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Inhibitory effects of green tea polyphenol (–)-epigallocatechin gallate on the expression of matrix metalloproteinase-9 and on the formation of osteoclasts

Jeong-Ho Yun[1], Eun-Kyoung Pang[1], Chang-Sung Kim[1], Yun-Jung Yoo[2], Kyoo-Sung Cho[1,3], Jung-Kiu Chai[1], Chong-Kwan Kim[1,3], Seong-Ho Choi[1,3]

[1]Department of Periodontology, Research Institute for Periodontal Regeneration, College of Dentistry, Yonsei University, Seoul, [2]Department of Oral Biology, College of Dentistry, Yonsei University, Seoul and [3]Brain Korea 21 Project for Medical Science, Yonsei University, Seoul, Korea

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Background: Alveolar bone resorption is a characteristic feature of periodontal diseases and involves the removal of both the mineral and organic constituents of the bone matrix, which is caused by either multinucleated osteoclast cells or matrix metalloproteinases (MMPs). The gram-negative bacterium, *Porphyromonas gingivalis* has been reported to stimulate the activity and expression of several groups of MMPs, whereas (–)-epigallocatechin gallate (EGCG), the main constituent of green tea polyphenols, has been reported to have inhibitory effects on the activity and expression of MMPs.

Objectives: In the present study, we investigated the effects of the green tea polyphenol, EGCG, on the gene expression of osteoblast-derived MMP-2, -9 and -13, stimulated by *P. gingivalis*, and on the formation of osteoclasts.

Methods: The effect of EGCG on the gene expression of MMPs was examined by treating mouse calvarial primary osteoblastic cells with EGCG (20 μM) in the presence of sonicated *P. gingivalis* extracts. The transcription levels of MMP-2, -9 and -13 were assessed by reverse transcription-polymerase chain reaction (RT-PCR). The effect of EGCG on osteoclast formation was confirmed by tartrate-resistant acid phosphatase (TRAP) staining in a co-culture system of mouse bone marrow cells and calvarial primary osteoblastic cells.

Results: Treatment with the sonicated *P. gingivalis* extracts stimulated the expression of MMP-9 mRNA and this effect was significantly reduced by EGCG, whereas the transcription levels of MMP-2 and MMP-13 were not affected by either the sonicated *P. gingivalis* extracts or EGCG. In addition, EGCG significantly inhibited osteoclast formation in the co-culture system at a concentration of 20 μM.

Conclusions: These findings suggest that EGCG may prevent the alveolar bone resorption that occurs in periodontal diseases by inhibiting the expression of MMP-9 in osteoblasts and the formation of osteoclasts.

Dr Seong-Ho Choi, DDS, PhD, Department of Periodontology, Research Institute for Periodontal Regeneration, College of Dentistry and Brain Korea 21 Project for Medical Science, Yonsei University, 134 Shinchon-Dong, Seodaemun-gu, Seoul, Korea
Tel: + 82 2 361 8833
Fax: + 82 2 392 0398
e-mail: shchoi726@yumc.yonsei.ac.kr

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Alveolar bone resorption is the clinically most important issue in human periodontitis, because it leads to tooth loss (1). Bone resorption involves the removal of both the mineral and organic constituents of the bone matrix. Osteoclasts, the cells principally responsible for this process (2), acidify the sub-osteoclastic resorption zone leading to the dissolution of minerals (3), while the organic matrix (mainly type I collagen) is degraded by proteolytic enzymes. Osteoblastic cells play a role in the initiation of bone resorption by releasing matrix metalloproteinases (MMPs) that degrade the non-mineralized osteoid layer (principally type I collagen) covering the bone surface, thereby exposing the underlying mineralized matrix to osteoclastic action (4–6).

