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In vitro evaluation of the anticancer effect of lactoferrin and tea polyphenol combination on oral carcinoma cells

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Abstract

We investigated the anticancer effects of green and black tea polyphenols alone and in combination with bovine milk lactoferrin (bLF) on human tongue squamous carcinoma (CAL-27) and normal human gingival fibroblast (HGF) cells. Both green (Polyphenon-E;P-E) and black tea polyphenols (Polyphenon-B;P-B) preferentially inhibit the growth of CAL-27 cells in a dose-dependent manner. Based on the IC_{50} values, P-E was found to be more effective than P-B and the combination of P-E and bLF (1:2 ratio) exhibited synergistic inhibition of CAL-27 cells. Analysis of the mechanism revealed nuclear fragmentation and condensation with appearance of the A_o peak indicative of apoptosis. Furthermore, tea polyphenols transduced the apoptosis signal via generation of reactive oxygen species and decrease in the Bcl-2/Bax ratio thereby inducing mitochondrial permeability transition with consequent activation of caspase-3. Overall, the potency of cytotoxic and apoptosis inducing effects of dietary agents on CAL-27 cells was in the order P-E and bLF combination (1:2 ratio) > P-E > P-B. These results suggest that a ''designer'' approach may be useful for oral cancer prevention strategies.

Keywords: Anticancer; Apoptosis; Tea polyphenols; Lactoferrin

1. Introduction

Chemoprevention by dietary agents has evolved as a promising approach to control the incidence of oral cancer, an important contributor to morbidity and mortality (Mignogna et al., 2004). Of late, chemoprevention by a combination of dietary phytochemicals with distinct molecular mechanisms has received growing consideration as a means to achieve higher efficacy and potency with reduced toxicity. In particular, combination regimens that use tea polyphenols as one of the constituents have been found to be potentially effective in chemoprevention (Sakamoto, 2000; Suganuma et al., 2001; Ohigashi and Murakami, 2004; Zhou et al., 2004).

In most parts of the world, tea is consumed together with milk. Both milk and tea are rich in bioactive compounds and nutraceuticals. Bovine milk lactoferrin (bLF), an 80 kDa iron-binding glycoprotein present in whey protein fraction of milk, and polyphenols in green and black tea are reported to exhibit a wide range of beneficial effects including chemopreventive activity by modulating multiple signal transduction pathways when administered as single agents (Yang et al., 2000; Tsuda et al., 2002; Moyers and Kumar, 2004). These findings suggest that a combination of bLF and tea polyphenols may have synergistic effects in inhibiting cancer development. However, it is important to establish the chemopreventive efficacies of agent combinations by evaluating cytotoxicity and apoptosis induction in cancer cell lines before embarking on whole animal bioassays or clinical trials.

Abbreviations: bLF, bovine lactoferrin; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; $\Delta\psi_m$, mitochondrial transmembrane potential; P-B, polyphenon-B; P-E, polyphenon-E; ROS, reactive oxygen species.

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Apoptotic cell death is characterized by distinct morphological features such as cell shrinkage, chromatin condensation, nuclear DNA fragmentation, membrane blebbing and breakdown of the cell into apoptotic bodies. The two major pathways of apoptosis mediated by the mitochondria and death receptors result in activation of caspases that cleave a variety of cellular substrates eventually leading to cell death (Wang, 2001; Gupta, 2003). Both the pathways are regulated by Bcl-2 family proteins that may be antiapoptotic or proapoptotic. Evasion of apoptosis increases the likelihood of sustaining gene mutations required for malignant transformation. Induction of apoptosis is currently recognized as an active strategy to arrest proliferation of cancer cells (Wang, 2001; Gupta, 2003; Vermeulen et al., 2005). A large number of dietary constituents including tea polyphenols have been reported to induce apoptosis in malignant cells (Wang et al., 1999; Bhattacharyya et al., 2005; Nakazato et al., 2005a; Shimizu et al., 2006). Increasingly, the mitochondrion has become the focus of attention as a potential target for chemointervention (Wang, 2001).

The present study was designed to evaluate the anticancer and apoptosis inducing effects of green and black tea polyphenols alone and in combination with bLF on human tongue squamous carcinoma (CAL-27) cells with special emphasis on the mitochondrial pathway of apoptosis.

