Epigallocatechin-3-gallate increases the formation of mineralized bone nodules by human osteoblast-like cells
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Abstract
Consumption of green tea, a rich source of epigallocatechin-3-gallate (EGCG), is associated with increased bone mineral density. However, the direct effects of this flavonoid on bone mineralization are not known. The present study demonstrates the effects of EGCG on the formation of mineralized bone nodules by SaOS-2 human osteoblast (HOB)-like cells. EGCG at concentrations of 1–5 μM caused a dose-dependent increase in the number and area of mineralized bone nodules as assessed by both von Kossa and alizarin red staining. EGCG also increased alkaline phosphatase activity, an early marker of osteoblastic differentiation. After 48 h of treatment, EGCG decreased protein levels of Runx2, a transcription factor that regulates osteoblast maturation by inhibiting late-stage differentiation. The observed effects of EGCG on bone formation by HOB-like cells suggest that EGCG may have beneficial effects on bone health.

1. Introduction
Osteoporosis is a complex disease influenced by genetic and environmental factors such as diet and lifestyle. Epidemiological studies have shown an association between a reduced risk of osteoporosis and the consumption of tea, including green tea [1–6]. Green tea is produced by drying fresh young leaves of the plant Camellia sinensis [7]. Unlike the preparation of black tea in which the leaves are fermented and oxidized, green tea is produced by steaming the leaves, which preserves their polyphenolic content [8]. The polyphenols comprise ~30–40% of the solid extract of leaves and are mostly categorized as catechins (also called flavanols). The main green tea catechins are epigallocatechin-3-gallate (EGCG), epicatechin-3-gallate, epigallocatechin and epicatechin [9]. EGCG is the most abundant catechin in green tea that contributes to many of the beneficial health effects associated with consuming this beverage [10].

Bone integrity requires a tight coupling between the activity of bone-forming osteoblasts and bone-resorbing osteoclasts [11]. The phenotype of mature osteoblasts is characterized by their ability to synthesize and secrete molecules of the extracellular matrix. Osteoblasts regulate the mineralization of the formed matrix by producing alkaline phosphatase (ALP) [12]. This enzyme hydrolyzes phosphate esters to increase the local phosphate concentration and enhance mineralization of the extracellular matrix [13]. One of the characteristics of a mature osteoblast phenotype is the ability of the cells to synthesize ALP, which is considered an early marker of osteoblast differentiation. Differentiation of osteoblasts from multipotent mesenchymal stem cells is regulated by a member of the runt-domain gene family, known as Runx2-related transcription factor-2 (Runx2) [14–16]. Runx2 has been shown to enhance osteoblast differentiation at an early stage but inhibit osteoblast maturation at late stages [17]. Runx2 is expressed as two isoforms, type I and type II, which differ only in their N-terminal sequences [18], and both isoforms are expressed in a number of osteoblast-like cell lines including SaOS-2 cells [19]. Although osteosarcoma cell lines such as SaOS-2 are transformed, they have a stable osteoblast-like phenotype in
culture, making them a good model for studying the
caracteristics and functions of osteoblasts [20,21].
EGCG has been shown to induce apoptotic cell death
in cultured mouse osteoclasts [22] and inhibit the
formation of osteoclasts in a coculture system of mouse
bone marrow cells and calvarial primary osteoblasts [23].
However, the effects of EGCG on osteoblastic bone
formation are not known. In the present study, we
investigated the direct effects of EGCG on osteoblastic
bone formation using a human osteoblast (HOB)-like cell
line. Our results demonstrate that EGCG causes a dose-
dependent increase in the formation of mineralized bone
nodules, which was associated with a marked increase in
osteoblast differentiation and a reduction in Runx2
protein levels.

