(−)-Epigallocatechin-3-gallate Inhibits Differentiation and Matrix Metalloproteinases Expression in Osteoclasts

Yuko IRIE1,2, Shinichi IWAI3, Hitoshi AMANO4, Kakei RYU1, Haruka EMORI1,2, Tomoko KAWAKAMI1, Hidehiro KOCHIDAIRA3, Yoshito KOBAYASHI1 and Katsuji OGUCHI1

Abstract: The osteoclast is a multinucleated giant cell differentiated from monocyte macrophages that has an important role in bone resorption. Several studies have reported a relationship between tea consumption and decreased risk of bone fracture. Matrix metalloproteinases (MMPs) play an important role in the degeneration of bone and cartilage matrix. Regulation of osteoclast activity is essential in the treatment of bone disease. Moreover, MMPs are associated with osteoclast formation and differentiation. We have reported previously that (−)-epigallocatechin-3-gallate (EGCG) inhibits MMP-2 and MMP-9 expression and activity. However, the effects of EGCG on osteoclasts and other MMPs are not clear. Therefore, in the present study we examined whether EGCG affects MMP expression, as well as osteoclast formation, differentiation and activity, in vitro. We used bone marrow cells from the femur and tibial bones of male ddY mice. Bone marrow cells were cultured in the presence of 1−100 μM EGCG for 6 or 8 days. EGCG decreased the number of mature osteoclasts, as determined by tartrate-resistant acid phosphatase staining. Concentrations as low as 1 μM EGCG clearly inhibited the differentiation of osteoclasts from bone marrow cells. EGCG also inhibited the number of osteoclasts with an actin-ring, as determined by rhodamine phalloidin staining, as well as osteoclast activity, as determined by the pit formation assay. Furthermore, EGCG concentration-dependently decreased MMP-9 and membrane type 1-MMP mRNA expression in mouse osteoclasts. However, EGCG had no changing on mRNA levels of tissue inhibitor of metalloprotease (TIMP)-1 and TIMP-3. Together, the results suggest that EGCG may be a suitable agent or lead compound for the development of treatments for bone resorption diseases associated with MMPs.

Key words: (−)-epigallocatechin-3-gallate (EGCG), mouse osteoclast, mouse bone marrow cell, matrix metalloproteinase (MMP), tissue inhibitors of metalloprotease (TIMP)
Introduction

It has been proposed that tea consumption can activate mechanisms that modulate and potentially slow the progression of age-related diseases, such as cardiovascular disease, cancer, obesity, Alzheimer’s disease and osteoporosis. Several recent studies have reported that tea consumption has a beneficial effect on orthopedic diseases. For example, tea consumption reportedly helps protect against hip fractures, and bone mineral density measurements are higher in older women who drink tea compared with those who do not. Green, black, and Oolong teas are made from the leaves of *Camellia sinensis*. Catechins are important components of green tea and include (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG), and (-)-epicatechin (EC). EGCG is the most abundant polyphenol in green tea. It is known that catechins, particularly EGCG, have health benefits. For example, EGCG has been reported to suppress osteoclast differentiation and ameliorate rheumatoid arthritis (RA) in a mouse model of the disease. Osteoclasts, which are essential in bone homeostasis, play a key role in the development of osteoporosis, inflammatory arthritis, and RA. Progressive bone destruction in RA involves the abnormal activation of osteoclasts. This activation is due to interactions with synovial fibroblasts and T helper cells that express receptor activator of nuclear factor-κB ligand (RANKL). To treat bone-associated diseases, it is important that osteoclast activity be inhibited.

There are several reports of the effects of EGCG on mouse osteoclasts. In a previous study, we demonstrated that theaflavin-3, 3’-digallate (TFDG) and EGCG, at concentrations of 10 or 100 μM, inhibit osteoclast formation and/or differentiation in rat osteoclast precursor cells, as well as in mature osteoclasts. In addition, TFDG and EGCG suppress matrix metalloproteinase (MMP)-2 and MMP-9 enzyme activity, and MMP-9 mRNA expression in osteoclast precursor cells. Activated osteoclasts exhibit polarized cytoskeletal structures known as actin rings, and the formation of these rings is important for bone resorption. TFDG and EGCG may suppress osteoclast differentiation by inhibiting the formation of cytoskeletal actin rings in mature osteoclasts as a result of the suppression of MMPs.

