the cells were dissociated with 0.25% trypsin and cell suspensions were placed into 75 mL

culture flasks and cultured for 14 days with 400 μg/mL G418 selection. The stable transfectants were established and cultured in G418-free RPMI-1640 for at least two passages before each experiment.

Western Blot Analysis for Cyclin D1, CDK4, E2F1, and E2F4

The expressions of cyclin D1, CDK4, E2F1, and E2F4 were determined by Western blot with corresponding antibodies. Cells were cultured in flasks with 10% FBS RPMI-1640. After cell density reached 70%-80% confluence, the medium was replaced with serum-free RPMI-1640 (supplemented with 0.5% FBS) and cultured for 24 h, pretreated with or without 100 μmol/L vitamin C for 1 h and then exposed to 2 μmol/L B[a]P for 24 h. At the time of harvest, cells were washed three times with ice-cold PBS, and lysed with 500 μL lysis buffer [50 mmol/L Tris-HCl (pH 6.8), 100 mmol/L DTT, 2% SDS, 0.01% bromophenol blue, 10% glycerol]. The samples were boiled for 10 min, sonicated for 1 min, and then centrifuged at 10 000 g for 10 min. Extracts prepared from equal numbers of cells were separated on 10% SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked in blocking buffer [5% (w/v) nonfat dry milk in Tris-buffered saline, pH 7.6, 0.1% (v/v) Tween-20 (TBST)] for 1 h at room temperature and incubated with the relevant primary antibodies (1:1000) in blocking buffer overnight at 4℃. After being washed with TBST, the blots were incubated with the appropriate peroxidase-conjugated secondary antibody (1:2000) for 1 h at room temperature and developed using the enhanced chemical luminescence detection system (ECL kit).

Cell Cycle Analysis

Cell cycle was analyzed by flow cytometry. Exponentially proliferating cells were digested, washed twice with cold phosphate-buffered saline (PBS) and fixed in 70% cold ethanol on ice for 30 min. Fixed cells were washed with PBS, incubated with 30 μL RNase A (1 mg/mL) for 30 min at 37℃, stained with 50 μL propidium iodide (1 mg/mL), and diluted by PBS to a final volume of 0.5 mL, stained for 40 min on ice without light. An Ortho Cytofluorograf 50H was used to analyze the cell cycle. Approximately 10 000 cells were examined for each sample. The percentages of cells in different phases of the cell cycle were analyzed using the Cell-FIT software. All experiments were repeated three times.

RESULTS

Association of Different Concentrations of Vitamin C with Expression Levels of Cyclin D1, CDK4, E2F1, and E2F4 in HELF Cells Stimulated by B[a]P

In order to find the appropriate concentration of vitamin C, we designed different concentrations of vitamin C (10, 100, 500, 1000, and 5000 μmol/L) to pretreat HELF cells for 1 h before stimulation with 2 μmol/L B[a]P for 24 h. Then we investigated the expression levels of cyclin D1, CDK4, E2F1, and E2F4. As shown in Fig. 1, B[a]P significantly elevated the expression levels of cyclin D1, E2F1, and E2F4 in HELF cells (Fig. 1, lane 4 *vs* lane 1) . The studies showed that 100 μmol/L vitamin C obviously inhibited 2 μmol/L B[a]P-induced overexpression of cyclin D1, E2F1, and E2F4 (Fig. 1, lane 6 *vs* lane 4). However, results did not display a dose-dependent relationship as expected.

