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Difference in Growth Suppression and Apoptosis Induction of EGCG and EGC on Human Promyelocytic Leukemia HL-60 Cells

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Growth suppression and apoptosis inducing effect of (-)-epigallocatechin 3-gallate (EGCG) and (-)-epigallocatechin (EGC) were studied against human promyeolcytic leukemia, HL-60 cells. EGCG showed higher growth suppression against HL-60 cells than EGC. IC_{50} values for EGCG were 60.0 μM and EGC was 107.7 μM, respectively. Both EGCG and EGC induced apoptosis evidenced by nuclei fragmentation. Nuclear fragmentation was observed as a time-dependent manner and the extent of nuclear fragmentation was slightly higher in EGCG-treated cells than EGC-treated cells. The expression level of Bcl-2 was decreased and caspase-3 was activated by EGCG or EGC treatment. The extent in decrease of Bcl-2 and activation caspase-3 were more extensively occurred in EGCG-treated cells than in EGC-treated cells. These data corresponded to the growth suppression data. EGC showed no cytotoxicity to a normal V79-4 cell line and EGCG showed slight cytotoxicity at higher concentrations.

Key words: EGCG, EGC, Growth suppression, Apoptosis, Bcl-2, Caspase-3

INTRODUCTION

Green tea has been a most consumed beverage next to water for many centuries in Asian countries. Since the beneficial effects of green tea are known, many studies have been carried out to identify the components of green tea and to investigate the mechanism of active ingredients (Aggarwal and Shishodia, 2006, Bode and Dong, 2006, Zaveri, 2006, Dorai and Aggarwal, 2004). The major components of green tea polyphenolic catechins include (-)-epigallocatechin 3-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), (-)-epichatechin (EC), (+) catechin and (+) gallocatechin. The beneficial effects of green tea are well-known including cancer preventive activity and other medical applications (Fujiki et al., 2002; Mukhtar and Ahmad, 2000; Zaveri, 2006).

Among polyphenolic catechins of green tea, EGCG, which is the most abundant component of green tea, has drawn attention of researchers mostly, so potential cancer preventive effect was suggested (Sugamura et al., 1999; Yang et al., 2002) based on its inhibitory effects on diverse cellular events associated with anti-mutagenic effect and tumorigenesis (Hernaez et al., 1998; Hour et al., 1999; Mimito et al., 2000). It is also reported that this compound has anti-oxidative and anti-inflammatory activities (Shi et al., 2000; Ahmed et al., 2002). EGC, the second most abundant catechin of green tea and constitute about 50% of the level of EGCG, has not much drawn attention to its beneficial medical effect; Apoptosis induction in human breast cancer cells (Vergote et al., 2002) and topoisomerase II inhibition activity of EGC were reported (Neukam et al., 2008). In this study we compared the growth suppression and apoptosis induction effect of EGC with those of EGCG in human leukemia cells in order to obtain data can be used for developing potential therapeutic or chemo-preventive agents for cancer patients.

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MATERIALS AND METHODS

Cell culture, Cell viability and cytotoxicity analysis

The human promyelocytic leukemia cell line HL-60 (ATCC CCL-240) was grown in RPMI-1640 (Gibco BRL, USA). The Chinese hamster lung cell line V79-4 (ATCC CCL-93) was grown in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, USA). Culture media was supplemented with 10% (v/v) heat inactivated fetal bovine serum, 2 mM glutamine, and antibiotics. Cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cytotoxicity of EGCG and EGC were estimated by the MTT assay (Hansen et al., 1989). HL-60 cells were treated with samples for the indicated time. V79-4 cells were treated with EGCG or EGC for 48 hr. The data are expressed as a mean percentage of viable cells as compared to the respective control cultures.

Nuclear morphology observation

HL-60 cells were treated with 50 μM of EGCG or EGC for 24 and 48 hr. Morphology of cellular nuclei were observed under a fluorescent microscope as described previously (Lee et al., 2003).

Western blot analysis

HL-60 cells were treated with samples and subjected to Western blot analysis, as described previously (Piao et al., 2001). Blots were probed with mouse monoclonal anti-human anti-Bcl-2 (Oncogene Science, USA) and anti-caspase-3 (Transduction Laboratory, USA) antibodies. Immunoreactivity was detected using either an anti-mouse (Santa Cruz Biotechnology, USA) or anti-rabbit (Amersham Biosciences, UK) peroxidase-conjugated secondary IgG antibody and an enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences, UK). The intensity of protein bands was measured using software, *Image J 1.41* and expressed as relative values to the control.