MMPs comprise a family of zinc-dependent endopeptidases and include collagenases (MMP-1, -8, -13 and -18), gelatinases (MMP-2 and -9), stromelysins (MMP-3 and -10), and membrane-type (MT1-MMP; MMP-14) endopeptidases. These MMPs can degrade the organic components of connective tissue matrices. MMPs play a crucial role in tissue remodeling and in the destruction of bone in arthritic joints because they are able to degrade a wide variety of extracellular matrix components. In particular, MMP-9 is essential for initiating the osteoclastic resorption process by removal of the collagenous layer from the bone surface prior to demineralization. Osteoclasts produce MMPs; furthermore, MMP-9 expression in osteoclasts is markedly higher than in other cell types. MMPs are secreted as proenzymes by many cell types. MMP activity is regulated by activation of precursorzymogens and is inhibited by endogenous inhibitors and tissue inhibitors of metalloproteinases (TIMPs). TIMPs tightly control MMP activity at a 1:1 ratio. TIMP-1 inhibits all known active forms of MMPs, as well as proMMP-9.
However, the relationship between EGCG and many MMPs and TIMPs in osteoclasts remains somewhat unclear.

In the present study we examined the ability of EGCG to suppress the expression, differentiation, maturation, and activity of individual MMPs and TIMPs in mouse osteoclasts obtained from bone marrow cells.

**Materials and methods**

**Animals and reagents**

In the present study we used 5-8-week-old male ddY mice (Sankyo Labo Service Corporation, Tokyo, Japan). All experiments were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals of The Japanese Pharmacological Society and were approved by the Showa University Animal Care and Use Committee (certificate no. 13041). EGCG was purchased from Wako Pure Chemical Industries (Osaka, Japan). Macrophage colony-stimulating factor (M-CSF/CSF-1) and RANKL were obtained from Peprotech (Rocky Hill, NJ, USA).

**Osteoclast formation assay**

Bone marrow cells were obtained from the femur and tibia of mice. The bone marrow cells were cultured to a high density (1.0 × 10^5 cells/well) in a 96-well plate for 6 days in medium containing 15% serum, 25 ng/mL M-CSF, and 100 ng/mL RANKL at 37°C in a humidified atmosphere of 5% CO₂. The medium was changed on Days 1 and 3. The cells were assigned to one of five experimental groups: (i) a negative control group (without M-CSF and RANKL); (ii) a control group (medium alone); and (iii-v) cells treated with 1, 10 or 100 μM EGCG, respectively. EGCG was added to the appropriate cultures each day for 6 days.

Bone marrow cells (1.0 × 10^6 cells/well) were also cultured in a six-well plate for 8 days in the same medium and under the same conditions as described above. The medium was changed on Days 2, 4 and 7. In this series of experiments, cells were assigned to one of four groups, namely a control group (medium alone) and cells treated with 10, 50 or 100 μM EGCG. EGCG was added to the appropriate cultures on each day for 8 days.

**F-Actin and tartrate-resistant acid phosphatase staining**

At the end of the culture period, cells were fixed with 4% paraformaldehyde for F-actin staining and then permeated with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 5 min. F-Actin was stained with 0.3 mM rhodamine-conjugated phalloidin. The ring-forming F-actin bands were detected under a Zeiss Axiophot fluorescent microscope (Carl Zeiss, Jena, Germany). Subsequently, cells were stained for tartrate resistant acid phosphatase (TRAP) using a final concentration of 50 mM tartrate. TRAP-positive multinucleated cells with more than three nuclei were considered to be osteoclasts and were counted under a light microscope.
Pit formation assay

Bone marrow cells (1.0 x 10^6 cells / well) were cultured in a Corning Osteo Assay Surface Polystyrene 1 x 8 Stripwell microplate (Corning, Corning, NY, USA) for 8 days in medium containing 15% serum, 25 ng / mL M-CSF, and 100 ng / mL RANKL at 37°C in a humidified atmosphere of 5% CO₂. The medium was changed on Days 2, 4, and 7. Cells were assigned to one of three groups, namely a control group (medium alone) and groups cultured in the presence of 10 or 50 μM EGCG. EGCG was added to the appropriate cultures each day for 8 days. Von Kossa staining was used to analyze the microplate surface for pit formation. Briefly, the medium was aspirated from the wells after 8 days and the wells were washed with distilled water. The wells were then incubated with 1 M ammonia solution overnight. After overnight incubation, the wells were washed with distilled water before being stained with 5% silver nitrate for 60 min at room temperature under a bright light. To stop the development of silver nitrate, cells were washed with water and treated with a 5% solution of sodium thiosulfate. Following another water wash and air drying, nodules were visualized as dark-stained patches under a light microscope. The resorbed area was measured using Adobe Photoshop Elements 9 (Adobe Systems, San Jose, CA, USA).

