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Inhibition of Proliferation and Induction of Apoptosis by EGCG in Human Osteogenic Sarcoma (HOS) Cells

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EGCG [(-)-epigallocatechin-3-gallate], a major component of green tea has been considered as a major antioxidant constituent. In addition to having been considered for cancer treatment as a chemopreventive and chemotherapeutic agent, EGCG has recently been attributed an anti-proliferative effect. We re-examined the latter finding in this study and added specific focus on the ability of EGCG to induce apoptosis in human osteogenic sarcoma (HOS) cells. Anti-proliferative action of EGCG (IC_{50} = 35.3 ± 6.0 μg/mL) appeared to be linked to apoptotic cell death based on morphological changes, chromosomal DNA degradation, and an increase in the sub-G_1 apoptotic cell population. Treatment of HOS cells with EGCG gradually activated caspase-3, an established inducer of apoptotic cell death.

Key words: EGCG, Apoptosis, Caspase-3, HOS cells

INTRODUCTION

Many plant-derived compounds and their derivatives have been identified useful in the treatment of cancer including paclitaxel (from *Taxus brevifolia* L.), vincristine (*Catharanthus roseus* G. Don), podophyllotoxin (*Podophyllum peltatum* L.), and camptothecin (*Camptotheca acuminata*) (Lilenbaum and Green, 1993; Pezzuto, 1997; Bertrand and Sané, 1999). More recently, naturally occurring cytotoxic compounds have been added to the list, such as neolignans from *Saururus chinensis*, squamocin from the *Annona reticlata* seed, and momordin I from *Amelopsis japonica* – all of which effective against bladder and other types of cancer (Kim *et al.*, 2002; Hahm *et al.*, 2005, Yuan *et al.*, 2005). It has also been reported that a metabolite of ginseng saponin induced apoptosis by activating cytochrome c and consequentially caspase-3 protease (Lee *et al.*, 2000).

Epigallocatechin gallate (EGCG), a polyphenol found in green tea and other foods, has been suggested as a potential cancer-preventive agent (Sugamura *et al.*, 1999; Yang *et al.*, 2002) based on its inhibitory effects on mutagenesis and tumorigenesis (Hernaez *et al.*, 1998; Hour *et al.*, 1999; Mimito *et al.*, 2000). It is further known that EGCG is anti-oxidative and anti-inflammatory (Shi *et al.*, 2000; Ahmed *et al.*, 2002). Recently, it was also found that EGCG is anti-mutagenic during 2-hydroxyamino-3-methylimidazo[4,5-f]quinolone and *N*-methyl-*N'*-nitroso-guanidine (MNNG) challenges (Hernaez *et al.*, 1998; Hour *et al.*, 1999). Additionally, EGCG appears to attenuate genotoxic damage caused by ultraviolet irradiation (Tobi *et al.*, 2002) and protect cellular membranes against oxidative damage (Saffari and Sadrzadeh, 2004).

Anti-proliferative and anti-cancer effects of EGCG have been demonstrated in various cancer cell lines and different animal models. Among the recorded effects are inhibition of cell growth and induction of apoptosis in prostate cancer cells, cervical cancer cells, ovarian carcinoma cells, colon carcinoma cells, and oral squamous carcinoma cells (Paschka *et al.*, 1998; Elatter and Virji, 2000; Jung *et al.*, 2001; Ahn *et al.*, 2003; Huh *et al.*, 2004). Chemopreventive action of EGCG has been shown in osteosarcoma cells (Roomi et al., 2005) and an arsenite-transformed osteosarcoma cell line (Yang *et al.*, 2005). Several mechanisms have been suggested to explain these effects, including general antioxidative action as well as interaction with enzymes or proteins implicated in cancer progression (e.g., telomerase, matrix metalloproteinase-2, collagenase, activator protein-1) (Makimura *et*

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al., 1993; Chen *et al.*, 2000; Cutter *et al.*, 2001; Naasani *et al.*, 2003). However, the precise mechanism by which EGCG exerts its anti-cancer effect is still not fully understood, as many EGCG studies focused only on selected targets. The purpose of this study was to examine the origin of the anti-proliferative and apoptosis-inducing properties of EGCG in human osteogenic sarcoma (HOS) cells. We found that EGCG inhibited cell growth and induced apoptosis in HOS cells via caspase-3 activation.

