Effects of Tea Catechins, Epigallocatechin, Gallocatechin, and Gallocatechin Gallate, on Bone Metabolism

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In this study, three tea catechins, epigallocatechin (EGC), gallocatechin (GC), and gallocatechin gallate (GCG), were investigated for their effects on bone metabolism. The effects of the tea catechins on bone formation were evaluated using cultured rat osteoblast-like osteosarcoma cell line UMR-106. EGC stimulated alkaline phosphatase activity significantly at concentrations of 10 and 20 μM. The amount of mineralization also increased significantly with EGC. On another cell culture platform, EGC significantly inhibited osteoclast formations from RAW 264.7 cells upon receptor activation of nuclear factor-κB ligand induction on the fourth day of treatment, at a concentration of 10 μM. EGC also dose-dependently inhibited the mRNA expression of tatrate-resistant acid phosphatase. GC and GCG could decrease osteoclastogenesis at 20 μM. The present study illustrated that the tea catechins, EGC in particular, had positive effects on bone metabolism through a double process of promoting osteoblastic activity and inhibiting osteoclast differentiations.

INTRODUCTION

Green tea is one of the most popular beverages consumed worldwide. Common green tea catechins include (−)-epigallocatechin-3-gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG), and (−)-epicatechin (EC) (1). These catechins have been shown to be epimerized to (−)-catechin (C), (−)-gallocatechin (GC), (−)-catechin gallate (CG), and (−)-gallocatechin gallate (GCG), respectively, after heat treatment (2). EGCG accounts for >50% of the total green tea ingredients, followed by EGC and ECG (3).

Most studies on tea and some polyphenolic components have been focused on their beneficial effects in preventing cancers (4), cardiovascular diseases (5), and neurodegenerative conditions (6). Apart from these, recent evidence showed that EGCG could increase the formation of mineralized bone nodules by human osteoblast-like, SaOS-2 cells (7), enhance osteogenesis in a bone marrow mesenchymal stem cell line (8), and induce apoptotic cell death of osteoclasts differentiated from RAW 264.7 cells (9) in vitro. EGCG may improve osteoporotic condition by inhibiting progressive bone loss due to both increased osteoclastic bone resorption and a decrease in osteoblastic bone formation. However, other than EGCG, there is no related study of other green tea catechins on bone metabolism.

In the present study, we evaluated the biological effects of EGC, GC, and GCG on the osteoblast-like osteosarcoma cell line UMR-106. We also investigated the effects of EGC, GC, and GCG on osteoclast differentiation in murine monocyte/macrophage RAW 264.7 cells under receptor activator of nuclear factor-κB ligand (RANKL) induction.

MATERIALS AND METHODS

Cell Culture and Cell Viability Assay. Osteoblast-like UMR-106 cells and murine monocyte/macrophage RAW 264.7 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and subcultured to confluence in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Carlsbad, CA) containing 10% fetal bovine serum (Life Technologies), penicillin (100 U/mL; Life Technologies), and streptomycin (100 μg/mL; Life Technologies) in a humidified 5% CO_2 atmosphere at 37 °C. Cell viability was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO) assay after 2 and 6 days of treatment with different tea catechins (Sigma) at various concentrations in 96-well plates (UMR-106, 5×10^3; and RAW 264.7, 5×10^2 cells/well). The relative amount of viable cells was determined by measuring the reduction of MTT dye in live cells to blue formazan crystals at optical density at 540 nm and expressed as the percentage of control samples without treatment.

Catechin Treatment in Vitro. Three tea catechins, (−)-EGC, (−)-GC, and (−)-GCG, powders were stored at 4 °C. Before the experiments, the catechins were dissolved in dimethyl sulfoxide with a concentration of 20 mM and kept at −20 °C for the remaining experiments. The catechin stocks were diluted with culture medium immediately before applications. Cells were treated with different catechins from 0 to 20 μM. In the study of osteoblastic differentiation, UMR-106 cells were seeded in 6-well plates at 5×10^4 cells/well in DMEM. Cells were harvested at 4 and 6 days for alkaline phosphatase activity and matrix mineralization assays, respectively. For the study of osteoclastic differentiation, RAW 264.7 cells were seeded in 96-well plates at a density of 5×10^2 cells/well in DMEM. After 1 day of incubation, the differentiation of osteoclasts from RAW 264.7 cells was induced with 50 ng/mL of recombinant

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mouse soluble RANKL (Sigma) in α-minimal essential medium (Life Technologies) with 10% FBS for 4 days.