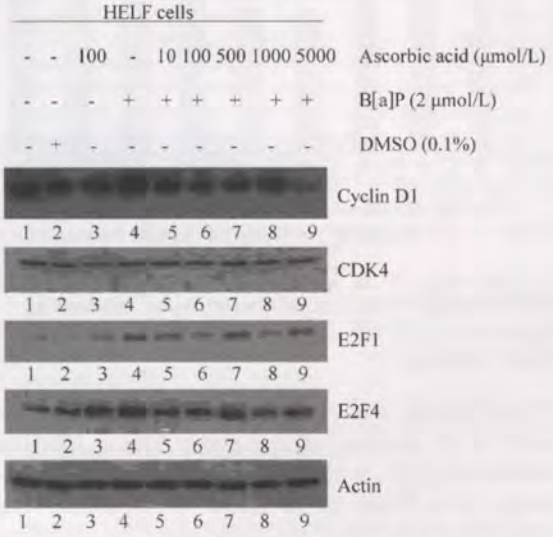

FIG. 1. Effects of ascorbic acid on B[a]P-induced overexpression of cyclin D1, CDK4, E2F1, and E2F4 in HELF cells.

Vitamin C Inhibited B[a]P-Induced Changes of Cell Cycle

Cancer cells differ from normal cells in many important characteristics, including low differentiation, increased invasiveness, and uncontrolled cellular growth[26]. Vitamin C is a potent antioxidant with cancer-preventive properties. The mechanism by which vitamin C imparts cancer-preventive effects has not been clearly defined. So we investigated the changes of cell cycle in HELF cells pretreated with vitamin C before stimulation

with B[a]P for 24 h. As shown in Fig. 2, B[a]P increased the percentages of S phase cells compared with control (Fig. 2, 2 *vs* 1). Both vitamin C and antisense cyclin D1 were capable of suppressing B[a]P-induced changes of cell cycle (Fig. 2, 3 *vs* 2, 4 *vs* 2). The antisense CDK4 did not attenuate the changes (Fig. 2, 8 *vs* 2). Vitamin C combined with antisense CDK4 markedly suppressed B[a]P-induced changes of cell cycle as compared with antisense CDK4. (Fig. 2, 9 *vs* 8), whereas, Vitamin C combined with antisense cyclin D1 and vitamin C alone (Fig. 2, 6 vs 3) or antisense cyclin D1 alone did not differ in suppressing B[a]P-induced changes of cell cycle (Fig. 2, 6 *vs* 5). These results suggested that the vitamin C might inhibite B[a]P-induced changes of cell cycle through cyclin D1 pathway.

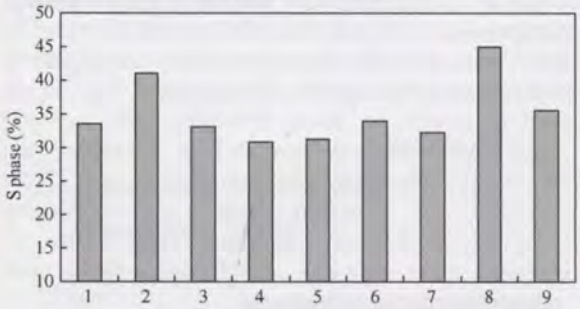

FIG. 2. Effects of vitamin C on B[a]P-induced changes of percentages of S phase cells.

Inhibitory Effects of Vitamin C on B[a]P-Induced Expression of Cyclin D1 and E2F in HELF Cells and Signal Pathway

An earlier study showed that antioxidant can result in an increase of the cyclin kinase inhibitor WAF1/CIP1/p21, which by inhibiting cyclin /Cdks results in a G0/G1phase arrest[27], suggesting that antioxidants can cause cell cycle arrest by inhibiting activity of cell cycle regulators in different phases of the cell cycle. Therefore, to understand the pathway mediating the changes of cell cycle, the effects of vitamin C were evaluated in HELF cells and cells transfected with antisense cyclin D1 and antisense CDK4. Cells were treated as indicated in Fig. 3. The results showed that the expression levels of cyclin D1, E2F1 and E2F4 in B[a]P-stimulated HELF transfectants were lower than those in B[a]P-treated HELF cells (Fig. 3, lane 2 *vs* lane 5, lane 2 *vs* lane 8), indicating that cyclin D1 and CDK4 were the upstream kinases of E2F in B[a]P-treated HELF cells. Vitamin C suppressed the overexpressions of cyclin D1, E2F1 and E2F4 in HELF cells induced by B[a]P (Fig. 3, lane 3 *vs* lane 2). The expression levels of cyclin D1 and E2F4 treated with vitamin C and antisense cyclin D1 decreased compared with those treated with antisense cyclin D1 alone (Fig. 3, lane 6 *vs* lane 5). The inhibitory effects of vitamin C combined with antisense CDK4 on the expression of cyclin D1 and E2F1/E2F4 were similar to those of antisense CDK4 alone (Fig. 3, lane 9 *vs* lane 8).

