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ORIGINAL ARTICLE

Green tea catechin enhances osteogenesis in a bone marrow mesenchymal stem cell line

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Abstract Green tea has been reported to possess antioxidant, antitumorigenic, and antibacterial qualities that regulate the endocrine system. Previous epidemiological studies found that the bone mineral density (BMD) of postmenopausal women with a habit of tea drinking was higher than that of women without habitual tea consumption. However, the effects of green tea catechins on osteogenic function have rarely been investigated. In this study, we tested (-)-epigallocatechin-3-gallate (EGCG), one of the green tea catechins, on cell proliferation, the mRNA expressions of relevant osteogenic markers, alkaline phosphatase (ALP) activity and mineralization. In a murine bone marrow mesenchymal stem cell line, D1, the mRNA expressions of core binding factors a1 (Cbfa1/Runx2), osterix, osteocalcin, ALP increased after 48 h of EGCG treatment. ALP activity was also significantly augmented upon EGCG treatment for 4 days, 7 days and 14 days. Furthermore, mineralizations assayed by Alizarin Red S and von Kossa stain were enhanced after EGCG treatment for 2–4 weeks in D1 cell cultures. However, a 24-h treatment of EGCG inhibited thymidine incorporation of D1 cells. These results demonstrated that long-term treatment of EGCG increases the expressions of osteogenic genes, elevates ALP activity and eventually stimulates mineralization, in spite of its inhibitory effect on proliferation. This finding suggests that the stimulatory effects of EGCG on osteogenesis of mesenchymal stem cells may be one of the mechanisms that allow tea drinkers to possess higher BMD.

Keywords Catechin · EGCG · Mesenchymal stem cell · Osteogenesis

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Introduction

Tea, brewed from the dried leaves of the plant *Camellia sinensis*, an evergreen shrub of the Theaceae family, is one of the most popular beverages in the world. About 3 billion kg of tea are produced and consumed annually. Green tea is produced from freshly harvested leaves that are rapidly steamed or pan fried to stop their enzymatic reactions, thereby preventing fermentation and generating a dry, stable product [1, 2]. More than 80% of green tea polyphenols are catechins (3, 3', 4', 5, 7-pentahydroxyflavan), which are derivatives of flavan-3-ol. The major catechins in green tea are (-)-epicatechin (EC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC) and (-)-epigallocatechin-3-gallate (EGCG). Among these catechins, EGCG has been demonstrated to have cancer-preventing effects [1–4].

Recent studies have verified the beneficial effects of catechins in decreasing serum lipid, reducing blood

pressure, and modulating immune responses. Catechins have also been reported to have antitumorigenic and antibacterial effects [1–3, 5, 6]. Many of the benefits of green tea depend on the antioxidant and free radical scavenging activities of catechins [6, 7]. Recent studies conducted in Europe have reported reduced risk of hip fractures in habitual tea drinkers [8, 9]. Another epidemiological study found that the bone mineral density (BMD) of habitual tea-drinking, postmenopausal women was higher than that of those who did not consume tea [10]. However, the effective components and the action mechanisms of tea on bone remodeling remain unclear.

Despite numerous reports on the effects of tea on the body, the osteogenic effects of tea have rarely been reported. In our previously published paper, we tested four catechins including EC, ECG, EGC and EGCG and found that EGCG possessed the most potent stimulatory effect on osteoprotegerin messenger RNA (mRNA) expression [11]. Accordingly, in this study, we attempt to elucidate the effects of EGCG on a bone marrow mesenchymal stem cell line, by screening the mRNA expressions of core binding factor a1 (Cbfa1/Runx2), osterix, osteocalcin (OC) and alkaline phosphatase (ALP). The effects of EGCG on cell proliferation, ALP activity and mineralization were also evaluated.

