Introduction

Osteoclasts, which are essential in bone homeostasis, play a key role in the development of osteoporosis, inflammatory arthritis, and rheumatoid arthritis (RA). Osteoporosis, or low bone mineral density (BMD), is the single most important risk factor for fracture in older women (1). Progressive bone destruction in RA involves the abnormal activation of osteoclasts, which is due to interactions with synovial fibroblasts and helper T cells that express the receptor activator nuclear factor-κB ligand (RANKL) (2, 3).

The health benefits associated with tea consumption with respect to the prevention of cancers and cardiovascular disease have been investigated intensively (4); additionally, tea consumption reportedly protects against hip fracture (5, 6). Older women who drank tea exhibited higher BMD measurements than did women who did not drink tea (7). Tea polyphenols may be primarily responsible for the health benefits associated with tea; (−)-epigallocatechin-3-gallate (EGCG), a tea polyphenol, suppressed osteoclast differentiation and ameliorated RA in a mouse model of the disease (8).

Tea Polyphenols Inhibit Rat Osteoclast Formation and Differentiation

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Abstract. Matrix metalloproteinases (MMPs) play an important role in degeneration of the matrix associated with bone and cartilage. Regulation of osteoclast activity is essential in the treatment of bone disease, including osteoporosis and rheumatoid arthritis. Polyphenols in green tea, particularly epigallocatechin-3-gallate (EGCG), inhibit MMPs expression and activity. However, the effects of the black tea polyphenol, theaflavin-3,3′-digallate (TFDG), on osteoclast and MMP activity are unknown. Therefore, we examined whether TFDG and EGCG affect MMP activity and osteoclast formation and differentiation in vitro. TFDG or EGCG (10 and 100 μM) was added to cultures of rat osteoclast precursor cells and mature osteoclasts. Numbers of multinucleated osteoclasts and actin rings decreased in polyphenol-treated cultures relative to control cultures. MMP-2 and MMP-9 activities were lower in TFDG- and EGCG-treated rat osteoclast precursor cells than in control cultures. MMP-9 mRNA levels declined significantly in TFDG-treated osteoclasts in comparison to control osteoclasts. TFDG and EGCG inhibited the formation and differentiation of osteoclasts via inhibition of MMPs. TFDG may suppress actin ring formation more effectively than EGCG. Thus, TFDG and EGCG may be suitable agents or lead compounds for the treatment of bone resorption diseases.

Keywords: theaflavin-3,3′-digallate (TFDG), epigallocatechin-3-gallate (EGCG), rat osteoclast, rat osteoclast precursor cell, matrix metalloproteinase (MMP)-9
EGCG is the most abundant polyphenol in green tea. Catechins, particularly EGCG, possess health benefits, functioning as antioxidants and anti-angiogenesis factors (12 – 16); moreover, catechins are reportedly beneficial in hyperlipidemia, atherosclerosis, Parkinson’s disease, and other diseases in animal models as well as in humans (17, 18).

Black tea contains catechins, theaflavin, and the theaflavin derivatives theaflavin-3-gallate, theaflavin-3′-gallate, and theaflavin-3,3′-digallate (TFDG) (Fig. 1: B, C) (11, 19). TFDG possesses two galloyl groups as a result of the polymerization of two EGCG molecules. Theaflavins in black tea and catechins in green tea are equally effective antioxidants (20). Theaflavins also exhibit antitumor effects (21); furthermore, TFDG, as demonstrated in vitro, displays antiangiogenic activity due to its inhibition of matrix metalloproteinase (MMP)-2 and MMP-9 (22).

MMPs comprise a family of zinc-dependent endopeptidases, which includes collagenases (MMP-1, -8, -13, and -18), gelatinases (MMP-2 and -9), and stromelysins (MMP-3 and -10). These MMPs possess the combined ability to degrade the organic components of connective-tissue matrices (23 – 25). MMPs play a crucial role in tissue remodeling and in the destruction of bone in an arthritic joint due to their ability to degrade a wide variety of extracellular matrix components (26). In particular, MMP-9 is essential with respect to the initiation of the osteoclastic resorption process via removal of the collagenous layer from the bone surface prior to demineralization (27, 28). Moreover, MMP-9–knockout mice display only transient disturbances in bone development (27, 29). An in vitro experiment involving calvarial bone revealed that digestion of the bone matrix is inhibited in the presence of an MMP inhibitor (27, 30). Osteoclasts produce MMPs; furthermore, MMP-9 expression in osteoclasts is markedly higher than in other cell types (31).

