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Anti-proliferative and differentiation-inducing activities of the green tea catechin epigallocatechin-3-gallate (EGCG) on the human eosinophilic leukemia EoL-1 cell line

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Abstract

A novel approach for the treatment of leukemia is the differentiation therapy in which immature leukemia cells are induced to attain a mature phenotype when exposed to differentiation inducers, either alone or in combinations with other chemotherapeutic or chemopreventive drugs. Over the past decade, numerous studies indicated that green tea catechins (GTC) could suppress the growth and induce apoptosis on a number of human cancer cell lines. However, the differentiation-inducing activity of GTC on human tumors remains poorly understood. In the present study, the effect of the major GTC epigallocatechin-3-gallate (EGCG) on the proliferation and differentiation of a human eosinophilc leukemic cell line, EoL-1, was examined. Our results showed that EGCG suppressed the proliferation of the EoL-1 cells in a dose-dependent manner, with an estimated IC_{50} value of 31.5 μM. On the other hand, EGCG at a concentration of 40 μM could trigger the EoL-1 cells to undergo morphological differentiation into mature eosinophil-like cells. Using RT-PCR and flow cytometry, it was found that EGCG upregulated the gene and protein expression of two eosinophil-specific granule proteins, the major basic protein (MBP) and eosinophil peroxidase (EPO), in EoL-1 cells. Taken together, our findings suggest that EGCG can exhibit anti-leukemic activity on a human eosinophilic cell line EoL-1 by suppressing the proliferation and by inducing the differentiation of the leukemia cells.
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Keywords: Differentiation; Green tea catechin; Epigallocatechin-3-gallate (EGCG); Human eosinophilic leukemia EoL-1 cells

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Introduction

Cancer has long been the number one killer in many parts of the world. Conventional cancer therapies such as surgery, chemotherapy and radiotherapy have a number of limitations such as relatively high cost, development of resistance and are often accompanied with many adverse side effects. Therefore, there is a pressing need to search for new drugs and to establish new therapeutic strategies that are safe and effective for cancer treatments. In recent years, a number of physiological compounds and natural products, including steroid hormones, vitamin analogs, cytokines and plant phytochemicals etc., have been identified to have the ability to induce differentiation, apoptosis and inhibition on the growth of certain cancer cells such as leukemia cells [1–5]. Differentiation therapy is a novel approach for the treatment of leukemia in which immature leukemia cells are induced to attain a mature phenotype when exposed to differentiation inducers, either alone or in combinations with other chemotherapeutic or chemopreventive drugs. One successful example is the use of all-trans-retinoic acid in the treatment of acute myeloid leukemia [6].

Green tea is a widely consumed beverage in Asian countries, especially in China and Japan. The polyphenolic compounds, which make up over 30% of the dry weight of green tea, are commonly known as flavan-3-ol or tea catechins. Over the past decade, numerous studies reported that green tea catechins (GTC) have significant anti-carcinogenic and anti-oxidative activities, which suggest that GTC may be a useful cancer chemopreventive agent in the human populations [7–9]. The biological activities of green tea are believed to be mediated by its major polyphenolic constituent, epigallocatechin gallate (EGCG). EGCG has been studied extensively due to its diverse physiological and pharmacological properties, including hypolipidemic, anti-inflammatory, anti-microbial, anti-oxidative, anti-carcinogenic and anti-tumor activities [10,11]. More recent studies indicated that EGCG could suppress the growth and induce apoptosis on a number of human cancers, including cancers of the breast, liver, lung, stomach, skin, colon, prostate gland and leukemia, both *in vitro* and *in vivo* [12–16]. Nevertheless, the differentiation-inducing activity of EGCG on human tumors remains poorly understood. In the present study, the effect of EGCG on the proliferation and differentiation of a human eosinophilc leukemic cell line, EoL-1, was examined. It was reported that EoL-1 cells can be induced to differentiate not only phenotypically but also functionally into mature eosinophil-like cells in response to a number of stimuli, such as cytokines [17], butyric acid [18] and dibutyryl cyclic AMP (dbcAMP) [19,20]. In the present study, the effect of EGCG on proliferation of the EoL-1 cells was investigated by the [^{3}H]-thymidine incorporation assay whereas the differentiation-inducing activity on EoL-1 cells was studied by observing the morphological change as well as the expression of the eosinophil-specific proteins, including the major basic protein (MBP) and eosinophil peroxidase (EPO), in EGCG-treated EoL-1 cells.