2. Materials and methods

2.1. Materials

SaOS-2 cells were obtained from the American Type
Culture Collection (Rockville, MD); Ham’s F-12 medium
was purchased from the Central Technical Services at the
University of Toronto, and fetal bovine serum (FBS) was
purchased from Cansera (Rexdale, Ontario). Reagents for
Western blotting were obtained from BIORAD (Missis-
sauga, Ontario). IgG–HRP-conjugated secondary antibody,
Cruz Marker molecular weight standards and luminol
reagent were purchased from Santa Cruz Biotechnology,
Inc. (Santa Cruz, CA). Runx2 antibody was generously
supplied by Dr. N. Elango (University of Texas Health
Science Center, San Antonio, TX) [19]. ALP liquid reagent
was purchased from TECO Diagnostics (Anaheim, CA).
EGCG from green tea (>95% pure) and all other chemicals
were purchased from Sigma (Oakville, Ontario).

2.2. Cell culture

SaOS-2 cells were grown in Ham’s F-12 medium
containing 10% FBS, 28 mmol/L HEPES buffer (pH 7.35), 1.4 mmol/L CaCl2, 2 mmol/L glutamine and 1%
antibiotic–antimycotic solution. Cells were seeded at a
density of 5×10^5 cells per well in 24-well plates and
1×10^5 cells per 75-cm² flask. The media were supple-
mented with 10 nM dexamethasone (Dex) and 50 μg/ml
ascorbic acid at the time of seeding (Day 0). On Day 8,
10 mM β-glycerophosphate was added to the culture
medium and the cells were treated with either vehicle
(0.1% PBS) or varying concentrations of EGCG. This
medium was replaced every 48 h until the end of the
culture period [24].

2.3. Determination and quantification of mineralized bone
nodules

On Day 17, cells seeded in 24-well plates were fixed in
4% paraformaldehyde overnight and the mineralized bone
nodules were visualized by von Kossa [25] and alizarin red
staining techniques. In order to detect calcium
deposits, we stained cells with alizarin red (40 mM, pH 4.2) for 10 min and then rinsed them with PBS. After the
stain was extracted by 10% cetylpyridinium chloride in
10 mM sodium phosphate (pH 7), the same nodules were
stained with 5% silver nitrate. The nodules were then
exposed to UV light for 30 min to detect phosphate
deposits using the von Kossa method. Background color
was removed by 5% sodium thiosulfate, and the cells were
kept in 50% glycerol. Nodules were visualized using an
inverted microscope, and the number and area of
mineralized nodules were quantified by a FluorChem
imaging system.

2.4. ALP activity

At different time points after treatment, cells seeded in
24-well plates were washed twice with 50 mM Tris–HCl
(pH 7.3) and kept in 0.05% Triton X-100 lysis buffer
overnight at −20°C. The cells were later thawed on ice and
harvested by scraping [24]. The ALP activity of the samples
was determined by a colorimetric assay using an ALP
reagent of p-nitrophenyl phosphate. The absorbance of
p-nitrophenol formed by the hydrolysis of p-nitrophenyl
phosphate, catalyzed by ALP, was measured at 410 nm by a
Fusion plate reader 15 times every 2 min. The protein
content of the cell lysates was determined using the
commercial Biorad protein assay [27]. The ALP activity
of each sample was then normalized to its protein
concentration and expressed as units per milligram of
protein, with each unit of activity being defined as the
amount of enzyme that produces 1 mmol p-nitrophenol/min.

