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Differentiation-associated alteration in sensitivity to apoptosis induced by (–)-epigallocatechin-3-*O*-gallate in HL-60 cells

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ABSTRACT

Green tea and its constituent (–)-epigallocatechin-3-*O*-gallate (EGCG) are known to have apoptosis-inducing activity on tumor cells including human leukemia HL-60 cells, providing an explanation for their anti-cancer effects. In the present study, we compared the sensitivity of undifferentiated cells and differentiated HL-60 cells with normal-like phenotypic characters. HL-60 cells treated with three differentiating agents were found to be resistant to EGCG-mediated apoptosis as compared with undifferentiated cells. Gene and protein expression of 67 kDa laminin receptor was down-regulated in differentiated HL-60 cells, suggesting its contribution to the difference in sensitivity in view of the fact that the receptor is a target of EGCG's action to induce apoptosis. The finding supports the view that EGCG induces apoptosis preferentially in cancer cells as compared with normal counterparts.

Necrosis and apoptosis are two distinct forms of cell death. Necrosis is a degenerative phenomenon that follows irreversible injury, while apoptosis is an active self-destructive process requiring protein synthesis for its execution (25, 27). Apoptosis involves condensation of the nuclear chromatin and cytoplasm, fragmentation of the nucleus, and blebbing of the whole cell to produce membrane-bounded bodies in which organelles are initially intact (18, 25, 27). There is a distinctive internucleosome cleavage of DNA in apoptosis (6, 16), which is quite different from the random DNA degradation observed in necrosis. Several anti-cancer drugs have been reported to induce apoptosis in cancer cells and apoptosis is believed to be a primary mechanism of their anti-cancer activity (6, 19). Therefore, any compound with the ability to induce apoptosis in cancer cells is potentially a chemopreventive agent. In 1996, Hibasami *et al*. (7) demonstrated the first evidence that catechins such as (–)-epigallocatechin-3-*O*-gallate (EGCG) induce programmed cell death or apoptosis. Since then, many studies have shown that catechins induce apoptosis in a variety of cancer cells including human leukemia HL-60 cells (2, 9, 14, 28).

It has been shown that EGCG's effects on cell growth differ between tumor cells and normal counterparts. For example, in a study comparing the effect of EGCG on the growth of SV40 virally transformed WI-38 human fibroblasts (WI-38VA) with that of normal WI-38 cells, the IC_{50} values of EGCG were shown to be 120 and 10 μM for WI-38 and WI-38VA cells, respectively (3). Employing human melanoma cell lines (A-375 amelanotic malignant melanoma and Hs-294T metastatic melanoma cells) and normal human epidermal melanocytes, it was demonstrated that EGCG caused a dose-dependent decrease in the viability and growth of both melanoma cell lines, whereas normal melanocytes were not affected (12).

The cell line HL-60 was established from a patient with promyelocytic leukemia and is known to

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respond to a variety of inducing agents by ceasing division and acquiring some of the characteristics of either granulocytes or monocytes (15). *all-trans*-retinoic acid (ATRA) and dimethylsulfoxide cause neutrophilic differentiation, while 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and 1α,25-dihydroxyvitamin D3 (DVD) induce monocytic differentiation in these cells (10, 11). It has been shown that DVD induces a differentiation program in HL-60 cells that in many ways resembles that of normal monocytic cells (15).

We were interested in knowing whether differentiated HL-60 cells have a different sensitivity to EGCG-mediated apoptosis. In the present study, we induced differentiation in HL-60 cells with ATRA, TPA, and DVD, and compared induction of apoptosis with EGCG between undifferentiated and differentiated cells.

MATERIALS AND METHODS

Cells and treatments. Human HL-60 cells were obtained from the Riken Cell Bank, Tsukuba, Ibaraki, Japan. HL-60 cells were cultured in 10% fetal bovine serum in RPMI 1640 medium containing 50 U/mL penicillin, 50 μg/mL streptomycin, and 2.5 μg/mL amphotericin B at 37°C under a 5% CO_2 atmosphere. HL-60 cells were treated with 1 μM ATRA (Wako Pure Chemical Industries, Ltd. Osaka, Japan) for 3 days, 0.5 nM TPA (Wako Pure Chemical Industries) for 1 day, or 100 nM DVD for 3 days (20–22) to the treatment with EGCG (Funakoshi Co. Ltd., Tokyo). The differentiated cells were then incubated with EGCG at various concentrations in the cell culture medium at 37°C for 24 h in the presence of each differentiation inducer, and compared with the results from undifferentiated cells. For detection of apoptotic bodies, HL-60 cells were incubated with 50 μM EGCG for 4 h and observed under a phase-contrast microscope as described previously (26).

