

Tea pigments induce cell-cycle arrest and apoptosis in HepG2 cells

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Abstract

AIM: To investigate the molecular mechanisms by which tea pigments exert preventive effects on liver carcinogenesis.

METHODS: HepG2 cells were seeded at a density of 5×10^5 /well in six-well culture dishes and incubated overnight. The cells then were treated with various concentrations of tea pigments over 3 d, harvested by trypsinization, and counted using a hemocytometer. Flow cytometric analysis was performed by a flow cytometer after propidium iodide labeling. Bcl-2 and p21^{WAF1} proteins were determined by Western blotting. In addition, DNA laddering assay was performed on treated and untreated cultured HepG2 cells.

RESULTS: Tea pigments inhibited the growth of HepG2 cells in a dose-dependent manner. Flow-cytometric analysis showed that tea pigments arrested cell cycle progression at G₁ phase. DNA laddering was used to investigate apoptotic cell death, and the result showed that 100 mg/L of tea pigments caused typical DNA laddering. Our study also showed that tea pigments induced upregulation of p21^{WAF1} protein and downregulation of Bcl-2 protein.

CONCLUSION: Tea pigments induce cell-cycle arrest and apoptosis. Tea pigments may be used as an ideal chemopreventive agent.

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Key words: Tea pigments; Cell cycle; Apoptosis

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INTRODUCTION

Liver cancer is the sixth most common cancer and the third

most common cause of death from cancer in the world. In general, the disease is relatively uncommon in developed countries. In the developing world, however, liver cancer is very common, accounting for more than 80% of the global cases, with China alone accounting for 55% of the worldwide incidence. Liver cancer is almost always fatal and has no effective treatment. The survival rate is low, with only a 6% 5-year survival rate in the USA. In developing countries, the survival rate is even lower. Therefore, high prevalence, high death rate, and ineffective therapy have spurred the search of novel strategies in the prevention rather than treatment of liver cancer.

Nowadays, chemoprevention is gaining more attention. This approach aims to decrease overall cancer morbidity and mortality by using substances that are capable of preventing cancer progression. Several classes of compounds have been evaluated for this purpose. Tea is one of the most popular beverages consumed in the world. The two major types of tea consumed worldwide are black tea and green tea. Green tea polyphenols and extracts have been shown in a number of animal models to prevent against chemically-induced carcinogenesis in lung, forestomach, esophagus, duodenum, pancreas, breast, and colon^[1-3]. Tea pigments, a major flavonol component in black tea, have strong antioxidant potency^[4]. In our previous studies, we found tea pigments to be effective in preventing the occurrence and progression of precancerous liver lesions in rats^[5]. However, studies on tea pigments are still limited, especially in terms of mechanism.

The purpose of this study was to investigate the molecular mechanisms by which tea pigments exert preventive effects on liver cancer cells and to provide the scientific rationale for using tea pigments as a chemopreventive agent against liver cancer.

MATERIALS AND METHODS

Tea pigments

Tea pigments were provided by the Institute of Tea Science and Research, Chinese Academy of Agricultural Sciences (Hangzhou, China).

Cell culture and growth inhibition studies

Human hepatoma cell line HepG2 was obtained from Beijing University Hospital (Beijing, China) and cultured in DMEM medium (Sigma, USA) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in a 50 mL/L CO₂ atmosphere at 37 °C. The cells were seeded at a density of 5×10^5 /well in six-well culture dishes and incubated overnight. The cells then were treated with various concentrations of tea pigments over 3 d, harvested by trypsinization, and counted using a hemocytometer.

Flow-cytometric analysis

HepG2 cells were seeded at a density of 5×10^5 /well in six-well culture dishes. To synchronize the cells at the G₀ phase, HepG2 cells were incubated in serum-free medium for 24 h before treatment with tea pigments. The cells were then treated with or without 100 mg/L tea pigments and harvested at one, or after 2 and 3 d by trypsinization. The cells were centrifuged at 2 000 r/min for 5 min, washed with phosphate-buffered saline (PBS), fixed with 70% ethanol, then subjected to flow cytometric analysis on a flow cytometer after propidium iodide labeling.

Protein extraction and Western blot analysis

Control cells, as well as cells treated with tea pigments, were harvested by scraping the cells from cultured dishes using a cell scraper and collected by centrifugation. Whole cell extracts were then prepared by lysing the cells using 4% sodium dodecyl sulfate (SDS) gel sample buffer. Cell extracts were boiled for 10 min and chilled on ice, subjected to 12% SDS-polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane. Each membrane was cut into two pieces, one piece was incubated at 4 °C overnight with p21^{WAF1} or Bcl-2 antibodies, and the other one with β-actin (used as a control for protein loading). All antibodies were obtained from Zymed Laboratories (USA). Then membranes were incubated at 37 °C for 1 h with secondary antibody conjugated with peroxidase, and the signal was detected using chemiluminescence detection reagent. The relative protein level was calculated as the ratio of the optical density of p21^{WAF1} or Bcl-2 and that of β-actin.