2.5. Western blot analysis

Cells seeded in 75-cm² flasks were harvested at 6, 24
and 48 h after treatment by trypsin and disrupted in RIPA
lysis buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium
deoxycholate, 0.1% SDS, 100 μg/ml PMSF, 45 μg/ml
aprotinin, 1 mM sodium orthovanadate and 50 μM
leupeptin) using a minihomogenizer. Cell lysates were

![Fig. 1. Dose-dependent effects of EGCG on the formation of mineralized
nodules in a 24-well plate fixed on Day 17 and stained by von Kossa (A) or
alizarin red (B). Cells were treated with either vehicle or EGCG at
concentrations of 1, 2.5 and 5 μM on Day 10.](image-url)
centrifuged for 10 min at 10,000 rpm, and the supernatants were stored at −20°C. Approximately 50 μg of total protein from each sample was solubilized in 2× Laemmli buffer with 2-mercaptoethanol, heated at 95°C for 5 min, electrophoresed using a 10% SDS-polyacrylamide gel and transferred to PVDF membranes using a semidy transfer apparatus. The membranes were blocked overnight with 5% nonfat milk in Tris-buffered saline/0.05% Tween 20 (TBST) at 4°C. The membranes were then incubated in anti-Runx2 antibody at a 1:1000 dilution for 2 h at room temperature. The antibody detects a 65-kDa band that represents both type I and type II Runx2. Membranes were washed with TBST and probed with horseradish-peroxidase-conjugated antirabbit IgG antibody for 1 h at room temperature. After a final wash in TBST, the signals were detected by enhanced chemiluminescence. Exposure times were optimized for each treatment time (6, 24 and 48 h), and band intensities were compared relative to the intensity of the corresponding control band. To ensure equal protein loading, we stained membranes with 0.1% Coomassie brilliant blue solution containing 50% methanol and 10% acetic acid. The membranes were destained using a solution containing 25% methanol and 7.5% acetic acid.

2.6. Statistical analyses

Results are expressed as mean±S.E.M. with six replicates in each group. Differences were analyzed using one-way and two-way analyses of variance followed by Bonferroni’s post hoc test for multiple comparisons with a significance level of P<.05. All data were analyzed using GraphPad Prism Software, Version 4.00.

3. Results

3.1. Effects of EGCG on the formation of mineralized bone nodules

The first mineralized bone nodules formed by SaOS-2 cells were observed after 2 days of treatment (Day 10).
The nodules appeared three-dimensional in appearance under a phase contrast microscope and continued to grow until the end of the culture period. After staining the nodules on Day 17, the discrete mineralized bone nodules could be visualized by the naked eye as dark black spots after von Kossa staining (Fig. 1A) and as red–purple spots after staining with alizarin red (Fig. 1B). The addition of EGCG at concentrations as low as 1 μM to the culture media increased the formation of mineralized nodules compared with control. EGCG caused a dose-dependent increase in the number and area of mineralized bone nodules as quantified using a FluorChem imaging system.

Analysis of the results from von Kossa staining showed that at 5 μM, EGCG increased the number of nodules by ~10-fold compared with untreated cells (12.6±1.5 vs. 138.7±5.5, P<.01; Fig. 2A) and the area of nodules by ~18-fold (100.0±17.0 vs. 1802.0±175.6, P<.01; Fig. 2C). Analysis of the results from alizarin red staining demonstrated that 5 μM EGCG resulted in an ~7-fold increase in the number of nodules (17.3±4.5 vs. 117.0±2.6, P<.01; Fig. 2B) and in an increase in the area of nodules by ~20-fold (100.0±37.5 vs. 1985.0±235.2, P<.01; Fig. 2D). A similar increase in both the number and area of nodules was also seen in cultures treated with 10 μM EGCG (data not shown). At this concentration, however, cells surrounding the nodules displayed marked changes in morphology and began to detach from the surface of the plate.

3.2. Effects of EGCG on ALP activity

Addition of EGCG to SaOS-2 cells cultured in 24-well plates showed a significant increase in the activity of ALP (Fig. 3). EGCG at a concentration of 5 μM significantly increased ALP activity after 2 days of treatment. After 8 days of treatment, 5 μM EGCG caused an ~35% increase in ALP activity (0.16±0.01 vs. 0.22±0.01 U/mg protein, P<.05).