RESULTS AND DISCUSSION

Growth suppression of EGCG and EGC

The growth suppression effect of EGCG and EGC on a human promyelocytic leukemia cell line, HL-60 was evaluated using MTT assay. At 48 hr exposure to EGCG and EGC, HL-60 cell growth was dramatically decreased in a dose-dependent manner, as shown in Fig. 1A. Growth suppression effect of EGCG was slightly higher than that of EGC in almost all ranges of the concentration used in this study. However, both EGCG and EGC revealed similar pattern of growth suppression of HL-60 cells. Control cells treated with vehicle alone showed no changes in cell growth or viability. The concentration required to inhibit growth by 50% (IC_{50}) for EGCG and EGC on HL-60 cells was 60.0 μM and 107.7 μM after 48 hrs of incubation, respectively (Fig. 1B). When the incubation time was increased to 72 hr, IC_{50} value for EGCG and EGC were decreased slightly to 57.5 μM and 97.5 μM, respectively even though these decreased values were not significantly different from the values of 48 hr incubation.

Induction of apoptosis by both EGCG and EGC treatment

To determine whether EGCG and EGC-meditated

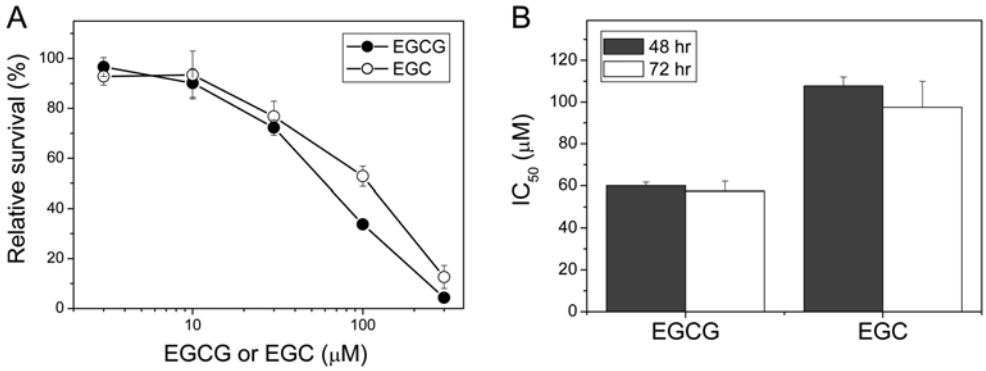

Fig. 1. Growth suppression effect of EGCG and EGC in HL-60 cells. **A**. Dose-dependent growth curve of EGCG or EGC-treated HL-60 cells. Cells were incubated with 0, 3, 10, 30 and 100 and 200 μM of EGCG (●) or EGC (○) for 48 hr. The relative cell survival rate was measured by the MTT assay. The percentage of cell growth in the control group was designated as 100%. **B**. IC_{50} values of EGCG and EGC against HL-60 cells after 48 or 72 hr incuation. Experiments were performed at least 3 times and data are presented as means ± S.D.

growth suppression of HL-60 cells was associated with apoptosis, we examined the EGCG and EGC-induced nuclei fragmentation. Cells were treated with 50 μM of EGCG or EGC and the nuclei of cells were stained with propidium iodide after 24 or 48 hr of incubation. Control cells exhibited intact nuclei, but EGCG and EGC-treated cells showed significant nuclear fragmentation (Fig. 2), which is one of typical makers of apoptosis induction. More cells with nuclei fragmentation were observed in cells treated with EGCG or EGC for extended period of time (48 hr in Fig. 2). Slightly more nuclei fragmentation was observed in cells treated with EGCG than cells treated with EGC. The induction of apoptotic cell death was accompanied by characteristic morphological and structural changes including a condensed and fragmented nuclear structure and decreased cell size (Kerr et al., 1994). In this experiment we showed the changes in the morphology of the nucleus of EGCG or EGC-treated cells. Thus, our data suggest that the growth suppression effect of both EGCG and EGC related to induction of apoptosis evidenced by the induction of nucleus fragmentation.