Alkaline Phosphatase (ALP) Activity Assay. ALP activity of treated UMR-106 cells was determined on day 4 using an Alkaline Phosphatase Microwell Substrate System (Sigma) according to the manufacturer's instruction. In brief, each sample was rinsed twice with PBS and extracted with lysis buffer consisting of 50 mM Tris-HCl (pH 7.2), 0.1% Triton X-100, and 2 mM MgCl$_2$. The lysate was then subjected to a freeze−thaw cycle at −70 °C twice. The supernatant was collected after centrifugation at 10000g for 10 min, and ALP activities were determined in assay buffer containing bromochloro indoyl phosphate analogue and nitro blue tetrazolium. The optical intensity of the reaction mixture was measured at 595 nm after 15 min of incubation. ALP activity was normalized by the protein content and expressed as percentage relative to the control group.

Matrix Mineralization Assay. The degree of matrix mineralization of UMR-106 cell was measured using an Alizarin Red S staining method after 6 days of catechin treatment. Each sample was rinsed twice with PBS and fixed with ice-cold 70% ethanol for 1 h. Then the sample was rinsed twice with distilled water, followed by a 10 min staining process using 40 mM Alizarin Red S solution (pH 4.2; Sigma). The unbonded stain was removed by washing with distilled water five times. The amount of stain on each sample was measured by shaking the sample with 10% cetylpyridinium chloride (Sigma) in 10 mM sodium phosphate (pH 7.0) for 15 min and then measured at an optical intensity of 562 nm. The relative matrix mineralization values were normalized by the relative cell viability and were expressed as percentage relative to the control group.

Osteoclast Staining. After treatment with RANKL and different catechins for 4 days, the RAW 264.7 cell differentiated osteoclasts were fixed and stained for tatrate-resistant acid phosphatase (TRAP), an osteoclast enzyme marker, by using an acid phosphatase kit (Sigma) according to the manufacturer's instructions. TRAP-positive multinucleated cells showing more than three nuclei were counted as osteoclasts.

Real-Time PCR on TRAP mRNA Expression. After treatment with RANKL and different catechins for 4 days, total mRNA of each treated osteoclastic culture was isolated by the RNeasy mini kit (Qiagen, GmbH) and stored at −20 °C. The amount of TRAP mRNA was determined using Quanti-Fast SYBR Green RT-PCR kit (Qiagen) with a validated primer set specific for TRAP mRNA (Qiagen; NM_001102404) on an ABI 7500 Fast Real-Time PCR system (Life Technologies). The threshold cycle (Ct), the cycle number at which the amount of amplified gene of interest reached a fixed threshold, was determined. Relative expression of the RT-PCR product was calculated using the comparative $2^{-\Delta\Delta Ct}$ method. Endogenous control glyceraldehyde-3-phosphate dehydrogenase mRNA was used for normalization. Fold difference was then determined by normalizing all values to the mean of the relative expression for the control group without treatment.

Statistical Analysis. The differences between treatment and control groups were tested with the Kruskal−Wallis test, followed by the post hoc Dunn test. All statistical analyses were performed by using the Statistical Package of Social Science (SPSS) version 15.0 for Windows and carried out at the 5% level of significance ($P < 0.05$). Data are expressed as mean ± standard error of the mean (SEM).

RESULTS

Effects of Tea Catechins on Osteoblastic Differentiation of UMR-106 Cells. As shown in **Figure 1**, there were no cytotoxic effects of EGC, GC, and GCG on UMR-106 cells upon treatment for 2 and 6 days at concentrations up to 20 μM. To determine whether these tea catechins could stimulate osteoblastic cell differentiation, their effects on ALP activity and matrix mineralization were studied. Our data illustrate that treatment of UMR-106 cells with EGC for 4 days stimulated ALP activity in a dose-dependent manner (**Figure 2**). ALP activity was significantly increased by 39.3 ± 2.3 and 78.7 ± 7.9% with EGC concentration at 10 and 20 μM, respectively, compared with respective control without treatment. However, there was a slight but insignificant decrease in ALP activity upon GC and GCG treatment. For matrix mineralization, the addition of EGC
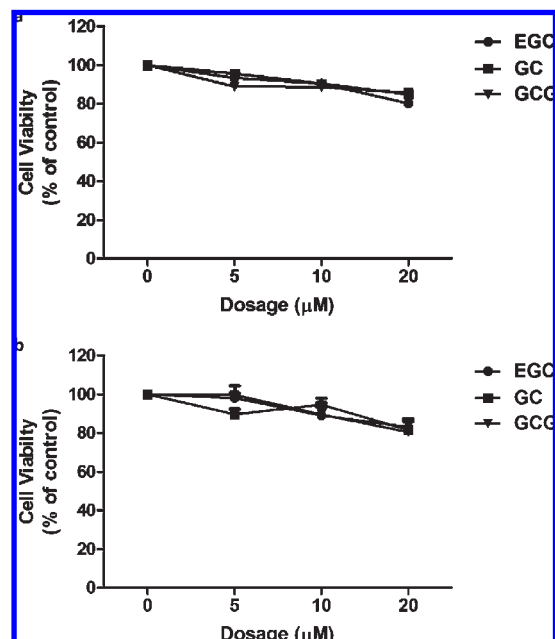