3.3. Effects of EGCG on Runx2 protein level

To determine whether the observed increase in the formation of mineralized nodules by EGCG-treated cells was associated with changes in the expression of Runx2 protein, we performed Western blot analyses on extracts of cells grown in the presence of increasing concentrations of EGCG after 6, 24 and 48 h. Quantification of blots by density scanning of band intensities showed no significant effects of EGCG on Runx2 protein levels after 6 or 24 h of treatment (Fig. 4A and B). However, a significant decrease in Runx2 protein was observed after 48 h of treatment with 1–5 μM EGCG. At 5 μM EGCG, Runx2 protein levels were reduced by ~65% compared with controls (100.0±21.9 vs. 35.1±15.4, P<.01; Fig. 4A and B). A Coomassie blue staining of the membrane confirmed equal loading of protein (data not shown).

3. Discussion

In the present study, we have shown that EGCG, the most abundant flavonoid found in green tea, increases the formation of mineralized bone nodules from HOB-like cells by increasing osteoblastic differentiation. Our data provide some cellular and molecular evidence in support of the epidemiological associations between green tea consumption and a reduced risk of osteoporosis. Tea consumption has been associated with higher bone mineral density (BMD) [1,2,5] and a lower risk of hip fractures in men and women [3,4], although the effect of different types of tea consumed was not ascertained. In a study by Wu and Yang [6], in which 90% of tea drinkers regularly consumed green tea, a positive association was observed between tea and BMD of the total body, lumbar spine and hip regions in both men and women. Animal studies have also provided some evidence of the protective effect of green tea catechins against bone loss [28,29]. Investigation of the effects of green tea catechins on bone metabolic disorders using a model of chronic cadmium-poisoned rats with increased bone resorption showed that a 0.5% catechin diet aided in normalizing decreased BMD and bone mineral content caused by cadmium intoxication [28]. Results from examining the role of whole aqueous C. sinensis extract on bone loss induced by estrogen deficiency in ovariectomized rats also suggest that tea catechins may be
effective in preventing bone loss due to ovarian hormone deficiency [29].

EGCG has been shown to inhibit bone resorption by inducing apoptotic death of mouse osteoclast-like cells [22]. It was also demonstrated that EGCG inhibits the formation of osteoclasts in a coculture system of mouse bone marrow cells and primary osteoblastic cells [23]. Although these two studies support the inhibitory effect of EGCG on osteoclastic bone resorption, no studies have examined the effects of EGCG on osteoblastic differentiation or bone formation. To our knowledge, the present study is the first to investigate the direct effects of EGCG on bone-forming osteoblasts. We demonstrated that EGCG has a strong stimulatory effect on the formation of mineralized bone nodules in long-term cultures of human SaOS-2 cells. Osteosarcoma cell lines such as SaOS-2 have been developed to overcome the limitations of working with normal HOBs obtained either by human bone explant technique [30] or from human bone marrow [31]. HOB cells need a long time to grow in culture to become confluent, and only the first few passages can be used for experiments [32,33]. HOB cells grow slowly, which results in prolonged and costly maintenance, and their phenotypic properties tend to change after several passages. Because of the heterogeneous nature of these cells, their responses are also age dependent [30,34]. Although SaOS-2 is a transformed cell line, these cells have a stable osteoblast-like phenotype [20]. They not only grow rapidly but also form bone nodule-like structures and have been shown to differentiate into various stages in culture [35,36]. SaOS-2 cells develop different stages of differentiation in the presence and absence of Dex. Absence of physiological concentrations of Dex results in the development of a less-differentiated (Dex –cells) and continuous presence of Dex to a more-differentiated (Dex +cells) osteoblastic phenotype. The more-differentiated cultures express higher levels of ALP [20,35,37]. It is not exactly known which stage of osteoblastic differentiation SaOS-2 cells represent. However, based on different levels of ALP expression, they can qualitatively represent different stages of osteoblastic phenotype [21].