Materials and methods

Reagent

The green tea epicatechin isomer (–)-epigallocatechin-3-gallate (EGCG) with purity >95% was purchased from Sigma Chem. Co. (U.S.A.).

Cell culture

EoL-1 cells were obtained from the Riken Cell Bank (Tsukuba Science City, Japan) and they were maintained in RPMI 1640 medium (Gibco BRL, USA.) supplemented with 10% fetal bovine serum (Gibco BRL, USA) in 5% humidified air at 37°C.

[^{3}H]-Thymidine incorporation assay

The [^{3}H]-Thymidine (^{3}H-TdR) incorporation assay was used to measure the proliferation of leukemia cells as described previously [3]. EoL-1 cells in quadruplicates, with the indicated cell number, were incubated at 37°C in the absence or presence of various concentrations of EGCG for 48 h. The cells were then pulsed with 0.5 μCi of [^{3}H]-TdR (specific activity = 5 Ci/mmol, Amersham-Pharmacia Ltd., UK) in 20 μl complete medium for 6 h. After one freezing and thawing cycle, the cells were harvested onto a glass microfiber filter. The radioactivity, in counts per minute (cpm), was measured by the Beckman LS6000 liquid scintillation counter. The results were expressed as the percentage inhibition of [^{3}H]-TdR incorporation, using the untreated cells as a control [3]. The percentage inhibition of [^{3}H]-TdR incorporation was calculated as follows:

$$\%\ inhibition = \{(\text{cpm of control} - \text{cpm of test sample}) / \text{cpm of control}\} \times 100\%$$

All cell proliferation results were expressed as % inhibition and data represent the arithmetic mean ± standard error of quadruplicate determinations of three separate experiments performed under the same conditions. Student's "t" test was used to determine the confidence limits in group comparison, and $p < 0.05$ was regarded as significantly different.

Cell morphology study

The ability of EGCG to induce morphological differentiation of EoL-1 cells was determined as described previously [20]. EoL-1 cells (5×10^4 cells/ml) were incubated with EGCG (40 μM) for 9 days. Cell morphology was then examined by the preparation of cytospin smears. The EoL-1 cells (5×10^4 cells) were fixed onto a microscopic slide by cytocentrifugation at 500 rpm for 5 min using the Shandon Cytospin 3 centrifuge (Shandon Scientific Ltd., UK). The cells were allowed to be air-dried. Then they were stained with the Hemacolor staining solutions (Diagnostica Merck, USA) for 15 sec and destained under running tap water. Finally, the air-dried cells on the slides were mounted with neutral mounting medium, *Canada balsam* (Sigma Chemicals Co., USA) and the cell morphology was examined under the light microscope. The percentage of differentiated cells was determined by counting at least 200 cells as described previously [20].

RNA isolation

EoL-1 cells (5×10^4/ml) were exposed to EGCG (40 μM) for various time periods as indicated in the text. Total RNA was then extracted from EoL-1 cells using the TRIZOL reagent (Gibco BRL, USA) as described previously [21]. The RNA concentration was determined by spectrophotometry at 260 nm. The purity was estimated by the ratio of A_{260}/A_{280} nm and was found to be 1.7 to 2.0.

Reverse transcription-polymerase chain reaction (RT-PCR)

One μg of total RNA was reverse-transcribed in a 20 μL reaction mixture containing 200 U of MMLV reverse transcriptase (Gibco BRL, USA), 0.5 mM of each dNTP, 40 U of RNase inhibitor (Promega Corp., USA), 0.1 μg oligo-dT_{12-18}, 10 mM dithiothreitol (Gibco BRL, USA), 50 mM Tris/HCl pH 8.3, 3 mM magnesium chloride and 75 mM potassium chloride. The mixture was incubated at 37 °C for 60 min, then heated to 99 °C for 5 min and cooled to 4 °C for 5 min. The RT sample was used immediately or stored at − 20 °C.

The sets of specific primers for PCR amplifications of specific cDNAs were purchased from Gibco BRL, USA (Table 1). The primer sets for MBP, EPO and GAPDH were designed on the basis of previous published data (MBP and EPO [22], GAPDH (glyceraldehyde-phosphate dehydrogenase) [23]), and these oligonucleotides were derived from the sequences of the corresponding human genes and cDNA, with nucleotide positions as indicated. In addition, the primer sequences were chosen from separate exons of the genes so that the RT-PCR product could readily be distinguished from any genomic DNA-induced PCR product. Each primer was diluted to 2.5 μM and stored at − 20 °C until use.