Figure 1. Effect of EGC, GC, and GCG on cell viability of osteoblast-like UMR106 cells for (**a**) 2 days and (**b**) 6 days at different concentrations (5−20 μM). Data are the means (SEM; error bars) from three independent experiments in triplicate. ∗, $P < 0.05$ for difference in cell viability from respective baseline culture without tea catechins.
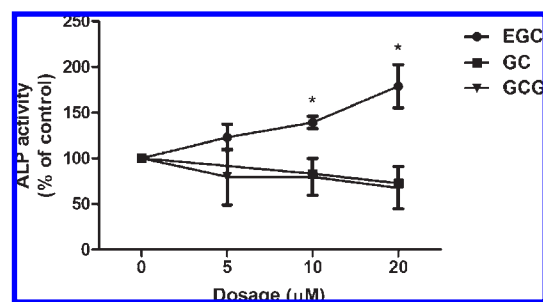

Figure 2. Dose effect of EGC, GC, and GCG on the alkaline phosphatase (ALP) activity of UMR106 cells at day 4. Data are the means (SEM; error bars) from three independent experiments in triplicate. ∗, $P < 0.05$ for difference in ALP activity from respective baseline culture without tea catechins.

significantly increased the matrix mineralization at 20 μM after 6 days of culture, whereas GC and GCG had no effect on matrix mineralization on UMR-106 cells at any concentration (**Figure 3**).

Effect of Tea Catechins on RANKL-Induced Osteoclast Differentiation of RAW264.7 Cells. Our data demonstrated that the viability of osteoclast precursor RAW 264.7 cells was not affected by EGC, GC, and GCG with concentrations up to 20 μM (**Figure 4**). To determine the effects of these catechins on RANKL-induced osteoclastogenesis of RAW 264.7 cells, TRAP-positive cells were stained and counted after 4 days of treatment. Compared with nontreated cells, concentrations of all studied tea catechins up to 5 μM showed no effects on RANKL-induced osteoclast differentiation. A slight but statistically insignificant decrease of TRAP-positive osteoclast number was observed at 10 μM GC and GCG ($P > 0.05$). In contrast, at the same concentration, the number of TRAP-positive osteoclasts was decreased significantly by 43.4 ± 6.9% when treated with EGC. At a higher concentration of 20 μM, the osteoclast number was effectively decreased by 30.1 ± 7.7 and 37.9 ± 3.1%,
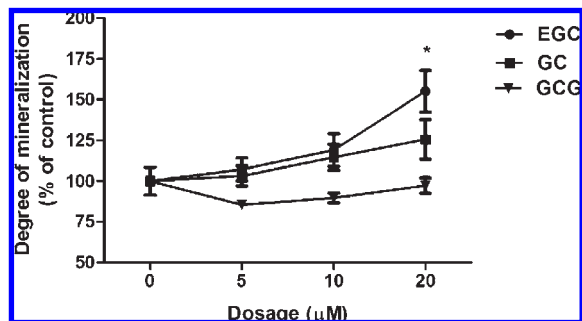
Figure 3. Effect of EGC, GC, and GCG on matrix mineralization of UMR-106 cells at day 6. Data are the means (SEM; error bars) from three independent experiments in triplicate. *, $P < 0.05$ for difference in matrix mineralization from respective baseline culture without tea catechins.
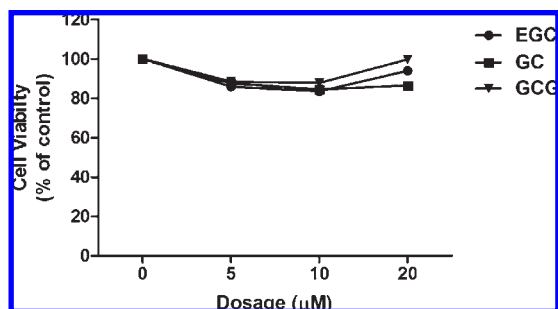