We used both von Kossa and alizarin red staining techniques to confirm the presence of hydroxyapatite \([\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]\), which is the mineral part of the bone that consists predominantly of calcium and phosphate. Silver nitrate used in von Kossa staining is known to react specifically with phosphate deposits [38]. As the presence of phosphate alone does not necessarily confirm the existence of hydroxyapatite, we used an additional staining method (alizarin red) that detects calcium deposits. Since each of these staining techniques detects a different deposit and since each may have different nonspecific binding, it is possible that the two methods produce slightly different outcomes. Our results, however, show very similar increases in nodule formation by both techniques. Although it is not possible to determine at which stage of differentiation SaOS-2 cells are on the day of initiating treatment, we observed that the cells at this stage were starting to form the extracellular matrix, and the addition of β-glycerophosphate on Day 8 contributed to their maturation. Addition of EGCG at this point clearly accelerated the rate of cell maturation as shown by the increase in ALP (Fig. 3) and by the increase in bone nodule area and number (Fig. 2).

The process of formation of mineralized nodules has three main developmental stages: proliferation, development of the extracellular matrix and mineralization [12]. ALP activity is considered an early marker of osteoblastic differentiation because it generally increases during the differentiation stage and decreases when mineralization has progressed [39]. We observed a significant increase in ALP activity in cells treated with 5 μM EGCG after 2 and 8 days of treatment. Although this effect was not significant on Days 4 and 6, probably due to the variability in ALP activity between samples within each group, a trend toward increased ALP activity in EGCG-treated cells was observed. The sampling time for ALP analysis was chosen based on our observation that the first mineralized nodules formed after 2 days of treatment with EGCG. These nodules continued to grow until the end of the culture period, which was 8 days after EGCG treatment. Although some studies using other osteoblastic cell lines have grown cells in differentiation media for up to 25 days [40], the SaOS-2 cells used in the present experiment begin to detach after a prolonged period in culture, which limited investigating the time to peak ALP activity. The stimulating effect of EGCG on ALP activity indicates an increase in the differentiation of SaOS-2 cells toward the mature bone-forming phenotype.

To determine a possible pathway through which EGCG may have increased the formation of bone nodules, we examined the levels of Runx2 protein in cells treated with EGCG at different time points using an antibody that detects both isoforms of Runx2. Differentiation of osteoblasts from multipotent mesenchymal cells is a process mainly controlled and regulated by the Runx2 transcription factor, a member of the DNA binding runt-domain family [14,41]. Runx2-deficient calvarial cells in vitro have been shown to differentiate into both adipocytes and chondrocytes but totally lacked the ability to differentiate into osteoblasts [42]. However, after cells commit to the osteoblast lineage, the function of Runx2 may become different. Transgenic mice that overexpress Runx2 in their osteoblast lineage have severe osteopenia and bone fractures, with an increase in osteoblast number from an early developmental stage, but impaired osteoblast function in matrix production and mineralization. Osteoblast maturation in these mice was shown to be inhibited at a late stage, and the failure in terminal differentiation resulted in bone fragility, demonstrating that Runx2 inhibits osteoblast differentiation at a late stage [17]. Our results showed a decrease in Runx2 after 48 h of treatment with EGCG. The reduction in the level of Runx2 protein by EGCG was observed with 1 μM and reached a maximal inhibitory effect with 5 μM. Since Runx2 is known to negatively regulate late-stage osteoblast
differentiation and restrain osteoblast maturation, the inhibitory effect of EGCG on Runx2 protein provides a potential mechanism by which this compound increases the formation of mineralized bone nodules. By blocking the synthesis of Runx2 protein, EGCG may enhance the differentiation of osteoblasts to progress to the maturation level, thereby increasing the mineralization of bone matrix and forming more bone nodules.

In summary, our results demonstrate that EGCG increases the differentiation and formation of mineralized bone nodules from HOB-like cells and suggest that green tea catechins may have beneficial effects on bone health.

References