2. Materials and methods

2.1. Chemicals

bLF (lot No. 020119) of purity ≥96.2% was obtained from Morinaga Milk Industry Co., Ltd, Tokyo, Japan. The iron content of bLF was 18 mg/100 g. Green tea polyphenols (Polyphenon-E:P-E) and black tea polyphenols (Polyphenon-B:P-B) were kindly provided by Mitsui Norin Co., Ltd., Tokyo, Japan. The composition of Polyphenon-E and Polyphenon-B is same as described previously (Chandra Mohan et al., 2005, 2006). Polyphenon-E (P-E) is a mixture of epigallocatechin-3-gallate (64.6%), epigallocatechin (4.3%), epicatechin (9.4%), epicatechin-3-gallate (6.4%), gallocatechin-3-gallate (3.5%), catechin-3-gallate (0.2%), gallactocatechin (0.2%), catechins (1.1%) and caffeine (0.7%). Polyphenon-B (P-B) has the following composition: epicatechin (0.4%), epigallocatechin-3-gallate (1.4%), epicatechin-3-gallate (0.1%), gallocatechin-3-gallate (0.2%), free theaflavins (0.32%), theaflavinmonogallate-A (0.14%), theaflavinmonogallate-B (0.15%), theaflavindigallate (0.21%), tannin (35.6%) and caffeine (4.9%). All other reagents used were of analytical grade. Stock solutions of bLF and P-E were prepared in phosphate buffered saline (PBS). P-B was dissolved in PBS containing 0.5% dimethyl sulfoxide (DMSO). The stock solutions were then diluted with the medium prior to use to obtain the desired concentration. The final concentration of DMSO in the medium was less than 0.01 per cent that proved to have no detectable effect on cell growth.

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from GIBCO. Dithiothreitol (DTT), 3,3-diaminobenzidine tetrahydrochloride (DAB), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), propidium iodide, proteinase K, phenylmethanesulfonyl fluoride (PMSF), rhodamine 123, and RNase A were purchased from Sigma Chemical Company, St. Louis, MO, USA.

2.2. Cell lines and cell cultures

Normal human gingival fibroblast (HGF) and human tongue squamous cell carcinoma (CAL-27) cell lines generously provided by Dr. D.A. Tipton, University of Tennessee, College of Dentistry, Memphis, TN, USA were used in this study. The cells were grown in DMEM supplemented with 10% FBS, 50 U/ml penicillin G, and 50 μg/ml streptomycin sulphate. The cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air. Exponentially growing cells were used for all the experiments.

2.3. Cytotoxicity assay

Cytotoxicity was assessed by the MTT assay based on the reduction of MTT by mitochondrial dehydrogenases of viable cells to a purple formazon product (Mosmann, 1983). Briefly, cells were diluted in growth medium and seeded in 24-well plates (5×10^4 cells/well). After overnight growth, the growth medium was replaced with exposure medium (DMEM without FBS) containing indicated doses of bLF, P-E, P-B alone and a combination of bLF and P-E or P-B. After 24 h, the cells in each well were washed with 200 μl of PBS, and incubated with 100 μl of 500 μg/ml MTT in PBS at 37 °C for 3 h. The MTT-formazon product dissolved in 200 μl of DMSO was estimated by measuring the absorbance at 570 nm in an ELISA plate reader. Cell survival was expressed as percentage of viable cells of treated samples to control samples. All the dietary agents were tested in triplicates and the experiments were repeated at least three times.

2.4. Nuclear morphology

CAL-27 cells were plated at a density of 5×10^4 cells/well into 6-well chamber slides. After 80% confluence, CAL-27 cells were treated with dietary agents alone and in combination for 24 h. The cells were then washed with PBS, fixed in methanol: acetic acid (3:1, v/v) for 10 min and stained with 50 μg/ml propidium iodide for 20 min. Nuclear morphology of apoptotic cells with condensed/fragmented nuclei was examined under a fluorescent microscope and at least 1×10^3 cells were counted for assessing apoptotic cell death (Keum et al., 2002).

2.5. Cell cycle analysis

Cell cycle distribution and measurement of the percentage of apoptotic cells were performed by flow cytometry (Tai et al., 2000). After treatment, floating cells in the medium were combined with attached cells harvested by trypsinisation. Cells were then washed with cold PBS and fixed in 80% ethanol in PBS at −20 °C. After 12 h, fixed cells were pelleted and stained with propidium iodide (50 μg/ml) in the presence of RNase A (20 μg/ml) for 30 min at 37 °C, and about 10^4 events were analysed on a Becton Dickinson FACScan flow cytometer. Cell cycle histograms were analysed using Cell Quest software. Apoptotic cells were distinguished form non-apoptotic intact cells by their decreased DNA content as determined by their lower propidium iodide staining intensity appearing in the area below subG_0/G_1 phase.

2.6. Determination of ROS generation

To assess the generation of intracellular ROS, the oxidation-sensitive fluorescent probe DCFH-DA was used. Briefly, after treatment, CAL-27 cells were harvested and suspended in 0.5 ml PBS containing 10 μM DCFH-DA for 15 min at 37 °C in the dark. DCFH-DA was taken up by the cells and deacetylated by cellular esterase to form a non-fluorescent product DCFH, which was converted to a green fluorescent product DCF by intracellular ROS produced by treated CAL-27 cells. The intensity of DCF fluorescence was measured by flow cytometry with excitation and emission settings of 488 and 530 nm respectively (Wang et al., 1999). A total of 10^4 events were counted and the histograms were analysed using Cell Quest software and compared with histograms of control untreated cells.

2.7. Determination of mitochondrial transmembrane potential

The changes in mitochondrial transmembrane potential ($\Delta\psi_m$) were measured by uptake of the mitochondrial specific lipophilic cation dye rhodamine 123 (Scaduto and Grotyohann, 1999). After treatment, CAL-27 cells