Environ Health Prev Med (2012) 17:246–251
DOI 10.1007/s12199-011-0239-5

SHORT COMMUNICATION

Epigallocatechin gallate inhibits sphere formation of neuroblastoma BE(2)-C cells

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Received: 9 June 2011 / Accepted: 16 August 2011 / Published online: 10 September 2011

Abstract
Objectives A growing number of epidemiological studies have demonstrated that the consumption of green tea inhibits the growth of a variety of cancers. Epigallocatechin gallate (EGCG), the most abundant catechin in green tea, has been shown to have an anti-cancer effect against many cancers. Most cancers are believed to be initiated from and maintained by a small population of tumor-initiating cells (TICs) that are responsible for chemotherapeutic resistance and tumor relapse. In neuroblastoma, an aggressive pediatric tumor that often relapses and has a poor prognosis, TICs were recently identified as spheres grown in a serum-free non-adherent culture used for neural crest stem cell growth. Although EGCG has been reported to induce growth arrest and apoptosis in neuroblastoma cells, its effect on neuroblastoma TICs remains to be defined.
Methods Gene expression was analyzed by real-time reverse transcription polymerase chain reaction (RT-PCR). The effects of EGCG on cell proliferation, apoptosis, and sphere formation were determined by cell counting, propidium iodide staining, and sphere (>100 μm in diameter) counting, respectively.
Results Neuroblastoma BE(2)-C cells showed increased expression of stem cell markers (nanog homeobox [NANOG] and octamer-binding transcription factor 4 [OCT4]), as well as decreased expression of neuronal differentiation markers (Cu^{2+}-transporting ATPase alpha polypeptide [ATP7A] and dickkopf homolog 2 [DKK2]) in spheres grown in serum-free non-adherent culture, compared to parental cells grown in conventional culture. Although EGCG induced growth arrest and apoptosis in the parental cells in a dose-dependent manner, it was not effective against spheres. However, EGCG potently inhibited sphere formation in the BE(2)-C cells.
Conclusions The present results suggest that EGCG may inhibit the development of TICs in BE(2)-C cells.

Keywords Epigallocatechin gallate · Neuroblastoma · Tumor-initiating cell · Sphere

Abbreviations

EGCG	Epigallocatechin gallate
TIC	Tumor-initiating cell
NANOG	Nanog homeobox
OCT4	Octamer-binding transcription factor 4
ATP7A	Cu^{2+}-transporting ATPase alpha polypeptide

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DKK2 Dickkopf homolog 2
PGK1 Phosphoglycerate kinase 1

Introduction

Green tea is one of the most popular beverages in the world, and receives considerable attention because of its beneficial properties on human health. A growing number of epidemiological studies have demonstrated that the consumption of green tea inhibits the growth of a variety of cancers [1, 2]. In a cohort study in China, tea consumption was inversely associated with colon cancer risk [3]. In a recent pilot study in Japan, green tea extract was also shown to prevent the recurrence of colorectal adenoma, the precursors of most sporadic colorectal cancers, after total endoscopic resection of the preceding lesion [4]. Green tea contains at least four catechins: epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), and epicatechin (EC). The anti-cancer effects of EGCG, the most abundant and active catechin in green tea, have been extensively characterized in many cell and animal models and in some human epidemiological studies [5]. Although these studies have revealed multiple signaling pathways targeted by EGCG, the molecular mechanisms of EGCG anti-cancer effects in humans remain to be further investigated.

Neuroblastoma is the most common extracranial solid tumor in children and accounts for $\sim$15% of pediatric cancer deaths [6–8]. Despite aggressive therapies, <40% of high-risk patients achieve long-term survival. The failure of current therapies is mainly caused by tumor relapse that is lethal in most cases. To achieve better prognosis for high-risk patients, new therapies that can prevent and/or treat tumor relapse are necessary. Accumulating evidence has revealed that a variety of cancers are initiated from and maintained by a small population of tumor-initiating cells (TICs) that generate the bulk of the tumor through continuous self-renewal and differentiation [9–11]. TICs also show inherent drug resistance that leads to tumor relapse [12, 13]. In neuroblastoma, TICs from a high-risk patient were recently identified as spheres grown in serum-free non-adherent culture used for neural crest stem cell growth and were shown to express stem cell markers, self-renew, and form metastatic tumors in immunodeficient mice [14].