DNA fragmentation. DNA was isolated from HL-60 cells incubated with EGCG at various concentrations at 37°C for 24 h and electrophoresed in 2% agarose gel. After staining with SYBR Green I (Molecular Probes, Inc., OR, USA), degraded DNA was imaged using FluoroImager (Molecular Dynamics, Japan, Inc., Tokyo) as described previously (26). For confirmation of caspase-dependent DNA ladder formation, the pan-caspase inhibitor benzyloxycarbonyl-L-aspar-1-tyl)[(2,6-dichlorobenzoyl]oxy]methane (Z-Asp-CH_2-DCB, Peptide Institute, Inc., Osaka) was added in the culture medium and incubated as described above (26).

Cell viability. A Trypan blue dye exclusion assay was utilized to determine the cell viability as described previously (8).

Quantitative reverse transcription polymerase chain reaction (Q-PCR). We examined gene expression of 67 kDa laminin receptor (67LR), a receptor for EGCG (23), by Q-PCR. Total RNA was extracted from cells and mRNA was prepared using a QIAamp RNA Blood Mini Kit (Qiagen Ltd., Tokyo, Japan) according to the manufacturer's directions. To prevent possible contamination, samples were treated with deoxyribonuclease (RT-grade, Wako Pure Chemical Industries Ltd.) as recommended by the manufacturer. Q-PCR was performed using the Thermal Cycler Dice (TaKaRa Bio., Tokyo, Japan) as described previously (22). Primers for 67LR designed using a software OLIGO4.0-s were 5'-GC CATTGAAAACCCTGCTG-3' and 5'-GCTGCCTG GATCTGGTTAGTG-3' (GenBank accession no. NM 002295.4), and obtained from Sigma-Aldrich Japan (Tokyo, Japan). Primers for β-actin, 5'-TG GCACCCAGCACAATGAA-3' and 5'-CTAAGT CATAGTCCGCCTAGAAGCA-3', were purchased from TaKaRa Bio.

Western blotting. Total proteins were extracted from undifferentiated HL-60 cells and those treated with ATRA for 3 days, TPA for 1 day, or DVD for 3 days using the lysis buffer, pH 7.4 containing 10 mM Tris-HCl, 1 mM sodium orthovanadate, and 1% sodium dodecylsulfate (SDS). The lysates were heated at 100°C for 3 min and sonicated for 30 s. After SDS-polyacrylamide gel electrophoresis, blotted proteins on nitrocellulose membrane were probed with goat anti-67LR antibody (F18) (24) which recognizes the 37 kDa precursor protein of 67LR (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-β-actin IgG (Affinity BioReagents, CO, USA), horseradish peroxidase-conjugated rabbit anti-goat IgG (Santa Cruz Biotechnology), and swine horseradish peroxidase-conjugated anti-rabbit IgG (DAKO A/S, Denmark). Chemiluminescence was detected using ECL plus Western Blotting Detection System (GE Healthcare Biosciences, Tokyo) according to the directions provided by the manufacturer and visualized using as described previously (20).

RESULTS

EGCG-mediated apoptosis

When undifferentiated HL-60 cells were treated with 50 μM EGCG for 4 h, microscopic observation showed formation of apoptotic bodies, suggesting that apoptosis was induced by EGCG (Fig. 1). Induction of apoptosis by EGCG was further demonstrated by concentration-dependent formation of DNA ladder (Fig. 2). Further support was provided by the fact that the caspase inhibitor Z-Asp-CH_2-DCB blocked the degradation of DNA in the presence of EGCG (Fig. 2). These results clearly show that EGCG induces apoptosis in undifferentiated HL-60 cells.

Sensitivity to EGCG-mediated apoptosis in differentiated cells

To test the possibility that sensitivity to EGCG-mediated apoptosis of HL-60 cells might change after differentiation, HL-60 cells were treated with ATRA, TPA or DVD. The differentiated cells were then incubated with EGCG at various concentrations for 24 h and cell viability was determined (Fig. 3). The IC_{50} value was 52, 68, and 88 μM for DVD, ATRA, and TPA, respectively. The results showed that all of three differentiation inducers lowered sensitivity to EGCG-mediated apoptosis as compared with the undifferentiated cells for which IC_{50} was 8 μM.

Expression of 67LR

The 67LR protein is a cellular receptor for cell adhesive protein laminin and a 37 kDa protein is the precursor of the receptor, although the exact manner by which it configures its mature form is not clear (4). Previous studies have shown that the cell surface 67LR is the target for EGCG and acts as the receptor for anti-tumor action of EGCG (23). To examine the possibility that differentiation may be accompanied by a change in 67LR expression, the gene expression levels were determined by Q-PCR. The results indicated that the gene expression of 67LR was decreased in differentiated HL-60 cells (Fig. 4).