PCR was performed in a total volume of 25 μL consisting of 2 μL of RT sample (equivalent to 100 ng of total RNA), 1 U of Thermoprime^{plus} DNA polymerase (Advanced Biotechnologies), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 μM of both sense and antisense oligonucleotides, 20 mM ammonium sulphate, 0.01% (w/v) Tween 20, 75 mM Tris/HCl pH 9.0 and 50 mM potassium chloride in a GeneAmp PCR System 9700 programmable thermal controller (Perkin-Elmer). The PCR condition was 94 °C for 5 min, then 22–35 cycles of 94 °C for 0.5 min, 59–61 °C for 1 min to 1 min and 15 sec, 72 °C for 1 min, followed by a final extension of 5 min at 72 °C. Besides, a negative control with no cDNA template was included to detect the presence of any contaminant in the PCR process.

A 5 μL-aliquot of PCR products was separated on a 2% agarose gel, stained with ethidium bromide and photographed under UV fluorescence, and the photographs were analysed by the ImageQuant (Microsoft) software on a densitometry system. The relative intensity of each band of the RT-PCR product was normalized after dividing by the relative intensity of the corresponding band of GAPDH, and the relative intensity of each GAPDH band in the experimental group was expressed as the fraction of the control GAPDH band which was taken as unity. Differences of relative intensity larger than 100% were considered as significant changes and less than 50% were regarded as non-significant variations. Differences of relative intensity between 50 to 100% were defined as slight changes in the present study [21].

Table 1
Primers used in RT-PCR and the predicted sizes of the PCR products

cDNA amplified	Primer sequences (5′ → 3′)	Size of amplified PCR product (bp)
MBP	Sense strand: AGT GGT GGG CAT CCC TGG	304
	Antisense strand: GGC CAC GCA GTG ACC ACC	
EPO	Sense strand: GCA TCT GCT CCC AGC CCT	669
	Antisense strand: GAA GGG TCC GGA CCG CTG	
GAPDH	Sense strand: TGA AGG TCG GAG TCA ACG GAT TTG GT	983
	Antisense strand: CAT GTG GGC CAT GAG GTC CAC CAC	

Flow cytometry of intracellular MBP and EPO

EoL-1 cells (5×10^4/ml) were incubated with EGCG (40 μM) for various time periods. They were then fixed with 4% paraformaldhyde in PBS for 30 min and then permeabilized with 0.2% Triton-X 100 in PBS for 10 min. The cells were first incubated with normal mouse IgG (Sigma Chemical Co., USA) (1 mg/ml, diluted 1:100) for 30 min before staining with mouse anti-human MBP or EPO monoclonal antibodies (Pharmingen Inc., USA) (0.5 mg/ml, diluted 1:200) for 2 h, and followed by incubation with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (Sigma Chemical Co., USA) (1 mg/ml, diluted 1:250) for 1 h. The stained cells were analyzed by flow cytometry (FACSORT flow cytometer, Becton Dickinson, CA, USA). A total of 10,000 events (gated to exclude nonviable cells) were collected in the log mode and expressed as histograms of relative fluorescence intensity.

Results

The anti-proliferative effect of EGCG on the human eosinophilic leukemia EoL-1 cells

The eosinophilic leukemia EoL-1 cells were incubated with different concentrations of the green tea epicatechin EGCG for 48 h and the proliferation of cells was then measured by the ^{3}H-TdR incorporation assay. As shown in Fig. 1, EGCG could significantly inhibit the proliferation of EoL-1 cells in a dose-dependent manner. The 50% inhibitory concentration (IC_{50}) of EGCG on EoL-1 cells was estimated to be 31.5 μM and up to ~ 80% inhibition could be observed at a concentration of 40 μM. It should be noted that the observed growth-inhibitory effect of EGCG on EoL-1 cells cannot be attributed directly or solely to the cytotoxic effect of EGCG on the leukemia cells, as our results showed that EGCG exhibited little, if any, cytotoxicity on EoL-1 cells at or below 40 μM concentrations, as measured by the trypan blue exclusion test (data not shown).