DNA laddering assay

Treated and untreated cultured HepG2 cells were harvested and centrifuged at 2 000 r/min for 5 min. The cells were resuspended in lysis buffer and centrifuged for 15 min at 15 000 g. DNA was extracted with phenol, precipitated with 100% cold ethanol, resuspended in TE buffer, electrophoresed on 1.5% agarose gel, stained with ethidium bromide, detected by ultraviolet transillumination, and photographed.

RESULTS

Effect of tea pigments on cell growth

Treatment of HepG2 cells with tea pigments for 24-72 h resulted in a dose-dependent inhibition of cell proliferation. The effects of tea pigments on the proliferation of HepG2 cells are depicted in Figure 1. Tea pigments inhibited HepG2 cell growth at >25 mg/L over 3 d. The IC₅₀, as measured by the number of viable cells 72 h after the addition of tea pigments, was 50 mg/L.

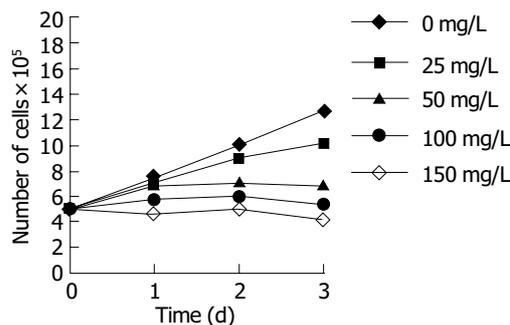


Figure 1 Effect of tea pigments on HepG2 cell growth.

Tea pigments induced G1 cell cycle arrest in HepG2 cells

Flow cytometric analysis of HepG2 cells treated with 100 mg/L tea pigments showed a G₁ arrest. The percentage of cells in the G₁ phase rose from a baseline of 38.0-55.2% after 24 h of treatment and increased from 37.5% to 76.7% after 72 h (Table 1).

Effect of tea pigments on p21^{WAF1} expression

Western blot analysis of cell extracts from tea pigments-treated HepG2 cells showed upregulation of p21^{WAF1}, an inhibitory protein that could modulate cell growth and cell cycle. The result of a typical experiment is shown in Figure 2. To obtain a quantitative value for the protein expression of p21^{WAF1}, the optical density of p21^{WAF1} was measured. The ratio of p21^{WAF1} to β-actin was calculated, and the relative protein level of p21^{WAF1} was measured as presented in Figure 2, which clearly shows the upregulation of p21^{WAF1}.

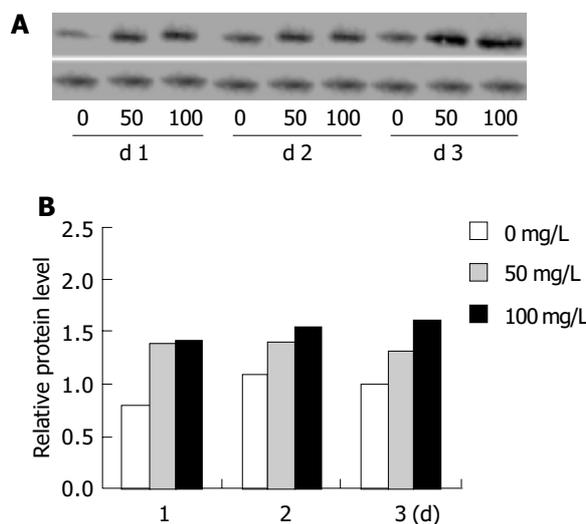


Figure 2 Upregulation of p21^{WAF1} protein expression in HepG2 cell lines treated with tea pigments visualized by ECL detection system (A) and plotted as relative protein level (B).

Table 1 Flow cytometric analysis of HepG2 cells treated with 100 mg/L tea pigments

Tea pigments (mg/L)	D 1			D 2			D 3		
	G ₁ (%)	S (%)	G ₂ /M (%)	G ₁ (%)	S (%)	G ₂ /M (%)	G ₁ (%)	S (%)	G ₂ /M (%)
0	38.0	48.0	14.0	47.1	39.5	13.4	37.5	50.3	12.2
100	55.2	27.8	17.0	68.7	22.0	9.3	76.7	10.3	13.0

Induction of apoptosis

Firstly, agarose gel electrophoresis was used to demonstrate low-molecular-weight DNA ladder formation. Nuclear DNA fragmentation, a classical feature of apoptotic cell death, was clearly shown in HepG2 cells with 100 mg/L tea pigments over 3 d (Figure 3). Then, we observed the expression of Bcl-2 protein, a known inhibitor of apoptosis. Western blot analysis showed downregulation of Bcl-2 protein. The result of a typical experiment is shown in Figure 4. The ratio of Bcl-2 to β -actin was calculated. The relative protein level of Bcl-2 was measured as presented in Figure 4.