MMPs are a family of zinc-dependent endopeptidases, including collagenases, gelatinases and stromelysins, which have the combined ability to degrade the organic components of connective-tissue matrices (7–9). Not only collagenase (MMP-1 and MMP-13), but also gelatinase A (MMP-2) and B (MMP-9) have been considered the principal MMPs in the digestion of bone collagen by osteoblasts (10–14). It was reported that although collagenase is responsible for the initial cleavage of native type I collagen, gelatinase A and B may play a significant role in the subsequent digestion of the denatured collagen fibrils (12).

Porphyromonas gingivalis, a gram-negative anaerobic bacterium, is implicated as an etiologic agent in periodontal diseases (15–17). Even though periodontal destruction is partly caused by proteinases secreted from this group of bacteria, it is now accepted that the host response to such bacterial products is the major cause of the pathogenesis (18). Furthermore, the products of *P. gingivalis*, such as lipopolysaccharide, membrane proteins and bacterial proteinases, have been shown to have the ability to induce and activate host MMPs (19–25).

Green tea is one of the most popular beverages in the world and it has received considerable attention because of its many scientifically proven beneficial effects on human health. Several epidemiologic and experimental observations have confirmed that there is a close relationship between green tea consumption and the prevention of both cancer development and cardiovascular disease (26). These effects have been largely attributed to the most prevalent polyphenol contained in green tea, (–)-epigallocatechin gallate (EGCG). Recently, EGCG has been shown to inhibit the activity (27–29) and expression (30, 31) of collagenase or gelatinase (MMP-2 and MMP-9). Furthermore, it has been reported that EGCG could induce the apoptotic cell death of osteoclasts (32).

However, the biological effect of EGCG on alveolar bone destruction has not been documented. Therefore, in order to examine whether EGCG has an inhibitory effect on alveolar bone resorption, we evaluated the effect of EGCG on the gene expression of MMP-2, -9 and -13 in mouse calvarial primary osteoblastic cells, which were stimulated by sonicated *P. gingivalis* extracts. We also investigated the inhibitory effect of EGCG on osteoclast formation in a co-culture system of mouse bone marrow cells and calvarial primary osteoblastic cells.

Materials and methods

P. gingivalis culture and preparation of sonicated *P. gingivalis* extracts

P. gingivalis strain ATCC 33277 was cultured in a brain heart infusion broth, which contained 5 mg/ml of hemin and 0.5 mg/ml of Vitamin K at 37°C in an anaerobic chamber in an atmosphere containing 80% N_2, 10% H_2 and 10% CO_2. After 2 days of culture, the bacteria were harvested by centrifugation at $3200 \times g$ for 20 min at 4°C and washed three times with phosphate buffered saline. The purity of the cultures was confirmed by phase-contrast microscopy and gram staining. The bacterial culture was subjected to sonication (Misonix Inc., Farmingdale, NY, USA). The insoluble debris was removed by centrifugation at $12{,}000 \times g$ for 5 min at 4°C. The supernatant was sterilized by filtering through a membrane filter with a pore size of 0.22 μm. The protein content of the sonicated *P. gingivalis* extracts was determined using a protein assay reagent kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. The sonicated *P. gingivalis* extracts were stored at −70°C until used. The concentration of sonicated *P. gingivalis* extracts (1 μg/ml) used in the present study was based on our pilot cytotoxicity experiment.