Materials and methods

D1 cell preparation

D1 cells used in this study were cloned from multipotent mesenchymal stem cells, as has been characterized previously [12]. D1 cells are found to be primarily osteogenic and are able to ''home'' back to bone marrow and participate in fracture repair, on either systemic or local injection [12]. Cells were maintained in the Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (Gibco, BRL, Bethesda, MD, USA), 50 mg/ml sodium ascorbate and antibiotics (Gibco BRL, Bethesda, MD, USA). The cells were seeded at a density of 1×10^4 cells per cm^2 in a six-well plate and cultivated in a humidified atmosphere of 5% carbon dioxide at 37°C. Experiments were performed after the cells reached about 80% confluence.

Catechin treatment

The EGCG powder was stored at 4°C. Before the experiments, EGCG was dissolved in dimethyl sulfoxide (DMSO) with a concentration of 10 mmol/l and kept at −20°C for the remaining experiments. The EGCG stock was diluted with culture medium immediately before treatment. Cells were treated by EGCG with concentrations of 1 μmol/l and 10 μmol/l. Accordingly, the concentration of DMSO was less than 0.1% in the experiments. The cultured medium was changed every other day. In the experiments of examining mRNA expressions of osteogenic markers, the D1 cells were treated with EGCG for 48 h. For the ALP activity assay, cells were harvested at 4 days, 7 days and 14 days after treatment. For thymidine incorporation assay, cells were collected after a 24-h treatment. In the mineralization assay, Alizarin Red S staining was performed 2 weeks after treatment, and von Kossa staining was done 3 and 4 weeks after treatment. The experiments were repeated at least 3 times.

Messenger RNA analysis

Total RNA was separated with chloroform and precipitated with isopropyl alcohol after homogenizing with Trizol reagent (Gibco BRL, Bethesda, MD). The RNA pellet was washed with 75% ethanol, and the RNA was redissolved in RNAse-free water. The concentration of RNA was quantified by measuring absorbance by a spectrophotometer at 260 nm and 280 nm. Total RNA 1 μg was reverse transcribed by Moloney murine leukemia virus RT and random hexamer primer (Applied Biosystems, Branchburg, NJ, USA). Polymerase chain reaction (PCR) was performed with a Perkin-Elmer Gene Amp 9700 PCR system (Applied Biosystems, Branchburg, NJ). The PCR reaction was carried out with the specific primers of each gene and a thermostable DNA polymerase (Gibco BRL, Bethesda, MD). Changes of the mRNA expressions of ALP, OC, osterix and Cbfa1/Runx2 were then analyzed. The following mouse primer pairs were used: OC (5'- CTTGGTGCACACC-TAGCAGA-3' forward and 5'-CTCCCTCATCGTGTT GTC CCT-3' reverse) and Cbfa1/Runx2 (5'-CGCTCCG CCCACAAATCTC-3' forward and 5'-CCGCACGA CAACCGCACCAT-3' reverse), ALP (5'-GTTCTGCT CAT GGACGCCGTGAAGC-3' forward and 5'- GCA GTGGAGATGGACCAGGCC-3' reverse) and osterix (5'-CACTTCTG TTCTTCGTTCTC-3' forward and 5'-TGAGGAAGAAGCCCATTCAC-3' reverse). The products of PCR were resolved by electrophoresis on a 2% agarose gel and visualized with ethidium bromide. The optical densities (OD) of the bands were quantified by densitometry for analysis. Quantitative differences were normalized with the QuantumRNA 18S (Ambion, Austin, TX, USA) PCR products.

Thymidine incorporation

Cells (2000 cells/well) cultured in 96-well plates were treated with EGCG for 24 h, and 2 mCi/well of ^{3}H-thymidine was pulsed 4 h before harvest. Incubations were terminated by washing with phosphate-buffered solution (PBS). Cells were detached by using 1% trypsin/EDTA, and collected in a 96-well UniFilter (Packard, Meriden, CT, USA) by using a FilterMate Harvester (Packard, Meriden, CT). The UniFilter was dried by 95% ethanol for 30 min. After sealing with a

TopSeal-A (Packard, Meriden, CT), liquid scintillant was added into the sealed UniFilter and counted in a TopCount Microplate Scintillation and Luminescence Counters (Packard, Meriden, CT). The results were shown as count per second (CPS)/well.