Green and black teas are rich sources of polyphenols; additionally, several epidemiological and animal model studies have shown that tea consumption is associated with health benefits. EGCG and TFDG can influence a number of cellular processes and inhibit the activities of MMP family members. Several reports indicated that the galloyl group contributes greatly to anti-proliferative activity in cancer cells (32 – 34). Therefore, TFDG may exhibit enhanced activity consequent to the presence of two galloyl groups. Several investigations regarding the effects of green tea polyphenols on osteoclasts have been conducted; however, few studies regarding the effects of black tea polyphenols, for example, TFDG, on osteoclasts have been reported.

The current study examined the ability of TFDG and EGCG to suppress individually MMP-2 and MMP-9 enzymatic activity, MMP-9 mRNA expression, and differentiation in rat osteoclasts.
Materials and Methods

Animals and reagents

Newborn Wistar rats were obtained from Saitama Experimental Animals Supply (Saitama). All experiments, which were conducted according to the “Guiding Principles for the Care and Use of Laboratory Animals” of The Japanese Pharmacological Society, were performed with the approval of the Showa University Animal Care and Use Committee (certificate number 10048). TFDG and EGCG were purchased from Wako Pure Chemical Industries, Ltd. (Osaka). Macrophage colony-stimulating factor (M-CSF/CSF-1) and RANKL were acquired from PeproTech, Inc. (Rocky Hill, NJ, USA).

Osteoclast formation assay

Primary cultures of osteoclast precursor cells were established from rat bone marrow employing the Osteoclast Culture Kit (Primary Cell, Co., Ltd., Sapporo). Osteoclast precursor cells (4.0 × 10^4 cells/well) were cultured for 7 days at 37°C in 5% CO2 in an osteoclast differentiation medium containing 50 ng/ml M-CSF and 50 ng/ml RANKL. The cells were assigned to five groups as follows: control (medium alone), 10 ng/ml RANKL. The cells were changed on the fourth day. TFDG and EGCG were added to the appropriate cultures each of the 7 days.

Quantitation of mRNA

Cells (4.0 × 10^4 cells/well) were collected; subsequently, total RNA was isolated and reverse transcribed using the Whole Transcriptome Amplification Kit (Takara Bio, Inc., Otsu). Real-time reverse transcribed polymerase chain reaction (real-time RT-PCR) served to quantify MMP-9 mRNA levels in the rat osteoclasts. cDNAs were amplified utilizing primers designed with the ProbeFinder software (Roche Diagnostics K.K., Tokyo). The primers and Roche Universal ProbeLibrary Probe numbers were as follows: MMP-9: sense, 5′-gaac caatctacgacag-3′ and antisense, 5′-gacaagggcaag-3′; accession no. NM_004994.2; probe no. #6. Amplification of MMP-9 cDNAs was performed with a LightCycler (Roche) employing a LightCycler TaqMan Master mix (Roche) according to the manufacturer’s protocols. The PCR reaction parameters were as follows: 95°C for 10 min, 40 cycles of 10 s at 95°C, 30 s at 60°C, and 1 s at 72°C. Fluorescence data were analyzed with LightCycler software (Roche) as described previously (35).

Gelatin zymography

The enzymatic activity of MMP-2 and MMP-9 was measured via gelatin zymography on 10% polyacrylam-}

ide gels containing 1 mg/ml gelatin in a polymerization mixture of SDS as described previously (36). Samples used for measurement of MMP-2 and MMP-9 enzymatic activity were prepared as follows: Culture supernatant, which was collected during the media exchange on the fourth and seventh days of cell culture, was diluted 10-fold for MMP-2 or 100-fold for MMP-9 with ddH2O. Samples (7 µl) were mixed with 7 µl of sample buffer. Upon completion of SDS-PAGE of samples through the gel matrix containing gelatin, the SDS was removed by washing with 2.5% Triton X-100 for 30 min at room temperature. Gels were then incubated in a solution of 50 mM CaCl2 and 0.02% sodium azide (pH 7.5) at 37°C for 1 day. Subsequently, gels were stained with 0.1% Coomassie blue and de-stained with ddH2O. The purified MMP-2 proenzyme (72 kDa) and the purified MMP-9 proenzyme (92 kDa) served as positive controls for enzymatic activity and as size standards (Oncogene Research Products, La Jolla, CA, USA). Zymography bands were digitized utilizing the ATTO imaging system (ATTO Co., Tokyo); data were analyzed with NIH imaging software (Scion Co., Frederick, MD, USA).