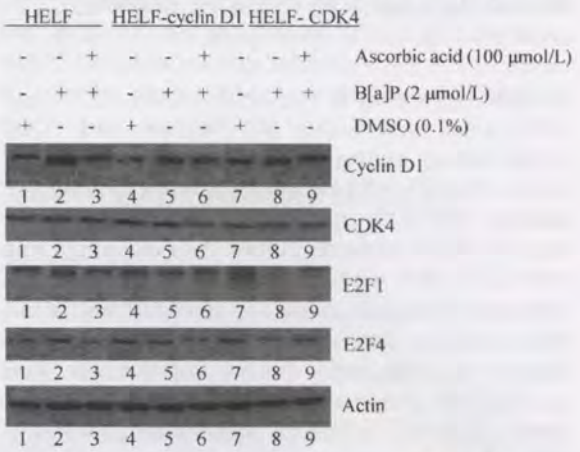

FIG. 3. Effects of ascorbic acid on B[a]P-induced overexpression of cyclin D1, CDK4, E2F1, and E2F4 in HELF, HELF antisense cyclin D1 and CDK4.

All the above results suggested that vitamin C attenuated B[a]P-induced changes of cell cycle partly *via* cyclin D1/ E2F pathway in HELF cells.

DISCUSSION

Polycyclic aromatic hydrocarbons (PAHs) are a well-characterized group of mutagens and carcinogens. Benzo[a]pyrene (B[a]P), the best known compound, exerts its geotaxis activity following metabolic activation, when it acquires the properties of an electrophilic reagent capable of interacting with DNA. Reactive oxygen species (ROS) can remerge during the PAH metabolic activation[28].

Vitamin C improves the quality of life and extends longevity in cancer patients, hence it could be considered as an adjuvant in cancer therapy. Vitamin C significantly decreases the adverse effect of ROS and inhibits the proliferation of all kinds of tumor cells *in vitro*. To date, the mechanism of vitamin C in protecting against cancer has been extensively studied[29-31]. However, these studies are mostly focused on its antioxidation and modulating AP-1 pathway.

Our present study provided a new insight into the signaling pathway involved in inhibitory effects of vitamin C on B[a]P-induced cell cycle progression and cell proliferation. Results showed that B[a]P obviously induced the overexpressions of cyclin D1, E2F1 and E2F4 in HELF cells and vitamin C

decreased the expression. In order to clarify whether cyclin D1 and CDK4 were involved in the inhibitory effects of vitamin C on B[a]P-induced cell cycle progression and cell proliferation in HELF cells, antisense cyclin D1 and CDK4 plasmids were transfected into HELF cells to inhibit the transcriptional levels of cyclin D1 and CDK4 separately. Western blot analysis demonstrated that the expression levels of cyclin D1, E2F1, and E2F4 in B[a]P-treated HELF transfectants were lower than those in B[a]P-stimulated HELF cells, suggesting that antisense cyclin D1 and CDK4 inhibit the transcription of cyclin D1 and CDK4 genes. The expression of E2F appeared to be dependent on cyclin D1 in B[a]P-treated HELF cells. The expression levels of cyclin D1 and E2F4 treated with vitamin C and antisense cyclin D1 were lower than those treated with antisense cyclin D1 alone.