Although EGCG was reported to induce growth arrest and apoptosis in neuroblastoma SH-SY5Y cells, its effect on neuroblastoma TICs remains to be defined [15, 16]. In the present study, we isolated spheres of neuroblastoma BE(2)-C cells and found that EGCG inhibited sphere formation in the BE(2)-C cells.

Materials and methods

Cell culture

Human neuroblastoma BE(2)-C cells were obtained from ATCC (Manassas, VA, USA). For parental cells, BE(2)-C cells were cultured in complete medium (CM), consisting of Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 (Wako Pure Chemical; Osaka, Japan), and 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), at 37°C in a 5% CO_2 tissue culture incubator and subcultured with 0.25% trypsin–ethylene diamine tetraacetic acid (EDTA) (Invitrogen). For spheres, BE(2)-C cells were cultured in sphere medium (SM), consisting of DMEM/Ham's F12 (3:1, Invitrogen), 100 units/ml penicillin/streptomycin (PC/SM; Invitrogen), 2% B27 supplement (Invitrogen), 40 ng/ml basic fibroblast growth factor (bFGF; R&D Systems, Minneapolis, MN, USA), and 20 ng/ml epidermal growth factor (EGF; R&D Systems), at 37°C in a 5% CO_2 tissue culture incubator, subcultured with non-enzymatic cell dissociation solution (Sigma, St Louis, MO, USA), and maintained for >4 weeks in ultra-low attachment culture dishes (Corning, Corning, NY, USA). Parental cell and sphere images were acquired using a BZ-9000E microscope (Keyence, Osaka, Japan).

Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA from parental cells and spheres was isolated with an RNeasy Mini kit (Qiagen, Valencia, CA, USA) and reverse transcribed using a Quantitect Reverse Transcription kit (Qiagen). Real-time PCR analysis was performed with an ABI 7500 Fast Real-time PCR System (Applied Biosystems, Foster City, CA, USA) using FastStart Universal SYBR Green Master (Roche, Mannheim, Germany) according to the manufacturer's instructions. Each sample was analyzed in triplicate. The relative mRNA expression of nanog homeobox (NANOG; NM_024865), octamer-binding transcription factor 4 (OCT4; NM_002701), Cu^{2+}-transporting ATPase alpha polypeptide (ATP7A; NM_000052), and dickkopf homolog 2 (DKK2; NM_014421) to phosphoglycerate kinase 1 (PGK1; NM_000291) was calculated by the comparative C_T method. Primer sequences are shown in Table 1.

Cell proliferation

Parental cells and spheres were seeded into a 24-well plate at a density of 1×10^4 cells per well, treated with 1, 10, 50, and 100 μM (−)-epigallocatechin-3-gallate (EGCG; Wako Pure Chemical) for 48 h, and harvested. The number of cells was counted manually with a KOVA slide (Thermo

Table 1 Real-time polymerase chain reaction (PCR) primers

Gene	Sense primer	Anti-sense primer	Product size (bp)
NANOG	agatgcctcacacggagact	tttgcgacactcttctctgc	127
OCT4	cttcgcaagccctcatttc	gagaaggcgaaatccgaag	88
ATP7A	tcttccaggattgtctgttatgaa	accagcctccgaaaaactg	74
DKK2	ggcagtaagaagggcaaaaa	cctcccaacttcacactcct	72
PGK1	ggagaacctccgctttcat	gctggctcggctttaacc	78

See list of abbreviations for the full names of the genes

Fisher Scientific, Waltham, MA, USA). Cell proliferation was defined as the percentage of cells in each sample in relation to a control sample (without EGCG).