Culture of authentic osteoclasts

Osteoclasts were isolated from tibiae and femurs of 1-day-old rats according to the method of Amano et al. (37, 38). The tibiae and femurs of 14 rats were dissected and cleaned of adherent soft tissue and epiphyseal cartilage. The bones were curetted with a scalpel in 1 ml of Medium 199 (pH 7.0) containing 10% heat inactivated fetal bovine serum (FBS). The cell suspension was collected. The bone fragments were washed twice with 1 ml of Medium 199 in order to remove the remaining cells. Bone fragments were permitted to settle for 90 s, after which the supernatant was re-suspended. Samples (70 µl) of the cell suspension were pipetted onto 13-mm circular plastic coverslips (Thermanox; Nunc Inc., Naperville, IL, USA), to which the cells were allowed to adhere for 1 h at 37°C; subsequently, non-adhering cells were removed by washing. The coverslips were transferred to 24-well plates. Osteoclasts on the coverslips were incubated in an osteoclast differentiation medium containing 50 ng/ml M-CSF and 50 ng/ml RANKL (PeproTech) at 37°C in normal atmosphere for 48 h.

F-actin and TRAP staining

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

Statistical analysis

Data were analyzed utilizing the Bonferroni test following one-way analysis of variance (ANOVA). All data were expressed as the mean ± standard error of the mean (S.E.M.). P-values of less than 0.05 were considered significant.

Results

Effect of tea polyphenols on osteoclast formation

To examine the effect of tea polyphenols on osteoclast formation, cultures of osteoclast precursor cells were treated with TFDG or EGCG at concentrations of 10 and 100 μM for 7 days (Fig. 2: A – E). Greater numbers of

Fig. 2. Effects of theaflavin-3,3′-digallate (TFDG) and (−)-epigallocatechin-3-gallate (EGCG) on osteoclast formation in vitro. A – E) Osteoclast precursor cells were cultured for 7 days in the absence (control) or presence of 10 or 100 μM TFDG or EGCG. Media were changed on the fourth day. Osteoclasts were identified via tartrate-resistant acid phosphatase (TRAP) staining (original magnification × 100). F) TRAP-positive multinucleated cells characterized by more than three nuclei (white arrows in panel A) were counted as osteoclasts as determined by light microscopy. Values are the mean ± S.E.M. from 5 independent experiments. *P < 0.05 and **P < 0.01, significantly different from the positive control. **P < 0.01, significantly different from TFDG (10 μM). ***P < 0.01, significantly different from EGCG (10 μM).
TRAP-positive multinucleated osteoclasts (white arrows) were observed in the control group in comparison with the TFDG- and EGCG-treated groups (Fig. 2: A and F). Osteoclasts treated with 10 μM TFDG or 10 μM EGCG were smaller and exhibited fewer nuclei than osteoclasts of the control group (Fig. 2: A, B, and D). Moreover, treatment with 10 μM TFDG resulted in markedly fewer multinucleated osteoclasts in cultures (as low as 79.4% of the number in control cultures). In contrast, treatment with 10 μM EGCG did not lead to significantly fewer multinucleated osteoclasts than were observed in control cultures (Fig. 2F). Treatment with 100 μM TFDG or 100 μM EGCG strongly suppressed cell–cell fusion among osteoclast precursor cells (Fig 2: C and E); moreover, the number of multinucleated osteoclasts was significantly lower in cultures treated with 100 μM TFDG or 100 μM EGCG than in control cultures (Fig. 2F). Tea polyphenols suppressed osteoclast formation in a dose-dependent manner.

Effects of TFDG and EGCG on MMP-9 mRNA levels in osteoclast precursor cells

Following treatment with TFDG or EGCG for 7 days, all cells in each well were harvested; subsequently, cells were used to reverse transcribe mRNA directly into cDNA with the Whole Transcriptome Amplification Kit (Takara). MMP-9 mRNA levels per well were significantly lower in TFDG- and EGCG-treated cells than in control cells (Fig. 3). Moreover, MMP-9 mRNA levels were substantially lower in cells treated with 100 μM TFDG in comparison with cells treated with 100 μM EGCG; TFDG affected MMP-9 mRNA levels in a dose-dependent manner.

