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Involvement of Caspase-3 in Epigallocatechin-3-gallate-Mediated Apoptosis of Human Chondrosarcoma Cells

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Green tea polyphenol—(−)epigallocatechin-3-gallate (EGCG)—is a potent chemopreventive agent in many test systems and has been shown to inhibit tumor promotion and induce apoptosis. In this study we describe a novel observation that EGCG displayed strong inhibitory effects on the proliferation and viability of HTB-94 human chondrosarcoma cells in a dose-dependent manner and induced apoptosis. Investigation of the mechanism of EGCG-induced apoptosis revealed that treatment with EGCG resulted in DNA fragmentation, induction of caspase-3/CPP32 activity, and cleavage of the death substrate poly(ADP-ribose)polymerase (PARP). Pretreatment of cells with a synthetic pan-caspase inhibitor (Z-VAD-FMK) and a caspase-3-specific inhibitor (DEVD-CHO) prevented EGCG-induced PARP cleavage. The induction of apoptosis by EGCG via activation of caspase-3/CPP32-like proteases may provide a mechanistic explanation for its antitumor effects. © 2000 Academic Press

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Chondrosarcomas may develop as a secondary change in pre-existing exostoses in tissues peripheral to cartilage or arise from a multipotent stem cell that subsequently undergoes chondrogenesis (1, 2). Although the cellular origin and the mechanism(s) leading to the development of malignant chondrosarcoma are at present not fully understood, a predisposition to developing chondrosarcoma has been found with the presence of hereditary multiple exostoses (3, 4). The proliferation of all cancer cells, including chondrosarcomas, results from a loss of genomic stability and tumor suppressor function of the endogenous cellular machinery. The p53 tumor suppressor protein normally keeps the cellular proliferation in check but in most of the human cancers, inactivation of this pathway due to mutations occurs and has been correlated with the tumorigenic phenotype (5). In this regard it is interesting to note that approximately 25% of high grade chondrosarcomas do contain a mutated p53 gene (6).

Green tea, a product of *Camellia sinensis,* has been shown to exert inhibitory effects against tumorigenesis and tumor growth and much of these effects are mediated by EGCG, the major polyphenol present therein (7–11). Oral consumption of green tea or its polyphenolic constituents has been shown to reduce cancer risk in tumor models of skin, lung, stomach, esophagus, colon, liver, and breast (reviewed in 8, 11). GTP is a mixture of several catechins including (−)-epigallocatechin-3-gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG), and epicatechin (EC) (12, 13). Several studies have suggested that EGCG is a major constituent of GTP and the principal mediator of the anti-carcinogenic properties of green tea (13–15). EGCG has been shown to inhibit tumor growth and to induce apoptosis in human cancer cells with little or no effect on normal cells (14–16). However, the mechanism of apoptosis induction by EGCG is poorly understood and similar studies analyzing the effect of green tea or its constituents on chondrosarcomas or malignant cells of chondrogenic/bone lineage have not been reported.

In this paper, we show that treatment of human chondrosarcoma cells HTB-94 with EGCG significantly ($P < 0.05$) reduced the viability and this correlated with induction of apoptosis. That the EGCG-induced apoptosis was caspase-dependent and involved the activation of caspase-3/CPP32 was shown by the ability of a pan-caspase inhibitor (Z-VAD-FMK) and a caspase-3-specific inhibitor DVD-CHO to block apoptosis. In addition, and importantly, our results also show that EGCG-treated cells (positive for PARP cleavage)

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exhibited significantly higher activity of the executioner caspase-3/CPP32-like proteases, in comparison to controls ($P < 0.05$). Thus, our data revealed a novel finding that induction of apoptosis by EGCG in human chondrosarcoma cells was mediated through the activation of caspase-3/CPP32-like proteases.

MATERIALS AND METHODS

Chemicals, biologicals, and antibodies. All culture media, fetal bovine serum (FBS) and nutritional supplements were purchased from GIBCO-BRL, Gaithersburg, MD. Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Protease inhibitor cocktail tablets were purchased from Boehringer Mannheim (Indianapolis, IN). The ECL system was from Amersham, Arlington Heights, IL. Anti-PARP antibody (Clone 7D3-6) and anti-mouse IgG were from Pharmingen (San Diego, CA). Polyacrylamide gradient gels for protein electrophoresis and Tris–glycine–SDS buffer were from Bio-Rad Laboratories, CA. Pan-Caspase inhibitor (Z-VAD-FMK) was purchased from R & D Systems (Minneapolis, MN). Caspase-3 inhibitor (DVD-CHO) was from Calbiochem (San Diego, CA).