Figure 4. Effect of EGC, GC, and GCG on cell viability of mouse macrophage RAW 264.7 cells. Cells were treated with different catechins for 48 h (5−20 μM). Data are the means (SEM; error bars) from three independent experiments in triplicate. *, $P < 0.05$ for difference in cell viability from respective baseline culture without tea catechins.
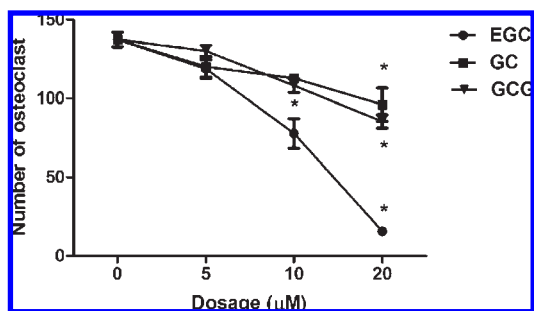

Figure 5. Effect of EGC, GC, and GCG on inhibition of osteoclastogenesis from RAW 264.7 cells upon RANKL induction for 4 days at different concentration (5−20 μM). TRAP-positive multinucleated cells with more than three nuclei were considered as osteoclasts. Data are the means (SEM; error bars) from three independent experiments in triplicate. *, $P < 0.05$ for difference in number of osteoclast from respective baseline culture without tea catechins.

respectively, when treated with GC and GCG ($P < 0.05$) (**Figure 5**). At the same concentration, EGC also significantly decreased the osteoclast formation by 88.6 ± 1.0% ($P < 0.05$). These data indicated that catechins EGC, GC, and GCG effectively regulated RANKL-induced osteoclastogenesis on RAW 264.7 cells. Among them, EGC was most potent in the inhibition of osteoclast development. Real-time RT-PCR analysis indicated that EGC could effectively down-regulate TRAP mRNA expression in a dose-dependent manner (5−20 μM) after 4 days of treatment (**Figure 6**).
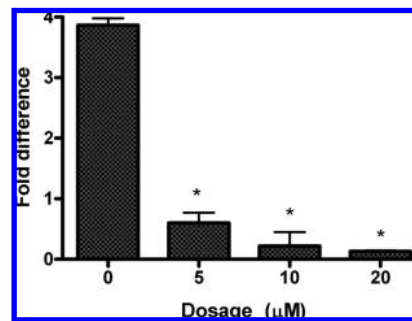

Figure 6. Inhibitory effect of EGC on TRAP mRNA expression of RAW 264.7 cells upon RANKL induction for 4 days in dose-dependent manner (5−20 μM). The expression level of TRAP gene was normalized on the basis of GAPDH expression. Fold difference was determined as the relative expression, compared with control cells without EGC and RANKL treatment. Data are the means (SEM; error bars) from three independent experiments in triplicate. *, $P < 0.05$ for difference in TRAP mRNA expression from culture with RANKL treatment only.

DISCUSSION

Green tea catechins have received considerable attention worldwide because of their many scientifically proven beneficial effects on human health (*10*, *11*). A number of epidemiological studies have been conducted on the beneficial effect of tea consumption on bone health (*12*, *13*). These epidemiological studies found that the bone mineral density of postmenopausal women who have a habit of tea drinking was higher than that of other women. Nowadays, bottled green tea drink is becoming increasing popular. It was reported that a considerable amount of green tea catechins was converted to their corresponding epimers in bottled green tea during heat pasteurization (*14*). It is possible that these epimers might possess properties similar to those of their catechin precursors; however, information related to their biological activities is limited. Most of the recent studies were focused on the comparison of antioxidative effects between different catechins and their epimers (*15*). Their effects on bone metabolism had not been a focus of attention.

In our present study, we showed that EGC stimulated osteoblastic differentiation in an osteoblast-like osteosarcoma cell line, UMR-106. This was indicated by the increase in ALP activity and matrix mineralization. Our result also demonstrated that EGC significantly inhibited RANKL-induced osteoclast differentiation from murine monocyte/macrophage RAW 264.7 cells as was revealed in the decrease of osteoclast number and mRNA expression of TRAP. These findings were similar to the biological activities of EGCG reported in bone metabolism in vitro (*7*−*9*). It was demonstrated that EGC-caused inhibition of TRAP mRNA expression was much stronger than that of number of TRAP-positive osteoclasts. The inhibitory actions of EGC might be time-dependent, and a lower dose of EGC would cause a significant effect on TRAP mRNA expression at day 3. There may be a time lag between the decrease in TRAP mRNA levels and the corresponding decrease in TRAP−protein synthesis. We found that GC and GCG also inhibited the osteoclastogenesis effectively, but that they did not promote the osteoblastic differentiation. All of the studied catechins were not toxic to the UMR-106 and RAW 264.7 cells after 6 days at increasing doses, up to a concentration of 20 μM.

