Effects of tea constituents on cell cycle progression of human leukemia U937 cells

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ABSTRACT

Tea and tea constituents are known to induce apoptosis in a variety of cancerous cells, suggesting their beneficial effects as chemopreventive agents. Previous studies have shown that low molecular weight constituent catechins and high molecular weight fractions of tea have the apoptosis-inducing activity, but that their action mechanisms may be different. Since cell cycle arrest is known to be one of the underlying mechanisms of apoptosis, we examined the effects of these tea constituents on cell cycle progression of human leukemia U937 cells. The results showed that the high molecular weight fractions of green tea and black tea caused G2/M arrest associated with up-regulation of p21/Waf1, but that epigallocatechin gallate, a major component of green tea catechins, gave little effects of cell cycle progression and p21/Waf1 expression. Thus, the present results suggest the difference in the apoptosis-induction mechanism between the two types of tea constituents.

Tea is a popular beverage in the world and many epidemiological studies have suggested that tea intake may be useful for cancer prevention (1, 19). *In vitro* cell culture studies have shown that several tea constituents induce apoptosis in cancerous cells, providing the basis of chemopreventive activity of tea (1, 2, 6, 14). *In vivo* animal studies have also shown that oral administration of tea polyphenols can reduce the incidence of carcinogenesis by inducing apoptosis (3, 13). In earlier studies, we demonstrated that (–)-epigallocatechin-3-*O*-gallate (EGCG), a major component of green tea polyphenols, and high molecular weight components of green tea and black tea induce apoptosis in human leukemia U937 cells and human stomach cancer

MKN45 cells (5, 16, 17). Later, we showed that EGCG and tea high molecular weight components cause differential expression of apoptosis-related genes in U937 cells (11), suggesting the difference in the apoptosis-induction mechanism between these two types of tea constituents.

Several studies have shown that cell cycle dysregulation is one of the mechanisms by which EGCG and other tea constituents induce apoptosis (1, 2, 6, 14, 20). In the present study, we compare the effects of EGCG and tea high molecular weight fractions on cell cycle progression of U937 cells and provide evidence for differential effects of these two types of tea constituents.

MATERIALS AND METHODS

Materials. High molecular weight fractions of green tea and black tea were prepared as described previously (5), and designated as GHF and BHF, respectively. Briefly, the extract of tea leaves in boiling water was sequentially extracted with chloroform,

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ethyl acetate, and n-butanol. The aqueous solution thus obtained was dialyzed exhaustively against water and the non-diffusible fraction was freeze-dried to give high molecular weight fractions. Some characteristics of these fractions have been reported elsewhere (9, 10). EGCG and resveratrol were obtained from Funakoshi Co., Tokyo, Japan, and Wako Pure Chemical Industries, Ltd., Osaka, Japan, respectively.

Cells. Human leukemia U937 cells and human epidermoid carcinoma A431 cells were obtained from the Health Service Research Resources Bank, Osaka, Japan. U937 cells were cultured in 10% fetal bovine serum in RPMI 1640 medium containing 50 U/ml penicillin, 50 µg/ml streptomycin, 2.5 µg/ml amphotericin B, and 50 µg/ml gentamycin at 37°C under a 5% CO₂ atmosphere as described previously (16). A431 cells were cultured in 10% fetal bovine serum in Dulbecco's minimum essential medium containing antibiotics.

Detection of apoptosis. U937 cells were incubated in a culture medium in the absence or presence of test compounds. For DNA fragmentation analysis, 5×10^5 cells were pelleted by centrifugation and DNA was isolated from the cell pellets as described by Sellins and Cohen (18). DNA was electrophoresed in 2% agarose gels, stained with SYBR Green, and imaged using FluorImager (Molecular Dynamics Japan, Inc., Tokyo, Japan) as described previously (5, 16, 17).

Cell cycle analysis. U937 cells (10^5 – 10^6 cells) incubated with tea constituents were pelleted and treated with 1.0 ml of cold 70% ethanol at –20°C for 2 h. After removal of ethanol, to the suspension was added a propidium iodide solution at 25 µg/ml (500 µl) containing ribonuclease A (10 µg/ml) in 0.15 M phosphate-buffered saline (pH 7.4) and the mixture was kept for 15 min at 37°C. Cell cycle analysis was performed using a flow cytometer EPICS XL System II (Beckman Colter, Tokyo, Japan) as described previously (7).