Alkaline phosphatase activity assay

The elevation of ALP activities of D1 cells reflects that those osteogenic cells were undergoing terminal differentiation. D1 cells were seeded at 1×10^4 cells per cm^2 in a six-well plate in the presence of 10 mmol/l beta-glycerophosphate. Cells were cultured for 4 days, 7 days and 14 days, media with or without EGCG and were changed every other day. D1 cells were harvested and washed twice with Ca^{2+}, Mg^{2+}, and NaHCO3 free-PBS. Cell lysate 100 µl was assayed for ALP activity by chemiluminescent method (Tropix, Applied Biosystems, Bedford, MA, USA). Total protein was determined by using a Bio-Rad protein assay kit. The specific activity of ALP was expressed as light unit/mg protein.

Mineralization assay: Alizarin Red S stain

After 2 weeks of EGCG treatment, mineralization was determined by Alizarin Red S stain. Cells were washed

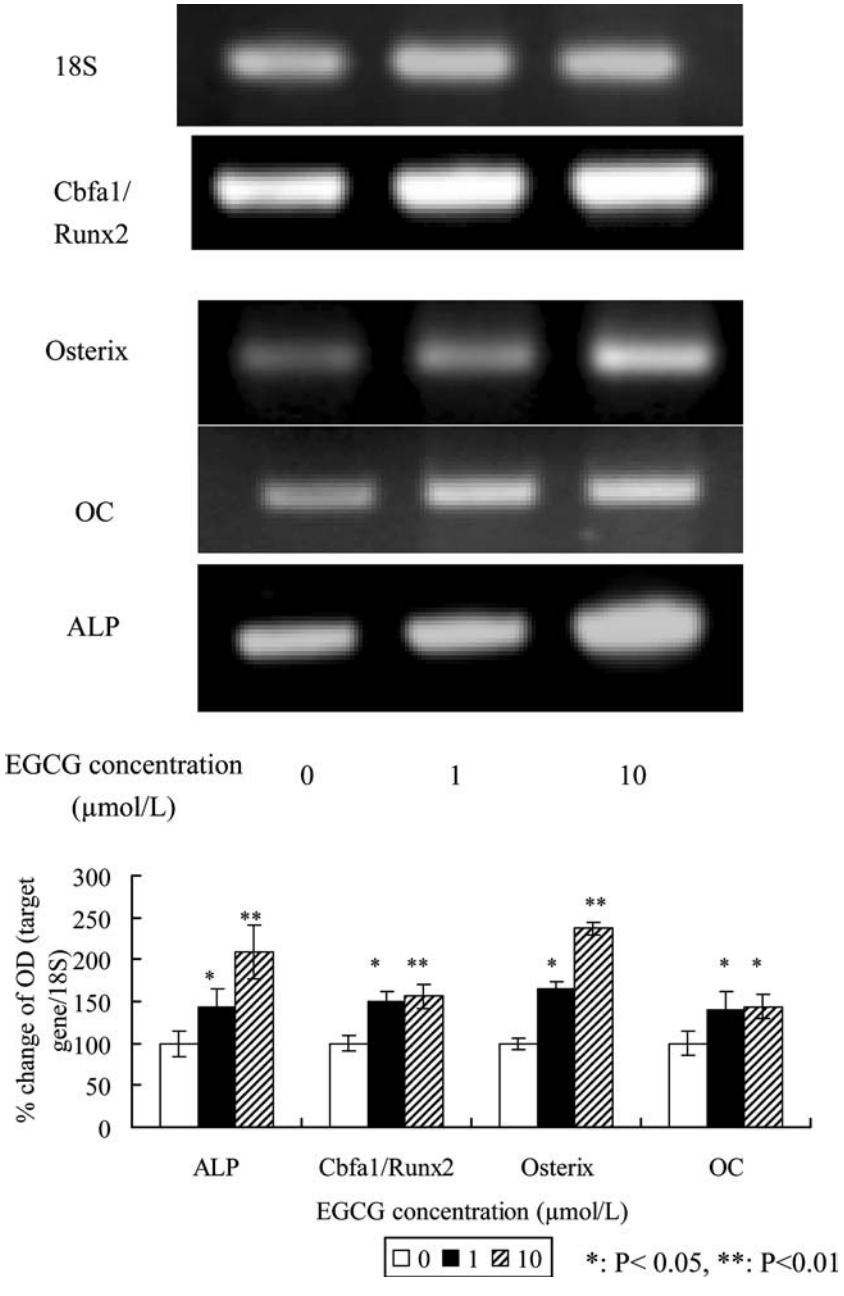

Fig. 1 Effects of EGCG on mesenchymal D1 cells in mRNA expression. The optical density (*OD*) of Cbfa1/Runx2, osterix, OC, ALP and 18S was semi-quantified by image-analysis software and presented as a *bar graph*. *Bars* represent the mean ± standard deviation, $n = 6$. Values are based on band density relative to internal control 18S rRNA and plotted as a percentage of the control (0 µmol/l EGCG). In Cbfa1/Runx2, mRNA expression 50% and 56% was increased by 1 µmol/l and 10 µmol/l. The osterix mRNA expression was enhanced 66% and 137% at concentrations of 1 µmol/l and 10 µmol/l of EGCG, respectively. There was 44% and 109% augmentation in ALP mRNA expression upon 1 µmol/l and 10 µmol/l of EGCG treatment, respectively. The mRNA expression in OC amplified 40% and 44% after EGCG treatment at concentrations of 1 µmol/l and 10 µmol/l, respectively (** $p < 0.01$; * $p < 0.05$) (*ALP* alkaline phosphatase, *EGCG* (-)-epigallocatechin-3-gallate, *OC* osteocalcin)