Changes in apoptosis-related molecules

In order to investigate the mechanism by which EGCG and EGC cause apoptosis, we observed the changes in levels of apoptosis-related proteins. The concentration of each protein lysate was measured and the same amount of protein was loaded for Western blot analysis. Protein integrity was confirmed by SDS-PAGE analysis (data not shown). Among apoptosis-related proteins, the changes in Bcl-2 protein level, an important regulator of apoptotic signaling pathways (Reed, 1998). As shown in Fig. 3A, Western blot analysis revealed that both EGCG and EGC treatment decreased Bcl-2 protein levels. The relative level of Bcl-2 protein was quantitatively compared and it was found that Bcl-2 protein level decrease was more intensively occurred in EGCG-treated HL-60 cells than EGC-treated cells (Fig. 3B).

Next, we observed the changes in caspase-3, an apoptosis execute molecule. Both EGCG and EGC induced the proteolytic processing of pro-caspase-3 as a dose-dependent manner (Fig. 3C). Caspase-3 is a cysteine protease that exists as an inactive zymogen in cells that is activated by sequential proteolytic events that cleave the 32-kDa precursor at aspartic acid residue to generate active heterodimer of 20 and 12 kDa subunits (Nicholson et al., 1995). Similar observation was made in green tea polyphenol treated-human osteosarcoma SAOS-2 cells, where the treatment of inhibitors of caspase-3 rescued SAOS-2 cells from GTP-induced apoptosis (Hafeez et al., 2006). The quantitative analysis showed that the proteolytic cleavage of procaspase-3, which implied the activation of caspase-3, was induced more intensively in EGCG-treated cells than EGC-treated cells (Fig. 3D). And the slope of the decrease in the level of procaspase-3 was sharper than that of Bcl-2. Therefore, it seemed that the downstream molecules such as caspase-3 in apoptotic pathway were more influenced by the EGCG-treatment. These results supported the growth suppression data pre-

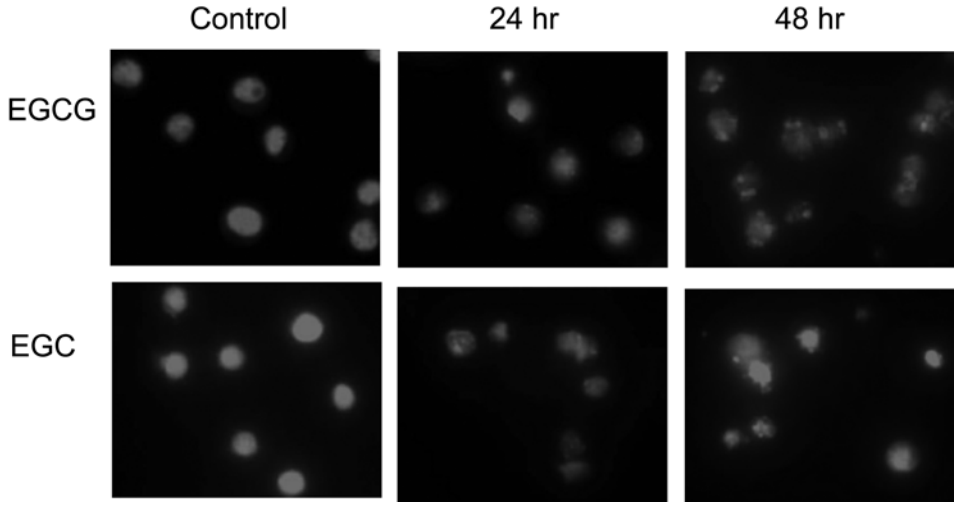

Fig. 2. Nuclear fragmentation was induced by EGCG or EGC treatment in HL-60 cells. HL-60 cells were treated with 50 μM of EGCG (upper panel) or EGC (lower panel) for 24 or 48 hr and stained with propidium iodide. Cellular morphological changes were observed using a fluorescence microscope at the magnitude of 600×.