Apoptosis

Parental cells and spheres were seeded into a 6-well plate at a density of 5×10^4 cells per well, treated with 1, 10, 50, and 100 μM EGCG for 48 h, and harvested. Collected cells were stained with 1 μg/ml propidium iodide (PI; Sigma) and analyzed with a MoFlo XDP flow cytometer and Summit v5.3 software (Beckman Coulter, Fullerton, CA, USA). Apoptosis was defined as the percentage of PI-positive cells in each sample.

Sphere formation

Parental cells were seeded into a 96-well ultra-low attachment culture plate (Corning) at a density of 1×10^3 cells per well and cultured in SM containing 0, 1, 10, 50, and 100 μM EGCG. Spheres were dissociated every 4–5 days with non-enzymatic cell dissociation solution (Sigma) and examined with a BZ-9000E microscope (Keyence) on days 14–17 (48 h after third passage). Sphere images covering a whole well were merged into a single image using a BZ-9000E microscope (Keyence) and the total number of spheres (>100 μm in diameter) was counted manually.

Results

To examine whether EGCG targeted neuroblastoma TICs, we used a neuroblastoma BE(2)-C cell line. Among the three major neuroblastoma cell types: N (neuroblastic), S (substrate-adherent and non-neuronal), and I (intermediate), BE(2)-C cells have a typical I-type phenotype that most closely resembles neuroblastoma TICs [17, 18]. We first cultured BE(2)-C cells in a serum-free non-adherent condition used for neural crest stem cell growth. In accordance with a previous report [14], BE(2)-C cells efficiently formed spheres (Fig. 1a). We next analyzed the expression of stem cell and neuronal differentiation markers in the spheres, as well as in the parental cells, by a quantitative real-time RT-PCR. Phosphoglycerate kinase 1 (PGK1) was used for normalization, as described previously [19]. Nanog homeobox (NANOG) and octamer-binding transcription factor 4 (OCT4, also known as OCT3 and POU5F1) are commonly used stem cell markers [20], while Cu^{2+}-transporting ATPase alpha polypeptide (ATP7A, also known as MK and MNK) and dickkopf homolog 2 (DKK2) are neuronal differentiation markers characterized in neuroblastoma [21]. Compared to the parental cells, spheres showed increased expression of stem cell markers (NANOG and OCT4) but decreased expression of neuronal differentiation markers (ATP7A and DKK2) (Fig. 1b). These results suggest that spheres of BE(2)-C cells are enriched in neuroblastoma TICs.

We then determined cell proliferation in BE(2)-C cells after 0, 1, 10, 50, and 100 μM EGCG treatment by directly counting the cell numbers. Consistent with previous observations in neuroblastoma SH-SY5Y cells, EGCG inhibited cell proliferation in BE(2)-C cells in a dose-dependent manner [22, 23]; 50 μM EGCG caused 93.8 and 25.2% inhibition of cell proliferation in parental cells and spheres, respectively (Fig. 2a). Next we analyzed the percentages of apoptotic cells treated with 0, 1, 10, 50, and 100 μM EGCG, by carrying out PI staining. EGCG also induced apoptosis in BE(2)-C cells in a dose-dependent manner, as reported previously [22, 23]; 50 μM EGCG induced apoptosis in 91.7 and 9.1% of parental cells and spheres, respectively (Fig. 2b). These results suggest that EGCG is not effective in inhibiting cell proliferation and inducing apoptosis in the spheres of BE(2)-C cells compared to the parental cells.

We further investigated the effect of EGCG on sphere formation in BE(2)-C cells by culturing the parental cells in a serum-free non-adherent condition for 14–17 days. In marked contrast to the effect of EGCG on cell proliferation and apoptosis of spheres, 50 μM EGCG completely inhibited sphere formation in BE(2)-C cells (Fig. 3). Notably, BE(2)-C cells showed 37.3% inhibition of cell proliferation and 67.2% inhibition of sphere formation in response to 1 μM EGCG (Figs. 2a, 3). These results suggest that EGCG is a potent inhibitor of sphere formation in BE(2)-C cells.