To confirm the differentiation-associated down-regulation at a protein level, Western blotting was performed. Since the antibodies against 67LR are not commercially available at present, we used the antibody F-18 which recognizes the 37 kDa precursor protein of 67LR according to the previous report (23). The results indicated the decreased protein expression of the precursor protein of 67LR after differentiation (Fig. 5). The protein expression appeared to be most markedly suppressed in the DVD-treated cells, followed by the ATRA-treated cells.

DISCUSSION

It has been well documented that EGCG induces apoptosis in a variety of cancer cells including HL-60 cells (2, 9, 14, 18). In the present study, we confirmed these previous findings. EGCG induced formation of apoptotic bodies (Fig. 1) and DNA ladder (Fig. 2) representing characteristic features of apoptosis (6, 16, 18). Administration of the caspase inhibitor blocked the DNA fragmentation, confirming EGCG-mediated apoptosis in these cells (26). *In vivo* experiments also have shown that green tea polyphenols containing EGCG exhibited anti-tumor activity through induction of apoptosis (5, 13). These findings support the view that green tea ingestion is beneficial for preventing certain cancers.

Previously, to investigate the different sensitivity

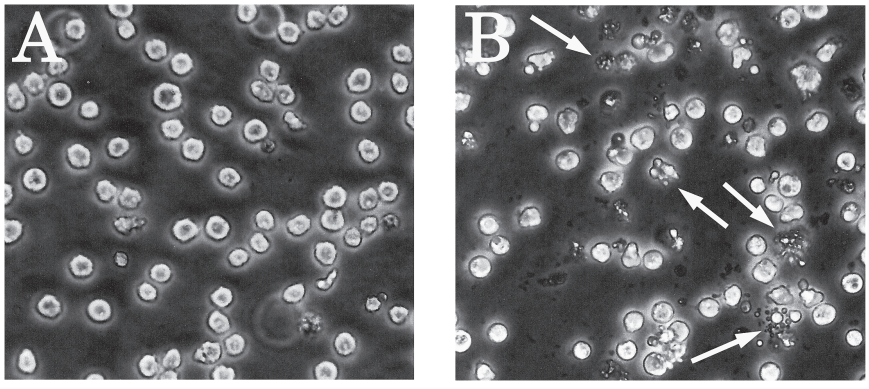

Fig. 1 Morphological differences between control HL-60 cells (**A**) and the cells treated with EGCG at 50 μM for 4 h (**B**). Apoptotic bodies are indicated by arrows.

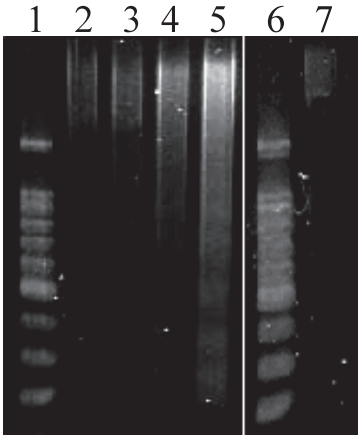

Fig. 2 Fragmentation of DNA from HL-60 cells treated with EGCG. HL-60 cells were incubated at 37°C for 24 h with EGCG at various concentrations. DNA isolated from the cell pellets was then electrophoresed in 2% agarose gel, stained with SYBR Green I, and imaged using FluoroImager. Lanes 1 and 6, DNA size markers; lane 2, untreated cells; lanes 3–5, cells treated with EGCG at 6.25, 12.5, and 25 μM, respectively. The fragmentation of DNA of the cells treated with 100 μM EGCG in the presence of the caspase inhibitor Z-Asp-CH_2-DCB at 200 μM was also examined (lane 7).

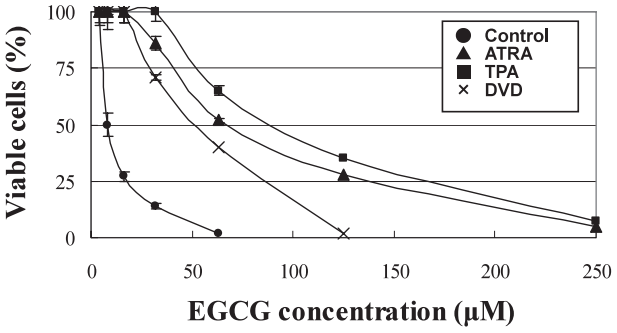

Fig. 3 Effects of EGCG on differentiated HL-60 cells. The cells treated with 1 μM ATRA (▲) for 3 days, 0.5 nM TPA (■) for 1 day, or 100 nM DVD (×) for 3 days were incubated with EGCG at various concentrations for 24 h in the presence of the respective differentiation inducer. Viable cells were determined by the Trypan blue dye exclusion assay. The results from three determinations are expressed as means ± SD relative to the value for the cells left untreated with EGCG (100%).