Morphological differentiation of EoL-1 cells induced by EGCG

One way that leads to the suppression of cell growth is the induction of cellular differentiation. As shown in Fig. 2, EoL-1 cells treated with EGCG (40 μM) for 9 days were found to contain more refractile vacuoles than control cells. These results suggested that the granule matrix was altered leading to the light microscope appearance of small refractile vacuoles. The percentage of differentiated cells containing refractile vacuoles was found to increase from 8 to 35% upon treatment with EGCG (Fig. 2). The ability of EGCG to induce morphological changes in EoL-1 cells was also found to be dose-dependent (the % of differentiated cells at 30 μM and 20 μM of EGCG are 21% and 12.7% respectively).

Effects of EGCG on the gene expression of MBP and EPO in EoL-1 cells

In order to confirm the induction of eosinophilic differentiation of EoL-1 cells by EGCG, the expression of the mature eosinophil markers MBP and EPO genes in the EGCG-treated cells were studied. EoL-1 cells were harvested on different days after the treatment with 40 μM EGCG, and the gene expression of MBP and EPO was detected by RT-PCR. Fig. 3 showed that untreated EoL-1 cells

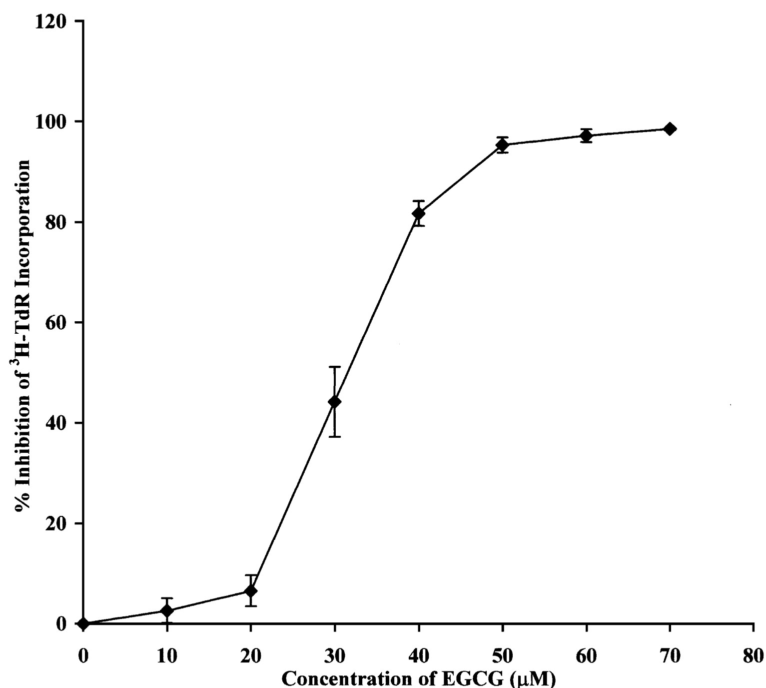

Fig. 1. Anti-proliferative effect of the green tea epicatechin EGCG on the human eosinophilic leukemia EoL-1 cells. EoL-1 cells (5×10^4 cells/ml) were incubated with various concentrations (0-70 μM) of EGCG at 37 °C for 48 h. Cultures were then pulsed with 0.5 μCi of ^{3}H-TdR for 8 h before harvest. Radioactivity in counts per minute (cpm) was measured using a liquid scintillation counter. Results were expressed as % inhibition of ^{3}H-TdR incorporation, using the untreated cells as a control. Each point represents the mean ± S.E. of quadruplicate cultures.

expressed relatively low levels of the two eosinophil granule protein mRNAs and the levels were up-regulated by EGCG treatment. It was found that the level of MBP gene expression was gradually increased from day 1 to day 5 in EGCG-treated EoL-1 cells and the expression remained high afterwards. On the other hand, the gene expression of EPO was increased to a maximum in EoL-1 cells exposed to EGCG for one day (a 3.5-fold increase) and the level remained high at day 3–5 but declined thereafter.