Discussion

In the present study, we isolated spheres of neuroblastoma BE(2)-C cells and obtained two novel findings. First, EGCG was not effective in inhibiting cell proliferation or in inducing apoptosis in the spheres of BE(2)-C cells compared to the parental cells. Second, EGCG potently

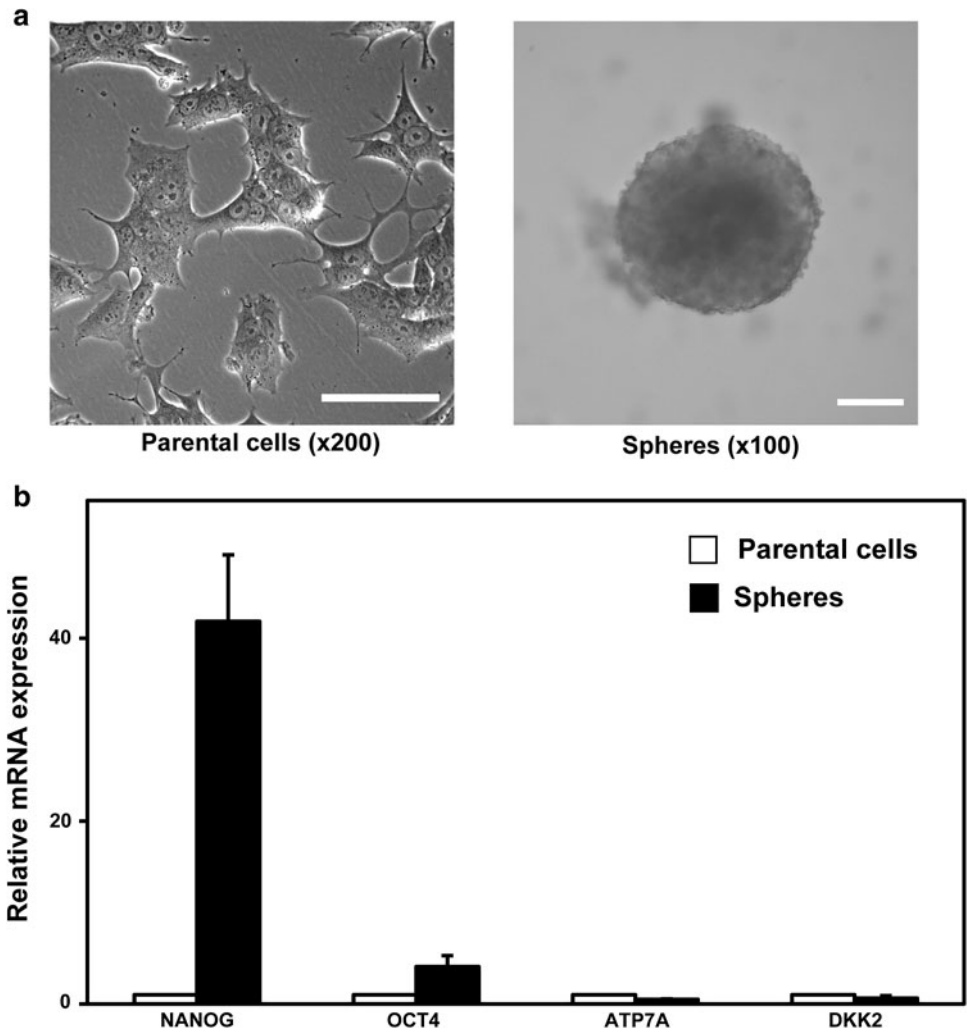

Fig. 1 Spheres from BE(2)-C cells. **a** BE(2)-C cells were cultured either in a 10% serum adherent condition for 3 days (parental cells) or in a serum-free non-adherent condition over 4 weeks (spheres) and examined by phase-contrast microscopy. The images shown are representative of three independent experiments. *Scale bars* show 100 μm. **b** Total RNA was prepared from parental cells and spheres. The relative mRNA expression of nanog homeobox (NANOG), octamer-binding transcription factor 4 (OCT4), Cu^{2+}-transporting ATPase alpha polypeptide (ATP7A), and dickkopf homolog 2 (DKK2) to phosphoglycerate kinase 1 (PGK1) was analyzed by quantitative real-time reverse transcription polymerase chain reaction (RT-PCR). The mean expression in parental cells was set at 1. The data shown are means ± SD of three independent experiments

inhibited sphere formation in BE(2)-C cells. As sphere formation likely represents the development of TICs leading to tumor relapse, EGCG may be effective in preventing recurrence of neuroblastoma.

To target TICs, several therapeutic strategies have been suggested [13]. Inhibiting the key signaling pathways active in TICs is one of the most promising strategies. Hedgehog, Notch, and Wnt signaling pathways are essential to regulate the self-renewal of TICs and are aberrantly activated in a variety of cancers [12]. An increasing number of studies have