twice with distilled water and fixed in ice-cold 70% (v/v) ethanol for 1 h. Cells were rinsed twice with deionized water and stained with Alizarin Red S. Alizarin Red S was prepared in deionized water and adjusted to pH 4.2 for 10 min at room temperature. After staining, excessive dye was washed gently with running water. Calcification deposits typically stained red.

Mineralization assay: von Kossa stain

The cultures were stained at the end of the third and fourth weeks to assess the mineralized matrix. The medium was removed, and the cell layers were rinsed with Tris-buffered saline (pH 7.4), fixed with 2% paraformaldehyde, and stained with 5% silver nitrate. After 30 min of UV exposure, the cell layers were washed three times in distilled water, and placed in 2.5% sodium thiosulfate for 5 min. Cell layers were washed another three times with distilled water and observed under a light microscope. Areas of mineral deposition were quantified by image processing system.

Statistical analysis

Data was presented as mean ± standard deviation. All data was evaluated by one-way analysis of variance (ANOVA) and Scheffer's method. $P < 0.05$ was considered significant.

Results

mRNA expression

The mRNA expressions of Cbfa1/Runx2, osterix, ALP and OC of the EGCG-treated cells were compared with the control cells. After EGCG 1 μmol/l and 10 μmol/l treatments, Cbfa1/Runx2 mRNA expression was increased by 50% ($p < 0.05$) and 56% ($p < 0.01$), respectively, in comparison to that of the non-treated control cells. The mRNA expression of osterix, ALP and OC also showed a marked increase with the addition of different concentrations (1 μmol/l or 10 μmol/l) of EGCG: an increase of 66% ($p < 0.05$) and 137% ($p < 0.01$) for osterix; 44% ($p < 0.05$) and 109% ($p < 0.01$) for ALP; and 40% ($p < 0.05$) and 44% ($p < 0.05$) for OC (Fig. 1). All the experiments were repeated at least three times and showed similar effects.