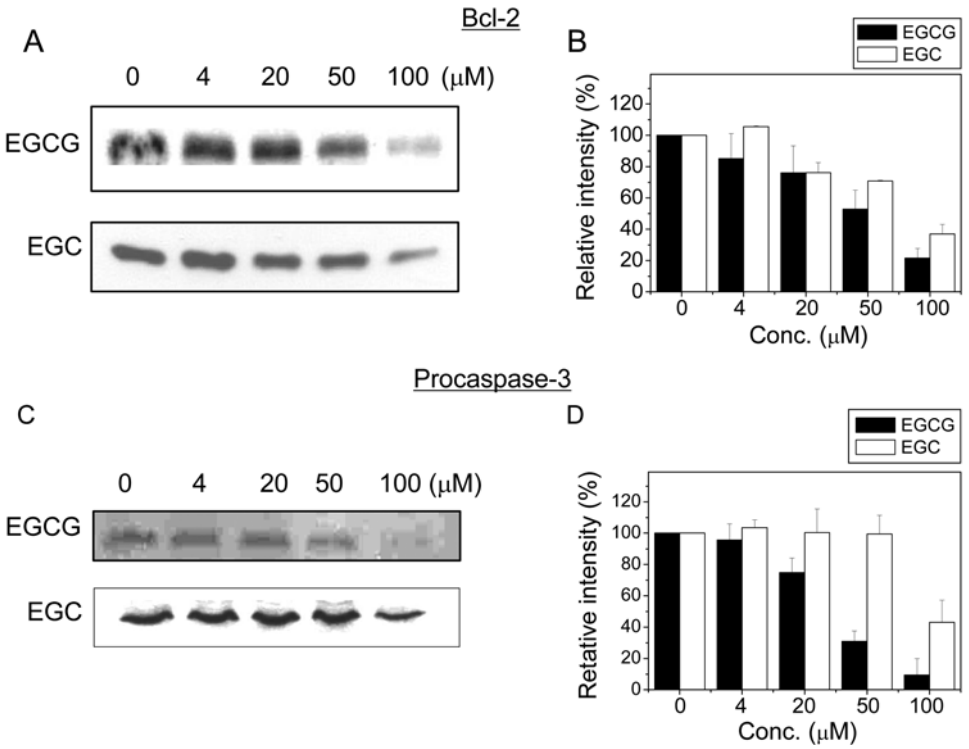

Fig. 3. Changes in the expression of apoptosis-related proteins in response to treatment with EGCG and EGC. HL-60 cells were treated with 0, 4, 20, 50 and 100 μM of EGCG and EGC for 48 hr. Cell extracts were subjected to Western blotting to determine immunoreactivity levels of Bcl-2 (A and B) and procaspase-3 (C and D) as described in Materials and Methods. Protein band intensity was measured and expressed as relative values (B for Bcl-2 and D for pro-caspase-3). Experiments were performed at least 3 times and representative Western blots are shown.

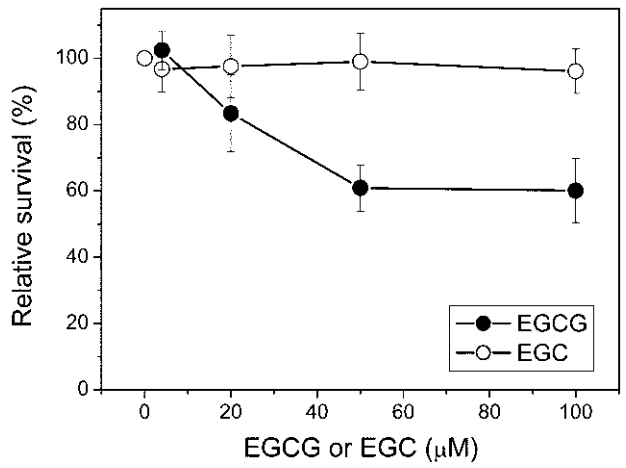

Fig. 4. Cytotoxicity of EGCG and EGC to a normal cells V79-4. V79-4 cells were incubated with 0, 4, 20, 50 and 100 μM of EGCG or EGC for 48 hr. The relative cell survival was measured by MTT assay. Data are presented as means±SD. The percentage of cell growth in the control group was designated as 100%.

sented above, where EGCG was more effective than EGC for the growth suppression of HL-60 cells.

Cytotoxicity to a normal cell line, V79-4

In order to observe cytotoxicity of EGCG and EGC on a normal cell line, we measured the relative cell survival of a Chinese hamster cell, V79-4 upon treatment of either EGCG or EGC (Fig. 4). EGC showed no cytotoxicity in 48 hr incubation. EGCG also showed no significant cytotoxicity at lower concentrations however, slight decrease in relative cell survival was observed at higher concentrations (50 μM) in EGCG-treated V79-4 cells. Similarly, EGC reduced the growth of human breast cancer cells but not that of normal breast epithelial cells (Vergote et al., 2002). These cytotoxicity data were obtained with a Chinese hamster cell line, the data may be used as a reference data for *in vivo* or clinical experiments to develop these phytochemicals as potential therapeutic agents.

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