Effects of TFDG and EGCG on MMP-2 and MMP-9 activity in osteoclast precursor cells

The gelatin zymographs of osteoclast precursor cell culture supernatant samples obtained after 7 days in culture are illustrated in Fig. 4, A and C. Pro-MMP-2 (72 kDa) and pro-MMP-9 (92 kDa) activity was detected in all samples. MMP-2 and MMP-9 samples were diluted 10- or 100-fold, respectively, with ddH2O prior to measurement of enzymatic activity; however, pro-MMP-9 activity, which was extremely high in osteoclast medium, was greater than pro-MMP-2 activity. Densitometric scanning of the bands corresponding to pro-MMP-2 or pro-MMP-9 served to quantify the gelatinolytic activity of the respective proteins in culture supernatant samples obtained from 4- or 7-day cultures (Fig. 4: B and D). Pro-MMP-2 and pro-MMP-9 activity was significantly lower in those samples derived from cultures treated with 100 μM TFDG or 100 μM EGCG than in those samples derived from control cultures. Moreover, TFDG and EGCG reduced pro-MMP-2 and pro-MMP-9 activity in a dose-dependent manner. TFDG appeared to suppress pro-MMP-2 and pro-MMP-9 activity to a greater extent than did EGCG.

Effect of tea polyphenols on authentic osteoclasts

Tea polyphenols inhibited mature osteoclasts in terms of formation of TRAP-positive multinuclear osteoclasts (Fig. 5: A, B and C). Numerous large multinucleated osteoclasts were detected in control cultures after 48 h in medium containing RANKL and M-CSF; in contrast, very few large multinucleated osteoclasts were observed in cultures also treated with TFDG or EGCG. Ring-formed F-actin bands were evident around TRAP-positive multinuclear osteoclasts in control cultures (Fig. 5: A and D); however, a few of the ring-formed F-actin bands in cultures treated with TFDG or EGCG disappeared from TRAP-positive multinuclear osteoclasts (EGCG; Fig. 5: C and F, TFDG; data not shown). TRAP-positive multinucleated osteoclasts decreased significantly in cultures treated with 10 μM TFDG (P < 0.05: 52.7% vs. control); the numbers of multinucleated osteoclasts did not differ significantly in either 10 μM EGCG–treated or control cultures (Fig. 5G). Treatment with TFDG or EGCG reduced the number of mature osteoclasts characterized by actin rings in a dose-dependent manner (Fig. 5H). The number of mature osteoclasts displaying actin rings (white arrows) was lower than that of TRAP-positive multinuclear osteoclasts.
Discussion

This study demonstrated that TFDG and EGCG at concentrations of 10 or 100 μM have the following effects: 1) inhibit osteoclast formation and/or differentiation in rat osteoclast precursor cells as well as in mature osteoclasts and 2) suppress MMP-2 and MMP-9 enzymatic activity and MMP-9 mRNA levels in osteoclast precursor cells. Activated osteoclasts exhibit polarized cytoskeletal structures known as actin rings; moreover, formation of these actin rings is important for bone resorption (39). TFDG and EGCG may suppress osteoclast differentiation as they inhibit the formation of cytoskeletal actin rings in mature osteoclasts via suppression of MMPs.

Several cohort studies conducted in western countries
Fig. 5. Effects of TFDG and EGCG on differentiation of primary osteoclasts in vitro. Primary osteoclasts were cultured for 48 h in the absence or presence of TFDG or EGCG at various concentrations (10 and 100 μM). Cells were stained for rhodamine-conjugated phalloidin (D – F), after which osteoclasts were identified by TRAP staining (A – C) (original magnification × 100). G) TRAP-positive multinucleated cells characterized by more than three nuclei were counted as osteoclasts. Cont, 10 μM TFDG, 100 μM TFDG, and 10 μM EGCG, n = 8; 100 μM EGCG, n = 6. Values are the mean ± S.E.M. *P < 0.05 and **P < 0.01, significantly different from the control. $P < 0.05, significantly different from EGCG (10 μM). H) Primary osteoclasts exhibiting clear F-actin rings (white arrows in panels D – F) were counted as activated osteoclasts. Cont, 10 μM TFDG, 100 μM TFDG, and 10 μM EGCG, n = 8; 100 μM EGCG, n = 6. Values are the mean ± S.E.M. ***P < 0.01, significantly different from the control.
indicated that tea consumption may exert osteoporosis-preventive effects (5 – 7). The most important practical issue involves low blood plasma concentration of tea polyphenols. Previous investigations demonstrated that consumption of green tea extract (1.5 – 4.5 g or 1 – 3 cups) by healthy individuals is associated with the appearance of EGCG, ECG, EGC, and EC in the plasma and that the plasma concentration of EGCG may reach 4 μM (40 – 42). Moreover, other studies revealed that the consumption of large amounts of green tea, particularly more than 5 cups daily, is correlated with EGCG plasma concentrations of 6.7 – 20 μM (43 – 45). It has been reported that locally high concentrations (up to 131.0 μM) of tea polyphenols occur when green tea leaves are held in the oral cavity (46). Therefore, the current results regarding EGCG are consistent (10 – 100 μmol) with the earlier findings. In contrast, fewer published reports pertaining to plasma concentrations of TFDG appear in the literature; to be sure, Henning et al. noted that theaflavins were absorbed more readily by the mouse prostate than was EGCG (47).