Cell culture. The human chondrosarcoma cell line HTB-94 (ATCC, VA) was expanded in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS, L-glutamine, penicillin–streptomycin, and Fungizone (all from GIBCO-BRL, Gaithersburg, MD) and grown in humidified air with 5% CO_2 at 37°C.

Treatment with EGCG and cell viability assay. HTB-94 cells (3×10^4 cells/well in 100 μl) were incubated in DMEM with low serum (5% FBS) containing EGCG (10 μM) overnight. Cell viability was then determined using the MTT Cell Viability Assay kit (R & D Systems) according to the instructions provided. Briefly, at the end of treatment MTT was added to each well and incubation was continued for an additional 2 h. Resulting formazon crystals were solubilized in kit supplied reagent and the absorbance of the resulting solution was read at 570 nm. Control cells were treated exactly the same except that no EGCG was added to the wells. In some experiments, cells were pretreated with a synthetic pan-caspase inhibitor (20 μM, Z-VAD-FMK) or caspase-3 inhibitor DEVD-CHO (20 μM) for 2 h prior to the addition of EGCG. The percentage of viable cells in these experiments was calculated by the formula [(Absorbance value of control/Absorbance value of treated) $\times$ 100] and the results are expressed as "Viable cells (% of control)." In some experiments, cell viability was also determined by Trypan blue exclusion assay. In other experiments, HTB-94 cells were treated with EGCG for 24 h and then cultured for an additional 24 h without EGCG.

Protein extraction and Western blot analysis of PARP cleavage. At the end of treatment, cells were suspended in ice cold RIPA-M buffer with 1% NP-40 and cell lysate prepared essentially as described above. Cell lysate protein (30 μg) was resolved by gradient (4–20%) SDS–PAGE and transferred to nitrocellulose membranes (Bio-Rad). The blots were blocked for at least 1 h at room temperature in blocking buffer [5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBST)]. Primary anti-PARP antibody was diluted in blocking buffer and incubated with the blots overnight at 4°C. The blots were washed three times with TBST and the bound antibody detected with a 1:5000 dilution of HRP-conjugated secondary antibody following the instructions provided with the ECL kit (Amersham). Blots were stripped and reprobed with anti-β-Actin antibody (Santa Cruz Biotechnology, CA) and detected by ECL.

DNA fragmentation assay. After treatment, HTB-94 cells (2×10^6) were harvested, washed with cold PBS and the DNA was isolated by lysing the cells in SDS-Proteinase-K digestion buffer, purified by organic extraction and ethanol precipitation. Precipitated DNA was pelleted by centrifugation (10,000*g*, 10 min), washed with 70% ethanol, dried and dissolved in 100 μl of TE buffer (Sigma). One-fifth of each sample was treated with DNase-free RNase (35 μg/ml) for 30 min at 37°C and resolved by electrophoresis through 1.2% agarose gels and visualized by staining with ethidium bromide.

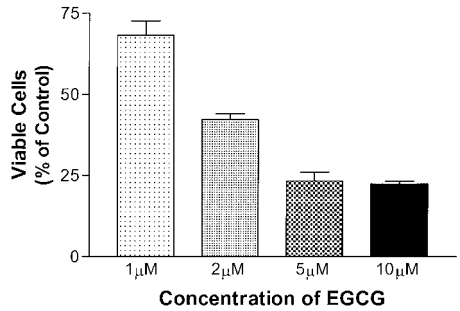

FIG. 1. EGCG affected the viability of HTB-94 cells in a dose dependent manner. HTB-94 cells were treated with indicated concentrations of EGCG as described under Materials and Methods. Data shown represent the analysis of three independent experiments performed in duplicate and the results are expressed as mean viable cells (±SD) percentage of controls.