Preparation of primary osteoblastic cells and bone marrow cells

Primary osteoblastic cells were prepared from the calvariae of 1- or 2-day-old newborn ICR mice (Samtako Inc., O-San, Kyung-gi-Do, Korea) by a previously reported method (33) with a slight modification. The calvariae removed from 10 mice were subjected to digestion four times at 20-min intervals using 0.2% collagenase (Wako Pure Chemical Industries, Ltd, Osaka, Japan) and 0.1% dispase (Gibco BRL, Life Technologies, Grand Island, NY, USA) in a shaking water bath at 37°C. The primary osteoblastic cells isolated in the first digestion were discarded, and those in the second to fourth digestions were collected and cultured to confluence in an α-minimum essential medium (α-MEM) (Gibco BRL, Life Technologies) containing 10% fetal bovine serum (Gibco BRL, Life Technologies) and antibiotic–antimycotic (100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate and 0.25 μg/ml amphotricin B) (Gibco BRL, Life Technologies) in a 10-cm culture dish at 37°C with 5% CO_2. The cells were then detached from the culture dish by treating them with trypsin-EDTA (Gibco BRL, Life Technologies) and collected by centrifugation. The bone marrow cells were collected from the femurs and tibiae of 4- to 8-week-old ICR mice. The ends of the femurs and tibiae were removed and the marrow cavity was flushed by slowly injecting media in at one end using a 25-gauge needle. The collected bone marrow cells were washed and treated with 10 mM Tris-HCl, 0.83% ammonium chloride to remove the red blood cells.

Cytotoxicity assay

The MTT colorimetric assay was used to measure the viability of the cells after treatment with EGCG. The number of viable cells was determined based on the reduction of MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-dipheyltetrazolium bromide) dye (Sigma Chemical Co., St. Louis, MO, USA) by mitochondria dehydrogenase in live cells to form blue formazan crystals (34). Primary osteoblastic cells (10^4 cells/well) were seeded in 96-well plates and grown in α-MEM containing 10% fetal bovine serum to sub-confluence. The cells were then treated with various concentrations of EGCG (Calbiochem, La Jolla, CA, USA) for an additional 3 days. In addition, after the primary osteoblastic cells (5×10^3 cells/well) had been co-cultured with bone marrow cells (5×10^4 cells/well) in α-MEM containing 10% fetal bovine serum in 96-well plates for 3 days, various concentrations of EGCG were added to each well and the cells cultured for an additional 3 days. After 3 days of culture, 50 μl of MTT solution (5 mg/ml) was added to each well and the cells incubated for 4 h at 37°C. The supernatant was discarded and 200 μl of dimethyl sulfoxide was added to each well to dissolve the formazan crystals. The optical density of the formazan solution was measured at 570 nm.

Reverse transcription-polymerase chain reaction (RT-PCR)

Primary osteoblastic cells were seeded in 35-mm culture dishes at a density of 2×10^5 cells/dish and grown to confluence in α-MEM containing 10% fetal bovine serum. The cells were precultured for 24 h in serum-free α-MEM containing 1 mg/ml bovine serum albumin (Gibco BRL, Life Technologies) and subsequently treated at the indicated concentrations for 24 h as follows: (i) no treatment, (ii) EGCG alone, (iii) sonicated *P. gingivalis* extracts alone and (iv) EGCG + sonicated *P. gingivalis* extracts.

The cells were collected and total RNA was extracted from the primary osteoblastic cells using a reagent (TRIzol; Gibco BRL, Life Technologies) according to the manufacturer's instructions. The concentration of the RNA obtained was determined by measuring the absorbance at 260 and 280 nm. RT-PCR for MMP-2, -9, -13 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was carried out with a commercial one-step RNA-PCR kit (Takara Shuzo Co., Ltd, Shiga, Japan). Total RNA (1 μg) isolated from each sample was used as a template for the cDNA synthesis. The reverse transcription of total RNA to cDNA and subsequent amplification were performed in a single tube containing 50 μl of reaction mixture (35). Each reaction tube contained 1 μg of total RNA, 0.1 U/μl of Avian Myeloblastosis Virus RTase, 0.1 U/μl of *Taq* DNA polymerase, 0.8 U/μl of RNase inhibitor, 5 mM $MgCl_2$, 1 mM dNTPs and 0.4 μM of each forward and reverse oligonucleotide primer (Table 1) in a one-step RNA-PCR buffer. RT-PCR was performed in a thermocycler (Tgradient, Biometra, Göttingen, Germany). The cycling conditions were 30 min at 50°C (reverse transcription) and 2 min at 94°C (reverse transcriptase inactivation) and then 35 cycles of standard PCR were performed with each cycle consisting of 30 s at 95°C, 45 s at 60°C and 1 min at 72°C. After amplification, 10 μl of each PCR product was analyzed by 1.5% agarose gel electrophoresis. The density of the bands was computer-analyzed by Tina Image software (Raytest, Wilmington, NC, USA). All the data were normalized according to the GAPDH mRNA level. The density ratios of MMP to GAPDH were calculated and then represented in the form of graphs.