RNA isolation and quantitative real-time reverse transcription-polymerase chain reaction

Quantitative polymerase chain reaction (PCR) was used to quantify mRNA levels of MMP-9, MMP-13, MT1-MMP, TIMP-1 and TIMP-3 in osteoclasts (after 8 days culture) and / or in bone marrow cells (after 2 days culture). Cells (1.0 x 10^6 cells / well) were harvested and total RNA was extracted with NucleoSpin RNA II (MACHEREY-NAGEL, Duren, Germany). Total RNA was reverse transcribed using PrimeScript RT Master Mix (TaKaRa Bio, Shiga, Japan). The cDNA was amplified using primers designed with ProbeFiber software (Roche Applied Science, Mannheim, Germany). Table 1 details the primers used, the Roche Universal ProbeLibrary Probe numbers, and accession numbers. Amplification was performed with a LightCycler

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using LightCycler TaqMan Master mix (Roche). The PCR reaction parameters were 95°C for 10 min, followed by 45 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 1 s. Fluorescence data were analyzed with LightCycler software (Roche). The mRNA levels were compared against those of 18s rRNA as a standard, and relative expression ratios were calculated.

Statistical analysis

Data were analyzed using the Bonferroni test following one-way analysis of variance (ANOVA). All data are expressed as the mean ± SEM. P<0.05 was considered significant.

Results

Effect of tea polyphenols on osteoclast formation

To examine the effect of EGCG on osteoclast formation, bone marrow cell cultures were treated with EGCG at concentrations of 1, 10, and 100 µM for 6 days (Fig. 1D, E). There were more TRAP-positive multinucleated osteoclasts in the control group (Fig. 1C) compared with the negative control group, cultured in the absence of RANKL and M-CSF (Fig. 1A). Osteoclasts treated with 1 µM EGCG were smaller and had fewer nuclei than osteoclasts in the control group (Fig. 1C, F). Moreover, treatment with 1 µM EGCG resulted in markedly fewer multinucleated osteoclasts in cultures (as low as 46.6% of the number in control cultures). Treatment with 100 µM EGCG strongly suppressed cell-cell fusion and the proliferation of bone marrow cells (Fig. 1D). Moreover, no multinucleated osteoclasts were observed in cultures treated with 100 µM EGCG (Fig. 1D). 100 µM was not toxic to bone marrow cells, because the number of bone marrow cells and/or osteoclast precursor cells following treatment with 100 µM EGCG was higher than that in the Day 3 control and in the negative control. EGCG suppressed osteoclast formation in a dose-dependent manner (Fig. 1G).

Effect of EGCG on F-actin bands of bone marrow cells

After 8 days culture in medium containing RANKL and M-CSF (control culture), numerous large multinucleated osteoclasts were detected. In contrast, very few large multinucleated osteoclasts were observed after cell culture in the presence of EGCG. As shown in Fig. 2, EGCG inhibited the formation of TRAP-positive multinuclear osteoclasts from mature osteoclasts. Furthermore, ring-forming F-actin bands were evident around TRAP-positive multinuclear osteoclasts in control cultures (Fig. 2A, E). However, there was a decrease in the ring-forming F-actin bands in the positive control and in 10 µM EGCG to 474% and 38.7% as compared with the number of TRAP-positive multinuclear osteoclasts, respectively (Fig. 2I, J). Figure 2F shows incomplete ring formation of the F-actin bands in the 10 µM EGCG-treated group. Exposure of cells to EGCG dose-dependently reduced the number of mature osteoclasts, as characterized by actin rings (Fig. 2F-H, J).

Effects of tea polyphenols on osteoclast activity

To examine the effect of EGCG on osteoclast activity, bone marrow cells were cultured in
the presence of 10 and 50 μM EGCG for 8 days (Fig. 3A-C). Treatment with EGCG dose-dependently reduced osteoclast activity (Fig. 3D).