revealed that several dietary compounds have potential to act against the self-renewal of TICs [24]. For example, curcumin is a well-known polyphenol present in the Indian spice turmeric and has been shown to target TICs, through its inhibitory effect on Wnt signaling, in breast cancer [25]. A dietary component of broccoli/broccoli sprouts, sulforaphane, has also been demonstrated to be effective in targeting breast cancer TICs and down-regulating Wnt signaling [26]. Potential signaling pathways targeted by EGCG in neuroblastoma are currently under investigation.

Neuroblastoma develops from primitive neural crest cells that normally differentiate into the sympathoadrenal lineage. Although the exact targets of transformation during neuroblastoma progression remain to be defined, TICs undergoing self-renewal as well as multi-lineage differentiation were isolated as spheres grown in serum-free non-adherent culture [14]. Consistent with the inherent drug-resistance of TICs, the ineffectiveness of EGCG in inducing growth arrest and apoptosis was detected in spheres of BE(2)-C cells (Fig. 2). In contrast, sphere formation in BE(2)-C cells was sensitive to EGCG (Fig. 3). Because the EGCG-induced growth arrest and apoptosis of parental cells also affects sphere formation, it will be crucial to clarify the molecular basis behind the EGCG-sensitive sphere formation in BE(2)-C cells.

Although the existence of TICs in most cancers is now widely accepted, it is currently unclear whether TICs arise directly from the transformation of normal stem/progenitor cells or whether they are derived from the de-differentiation of more mature transformed cells. Using H3K4 demethylase JARID1B as a marker for melanoma TICs,