Effects of EGCG on the intracellular protein expression of MBP and EPO in EoL-1 cells

To examine whether there is any enhancement in the expression of the eosinophil-specific proteins in response to EGCG, the effects of EGCG on the intracellular protein expression of MBP and EPO in EoL-1 cells were measured by flow cytometry, using MBP and EPO-specific monoclonal antibodies. Fig. 4 showed that there was very little, if any, elevation of the levels of MBP and EPO protein at day 5 after 40 μM of EGCG treatment. A more significant increase in the expression of MBP and EPO protein could be observed at day 7 and 9 after EGCG treatment. These findings are in agreement with the increased expression of the MBP and EPO genes in response to EGCG treatment in EoL-1 cells (Fig. 3), however, in case of EPO, the mRNA expression did not parallel with the protein expression as they have

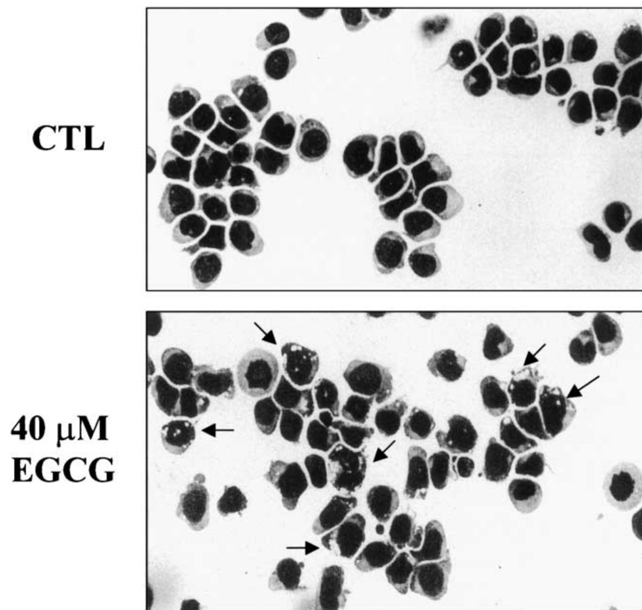

Fig. 2. Effect of EGCG on the morphological differentiation of EoL-1 cells. EoL-1 cells (5×10^4 cells/ml) were treated with EGCG (40 μM) for 9 days and the untreated cells served as the control (CTL). The cells were cytocentrifuged onto the microscopic slides and stained with Hemacolor staining solutions. Arrows indicate the differentiated cells (Magnification $\times$ 400).

slightly different kinetics of expression. Taken together, a nine-day treatment of EoL-1 cells with 40 μM EGCG could result in significant morphological changes and an induction of the eosinophil-specific proteins MBP and EPO in EoL-1 cells.

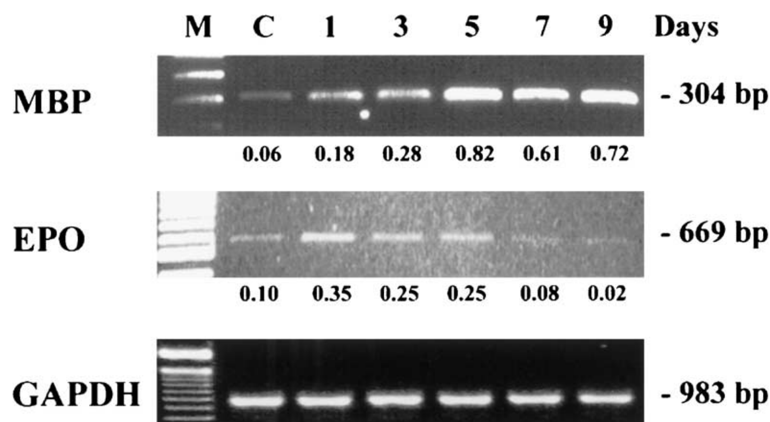

Fig. 3. Time course experiment of the effect of EGCG on the levels of MBP, EPO and GAPDH mRNAs in EoL-1 cells. EoL-1 cells (5×10^4 cells/ml) were treated with 40 μM EGCG for 1 to 9 days and the untreated cells were used as the control (C). Total RNA was isolated and subjected to RT-PCR. The 100 base-pair DNA markers (M) were run on the same gel and the sizes of the PCR bands were indicated on the right (bp).