In vitro formation of osteoclasts

Primary osteoblastic cells (10^4 cells/well) were co-cultured with bone marrow cells (10^5 cells/well), using a previously described procedure (36), in 48-well plates (200 μl/well) in α-MEM containing 10% fetal bovine serum. All cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. EGCG or $1\alpha,25(OH)_2D_3$ (10^{-8} M) (Sigma Chemical Co.) were added to the co-culture immediately after exchanging the medium on day 3. After an additional 4 days, the cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP), an enzyme generally accepted as a marker for osteoclasts (37), using an acid phosphatase kit (Sigma Chemical Co.). TRAP-positive multinucleated cells showing more than three nuclei were considered to be osteoclasts and were counted as such.

Statistical analysis

Data were expressed as means ± standard deviation (SD). Statistical differences were determined by analysis of variance (ANOVA) using the SAS 8.02 program. Tukey's test was used for the *post hoc* comparison of specific groups. Statistical significance was determined at the $p < 0.05$ level.

Results

Effect of EGCG on cell viability

The MTT assay was performed to assess the effect of EGCG on cell viability of EGCG and to determine the appropriate concentration to be used

Table 1. Synthetic oligonucleotide primers used for reverse transcription-polymerase chain reaction (RT-PCR)

Target gene	Primer sequence	Length of PCR product (base pairs)
MMP-2	Forward: 5′-CTGTCCTGACCAAGGATATAGCCT-3′ Reverse: 5′-ACCTGTGGGCTTGTCACGTGGTGT-3′	355
MMP-9	Forward: 5′-CTGTCCAGACCAAGGGTACAGCCT-3′ Reverse: 5′-GTGGTATAGTGGGACACATAGTGG-3′	263
MMP-13	Forward: 5′-CATTCAGCTATCCTGGCCACCTTC-3′ Reverse: 5′-CAAGTTTGCCAGTCACCTCTAAGC-3′	250
GAPDH	Forward: 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′ Reverse: 5′-CATGTAGGCCATGAGGTCCACCAC-3′	983

for the treatment of cells. EGCG showed no effect on the viability of primary osteoblastic cells or co-culture cells up to a concentration of 20 μM after 3 days of treatment, as compared with the non-treated cells (Fig. 1). However, inhibition of cell growth was observed at the higher concentrations of EGCG (50 μM and 100 μM for the primary osteoblastic cells and co-culture cells, respectively). Therefore, EGCG was used at a concentration of less than 20 μM in the subsequent studies.

Effect of sonicated *P. gingivalis* extracts and EGCG on the expression of MMP-2, -9 and -13 mRNA in primary osteoblastic cells

Mouse calvarial primary osteoblastic cells were cultured to confluence. At confluence, the cells were treated for 24 h as follows: (i) no treatment, (ii) 20 μM of EGCG alone, (iii) 1 μg/ml of sonicated *P. gingivalis* extracts alone and (iv) 20 μM of EGCG + 1 μg/ml of sonicated *P. gingivalis* extracts. As shown in Fig. 2(A), the expression of MMP-2, -9 and -13 was detected in all treatment groups by RT-PCR analysis. The treatment with sonicated *P. gingivalis* extracts (1 μg/ml) stimulated the expression of MMP-9 mRNA, showing a 215% increase in the density ratio analysis when compared to the untreated cells. In addition, this effect was significantly inhibited by EGCG (20 μM), reaching the same level of expression of MMP-9 mRNA as that observed in the untreated cells. However, EGCG alone had no effect on the expression of MMP-9 mRNA, and the expression of MMP-2 and MMP-13 mRNA was almost never affected by sonicated *P. gingivalis* extracts and EGCG (Fig. 2B).