Thymidine incorporation

A 24-h treatment of EGCG decreased thymidine incorporation by 12% (1 μmol/l) and 24% (10 μmol/l) ($p < 0.05$). The experiments were repeated three times and showed similar effects.

Alkaline phosphatase activity

In comparison with control cultures, the ALP activities of EGCG-treated cultures were increased by 102% and 178% ($p < 0.01$) on the fourth day, 61% and 83% ($p < 0.01$) on the seventh day, and 34% and 48% ($p < 0.01$) on the 14th day, respectively (Fig. 2). The experiments were repeated at least three times and showed similar effects.

Mineralization

The cytological results of D1-cell cultures were convincingly positive, when stained by either Alizarin Red S on the second week or von Kossa on the third and fourth weeks, with EGCG treatments of 1 μmol/l or 10 μmol/l (Fig. 3). On the third week, different concentrations of

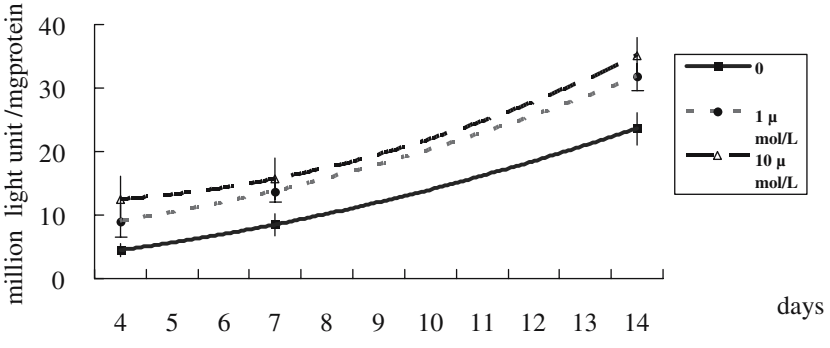

Fig. 2 Concentration and time response of ALP activity up-regulation by EGCG. The unit in the *vertical axis* is light unit/mg protein. On the fourth day, ALP activity rose to 202% and 278% of control group at concentrations of 1 μmol/l and 10 μmol/l EGCG, respectively ($p < 0.01$). Escalation of ALP activity to 161% and 183% of control group by 1 μmol/l and 10 μmol/l of EGCG, respectively, were noted on the seventh day ($p < 0.01$). Activity intensified to 134% and 148% of control group by 1 μmol/l and 10 μmol/l of EGCG was found on the 14th day ($p < 0.01$) (*ALP*alkaline phosphatase, *EGCG* (-)-epigallocatechin-3-gallate)