The changes of cell cycle were consistent with the changes of the cyclin D1 and E2F expressions in HELF cells. B[a]P increased the percentages of S phase cells, suggesting that B[a]P progressed cells from G1 to S phase. Both vitamin C and antisense cyclin D1 are capable of suppressing the changes of cell cycle induced by B[a]P. Moreover, vitamin C combined with antisense CDK4 significantly suppressed B[a]P-induced changes of cell cycle compared with antisense CDK4 alone. These results suggest that vitamin C inhibits B[a]P-induced cell cycle changes in HELF cells and decreases the overexpressions of cyclin D1, E2F1 and E2F4. The signaling mechanism of vitamin C in protecting against B[a]P-induced cell cycle progression appears to be partly *via* cyclin D1/ E2F pathway.

Taken together, these data indicate that B[a]P promotes cell cycle progression *via* intracellular signaling pathway involving cyclin D1/E2F. Vitamin C, in addition to its antioxidant properties, may modulate this signaling pathway to protect cells from injury caused by B[a]P. The intracellular signaling mechanisms at multiple stages remain to be further elucidated.

REFERENCES

1. Dipple A (1983). Formation, metabolism, and mechanism of action of polycyclic aromatic hydrocarbons. *Cancer Res* **43** (Suppl), 2422s-2425s.
2. Gould M N, Grau D R, Seidman L A, *et al.* (1986). Interspecies comparison of human and rat mammary epithelial cell-mediated mutagenesis by polycyclic aromatic hydrocarbons. *Cancer Res* **46**, 4942-4945.
3. Ethier S P, Ullrich R L (1982). Induction of mammary tumors in virgin female BALB/c mice by single low doses of 7,12-dimethylbenz[a]anthracene. *J Natl Cancer Inst (Bethesda)* **69**, 1199-1203.
4. Jeffrey A M, Jennette K W, Blobstein S H, *et al.* (1976). Benzo[a]pyrene-nucleic acid derivative found *in vivo*: structure of a benzo[a]pyrene tetrahydrodiol epoxideguanosine adduct. *J Am Chem Soc* **98**, 5714-5715.
5. Sato Y, Itoh F, Hareyama M, *et al.* (1999). Association of cyclin D1 expression with factors correlated with tumor progression in human hepatocellular carcinoma. *J Gastroenterol* **34**, 486-493.
6. Lim S C, Lee M S (2002). Significance of E-cadherin/ beta-catenin complex and cyclin D1 in breast cancer. *Oncol Rep* **9**, 915-928.
7. Sgambato A, Migaldi M, Faraglia B, *et al.* (2002). Cyclin D1 expression in papillary superficial bladder cancer: its association with other cell cycle-associated proteins, cell proliferation and clinical outcome. *Int J Cancer* **97**, 671-678.
8. Liang S B, Furihata M, Takeuchi T, *et al.* (2000). Overexpression of cyclin D1 in nonmelanocytic skin cancer. *Virchows Arch* **436**, 370-376.
9. Keum J S, Kong G, Yang S C, *et al.* (1999). Cyclin D1 overexpression is an indicator of poor prognosis in resectable non-small cell lung cancer. *Br J Cancer* **81**, 127-132.
10. Coco Martin J M, Balkenende A, Verschoor T, *et al.* (1999). Cyclin D1 overexpression enhances radiation-induced apoptosis and radiosensitivity in a breast tumor cell line. *Cancer Res* **59**, 1134-1140.
11. Hosokawa Y, Arnold A (1998). Mechanism of cyclin D1 (CCND1, PRAD1) overexpression in human cancer cells: analysis of allele-specific expression. *Genes Chromosome Cancer* **22**, 66-71. (Review)
12. Wang S J, Mintz L E, Natarajan V, *et al.* (2001). Third place--Resident Research Competition, AAO-2000. Antisense cyclin D1 inhibits growth of head and neck cancer xenografts in nude mice. *Otolaryngol Head Neck Surg* **124**, 656-662.
13. Johnson D G, Coleman A, Powell K L, *et al.* (1997). High-Affinity Binding of the Cell Cycle-regulated Transcription Factors E2F 1 and E2F 4 to Benzo[a]pyrene Diol Epoxide-DNA Adducts. *Mol Carcinog* **20**, 216-223.
14. Trimarchi J M, Lees J A (2002). Sibling rivalry in the E2F family. *Mol Cell Bio* **13**, 11-20.
15. Gregori J D (2002).The genetics of the E2F family of transcription factors: shared functions and unique roles. *BBA* **1602**, 131-150.
16. Sauberlich H E (1994). Vitamin C and Cancer. *Nutrition Journal* 111-157.
17. Schorah C J, Sobala M, Collins N, *et al.* (1991).Gastric juice ascorbic acid: effects of disease and implications for gastric carcinogenesis. *Am J Clin Nutr* **53**, 287S-293S.
18. Sobala G M, Pignaetelli B, Schorah C J, *et al.* (1991). Levels of nitrite, nitrate, N-nitroso compounds, ascorbic acid in gastric juice of patients with and without precancerous conditions of the stomach. *Carcinogenesis* **12**, 193-198.
19. Drake I M, Davies M J, Mapstone N P, *et al.* (1996). Ascorbic acid may protect against human gastric cancer by scavenging mucosal oxygen radicals. *Carcinogenesis* **17**, 559-562.
20. Brock K E, Berry G, Mock P A, *et al.* (1988). Nutrients in diet and plasma and risk in situ cervical cancer. *J Natl Cancer Inst* **80**, 580-585.
21. Verreault R, Chu J, Mandelson M, and Shy K (1989). A case study of diet and invasive cancer. *Int J Cancer* **43**, 1050-1054.
22. Potischman N, Brinton L A (1996). Nutrition and cervical neoplasia. *Cancer Causes Control* **7**, 113-126.
23. Rock C L, Michael C W, Reynolds R K, *et al.* (2000). Prevention of cervix cancer. *Crit Rev Oncol Hematol* **33**, 169-183.
24. Fang Q, Naidu AK, Naidu KA, *et al.* (2006). Ascorbyl stearate inhibits cell proliferation and tumor growth in human ovarian carcinoma cells by targeting the PI3K/AKT pathway. *Anticancer Res* **26**, 203-209.
25. Yan K X, Liu B C, Xu M, *et al.* (2003). Recombination and Identification of Sense and Antisense Cyclin D1 Eukaryotic Expression Vectors. *J Hygiene Res* **32**, 180-182.
26. Hartwell L H, Kastan M B (1994). Cell Cycle Control and

