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Green tea catechin inhibits lipopolysaccharide-induced bone resorption *in vivo*

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Background and Objective: Bone resorption is positively regulated by receptor activator of nuclear factor-κB ligand (RANKL). Pro-inflammatory cytokines, such as interleukin (IL)-1β, promote RANKL expression by stromal cells and osteoblasts. Green tea catechin (GTC) has beneficial effects on human health and has been reported to inhibit osteoclast formation in an *in vitro* co-culture system. However, there has been no investigation of the effect of GTC on periodontal bone resorption *in vivo*. We therefore investigated whether GTC has an inhibitory effect on lipopolysaccharide (LPS)-induced bone resorption.

Material and Methods: Escherichia coli (*E. coli*) LPS or LPS with GTC was injected a total of 10 times, once every 48 h, into the gingivae of BALB/c mice. Another group of mice, housed with free access to water containing GTC throughout the experimental period, were also injected with LPS in a similar manner.

Results: The alveolar bone resorption and IL-1β expression induced by LPS in gingival tissue were significantly decreased by injection or oral administration of GTC. Furthermore, when GTC was added to the medium, decreased responses to LPS were observed in CD14-expressing Chinese hamster ovary (CHO) reporter cells, which express CD25 through LPS-induced nuclear factor-κB (NF-κB) activation. These findings demonstrated that GTC inhibits nuclear translocation of NF-κB activated by LPS. In addition, osteoclasts were generated from mouse bone marrow macrophages cultured in a medium containing RANKL and macrophage colony-stimulating factor with or without GTC. The number of osteoclasts was decreased in dose-dependent manner when GTC was added to the culture medium.

Conclusion: These results suggest that GTC suppresses LPS-induced bone resorption by inhibiting IL-1β production or by directly inhibiting osteoclastogenesis.

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Lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria, has been identified as an important factor in the pathogenesis of periodontitis (1). Lipopolysaccharide stimulates inflammatory cells, such as neutrophils, macrophages and fibroblasts, to secrete interleukin (IL)-1, IL-6 and tumor necrosis factor-α (TNF-α) (2–4), and these mediators have been reported to accelerate osteoclast formation and bone resorption potently *in vivo* and *in vitro* (5–8). Several studies have shown that receptor activator of nuclear factor-κB (NF- κB) ligand (RANKL) is essential for osteoclastogenesis (9–11). The RANKL binds to the receptor activator of

nuclear factor-κB (RANK) expressed on the membranes of osteoclast precursor cells. Signaling by RANK/RANKL is mediated by tumour necrosis factor receptor-associated factor 6 (TRAF6), which activates downstream signaling pathways that activate transcription factor NF-κB, resulting in osteoclastogenesis (12,13). In contrast, osteoprotegerin (OPG), a soluble decoy receptor for RANKL, inhibits the formation, activation and survival of osteoclasts (14,15).

A great number of studies have shown beneficial effects of green tea catechin (GTC) on human health (16–20). Epigallocatechin-3-gallate (EGCG), a major ingredient of GTC, has been reported to exert a variety of biological effects, including anti-oxidant, antibacterial, anti-inflammatory and anticarcinogenic activities (21–25). Additionally, it has been reported that EGCG inhibits LPS-induced inflammatory cytokine production *in vitro* (26). However, only a few *in vivo* studies have confirmed the inhibitory effect of GTC on cytokine production. For instance, Yang *et al.* (27) examined the inhibitory effect of orally administrated green tea polyphenols on LPS-induced TNF-α production. They reported that mice fed an extract of green tea polyphenols had decreased TNF-α production in response to an intraperitoneal injection of LPS. Recently, it has also been reported that EGCG inhibited osteoclast formation induced by 1α,25 $(OH)_2$ vitamin D_3 in an *in vitro* co-culture system (28). Furthermore, EGCG was found to induce apoptotic cell death of osteoclast-like multinucleated cells in a dose-dependent manner (29). Such pharmacological effects of catechins would be useful for prophylaxis or treatment of inflammatory bone disease. However, there has been no study on the inhibitory effect of GTC on LPS-induced bone resorption *in vivo*.