In the case of A431 cells, EGCG-treated cells were detached by trypsinization and analyzed by the flow cytometer.

p21/Waf1 gene expression. Total RNA was prepared from 1×10^6 cells treated with or without tea constituents using Catrimox-14 RNA Isolation Kit ver. 2.11 (Takara Bio Co., Tokyo, Japan) according to the manufacturer's instructions. Reverse transcrip-

tion-polymerase chain reaction (RT-PCR) was performed using extracted total RNA and Ready-To-Go RT-PCR Beads (Amersham Biosciences K. K., Tokyo, Japan). Amplified DNA was subjected to electrophoresis in 2% agarose gels, stained with SYBR Green I, and imaged and calculated using FluorImager as described previously (7). Primers for p21/Waf1 designed according to information in the literature (4) were 5'-GAC ACC ACT GGA GGG TGA CT-3' and 5'-GGC GTT TGG AGT GGT AGA AA-3', and those for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 5'-AAG GTC ATC CCT GAG CTG AA-3' and 5'-CCC CTC TTC AAG GGG TCT AC-3', respectively (7). Expected lengths of PCR products for p21/Waf1 and GAPDH were 299 bp and 495 bp, respectively. The products of RT-PCR were then subjected to electrophoresis in 2% agarose gels, stained with SYBR Green I, and imaged and calculated using FluorImager as described previously (7).

p21/Waf1 protein expression. For flow cytometric analysis, U937 cells treated with tea constituents were carried out using a Dako Cytomation Intra Stain kit (Dako, Glostrup, Denmark) as follows. After incubation with or without tea constituents, U937 cells were washed with phosphate-buffered saline, fixed and permeabilized according to the manufac-

Control EGCG GHF BHF

Fig. 1 DNA fragmentation in U937 cells treated with EGCG (100 µM), GHF (0.6 mg/ml), and BHF (0.6 mg/ml). Extracted DNA was electrophoresed in 2% agarose gels, stained with CYBR Green, and imaged using FluorImager.

Fig. 2 Effects of tea constituents on cell cycle progression of U937 cells. Cells incubated with EGCG at 100 μ M (b), GHF at 0.6 mg/ml (c), or BHF at 0.6 mg/ml (d) at 37°C for indicated periods were stained with propidium iodide and analyzed using a flow cytometer. For comparison, data for the cells treated with resveratrol (Res) at 30 μ M are included (e). Ordinate, cell number; abscissa, fluorescence intensity for propidium iodide staining in the histograms. Apo, apoptotic cells.

turer's directions. The cells were incubated with a 10-fold dilution of a mouse anti-p21/Waf1 monoclonal antibody solution (SC-817) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.) and then with a 10-fold dilution of fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin G (Dako). The stained cells were analyzed using a flow cytometer as above.

RESULTS

Apoptosis induction by tea constituents

Incubation of U937 cells with EGCG at 100 μ M, GHF at 0.6 mg/ml, or BHF at 0.6 mg/ml at 37°C for 24 h caused DNA fragmentation (Fig. 1), one of the characteristic features of apoptosis (18), confirming the previous results (5, 16, 17).

Cell cycle arrest

The results of flow cytometric analysis indicated that the cell cycle of U937 cells was arrested in the presence of GHF or BHF at a G2/M phase (Fig. 2).

In contrast, no distinct cell cycle arrest was observed in the cells treated with EGCG. In the control experiment, U937 cells were treated with a grape-derived polyphenol, resveratrol at 30 μ M. The results indicated that resveratrol caused S phase arrest, confirming the previous finding reported by Park *et al.* (15).

Expression of p21/Waf1 mRNA and protein in U937 cells

p21/Waf1 is an inhibitor for kinase activities of cyclin/cyclin dependent kinase complexes, and is known to regulate cell cycle progression at both the G1/S and the G2/M transitions (12). The results of RT-PCR showed a marked elevation of mRNA expression for p21/Waf1 of U937 cells incubated with GHF or BHF compared to the control (Fig. 3). Resveratrol was also found to up-regulate p21/Waf1 mRNA expression. In contrast, no significant up-regulation of p21/Waf1 was observed for expression in EGCG-treated cells.