Caspase activity assay. Caspase-3-related protease activity in cell lysates was determined using a commercially available kit (Biomol). Briefly, cell lysate protein (nuclei free) was mixed with assay buffer (containing 10 mmol of dithiothreitol) and the colorimetric substrate DEVD-*p*NA (50 μmol) followed by incubation at 37°C for 1 h. Absorbance was then read with a plate reader at 405 nm and the activity units were determined according to the instructions provided with the kit.

Protein determination. For all of the experiments, protein content was quantitated using a detergent compatible kit (Bio-Rad).

Statistical analysis. All experiments were repeated twice to ensure for reproducibility. All assays and quantitative measurements were performed in triplicate and the data was analyzed for statistical significance by Student's *t* test using Graph Pad Prism software.

RESULTS

EGCG inhibited the growth of HTB-94 cells in a dose dependent manner. To assess whether EGCG inhibited the growth and proliferation of HTB-94 cells, cells were treated with various concentrations of EGCG (1–10 μM) and the cell viability was determined by MTT assay and by trypan blue exclusion assay. The results obtained with the MTT assay are shown in Fig. 1 and the cell viability is expressed as percent mean (±SD) viable cells compared to untreated cells (taken as 100% viable) at different concentrations of EGCG. As can be seen, EGCG was a potent inhibitor of HTB-94 cell proliferation and the cell viability was less than 20% after 24 h of treatment with 5 μM of EGCG. Based on this data, EGCG was used at a concentration of 5 μM in subsequent experiments as this concentration produced ~80% inhibition of chondrosarcoma cell proliferation when compared to controls ($P < 0.05$).

Treatment with EGCG induced apoptosis of chondrosarcoma cells. Apoptotic cell death is characterized by chromatin condensation, membrane blebbing, intranucleosomal fragmentation of DNA, activation of

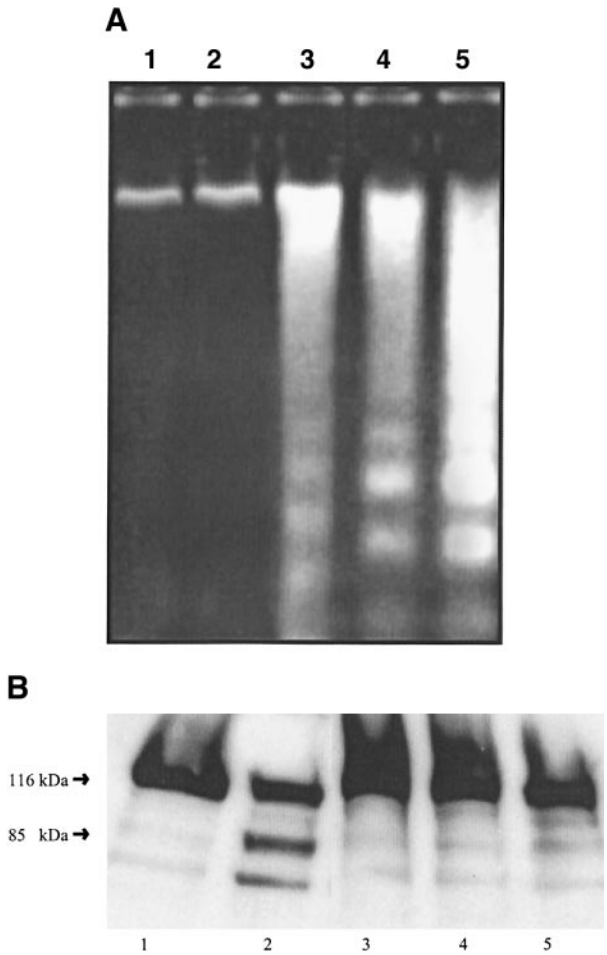

FIG. 2. (A) Induction of apoptosis in human chondrosarcoma cells HTB-94 by EGCG. Cells were incubated with different doses of EGCG for 24 h. Cells were subsequently harvested for DNA fragmentation analysis as described under Materials and Methods (lane 1, control; lane 2, 1 μM EGCG; lane 3, 2 μM EGCG; lane 4, 5 μM EGCG; lane 5, 10 μM EGCG). (B) Treatment with EGCG induced PARP cleavage which was blocked by caspase inhibitor Z-VAD-FMK. Cell lysates were prepared from untreated control (lane 1), EGCG treated (lane 2), HTB-94 cells pretreated with pan-caspase inhibitor Z-VAD-FMK (lane 3), HTB-94 cells + leupeptin (lane 4), and HTB-94 cells treated with Z-VAD-FMK and EGCG (lane 5). Total protein (30 μg/lane) was resolved by SDS–PAGE, transferred to nitrocellulose membrane and probed with anti-PARP antibody 7D3-6.