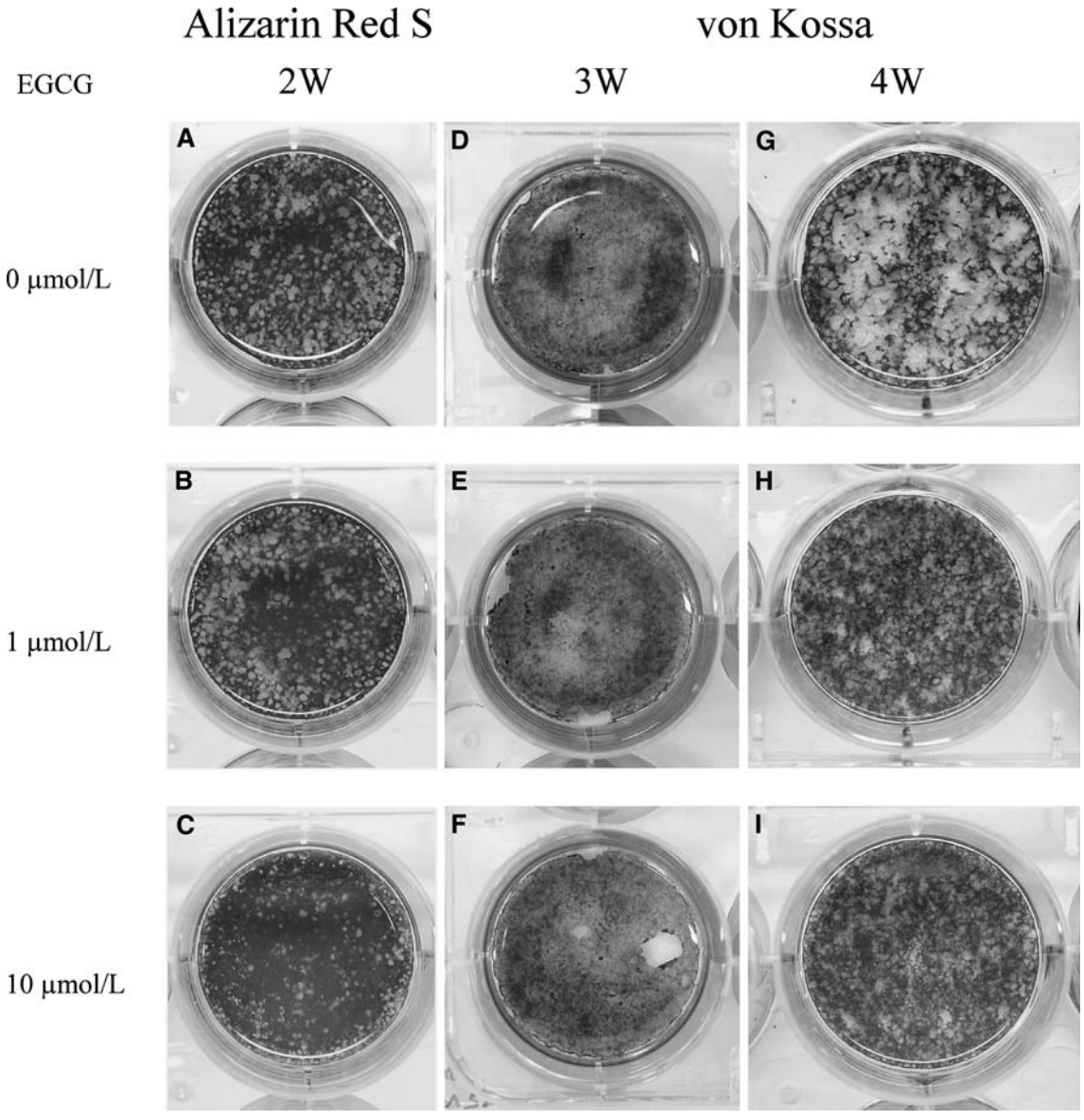

Fig. 3 Effects of EGCG on mesenchymal D1 cell mineralization. Increased mineralization was obvious upon EGCG treatment at concentrations of 1 μmol/l and 10 μmol/l in Alizarin Red S stain (*A*, *B*, and *C*) on the second week and von Kossa stain on the third week (*D*, *E* , and *F*) and the fourth week (*G*, *H* and *I*). With different concentrations of EGCG, 1 μmol/l and 10 μmol/l, mineralization increased by 5% and 29%, at the end of third week; 25% and 44% at the end of the fourth week, respectively (*EGCG* (-)-epigallocatechin-3-gallate)

EGCG, 1 μmol/l and 10 μmol/l, increased mineralization by 5% and 29%, compared with the control, respectively. The differences extend on the fourth week to 25% and 44%, respectively, compared with the control, by addition of EGCG, 1 μmol/l and 10 μmol/l. The experiments were repeated at least three times and showed similar effects.