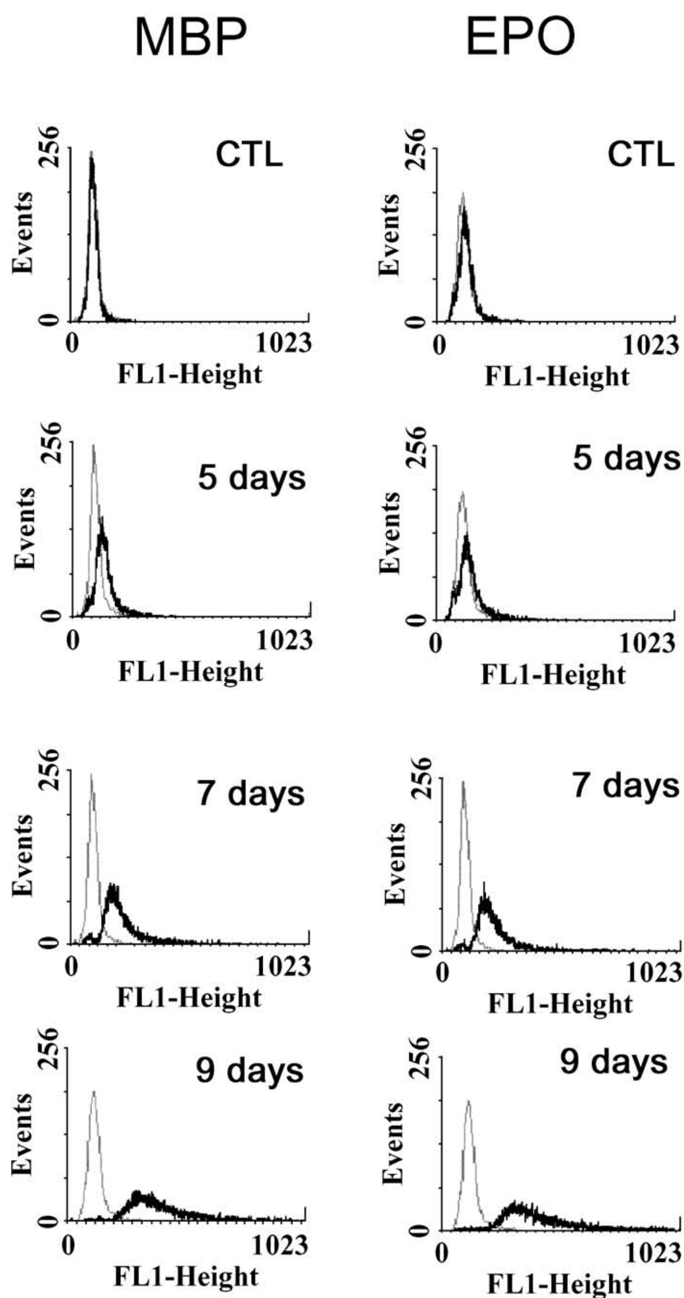

Fig. 4. The effects of EGCG on the expression of the intracellular MBP and EPO in EoL-1 cells as detected by flow cytometry. EoL-1 cells (5×10^4 cells/ml) were treated with 40 μM EGCG for 5, 7 and 9 days and the untreated cells were used as the control (CTL). Cells were then incubated with either mouse IgG isotype (grey profile), mouse anti-human MBP or EPO monoclonal antibody (black profile). The expression level of MBP and EPO was determined by the enhanced fluorescence following incubation with the mouse anti-human monoclonal antibody compared with cells incubated with mouse IgG isotype.

Discussion

A marked accumulation of eosinophils occurs in several important disorders such as allergic diseases, parasitic infections, and cancers. Abnormal morphologic features of eosinophils, an increase in immature cells in the bone marrow or blood, or a karyotypic abnormality indicates the presence of eosinophilic leukemia [24]. The EoL-1 cell line is a well established human eosinophilic leukemia cell line which has been commonly used as an *in vitro* model for the studies of human eosinophil functions and their regulation, and is particularly useful for analyzing leukemic cell differentiation [25]. It has been demonstrated that EoL-1 cells can be induced to differentiate into mature, eosinophilic granule-containing cells in response to a number of diverse stimuli, including cytokines such as granulocyte colony stimulating factor (G-CSF) and tumor necrosis factor-α (TNF-α) [17], butyric acid [18], dbcAMP [19,20], prostaglandin E2 and forskolin [19]. In the present study, we investigated the anti-proliferative and differentiation-inducing activities of the major green tea catechin EGCG on the human eosinophilic leukemic EoL-1 cells. From the results of the ^{3}H-TdR incorporation assay, we found that the estimated IC_{50} value for EGCG on EoL-1 cells was 31.5 μM. Interestingly, the observed growth-inhibitory effect of EGCG on EoL-1 leukemia cells cannot be attributed directly or solely to the cytotoxic effect of the green tea catechin on the leukemia cells, as our results showed that EGCG exhibited little, if any, cytotoxicity on EoL-1 cells at or below 40 μM concentrations. The underlying mechanisms for the growth-inhibitory effect of EGCG on EoL-1 leukemia cells remain obscure. The earlier work of Zhen *et al.* showed that green tea extract was an active nucleoside transport inhibitor which could prevent the supply of nucleosides for DNA synthesis [26]. Others have shown that EGCG can cause cell cycle arrest and induce apoptosis in a number of human carcinoma cell lines [27]. More recently, Liang *et al.* demonstrated that EGCG could inhibit the activities of cyclin-dependent kinases (cdk2 and 4) as well as inducing the expression of Cdk inhibitor p21 and p27 proteins during growth arrest of the human breast carcinoma cells [28]. However, our preliminary work showed that EGCG failed to induce apoptosis in the EoL-1 cells under similar experimental conditions, as judged by the failure of EGCG to induce DNA fragmentation and by morphologic criteria (data not shown). Despite of these findings, the molecular mechanisms by which EGCG could exert its anti-proliferative effect on EoL-1 leukemia cells remain to be further elucidated.