Inhibitory effect of EGCG on osteoclast formation

To examine the effect of EGCG on osteoclast formation, the co-culture of primary osteoblastic cells and bone marrow cells was treated with various concentrations of EGCG in the presence of $1\alpha,25(OH)_2D_3$ (10^{-8} M). $1\alpha,25(OH)_2D_3$ was used as a positive control to induce the formation of the osteoclasts. The largest number of TRAP-positive osteoclasts was detected following the treatment with EGCG in the presence of $1\alpha,25(OH)_2D_3$ (10^{-8} M) after 4 days of culture (Fig. 3B). α-MEM containing 10% fetal bovine serum was used as a negative control. No TRAP-positive osteoclasts were formed in the negative control (Fig. 3A). The addition of 20 μM EGCG to the co-culture resulted in a significant decrease in the number of osteoclasts to approximately 32% of the level of positive control. In addition, the effect of EGCG on the viability of the co-culture was re-evaluated by MTT assay on the duplicate 48-well culture plate. The MTT assay confirmed that EGCG had no cytotoxic effect on the co-culture at concentrations lower than 20 μM (Fig. 3C).

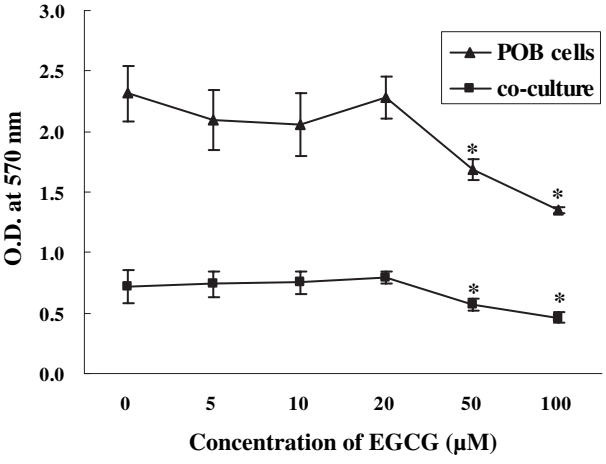

Fig. 1. The effect of (–)-epigallocatechin gallate (EGCG) on the viability of primary osteoblastic cells and the co-culture. Primary osteoblastic cells and a co-culture of primary osteoblastic cells and bone marrow cells were treated with various concentrations of EGCG for 3 days. The cellular activity was then estimated by MTT assay, and the results are expressed as the mean ± SD of six cultures. The data are representative of three separate experiments. *$P < 0.05$; significantly different from the non-treated group. POB cells, primary osteoblastic cells.

Discussion

Green tea consists mainly of polyphenols (catechins) which constitute up to 30% of the dry weight. The major catechins in green tea are (–)-epigallocatechin gallate (EGCG), (–)-epigallocatechin (EGC), (–)-epicatechin gallate (ECG), (–)-epicatechin (EC) and (+)-catechin (C). Among these polyphenols, EGCG is the most abundant catechin and the one that has been the most extensively studied (38). Numerous biological effects of green tea and its constituents have been reported. These include an anti-carcinogenic activity, anti-inflammatory effect, the alleviation of cardiovascular disease, etc. (26, 39). Recent studies demonstrated that EGCG inhibited the activity (27–29) and expression (30, 31) of collagenase or gelatinase (MMP-2 and MMP-9). Furthermore, it has been reported that EGCG could induce the apoptotic cell death of osteoclasts (32). In periodontitis, periodontal tissues are continuously challenged by periodontopathogens, such as *P. gingivalis*. It was shown that *P. gingivalis* could mediate the destruction of periodontal tissue and could be involved in alveolar bone resorption in various ways (40–44). In addition, the products of *P. gingivalis* were reported to have the ability to induce and activate host MMPs (19–25). However, the effects of EGCG and *P. gingivalis* on MMPs listed above have not been examined in osteoblastic cells. Moreover, no findings have been reported for the effect of EGCG on the gene expression of MMP in osteoblasts.