Effects of EGCG on mRNA levels of MMPs and TIMPs in osteoclasts

Figure 4 shows the expression of MMP and TIMP mRNA following treatment with 10, 50, and 100 μM EGCG for 8 days, normalized against mRNA expression in the control group at
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Fig. 2. Effects of (-)-epigallocatechin-3-gallate (EGCG) on the maturation of osteoclasts in vitro. Bone marrow cells (1.0 × 10^5 cells/well) were cultured in a six-well plate for 8 days. The low condensed bone marrow cells become the mature osteoclasts at 8 days of culture. Cells were stained with rhodamine-conjugated phalloidin (E-H), after which osteoclasts were identified by tartrate-resistant acid phosphatase (TRAP) staining (A–D). Red arrows (A, B) indicate TRAP-positive multinucleated osteoclasts; the white arrow (E) shows clear F-actin rings, the yellow arrow (F) indicates unclear F-actin rings. (I) TRAP-positive multinucleated cells characterized by more than three nuclei were determined to be osteoclasts and counted. (J) Osteoclasts exhibiting clear F-actin rings (white arrow) were determined to be activated osteoclasts and were counted. Cont, control. Data are the mean ± SEM (n = 6 in each group). *P < 0.05, **P < 0.01 compared with control; ††P < 0.01 compared with 10 μM EGCG.
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Resorption Pit Assay

![Resorption Pit Assay](image)

Fig. 3. Effects of (−)-epigallocatechin-3-gallate (EGCG) on osteoclast activity in vitro. Bone marrow cells \((1.0 \times 10^5 \text{ cells/well})\) were cultured in a Corning Osteo Assay Surface Polystyrene \(1 \times 8\) Stripwell microplate for 8 days. The plate was stained with Von Kossa stain (A–C) and the resorbed area was then measured (D). Cont, control. Data are the mean±SEM \((n = 7–8)\). **P < 0.01 compared with control; ††P < 0.01 compared with 10 μM EGCG.

8 days (set at 100%). EGCG treatment dose-dependently reduced MMP-9 mRNA levels (Fig. 4A). MMP-9 mRNA levels of cultured bone marrow cells after 2 days were significantly different compared with the 8 days control \((P < 0.01)\) and 100 μM EGCG-treated group \((P = 0.010)\).

There was no significant change in MMP-13 mRNA levels from Day 2 to Day 8 of culture in either the control or 10 μM EGCG-treated group. In contrast, after 8 days culture, 50 and 100 μM EGCG suppressed MMP-13 mRNA expression, although the suppression in the presence of 50 μM EGCG was greater (Fig. 4B). Exposure of cells to 10, 50, and 100 μM EGCG for 8 days dose-dependently reduced MT1-MMP mRNA levels (Fig. 4C). On Day 2 of in the control culture, TIMP-1 mRNA levels were significantly higher in the control group compared with the 10, 50, and 100 μM EGCG-treated groups \((P < 0.05, P < 0.01, \text{and} \ P < 0.01, \text{respectively})\). However, there were no significant changes in TIMP-1 mRNA expression from Day 2 to Day 8 \((P = 0.10, \text{Fig. 4D})\). As indicated in Fig. 4E, there were no significant differences among the control and EGCG-treated groups after 8 days in culture.

Discussion

We have demonstrated in the present study that 1 μM EGCG: (i) inhibits osteoclast forma-
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EGCG inhibits osteoclast activity and suppresses MMP-9, MMP-13, and MT1-MMP mRNA expression in osteoclasts. EGCG inhibited all the processes associated with MMPs and TIMPs involved in the maturation of osteoclasts from bone marrow cells.

Several cohort studies in Western countries have indicated that tea consumption may have osteoporosis-preventive effects. In a case-control study of 632 Japanese women aged 60 years or older, green tea intake was shown to increase bone density. Previous studies have demonstrated that the consumption of green tea extract (1.5–4.5 g, or 1–3 cups) by healthy individuals results in EGCG, ECG, EGC and EC in the plasma, and that the plasma concentration of EGCG may reach 4 μM. The results of the present study regarding EGCG (1 μM) are consistent with the results of these earlier studies. This plasma concentration of EGCG can be achieved by drinking green tea and it may help prevent osteoporosis. In recent years there have been various clinical studies on the effects of green tea...
on human health. Some reports focused on the benefits of benifuuki green tea, which contains \( O \)-methylated catechin. Because the ingestion of benifuuki tea results in higher blood concentrations of EGCG, it may have a more beneficial effect than other green teas\(^{21}\).