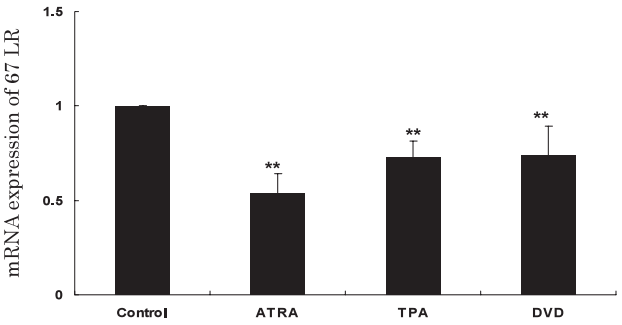

Fig. 4 67LR gene expression in differentiated HL-60 cells. The cells were incubated with 1 μM ATRA for 3 days, 0.5 nM TPA for 1 day or 100 nM DVD for 3 days. The gene expression level of 67LR was determined by Q-PCR and normalized using that of β-actin. Results are expressed as means ± SD relative to the level in undifferentiated cells (control) from four determinations. The statistical significance of the difference was determined by Student's *t*-test. $^{**}P < 0.01$.

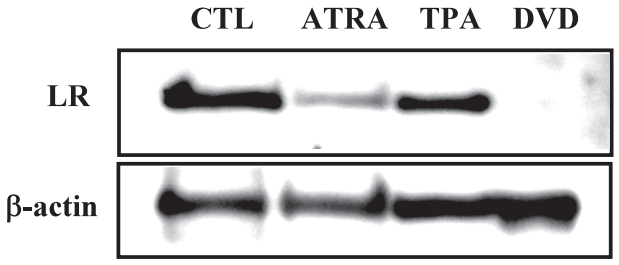

Fig. 5 67LR protein expression in differentiated and undifferentiated HL-60 cells. Lysates from untreated HL-60 cells (CTL) and the cells treated with 1 μM ATRA for 3 days (ATRA), 0.5 nM TPA for 1 day (TPA), or 100 nM DVD for 3 days (DVD) were subjected to SDS-polyacrylamide gel electrophoresis, and the blotted membrane was probed with anti-67LR and anti-β-actin. LR, the precursor protein of 67LR.

to apoptosis between tumor and normal cells in the same cell type, virus-transformed cells (WI-38 VA) and normal counterparts (WI-38) were compared (3). In the present study, we examined the sensitivity to EGCG-mediated apoptosis in leukemia HL-60 cells before and after differentiation. Differentiated HL-60 cells are known to exhibit normal cell-like phenotype. DVD induces a differentiation program in HL-60 cells similar to that of normal monocytic cells (1). When HL-60 cells were treated with 0.1 nM TPA for 1 day or 100 nM DVD for 3 days, a number of the cells became attached to the culture flask (data not shown). We reported that DVD suppressed cell growth (21) and that DVD and ATRA caused the change in gene expression of proteins involved in nucleocytoplasmic transportation (22). Thus, the present conditions appear to be sufficient to induce differentiation in HL-60 cells. These differentiated cells were shown to be less sensitive to EGCG-mediated apoptosis, whenever the differentiation is monocytic or granulocytic lineage (Fig. 3).

To know the molecular basis for the difference in the sensitivity, we examined gene expression of 67LR, which has been suggested to be the target for

EGCG (23). The results indicated that its gene expression was down-regulated in differentiated cells as compared with non-differentiated cells (Fig. 4). Consistent with the finding, the result of immunoblotting showed that the precursor protein of 67LR was reduced upon differentiation (Fig. 5). When human lung cancer A549 cells were transfected with the gene encoding 67LR, they showed EGCG-mediated growth inhibition more efficiently than those transfected with empty vector, suggesting that the cell surface 67LR acts as the receptor for the antitumor action of EGCG (23). Shammas *et al*. (17) showed that 67LR levels were significantly elevated in myeloma cell lines and multiple myeloma patient samples relative to normal peripheral blood mononuclear cells. RNAi-mediated inhibition of 67LR resulted in abrogation of EGCG-induced apoptosis, indicating that 67LR plays an important role in mediating EGCG activity in myeloma cells.

Thus, we believe that differentiation-associated down-regulation of 67LR is involved, at least partly, in acquisition of resistance for EGCG-mediated apoptosis in differentiated HL-60 cells. However, it appeared that sensitivity to apoptosis did not necessarily parallel with the degree of gene and protein expression among HL-60 cells treated with the three differentiation inducers, suggesting that 67LR is not the sole factor involved in the change in the sensitivity.

The present findings provide a further support for the view that cancer cells are more susceptible to the action of EGCG as compared with the normal counterparts and that EGCG is useful as a chemopreventive agent by taking advantage of this difference.

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