These findings led us to investigate the effect of EGCG and *P. gingivalis*

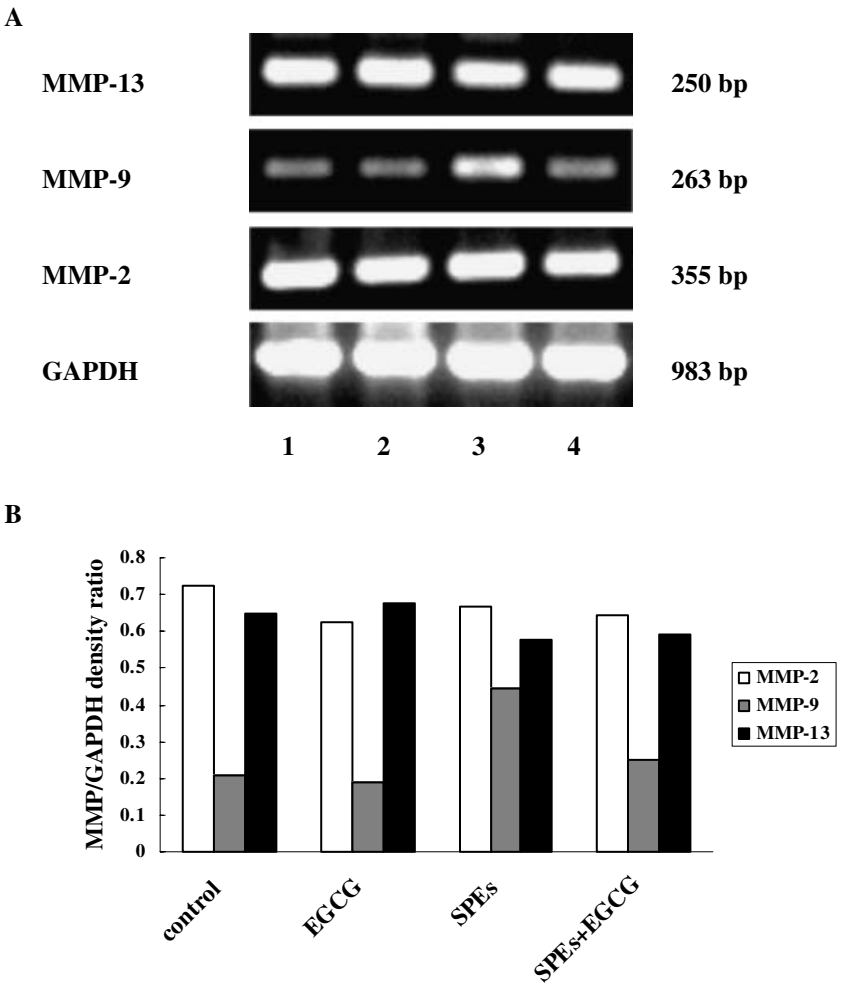

Fig. 2. The effect of sonicated *Porphyromonas gingivalis* extracts and (–)-epigallocatechin gallate (EGCG) on the expression of matrix metalloproteinases (MMP)-2, -9 and -13 mRNA. Primary osteoblastic cells were treated for 24 h with (1) no treatment, (2) 20 μM of EGCG alone, (3) 1 μg/ml of sonicated *P. gingivalis* extracts (SPEs) alone and (4) 20 μM of EGCG + 1 μg/ml of SPEs. The expression of MMP-2, -9 and -13 mRNA was analyzed by RT-PCR (A). The density ratios of MMPs to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are represented in graph (B). The data are representative of duplicate experiments.

on the expression of MMPs in osteoblasts. We also examined the effect of EGCG on the formation of osteoclasts using a co-culture of primary osteoblastic cells and bone marrow cells.