caspases, and apoptotic body formation. To study whether treatment with EGCG induced apoptosis in human chondrosarcoma cells HTB-94, cells were treated with different concentrations of EGCG for 24 h, genomic DNA was isolated and analyzed by agarose gel electrophoresis. In each case, nucleosomal DNA fragmentation, which is typical of apoptosis, was visible after staining of agarose gels with ethidium bromide (Fig. 2A). DNA fragmentation was most prominent in cells treated with higher concentrations of EGCG (lanes 3 and 4).

Another feature of apoptotic cell death is the activation of caspase-3-like proteases in response to death-inducing stimuli resulting in the cleavage of PARP producing 2 fragments of ~85 and 25 kDa. This causes the loss of normal PARP function which irreversibly commits the cell to die (17, 18). To determine whether EGCG-induced apoptosis in HTB-94 cells results in PARP cleavage, HTB-94 cells were grown to confluence (80–90%) and treated with EGCG (5 μM) for 24 h. The EGCG-containing medium was then replaced with fresh medium and the cells allowed to grow overnight. Cells were washed and the cell lysate prepared and analyzed for PARP cleavage as an indicator of caspase activation by Western blotting. The antibody used (clone 7D3-6) recognizes the intact 116 kDa and the cleaved 85-kDa fragments but does not react with the 25-kDa fragment. Previous studies have shown that the pattern of PARP cleavage differs in necrotic and apoptotic cells (19) and, thus, this assay can be used to determine whether treatment with a particular agent induced apoptosis or necrosis. The results shown in Fig. 2B clearly show the presence of the predominant ~85-kDa fragment of PARP in EGCG treated cultures but not in untreated control cultures. The lower fragment, also present in control cells at low intensity, probably represent the spontaneous degradation product of PARP recognized by this antibody (20). That the observed cleavage of PARP was due to the activation of caspase pathway, and not due to necrosis, was further confirmed by pre-treating the HTB-94 cells with the pan-caspase inhibitor Z-VAD-FMK. The results (Fig. 2B) showed that pre-treatment with the pan-caspase inhibitor blocked PARP cleavage. In some experiments leupeptin, a tripeptide aldehyde LLR-CHO, was used as a specificity control. It did not induce PARP cleavage (Fig. 2B). Similar results were obtained when DEVD-CHO, a caspase-3-specific inhibitor, was used in place of Z-VAD-FMK (results not shown). Trypan blue assay of cells treated with caspase inhibitors and EGCG showed no difference in the number of necrotic cells

FIG. 3. EGCG treated chondrosarcoma cells HTB-94 had high activity levels of caspase-3/CPP32-like proteases. HTB-94 cells (2×10^6) were left untreated or treated with 5 μM of EGCG as described under Materials and Methods. Nuclei free cell lysates were prepared from each sample and used in the caspase-3 activity assay using the colorimetric substrate DEVD-pNA. Absorbance was read at 405 nm. One unit of caspase activity is the amount of enzyme activity liberating 1 pmol of pNA/min. Values shown are means (±SD) of three independent experiments performed in triplicate.

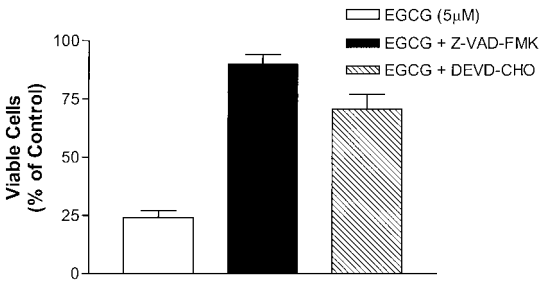

FIG. 4. Caspase inhibitors blocked the effect of EGCG on the viability of HTB-94 cells. Chondrosarcoma cells HTB-94 (1×10^4/100 μl) were pre-treated for 2 h with caspase inhibitors prior to the addition of EGCG as described above. Cell viability was then determined by the MTT assay. Results shown are means (±SD) of triplicate assays.