Cancer. *Science* **266**, 1821-1828.
27. Ahmad N, Adhami V M, Afaq F, *et al.* (2001). Resveratrol causes WAF-1/p21-mediated G 1-phase arrest of cell cycle and induction of apoptosis in human epidermoid carcinoma A431 cells. *Clin Cancer Res* **7**, 1466-1473.
28. Yang D, Louden C, Reinhold D S, *et al.* (1992). Malignant transformation of Human Fibroblast Cell Strain MSU-1.1 by (±) -7 β, 8 α- dihydroxy- 9 α, 10 α- epoxy- 7, 8, 9, 10, -tetrahydrobenzo(a) pyrene. *Proc Natl Acad Sci USA* **89**, 2237-2241.
29. Catani M V, Rossi A, Costanzo A, *et al.* (2001). Induction of gene expression via activator protein-1 in the ascorbate protection against UV-induced damage. *Biochem J* **356**(Pt 1), 77-85.
30. Kyaw M, Yoshizumi M, Tsuchiya K, *et al.* (2002). Antioxidants inhibit endothelin-1 (1-31)-induced proliferation of vascular smooth muscle cells via the inhibition of mitogen-activated protein (MAP) kinase and activator protein-1 (AP-1). *Biochem Pharmacol* **64** (10), 1521-31.
31. Gajecka M, Kujawski L M, Gawecki J, *et al.* (1999). The protective effect of vitamins C and E against B[a]P-induced genotoxicity in human lymphocytes. *J Environ Pathol Toxicol Oncol* **18**(3), 159-167.

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