In the present study, we showed by RT-PCR analysis that sonicated *P. gingivalis* extracts stimulated the expression of MMP-9 mRNA in mouse calvarial primary osteoblastic cells and that EGCG (20 μM) significantly inhibited this stimulatory effect (Fig. 2). Since EGCG alone did not affect the transcription level of MMP-9, we concluded that the effect of EGCG was limited to preventing the increase of MMP expression induced by sonicated *P. gingivalis* extracts. These findings suggest that *P. gingivalis* may contribute to the alveolar bone loss observed in periodontal disease by stimulating host osteoblastic cells to produce MMP. In addition, this inhibitory effect of EGCG indicates the potential of EGCG to prevent the bone resorption caused by periodontal pathogens, such as *P. gingivalis*. On the other hand, although the mRNA of MMP-2 and MMP-13 appeared to be expressed in primary osteoblastic cells, as shown in previous studies (45), the transcription levels of these MMPs were not affected by either sonicated *P. gingivalis* extracts or EGCG. In a recent study, the molecular basis for the EGCG-mediated regulation of MMP-2 and MMP-9 was investigated in cancer cells (31). This study suggested that EGCG caused the reduction in MMP activities by inhibiting the gene expression of MMP-2 and MMP-9 by suppressing the phosphorylation of extracellular signal-regulated kinase (ERK) in cancer cells. This protein kinase belongs to the mitogen-activated protein kinase (MAPK) family of enzymes, which is known to regulate MMP expression (46–48). In a previous study, it was reported that the inhibition of MAPK suppressed MMP expression (49). Furthermore, several studies have provided evidence suggesting that EGCG inhibits MAPK, resulting in the down-regulation of MMPs (50, 51). It was also reported that EGCG inhibits MAPK signal transduction to the nucleus, by preventing the association of Raf-1 with MEK1, as well as the phosphorylation of Elk-1 by ERK1/2 (50). In addition, it was found that [^{3}H]EGCG was incorporated into the cytosol, as well as the nuclei (52). Our finding is basically consistent with these results. Therefore, it may be inferred that EGCG inhibits the stimulatory effect of sonicated *P. gingivalis* extracts on MMP expression by intracellularly blocking the MAPK signaling pathway. However, because of the large gaps in our knowledge of the mechanisms underlying the inhibition of MMP by EGCG, more extensive studies are needed to obtain conclusive evidence. Therefore, it remains to be determined exactly how EGCG influences the MAPK signal transduction pathway.

We also found that EGCG inhibited the osteoclast formation induced by $1\alpha,25(OH)_2D_3$ (10^{-8} M), based on TRAP staining in the co-culture system. In the present study, EGCG remarkably reduced the number of TRAP-positive multinucleated cells at a concentration of 20 μM (Fig. 3).

In addition, we examined the effect of sonicated *P. gingivalis* extracts (1 μg/ml) on the osteoclast formation in the present study. However, sonicated *P. gingivalis* extract itself did not induce the osteoclast formation in our