(results not shown) suggesting that treatment with EGCG did not induce necrosis of HTB-94 cells.

The activity of caspase-3-like proteases in EGCG treated and untreated HTB-94 cells was also measured. These results (Fig. 3) showed that cells treated with EGCG had significantly higher activity of caspase-3/CPP32 indicating that activation of caspase-3/CPP32-like proteases was associated with reduced cell survival and apoptotic death of EGCG-treated HTB-94 cells. Thus, the effect of EGCG on cell viability could be blocked by inhibiting the activation of caspase-3/CPP32-like proteases. To test this hypothesis, we pre-treated the HTB-94 cells with the cell permeable inhibitor DEVD-CHO followed by EGCG for 24 h. Thereafter, the cell viability was determined as described above. As shown in Fig. 4, the presence of DEVD-CHO blocked the effect of EGCG on the viability of HTB-94 cells. Under these conditions, no PARP cleavage was evident (data not shown). Taken together, these results show for the first time that EGCG induced apoptosis in HTB-94 cells and that the induction of apoptosis was dependent on the activation of caspase-3/CPP32-like proteases.

DISCUSSION

Several studies in the past have shown growth inhibitory properties of green tea and green tea polyphenols (21). To our knowledge, this is the first study which investigated the effects of EGCG on the proliferation of human chondrosarcoma cells HTB-94 and to demonstrate that EGCG mediated apoptosis involved the activation of caspase-3/CPP32-like proteases. Our data clearly show that EGCG was highly effective at inhibiting the growth of this cell line *in vitro* and indicate that this inhibition was possibly achieved via apoptosis of HTB-94 cells. We further show that EGCG-mediated apoptosis in HTB-94 cells was caspase-dependent as PARP cleavage was inhibited by caspase inhibitors (Fig. 2). Multiple lines of evidence implicate capase-3 (CPP32/Yama/Apopain) as an executioner caspase. In many biological systems caspase-3 cleaves intracellular proteins vital to cell survival and growth such as poly(adenosine diphosphate ribose)-polymerase (PARP) and inhibition of caspase like proteases prevents apoptosis (22).

Apoptosis is the most common and distinct form of cell death involving a series of steps and acts as physiological suicide mechanism to preserve tissue homeostasis through proper cell turnover. There is ample evidence that naturally occurring compounds with anti-tumor effects can trigger the apoptosis of cancer cells. Indeed, induction of apoptosis has been speculated to be the mechanism of action of green tea polyphenols for the inhibition of several cancer cell lines such as HL-60 cells, A431 cells and prostate cancer cells (15). The growth inhibitory effect of EGCG was dose-dependent and even low concentrations were effective in inhibiting the proliferation of HTB-94 cells. Our results further indicate that the induction of apoptosis in HTB-94 cells involved the induction and activation of caspase-3/CPP-32-like proteases as EGCG treated cells showed high levels of caspase-3/CPP32-like protease activity. These results also suggest that apoptosis induced by different agents (22, 23) may share the common downstream pathway and effector molecules.

In conclusion, the two novel findings of this study are that (1) EGCG is a potent inducer of apoptosis in human chondrosarcoma cells HTB-94; and (2) that the induction of EGCG-mediated apoptosis was dependent on the activation of caspase-3/CPP32-like proteases. Tea polyphenols are generally recognized to be antioxidant (15), but they can be pro-oxidative and it is possible that the observed apoptosis in HTB-94 cells occurred via this pathway. Indeed, EGCG can induce the production of H_2O_2 in cancer cells (11). Thus, it is possible that the activation of caspase-3/CPP32-like proteases in EGCG treated HTB-94 cells may be due to a rise in the cellular load of peroxides. Another possibility is that the treatment with EGCG degrades or inactivates caspase inhibitor(s) present in chondrosarcoma cells resulting in an increased rate of production of active caspases. It is also possible that EGCG might affect the mitochondrial integrity resulting in the release of cytochrome-c. Which mechanism predominated in EGCG treated HTB-94 cells is not yet known and remains to be elucidated. Nevertheless, based on our findings EGCG or a derivative of EGCG could prove to be useful in the therapy of human chondrosarcoma.

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