MMPs are enzymes responsible for the degradation of collagen fibrils. Evidence indicates that MMPs play a critical role in osteoclastic bone resorption, and that MMPs facilitate the migration of osteoclasts to the bone surface via the extracellular matrix\(^{22}\). Ishibashi \textit{et al.} reported that MMP-9 is expressed in osteoclasts at very high levels\(^{23}\). In the present study, we demonstrated that \( \text{MMP-9} \) mRNA expression in osteoclasts increased 2.93-fold compared with bone marrow cells after 2 days of culture (Fig. 4A). EGCG may inhibit \( \text{MMP-9} \) mRNA expression in osteoclasts by one of two methods. First, EGCG may inhibit the development of bone marrow cells (osteoclast precursor cells) to osteoclasts. It has been reported that inhibition of MMP-9 suppresses preosteoclast migration\(^{24}\), and EGCG has been shown to directly inhibit MMP-9 activity\(^{9}\). EGCG may inhibit MMP-9 expression and activity, which, in turn, inhibits osteoclast precursor cell migration, ultimately resulting in suppression of the formation of multinucleated osteoclasts. Consequently, EGCG suppression of \( \text{MMP-9} \) mRNA expression reduces the number of multinucleated osteoclasts. In the present study, we demonstrated that EGCG up to a concentration of 100 \( \mu M \) was not toxic to bone marrow cells, because the number of bone marrow cells and/or osteoclast precursor cells following treatment with 100 \( \mu M \) EGCG was higher than that in the Day 3 control and in the negative control (without M-CSF and RANKL; Fig. 1A, B, D).

Second, EGCG may directly suppress \( \text{MMP-9} \) mRNA expression. \( \text{MMP-9} \) mRNA expression following treatment with 100 \( \mu M \) EGCG was lower than that in the Day 2 control culture. TIMPs tightly control MMP activity in a 1:1 ratio\(^{12}\). TIMP-1 inhibits all known active forms of MMPs, as well as proMMP-9\(^{11}\). Thus, the balance between MMP-9 and TIMP-1 is important for approximating absolute MMP-9 activity\(^{15}\). TIMP-3 inhibits all known active forms of MMPs and some of the members of the ADAM (a disintegrin and metalloproteinase) family\(^{25, 26}\). In the present study, \( \text{TIMP-1} \) and \( \text{TIMP-3} \) mRNA expression in osteoclasts did not change with EGCG treatment, but there was a decrease in \( \text{MMP-9} \) mRNA expression. Thus, dose-dependent decreases in MMP-9 activity were observed following EGCG treatment. MMP-13 is more active against type II collagen than other MMPs\(^{27}\), and is obligatory for the migration of preosteoclasts\(^{24}\). Without the migration of preosteoclasts, multinucleated osteoclasts do not form. Of the TIMPs, TIMP-3 is the most effective inhibitor of MMP-13 activity, and it can also inhibit the activity of MMP-3, MMP-9, and ADAM-17\(^{25, 26}\). In the present study, there was no change in the mRNA expression of \( \text{TIMP-1} \) and \( \text{TIMP-3} \) in osteoclasts after EGCG treatment, but there was a decrease in \( \text{MMP-13} \) mRNA expression following 50 \( \mu M \) EGCG treatment. MT1-MMP associates with MMP-2 and TIMP-2 at the cell surface and regulates MMP-2 activation\(^{11}\), and MT1-MMP is necessary for bone marrow cell fusion during osteoclast and giant cell formation \textit{in vitro} and \textit{in vivo}\(^{28}\). However, these functions of MT1-MMP do not depend on the catalytic activity of MT1-MMP or on the downstream activation of proMMP-2\(^{20}\). MT1-MMP participates in bone marrow cell fusion by regulating Rac1 signaling\(^{29}\).
Regulation of osteoclast differentiation may be an important strategy in the treatment of bone resorption and osteoporosis. The present study demonstrated that at concentrations as low as 1 µM, EGCG can suppress differentiation in mouse osteoclasts. In addition, EGCG regulates MMPs and TIMPs mRNA expression in mouse osteoclasts. Thus, the results of the present study suggest that the intake of green tea, such as benifuuki green tea, may be effective in preventing osteoporosis.

Disclosure

The authors report no potential conflicts of interest.

References


[Received January 15, 2014 : Accepted January 29, 2014]