MATERIALS AND METHODS

Cell culture and cytotoxicity test

Human osteogenic sarcoma cells (HOS, ATCC #CRL-1543) were cultured in Dulbecco's modified Eagle's media (Gibco, U.S.A.) supplemented with 10% fetal bovine serum, 2 mM L-glutamine and antibiotics in a humidified incubator with 5% CO_2/95% air at 37°C. Human oral epidermoid carcinoma (KB, ATCC #CCL 17) and human promyelocytic leukemia (HL-60, ATCC #CCL240) cells were cultured as recommended by the supplier. The trypan blue dye exclusion test was used to assess cell viability. Exponentially growing HOS cells were seeded at 5×10^4 cells/well in a 96-well plate and treated with the indicated concentrations of EGCG (Sigma, U.S.A.) or vehicle. Cell viability was determined by MTT assay which detects the reduction of 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazan (Monks *et al.*, 1991). Experiments were conducted at least in triplicate.

Purification of DNA and electrophoresis

Cells were grown to 60-70% confluency and treated with various concentrations of EGCG for 0 to 72 h. Subsequently, DNA was purified as previously described (Hyun *et al.*, 1997). The resulting DNA fragments were separated via electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining. The results are representative for three independent experiments.

Flow cytometry analysis of apoptosis

Cells were treated with various concentrations of EGCG for 0 to 72 h and harvested by centrifugation at 750 ×g for 5 min. The generated pellets were rinsed with phosphate buffer saline (PBS), mixed with a 1:1 (*v/v*) solution of 0.2 M Na_2HPO_4 and 0.1 M citric acid (pH 7.5), and fixed with cold ethanol at 4°C for 1 h. Following a wash with PBS, the fixed cells were then resuspended in a staining solution containing 10 μg/mL propidium iodide and 100 μg/mL DNase-free RNase. The cell suspensions were incubated in the dark at 37°C for 1 h and analyzed on a FACScaliber fluorescence-activated cell sorter flow cytometer (Becton Dickinson, Franklin Lakes, NJ, U.S.A.).

Western blot analysis

Following the EGCG treatment, the cells were washed with PBS and lysed in a buffer containing: 20 mM Tris-Cl, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM $NaVO_3$, 100 mM NaF, 1 mM EGTA, 10 mM Na-pyrophosphate, and 1 mM phenylmethylsulfonylfluoride as well as 1% Triton X-100 and 10% glycerol at pH 7.5. The cell lysates were cleared of insoluble material by centrifugation and the protein content determined according to the method described by Lowry and colleagues (1951). Samples containing equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH, U.S.A.) at 80 mA for 2 h. Blots were probed with a monoclonal mouse anti-human caspase-3 antibody (Transduction Laboratories, Lexington, KY, U.S.A.). Immunoreactivity was detected by enhanced chemiluminescence (ECL, Amersham, Little Chalfont, Buckinghamshire, UK) using an anti-mouse peroxidase-conjugated secondary immunoglobulin-G antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). Experiments were repeated at least three times.

RESULTS

Effect of EGCG on cell survival

The structure of epigallocatechin gallate (EGCG) - the most abundant polyphenol in green tea - is shown in Fig. 1. We first evaluated the effect of EGCG on HOS cells by using an MTT assay. As shown in Fig. 2A, EGCG significantly decreased the rate of cell survival in a dose-dependent manner at concentrations between 0 and 100 μg/mL. The concentration required for 50% growth inhibition (IC_{50}) after 48 h of treatment was 35.3 ± 6.0 μg/mL. Cell survival was decreased as the duration of EGCG treatment increased (Fig. 2A) regardless of the EGCG concentration. To determine the cytotoxic effects of EGCG on other cancer cells, cell survival was analogously determined for human oral carcinoma (KB) and human leukemia (HL-60, Fig. 2B and C). Compared to KB, HL-60 cells were more sensitive to EGCG, yielding IC_{50} values (48 h) of 64.4 ± 3.0 and 11.2 ± 1.1 μg/mL, respectively.