Discussion

The drinking of green tea has been related to lower incidence of various diseases, including cancer, cardiovascular disease, diabetes, allergy, asthma, arthritis, nervous system problems and obesity [1–3, 5]. Previous reports have indicated that many of the benefits of green tea, such as the regulation of the endocrine system, the immune system, appetite and brain functions, are due to the antioxidant and free radical-scavenging activities of catechins [1, 5, 7]. A positive correlation between tea consumption and body BMD has been reported among postmenopausal women in the UK [10], Canada [13] and the USA [14]. A positive correlation has also been reported between the increase of BMD and habitual tea drinkers in Taiwan [15]. Other studies verified that tea can have a protective effect against hip fracture [8, 9].

Fermented black tea is consumed more in western countries, whereas, unfermented green tea is consumed primarily in Asian countries. Fermentation of tea involves air oxidation and polymerization of tea components including polyphenolic catechins. The chief fermentation products of black tea are theaflavins and thearubigins, a complex mixture of catechins and condensed products with diverse molecular weight distributions [1]. Although there are differences in the ingredients between black tea and green tea, habitual tea drinkers of either kind seem to exhibit similar results when correlated with BMD [15]. As a first step, using green tea, we have investigated the effects of catechins on bone marrow stem cells. As a result, we were able to demonstrate that EGCG increases the mRNA expression of Cbfa1/Runx2, osterix, OC and ALP. Cytological evidence also indicated that EGCG increases ALP activity and mineralization in a cultured mesenchymal stem cell line derived from bone marrow.

Cbfa1/Runx2 is a critical regulator during the differentiation of osteoblast linage, as it is a transcription factor that regulates the expression of bone specific extracellular matrix [16]. These bone specific proteins include OC, type I collagen, ALP, etc. Cbfa1/Runx2 have been demonstrated to bind to the osteoblast-specific cis-acting element 2 (OSE2) of the OC promoter region and regulate OC expression [17]. Both Cbfa1/Runx2 and osterix are essential for mesenchymal cells to differentiate to osteoblasts [18]. Therefore, augmentation of the expression of Cbfa1/Runx2 and osterix by EGCG may lead to an increase in osteogenesis. Although we cannot conclude that the elevation of ALP and OC expressions by EGCG found in this study were through the enhancement of the expressions of Cbfa1/Runx2 and osterix, the elevation of these osteogenic markers should contribute to the increase of osteogenesis.

EGCG's stimulatory effects on ALP activity and the mineralization in D1-cell cultures further confirm its post-transcriptional influences on osteogenesis. Up-regulation of ALP activities was noted after 4–14 days of 1 μmol/l and 10 μmol/l EGCG treatments. Our unpublished data showed that EGCG also increased ALP activities in 3T3-E1 cells after treatment for 14 days. With the addition of different concentrations (1 μmol/l or 10 μmol/l) of EGCG, with respect to the control, ALP activities increased by 45% and 94%, respectively. This shows that the stimulatory effect of EGCG in ALP activity was not a unique response of D1 cells.

Previous reports have indicated that the drinking of one cup of green tea could accumulate the circulating level of EGCG to 1 μmol/l [1, 19, 20]. Our results illustrated that the effective concentration of EGCG is in a range of 1–10 μmol/l. This implies that regular green tea drinking may increase the gene expressions of Cbfa1/Runx2, osterix, ALP and OC. Our results further demonstrated that EGCG can increase the potential of osteoblastic terminal differentiation by increasing ALP activity, and eventually stimulating mineralization. However, a 24-h treatment of EGCG inhibited thymidine incorporation of D1 cells. These results demonstrated that long-term treatment of EGCG increases the expressions of osteogenic genes, elevates ALP activity and eventually stimulates mineralization, in spite of its inhibitory effect on proliferation. Elucidations on the detail mechanisms of EGCG in mesenchymal stem cells and osteoblasts, both in mice and in humans, require further investigation.

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