Flow cytometric analysis of p21/Waf1 protein ex-

Fig. 3 Effects of tea constituents on p21/Waf1 gene expression in U937 cells. Cells were incubated with EGCG at 100 μ M, GHF at 0.6 mg/ml, or BHF at 0.6 mg/ml at 37°C for indicated periods and extracted RNA was analyzed by RT-PCR. Amplified cDNA separated by agarose gel electrophoresis was detected by staining with CYBR Green and imaged using FluorImager (A). The results in A are normalized using data for GAPDH and are expressed as the value relative to that for control (100%) from three experiments (B). For comparison, data for the cells treated with resveratrol (Res) at 30 μ M are included. *Different from the control with statistical significance at $p < 0.05$ (Student's *t*-test). **Different from the control with statistical significance at $p < 0.01$ (Student's *t*-test).

pression demonstrated that GHF, BHF, and resveratrol caused the increased expression of p21/Waf1 protein in U937 cells (Fig. 4). In contrast, EGCG showed little effects on p21/Waf1 protein expression.

Effects of EGCG on cell cycle progression of A431 cells

In this study, no distinct cell cycle arrest was observed in EGCG-treated U937 cells. Previously, EGCG was reported to induce G0/G1 phase cell cycle arrest in A431 cells (1).

To test the possibility that the difference might be due to the difference in the cell type, we included the experiment employing A431 cells. The results indicated that EGCG caused G0/G1 phase arrest (Fig. 5) as reported previously (1). These results indicate that the effect of EGCG on cell cycle progression is cell type-dependent.

DISCUSSION

In our earlier studies, we demonstrated that EGCG and tea high molecular weight fractions derived from green tea and black tea (GHF and BHF, respectively) induced apoptosis in human leukemia U937 cells and human stomach cancer MKN45 cells (5, 16, 17). GHF and BHF have been shown to exhibit the broad spectra of a molecular weight larger than 12,000 and contain polyphenolic compounds such as catechins and gallate, quinic acid, and saccharides including glucose and galactose as the components (9). Thus, these high molecular weight tea constituents are chemically considered to be a heterogeneous complex of tannins with polyphenols (70–80% w/w) and polysaccharides (20–30% w/w).

Recently, we reported that EGCG and BHF affected differently gene expression of cancer- and apoptosis-related proteins, suggesting the difference in apoptosis induction mechanism in the two types of tea constituents (11).

In the present study we found that the tea high

Fig. 4 Flow cytometric analysis for the effects of tea constituents on p21/Waf1 protein expression. U937 cells incubated with EGCG at 100 μ M, GHF at 0.6 mg/ml, or BHF at 0.6 mg/ml at 37°C for 24 h were stained with anti-p21/Waf1 monoclonal antibody and fluorescein isothiocyanate-labeled anti-mouse immunoglobulin G. For comparison, datum for the cells treated with resveratrol at 30 μ M is included. Ordinate, cell number; abscissa, fluorescence intensity for fluorescein isothiocyanate-labeled protein.

Fig. 5 Effects of EGCG on cell cycle progression of A431 cells. A431 cells were incubated with EGCG at 200 μ M or 400 μ M for 24 h and stained with propidium iodide for flow cytometric analysis. Ordinate, cell number; abscissa, fluorescence intensity for propidium iodide staining.

molecular weight fractions induced G2/M phase arrest in U937 cells, whereas EGCG caused no distinct cell cycle arrest in these cells. These findings were consistent with those obtained from the experiments for the expression of p21/Waf1. GHF and BHF caused the increase in p21/Waf1 both at mRNA and protein levels. Therefore, its enhanced expression would explain cell cycle arrest at a G2/M phase (12). Since U937 cells are p53-negative (8), the cell cycle arrest caused by GHF and BHF is p53-independent in these cells. In contrast, EGCG showed no or little effects on these levels, being consistent with its little effects on the cell cycle progression of U937 cells. These results support the previous suggestion that the tea high molecular weight fractions induce apoptosis in a way different from that of EGCG, a major polyphenolic compound in green tea.

Previously, EGCG has been reported to cause cell cycle arrest at G0/G1 phase in A431 cells in a p53-independent manner (1) and at G2/M phase in human lung carcinoma PC-9 cells (14). In the present study, we confirmed the former result using A431 cells. Recently, Huh *et al.* (6) demonstrated that the cell cycle was arrested differently by EGCG in three human ovarian cancer cell lines. Thus, it appears that the effect of EGCG on cell cycle progression is cell type-specific.

It would be interesting to know if these different types of tea constituents affect differently cell cycle progression of other cell types including stomach cancer MKN45 cells. Moreover, the molecular basis on which two polyphenolic compounds, EGCG and resveratrol, affect differently the cell cycle progression in U937 cells remains to be clarified in future

studies.

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