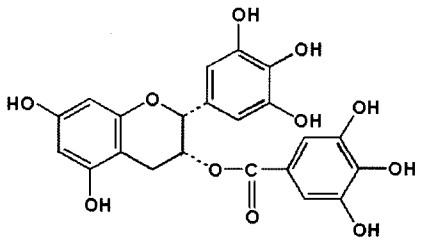

Fig. 1. Chemical structure of (-)-epigallocatechin-3-gallate (EGCG)

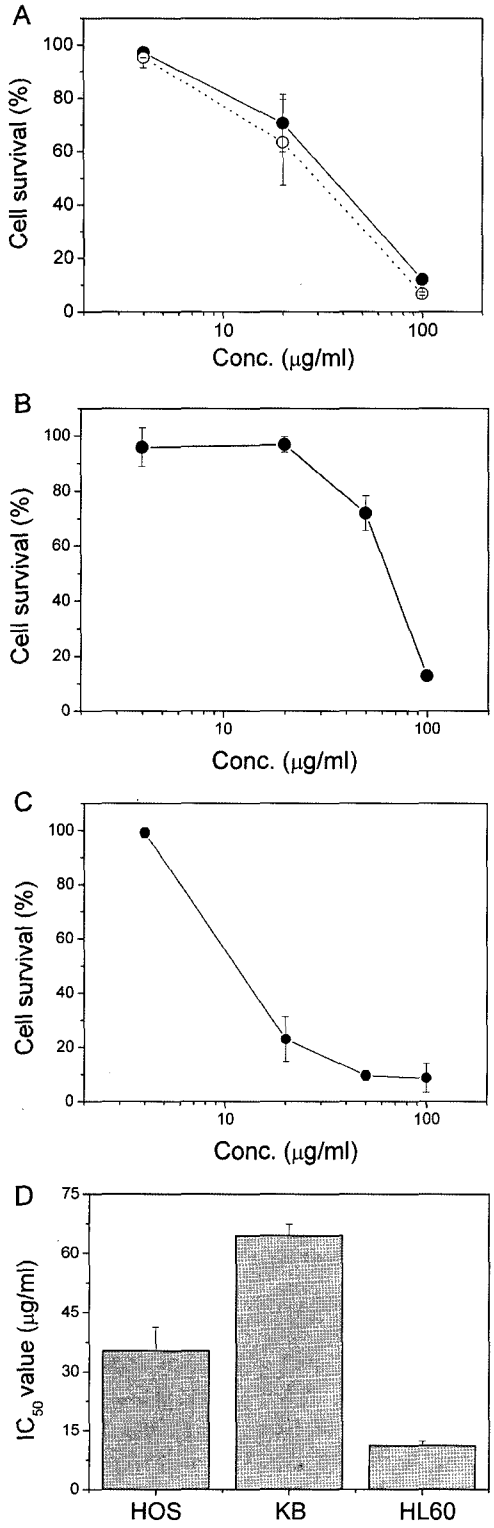

Fig. 2. Anti-proliferative effect of EGCG. **A.** Human osteogenic sarcoma (HOS) cells were incubated with 0, 5, 20, or 100 μg/mL EGCG for 48 (●) or 72 h (○) and cell survival was measured by the MTT assay. **B** and **C.** Human oral epidermoid carcinoma (KB, B) and human promyelocytic leukemia (HL-60, C) cells were treated with EGCG as in (A) and cell survival was measured. **D.** IC_{50} values of EGCG on HOS, KB and HL-60 cells. Data are expressed as mean ± S.D., N=3.