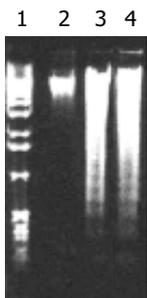


Figure 3 Induction of apoptosis by tea pigments in HepG2 cells. Lane 1: DNA molecular weight marker; lane 2 (control): DNA collected from untreated HepG2 cells after 3 d; lanes 3 and 4: DNA collected from HepG2 cells treated with 100 mg/L after 2 and 3 d.

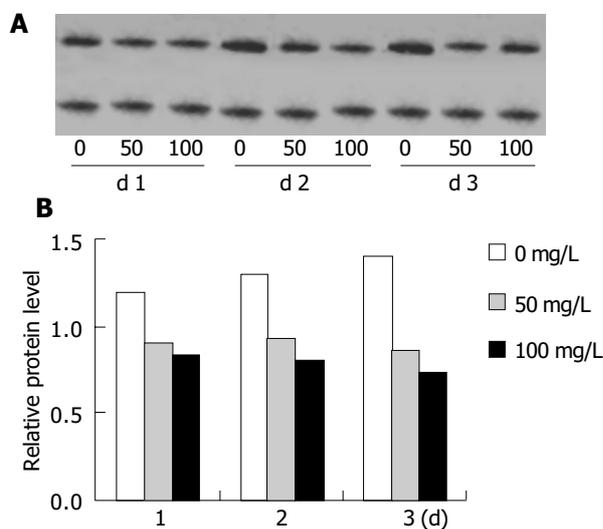


Figure 4 Downregulation of Bcl-2 protein expression in HepG2 cell lines treated with tea pigments visualized by ECL detection system (A) and plotted as relative protein level (B).

DISCUSSION

Green tea and its polyphenols have been demonstrated as chemopreventive agents in a number of experimental models^[6]. However, the mechanism of chemoprevention by black tea compared with that of green tea is not quite clear. Tea pigments used in this study are the main constituents of black tea, mainly theaflavins and thearubigins. Morse *et al.*^[7], demonstrated that theaflavins inhibit

NMBA-induced esophageal tumorigenesis in rats. Another study showed that black tea constituents inhibit NNK-induced lung tumorigenesis in A/J mice^[8]. Previously, we also found that tea pigments had an inhibitory effect on liver precancerous lesions in rats^[5]. However, the exact mechanism is unclear.

Eukaryotic cell cycle is regulated by signal transduction pathways mediated by a series of cell-cycle regulators. Cyclins are positive regulators of cell-cycle progression and function by forming a complex with and activating cyclin-dependent kinases (CDKs). CDK inhibitors are negative regulators of cell cycle and bind to and inhibit the activity of cyclin-CDK complexes^[9]. p21^{WAF1} has gained much attention as a universal inhibitor of cyclin-dependent kinases, and overexpression of p21^{WAF1} has been shown to induce tumor cell growth arrest and apoptosis^[10]. In this study, we have demonstrated that the inhibition of HepG2 cell growth by tea pigments is accompanied with a G₁ cell cycle arrest and upregulation of p21^{WAF1} protein. The modulation of cell-cycle regulatory protein is thus a novel effect of tea pigments, suggesting the possible molecular mechanism by which tea pigments inhibit cell growth in HepG2 cells. Lee *et al.*^[11], showed that doxorubicin, a commonly used anticancer drug, induces G₁ arrest and upregulation of p21^{WAF1} in HepG2 cells. Qin *et al.*^[12], also demonstrated that cisplatin induces a transient G₁ arrest and upregulation of p21^{WAF1} expression in HepG2 cells. These results are similar to the effects of tea pigments in this study. Although we have recognized that tea pigments can modulate the expression of p21^{WAF1}, we do not fully understand how p21^{WAF1} and cyclin-CDK complexes are working to elicit cell growth. Experiments are being conducted in our laboratory to elucidate the exact molecular mechanism by which tea pigments inhibit cell growth.

After treatment with tea pigments, the number of HepG2 cells decreased over time, suggesting the possibility of an increase in the rate of cell death. Agarose gel electrophoresis verified this suggestion. Nuclear DNA fragmentation, a classical feature of apoptotic cell death, was clearly shown in HepG2 cells with 100 mg/L tea pigments over 3 d. Additionally, we observed the expression of Bcl-2 protein. Modern molecular biological investigations indicate that apoptosis is regulated by many oncogenes, such as bcl-2, bax and p53^[13]. Bcl-2 protein is an apoptosis-related protein and plays an important role in regulating apoptosis^[14]. In our study, Western blot analysis showed that Bcl-2 protein expression was significantly downregulated in HepG2 cells after treatment with tea pigments. Bax is a homologous protein of Bcl-2, in the form of homopolymer (Bax/Bax) or isodipolymer (Bcl-2/Bax). Studies have demonstrated that the ratio of Bax and Bcl-2 protein influences the apoptotic rate of cells^[15].

In conclusion, tea pigments inhibit cell growth and induce apoptosis in HepG2 cells, suggesting that tea pigments may be used as an ideal chemopreventive agent.

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