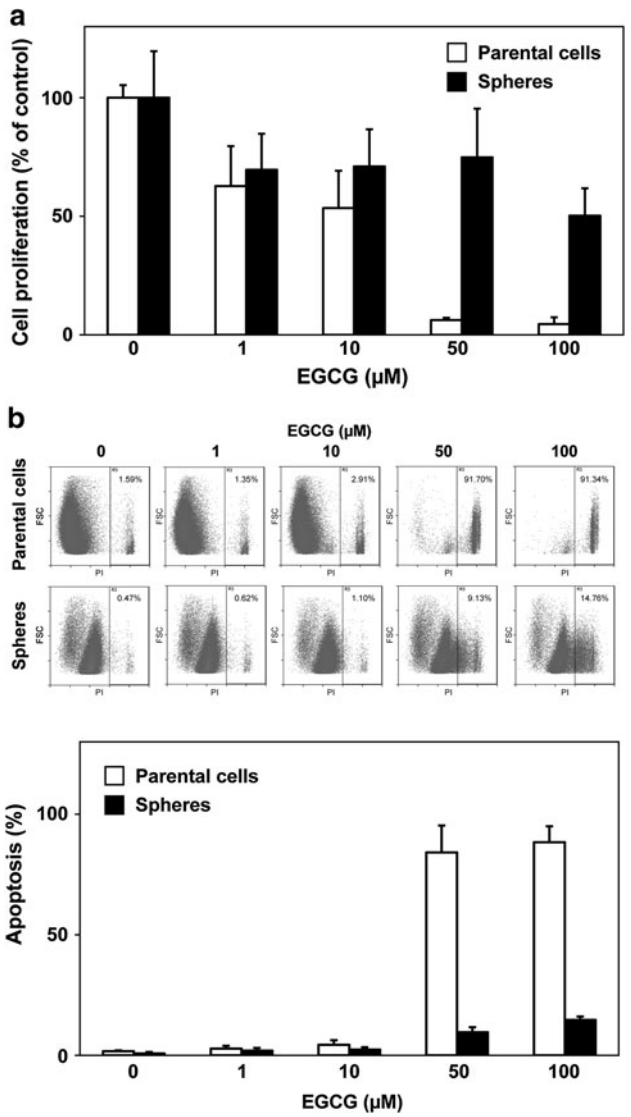

Fig. 2 Effect of epigallocatechin gallate (EGCG) on cell proliferation and apoptosis of BE(2)-C cells. **a** Parental cells and spheres were seeded into a 24-well plate and treated with the indicated concentrations of EGCG. After 48 h, the number of cells was counted manually. Cell proliferation was defined as the percentage of cells in each sample in relation to that in a control sample (without EGCG). The data shown are means ± SD of three independent experiments. **b** Parental cells and spheres were seeded into a 6-well plate, treated with the indicated concentrations of EGCG for 48 h, and stained with propidium iodide (PI). Apoptosis was defined as the percentage of PI-positive cells in each sample analyzed by flow cytometry. The data shown are means ± SD of three independent experiments

JARID1B-negative cells (non-TICs) were shown to become positive (TICs) [27]. In breast cancer, non-TICs were also demonstrated to de-differentiate into TICs [28]. Furthermore, the conversion of non-TICs into TICs was found to be regulated by interleukin (IL)-6 in breast and prostate cancer cells [29]. Accordingly, it is tempting to speculate that TICs may be generated from non-TICs in neuroblastoma [30]. In summary, the present study

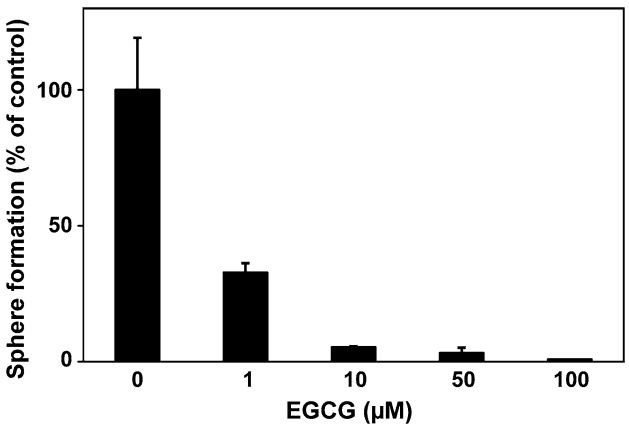

Fig. 3 Effect of EGCG on sphere formation in BE(2)-C cells. Parental cells were seeded into a 96-well plate, cultured in sphere medium (SM) containing the indicated concentrations of EGCG for 14–17 days, and examined by bright-field microscopy. The number of spheres (>100 μM in diameter) was counted manually. Sphere formation was defined as the percentage of spheres in each sample in relation to that in a control sample (without EGCG). The data shown are means ± SD of three independent experiments

supports the further evaluation of EGCG as a dietary compound targeting neuroblastoma.

Acknowledgments This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and grants from the Showa-hokokai and the Hyogo Prefecture Health Promotion Association.

Conflict of interest The authors declare no conflict of interest.

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