Induction of apoptosis by EGCG

To determine whether EGCG-meditated inhibition of growth and proliferation was associated with programmed cell death or apoptosis, we examined the EGCG-induced morphological changes in HOS cells. Microscopic analysis demonstrated that the cells had undergone gross morphological changes including cell shrinkage, chromatin condensation, and formation of apoptotic bodies (data not shown). Analysis of DNA extracted from EGCG-treated HOS cells additionally revealed a progressive, time-dependent increase in chromosomal DNA degradation, which may have been associated with the degradation of chromosomal DNA at the linker regions characteristic for apoptotic cells (Fig. 3).

Flow-cytometric analysis of EGCG-induced cell death

Apoptosis is commonly associated with loss of DNA. This occurs as degraded DNA diffuses out of fixed cells after endonuclease cleavage. We therefore analyzed the DNA content of EGCG-treated HOS cells by flow-cytometry. Histograms of DNA content obtained from propidium iodide-stained HOS cells showed that the percentage of cells with reduced DNA content progressively increased over time. Apoptosis was negligible within 24 h of the treatment. Forty-eight and 72 h into the EGCG treatment (30 μg/mL), however, the percentage of apoptotic cells increased to 19.0 and 30.1%, respectively (Fig. 4). The profile for the EGCG-induced increase in hypodiploid DNA content closely correlated with the chromosomal DNA degradation. Cells treated with increasing concentrations of EGCG (0 to 50 μg/mL for 24 h) presented with a gradual increase in the apoptotic cell population up to

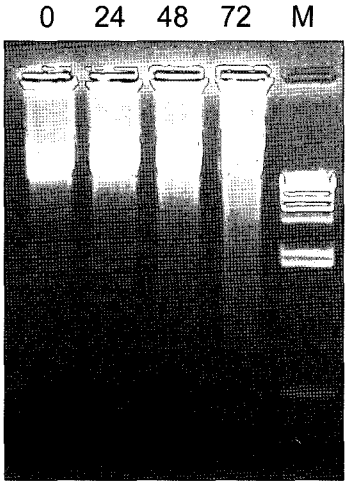

Fig. 3. Induction of chromosomal DNA degradation. HOS cells were incubated with 30 μg/mL EGCG for 0, 24, 48, or 72 h. Cellular DNA was isolated and analyzed on a 1.5% agarose gel with by ethidium bromide staining. M, molecular weight marker.

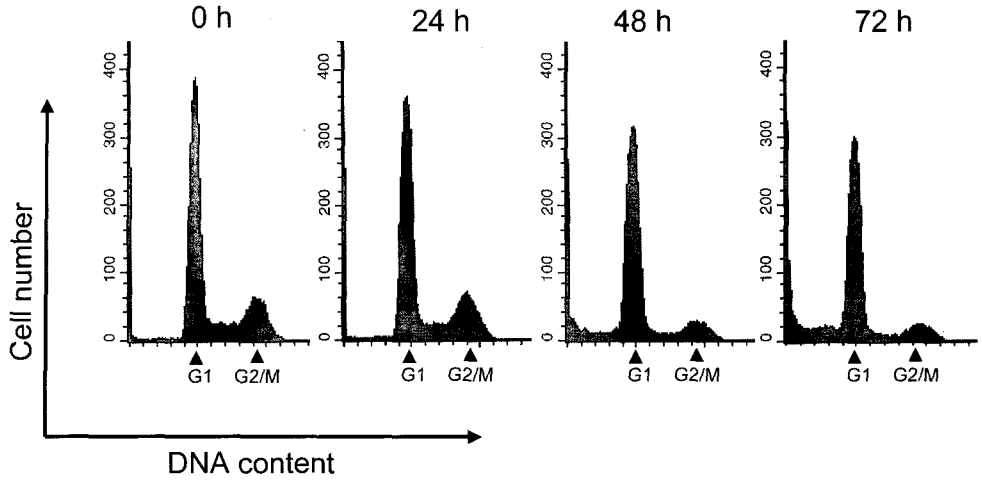

Fig. 4. Flow cytometric analysis of EGCG-treated HOS cells. Cells were exposed to 30 mg/ml EGCG for 0, 24, 48, and 72 h. Cells were stained with propidium iodide to assess DNA content.