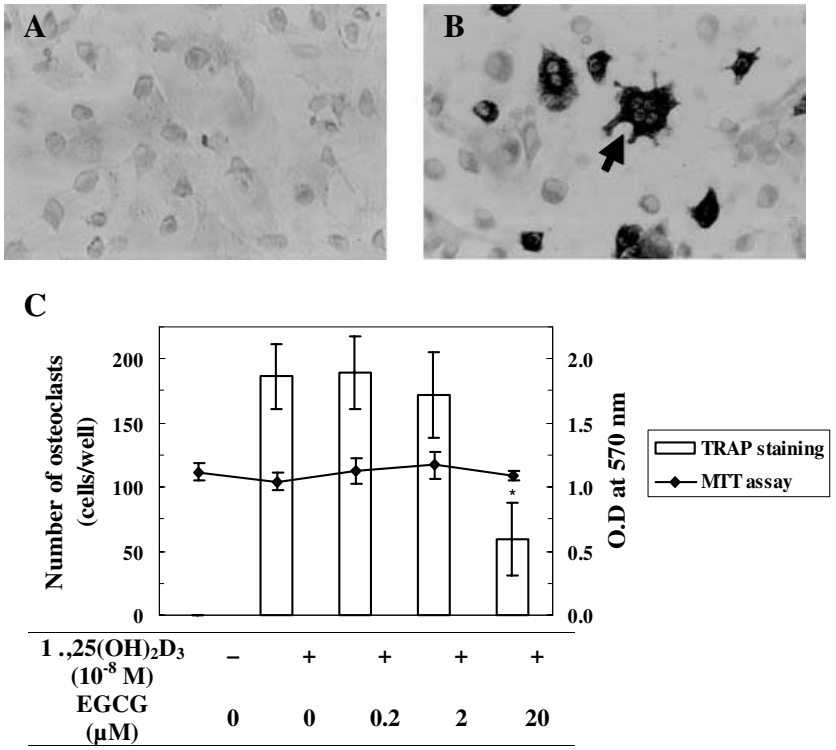

Fig. 3. The effect of (–)-epigallocatechin gallate (EGCG) on osteoclast formation. Mouse bone marrow and primary osteoblastic cells were co-cultured for 3 days. After changing the medium, the cells were treated with various concentrations of EGCG for an additional 4 days in the presence of $1\alpha,25(OH)_2D_3$ (10^{-8} M). Non-treated cells (A) or $1\alpha,25(OH)_2D_3$-treated cells (B) without EGCG were used as the negative and positive control, respectively (original magnification × 200). After co-culture, cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP). Arrow indicates TRAP-positive multinucleated cell. (C) The TRAP-positive multinucleated cells with more than three nuclei were counted as osteoclasts. At the same time, the effect of EGCG on the viability of the co-culture was re-evaluated by MTT assay on the duplicate 48-well culture plate. The results are expressed as the mean ± SD of four cultures. The data are representative of three separate experiments. $^*P < 0.05$; significantly different from the positive control.

pilot experiment (data not shown). Therefore, further studies are needed to evaluate exactly the effect of sonicated *P. gingivalis* extracts on the osteoclast formation. Although we have no ready explanation for the mechanism underlying the inhibitory effect of EGCG on osteoclast formation at present, in a previous study it was reported that EGCG inhibited bone resorption by inducing apoptotic cell death of osteoclast-like multinucleated cells in a dose-dependent manner (25–100 μM) without any significant effect on osteoblastic cells (32). In addition, various studies have reported that EGCG induces apoptosis in tumor cells (53–55). However, in the present study, we found that EGCG had no inhibitory effect on the cell viability of either the co-culture system or primary osteoblastic cells, at concentrations of up to 20 μM. Therefore, the observed reduction in the number of osteoclasts is thought to be mediated via a mechanism other than apoptosis. Therefore, the precise mechanism of action of EGCG on osteoclasts remains to be determined. In addition, since the bone resorbing activity of osteoclasts plays a crucial role in bone resorption, EGCG, with its ability to inhibits the formation of osteoclasts, might have the potential to be used in the treatment of bone diseases such as periodontitis. In addition, the low toxicity of EGCG and its distribution in bone tissue, as shown previously (56), supports its effectiveness *in vivo*. However, because we examined only the *in vitro* effects of EGCG in the present study, it remains to be determined whether EGCG exerts these effects *in vivo*.

In conclusion, in this study, we have shown that EGCG has an inhibitory effect on the gene expression of MMP-9 in osteoblasts and on the formation of osteoclasts. These findings suggest that EGCG may inhibit bone resorption by preventing the action of osteoblasts and osteoclasts. Therefore, our data indicate that the inhibitory activity of EGCG could be usefully applied to the development of a therapeutic agent for the treatment of bone diseases such as periodontitis.

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