MMPs, matrix metalloproteinases, are enzymes responsible for the degradation of collagen fibrils. Accumulated evidence indicates that MMPs play a critical role in osteoclastic bone resorption and that MMPs facilitate migration of osteoclasts to bone surfaces via the extracellular matrix (48). The level of MMP-9 mRNA expression in osteoporotic bone tissues is significantly higher than that in normal control tissue (49); additionally, MMP-9 is expressed in osteoclasts at very high levels (50). Moreover, an MMP inhibitor, an MMP-9 antisense RNA, and an MMP-9 siRNA each suppressed pre-osteoclast migration (51, 52). Therefore, MMP-9 is an important factor in terms of migration of osteoclast precursor cells. TFDG and 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. Furthermore, in the current study, maturation of osteoclasts, as indicated by the presence of actin rings, was inhibited by TFDG and EGCG. The 67-kDa laminin receptor (67LR), a high-affinity non-integrin laminin receptor, is also an EGCG receptor (53, 54). EGCG disrupted stress fibers via 67LR and inhibited F-actin assembly at the cleavage furrow between dividing cells (55); thus, EGCG may have inhibited actin ring formation via 67LR in osteoclasts. On the other hand, the affinity of TFDG for 67LR is unknown.

TFDG and EGCG inhibit MMP-2 and MMP-9 via two potential pathways. The first pathway involves direct inhibition of MMP-2 and MMP-9 enzymatic activity (19). Tea polyphenols can form complexes with metal ions (56). As a result, TFDG and EGCG may inhibit MMPs activity by forming complexes with zinc ions; these complexes will inhibit MMPs as MMPs are zinc-dependent proteinases. The second pathway in which EGCG or TFDG may influence MMPs is via inhibition of their secretion (19). The promoter of MMP-9 possesses binding sites for activator protein-1 (AP-1) and nuclear factor-κB (NF-κB); moreover, transcription of the gene is regulated by these transcription factors (57). TFDG and EGCG inhibit AP-1 and NF-κB activation (58). Thus, it is possible that TFDG and EGCG inhibit MMP-9 mRNA expression in osteoclasts via inhibition of these transcription factors. A recent study demonstrated that induced expression of 67LR (EGCG receptor) increases MMP-9 expression in gastric cancer cells and that the ERK and JNK signal pathways are involved (59).

However, TFDG and EGCG do not inhibit signal transduction pathways equally. For example, EGCG disrupts the association of MEK1 [MAPK (mitogen-activated protein kinase) / ERK (extracellular signal-regulated kinase) kinase] with Raf-1, possibly by binding to the proline-rich sequences on MEK1 (21). On the other hand, TFDG promotes lysosome-mediated degradation of the Raf-1 protein (21). These signal pathways of TFDG or EGCG lead to decreased phospho-MEK1 and phospho-ERK1/2 levels within the cell, which, in turn, inhibits AP-1 (60).

The regulation of osteoclast differentiation might be an important strategy for the treatment of bone resorption and osteoporosis. The current investigation demonstrated that TFDG and EGCG suppress differentiation and MMP mRNA expression as well as enzymatic activity in rat osteoclasts. Our results revealed that TFDG may have suppressed osteoclast formation and differentiation more effectively in comparison to EGCG. Thus, TFDG and EGCG may serve as suitable agents or lead compounds in the treatment of bone resorption and osteoporosis.

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References

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