Previous works in our laboratory have demonstrated that plant flavonoids such as biochanin A could exhibit potent differentiation-inducing activity on myeloid leukemia *in vitro* [29]. It is of interest to examine whether the green tea epicatechin can also induce cellular differentiation of the eosinophilic leukemia EoL-1 cells. Our results showed that 40 μM of EGCG could trigger the EoL-1 cells to undergo morphological differentiation *in vitro*. Whether EGCG can exhibit differentiation-inducing activity *in vivo* has yet to be determined. Nevertheless, our results are in line with others who recently showed that plant-derived polyphenols are capable of inducing or enhancing the differentiation of myeloid leukemia cells. For example, Rodrigue *et al.* showed that resveratrol, a polyphenolic compound found in grape juice and red wine, could induce the differentiation of the human erythroleukemic K562 cells [30]. Moreover, it has been reported that carnosic acid, an antioxidant polyphenol derived from the plant rosemary, could enhance the effects of 1α,25-dihydroxy-vitamin D3 and all-*trans*-retinoic acid on the differentiation of the human promyelocytic leukemia HL-60 cells [31]. To further confirm the morphological differentiation of EoL-1 cells induced by EGCG, the effects of EGCG on the gene expression of two eosinophil-specific granule proteins (MBP and EPO) were determined since eosinophils have the capacity to secrete a number of potent inflammatory mediators. The eosinophil-

specific MBP and EPO proteins were shown to be strong agonists for platelet activation as well as inducing the activation of mast cells, basophils and neutrophils [32]. In the present study, we found that the mRNA levels of MBP and EPO were significantly up-regulated by EGCG treatment. In addition, the intracellular protein expression of MBP and EPO was also upregulated by 40 μM EGCG in the EoL-1 cells after 9 days of culture. To our knowledge, this is the first report showing that EGCG, a plant flavonoid derived from green tea, can trigger eosinophilic differentiation of myeloid leukemia cells. Whether EGCG can induce monocytic or granulocytic differentiation of myeloid leukemia cells is an intriguing aspect that awaits further investigation.

Although in this study we showed that the green tea epicatechin EGCG could exhibit anti-proliferative and differentiation-inducing activities on a human eosinophilic leukemic cell line, however, the signaling pathways by which EGCG could exert its effects on the EoL-1 cells remain obscure. It had previously been reported that EoL-1 cells could be induced to differentiate into mature eosinophils by a 9-day treatment with dbcAMP [19,20]. dbcAMP will be metabolized into butyrate and cAMP in cells and it has been suggested that cAMP may be primarily responsible for the differentiation of EoL-1 cells into eosinophilic granule-containing cells [19]. In the current study, whether the EGCG-induced differentiation of EoL-1 may be mediated through the second messenger such as cAMP and the activation of protein kinase A has yet to be determined. On the other hand, since protein kinase C isozymes are known to play important roles in many cellular events that include cell growth and differentiation [33], and the PKC activator phorbol 12-myristate 13-acetate was found to up-regulate the expression of CD11b in EoL-1 cells [34], therefore, the possible involvement of PKC in the EGCG-induced EoL-1 differentiation is also worthy of future investigation.

Acknowledgements

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