The osteosarcoma cell line UMR-106 has been extensively used as a model for the study of bone formation. Although it is a transformed cell line, the cells have a stable osteoblast-like phenotype, making them a good model for studying the characteristics and functions of osteoblast (*16*, *17*). They grow rapidly

and exhibit their ability to differentiate into different stages in culture. The more differentiated cultures express higher ALP levels and greater extent of matrix mineralization (*18*). Our results indicated that EGC not only significantly increased ALP activity in a dose-dependent manner but also led to an increase of bone matrix mineralization in UMR-106 cells. The stimulating effects of EGC indicated an increase in differentiation toward the mature bone forming phenotype. However, the mechanism of EGC on the promotion of osteoblastic differentiation remains to be elucidated. Our result demonstrated that GC and GCG did not have any effect on osteoblastic differentiation. It might be suggested that because of the structure alteration during heat epimerization, GC and GCG might undergo structural alterations which diminish their ability to promote osteoblast differentiation.

An abnormal increase of osteoclast differentiation is one of the major causes in pathological bone erosion as well as osteoporosis (*19, 20*). Osteoclasts, cells specialized for bone resorption, are formed from a discrete population of hematopoietic cells under RANKL induction (*21*). Recently, it has been reported that reactive oxidative species (ROS) might play an important role as osteoclastic differentiation signaling intermediates and that bone resorption in vivo could be controlled by antioxidants (*22*). RANKL treatment led to ROS production through NADPH oxidase and activated NF-κB downstreaming osteoclastogenesis in osteoclast precursor cells (*23*). The green tea catechins EGC, GC, and GCG had been proven as potent antioxidants (*14*), and their antioxidative property may confer on them the potential to block osteoclast differentiation in vitro by ablating RANKL-initiated ROS production. Xu et al. reported that epimers of EGC, EGCG, GC, and GCG were less effective antioxidants than their precursors (*14*), and therefore they may also exert less inhibitory effects on osteoclast formation. Our study showed that EGC, GC, and GCG exerted their effects on differentiated osteoclasts but not on the undifferentiated RAW 264.7 cells. We also showed that all studied catechins had no inhibitory effect on the cell viability on UMR-106 cells at concentrations up to 20 μM. Taking these results together it was observed that EGC, GC, and GCG, at appropriate concentrations, could inhibit osteoclast differentiation. Therefore, their potential use as a chemopreventive agent against osteoporosis could be studied.

There has been accumulating evidence showing that EGCG can effectively inhibit osteoclastgenesis from RAW 264.7 cells upon RANKL induction (*24, 25*). Our pilot study was in line with those reports that EGCG can decrease the osteoclast number at 10 μM (data not shown). In our present study, GC and GCG were significantly less effective than their structural precursors, EGC and EGCG, in osteoclastgenesis inhibition, suggesting that epimerization may decrease the efficacy of these catechins. Recently, it was reported that there were significant differences in pharmacokinetics and bioavailability between catechins and their epimers in some cell culture systems (*26*). Epimerization of catechins may significantly affect the biological activities and cellular uptake. Further comparative study is needed to elucidate the influence of epimerization in biological systems.

In summary, our study has provided the first laboratory evidence on the bone promotion effects of the green tea catechin EGC as was demonstrated by the promotion of osteoblastic differentiation and inhibition of osteoclast formation. Moreover, GC and GCG can effectively inhibit osteoclastgenesis. However, the mechanisms of action of EGC, GC, and GCG must be complex. Our observations would serve as groundwork for further studies. Studies could explore the signaling pathways and gene expressions upon administration of these catechins on in vitro systems. Furthermore, the efficacy of these tea catechins on

bone metabolism in vivo, using appropriate osteoporotic animal models, could be evaluated.

ABBREVIATIONS USED

EGC, (−)-epigallocatechin; EGCG, (−)-epigallocatechin-3-gallate; GC, (−)-gallocatechin; GCG, (−)-gallocatechin gallate; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; TRAP, tatrate-resistant acid phosphatase.

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