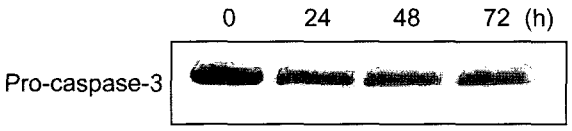

Fig. 5. Activation of caspase-3 by EGCG treatment. Cells were lysed and samples containing equal amounts of protein were subjected to immunoblotting with anti-caspase-3 antibody.

55.7%. In parallel to the increase in the number of cells with sub-G_1 hypodiploid DNA content, there was a decrease in the number of cells with diploid DNA content.

Effect of EGCG treatment on caspase-3 activation

We tested whether caspase-3 protease was involved in the mechanism by which EGCG caused apoptosis. Western blot analysis 24-72 h after the treatment showed an intensity reduction in a 32 kD protein band corresponding to the molecular weight of pro-caspase (Fig. 5), suggesting that EGCG induced proteolysis of this caspase-3 precursor. A decrease in precursor indicates concomitant activation of caspase-3, which is critical to the induction of apoptotic cell death.

DISCUSSION

Flavan-3-ols (catechins) are major constituents of green tea that include (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG), and (-)-epicatechin (EC). These polyphenol compounds have antioxidative and anti-mutagenic properties. A variety of antioxidants and chemopreventive agents are cytotoxic to cancer cells. Cellular growth inhibition by green tea has been established in many tumor cells and. EGCG has been a prime candidate for mediating this effect. Recently, anti-proliferative and anti-cancer action of EGCG has been reported in cancer cell lines (Paschka *et al.*, 1998; Elatter and Virji, 2000; Jung *et al.*, 2001; Ahn *et al.*, 2003; Huh *et al.*, 2004). However, no data is available on the direct effects of individual green tea components on HOS cells. Our observations demonstrate that EGCG, a major component of green tea, possesses HOS cell growth inhibitory properties. A vast variety of naturally occurring substances are known to protect against experimental carcinogenesis. It is becoming increasingly evident that certain phytochemicals, particularly those included in our daily diet, may have important cancer chemopreventive properties (Sanaha *et al.*, 1997). Some anti-inflammatory chemopreventive agents have been found to suppress growth and proliferation of transformed or malignant cells through induction of programmed cell death or apoptosis (Bellosillo *et al.*, 1998).

It is likely that EGCG inhibits cancer cell growth through many different regulatory pathways, along with apoptosis and cell cycle arrest. Our findings are consistent with EGCG inhibiting cell growth and inducing apoptosis in HOS cells. The cytotoxic properties of EGCG seemed to be more effective against leukemia (HL-60) cells than sarcoma (HOS) or carcinoma (KB) cells based on IC_{50} values. At this time, there is no explanation for these differences and more experiments are needed for further analysis. The induction of apoptotic cell death by EGCG was accompanied by characteristic morphological and structural changes (data not shown) as well as internucleosomal DNA degradation. Flow cytometry clearly confirmed apoptosis induction in EGCG-treated HOS cells.

Apoptosis is a tightly regulated process which involves changes in the expression of distinct genes. One group of genes regulating apoptosis are cytoplasmic aspartate-specific cysteine proteases of the ICE/CED-3 family, better known as caspases (Klaus *et al.*, 1998). Caspase-

3, for example, inactivates poly(ADP-ribosyl)polymerase by proteolytically degrading the 116 kDa enzyme into an 85 kDa fragment (Duriez and Shah, 1997; Li and Darzynkiewicz, 2000; Nigata, 2000). When HOS cells were treated with EGCG, the expression level of pro-caspase-3 was decreased indicating that the active form of caspase-3 was generated. We therefore conclude that EGCG-induced apoptotic death in HOS cells was mediated in part by activation of caspase-3. Induction of apoptosis in human osteogenic sarcoma cells by EGCG is hence supported *in vitro*. More research is needed to establish, whether this property is maintained in living tissue.

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