We previously reported that repeated injections of *Escherichia coli* (*E. coli*) LPS into mouse gingiva induced alveolar bone resorption *in vivo* (30–34). The purpose of the present study was to determine the effect of GTC on LPS-induced bone resorption *in vivo* and on osteoclastogenesis *in vitro*.

Material and methods

Lipopolysaccharide-induced alveolar bone resorption in mice

Seven-week-old male BALB/c mice were used in the present study. The mice were purchased from Charles River, Yokohama, Japan and maintained under specific pathogen-free conditions in the Laboratory Animal Center for Biomedical Research (Nagasaki University School of Medicine). The experimental protocol was approved by the Local Institutional Animal Care and Use Committee of Nagasaki University.

Alveolar bone resorption was induced as in a previously described model (30–36). Briefly, 5 μg of *E. coli* LPS (*E. coli* 0111: B4; Difco, Detroit, MI, USA) in 3 μL phosphate-buffered saline (PBS) was injected a total of 10 times, once every 48 h, into mice gingivae on the mesial side of the first molar of the left mandible under ether anesthesia (LPS group). Lipopolysaccharide with 1% Sunphenon BG (containing 91.3% polyphenol and 76.6% catechins, consisting of 45.9% EGCG, 9.6% epigallocatechin, 8.6% epicatechin gallate, 5.3% epicatechin and others; Taiyo Kagaku, Mie, Japan; catechin group), LPS with 1% indomethacin (Wako Pure Chemical Industries, Osaka, Japan; indomethacin group) or PBS alone (control group) was injected in the same way as in the LPS group. Mice housed with free access to water containing 1% Sunphenon BG throughout the experimental period were also injected with LPS as previously described (catechin-drinking group). Although the consumption of water in the catechin-drinking group was less than that in the LPS group (3–4 vs. 4–5 mL/day) a noticeable difference in body weight was not observed between the experimental groups. Each experimental group consisted of six mice.

Mice were killed 24 h after the 10th injection of LPS or PBS. Then the left mandible of each mouse was removed and fixed in 4% paraformaldehyde in PBS at 4°C for 6 h, decalcified with 10% EDTA for 1 week, and then embedded in paraffin using the AMeX method (37). Serial sections of 4 μm thickness were prepared in order to examine the mesio-distal section of the left first molar. Five groups of serial sections, each containing 10 subsections, were obtained from each specimen and were subjected to hematoxylin and eosin (H&E) staining, tartrate-resistant acid phosphatase (TRAP) staining and immunohistological staining. The first subsection from each group of serial sections was stained with H&E for histopathological observation of the surface of alveolar bone.

Histochemical staining and bone histomorphometry

In order to identify the osteoclasts, the second subsection from each group was stained with TRAP according to the procedure described by Katayama *et al.* (38). Briefly, a staining solution was made by mixing 0.5 mL pararosanilin solution, 0.5 mL of 4% sodium nitrite solution, 10 mL of 0.1 M acetate buffer, pH 5.0, and 10 mg naphthol AS-BI phosphate (Sigma, St Louis, MO, USA), dissolved in 8 mL of distilled water. The mixture was adjusted to pH 5.0 using concentrated NaOH and filtered through no. 1 Whatman filter paper. Then 150 mg L(+)-tartaric acid was added to a 10 mL aliquot of the solution. After the second subsection from each group had been incubated in the staining solution for 30 min at 37°C, it was counterstained with haematoxylin. The osteoclasts were identified as multinucleated TRAP-positive cells on the bone surface.

Owing to the difficulty of quantitatively evaluating total bone resorption, the percentage of active resorption sites (ARS) was evaluated as described previously (31,34–36). Briefly, ARS were defined as points of contact between osteoclasts and the alveolar bone surface. After counting the number of points of intersection of the bone surface by a line of a micrometer (Olympus, Tokyo, Japan) in 25 μm graduations at ×400 magnification, the rates of ARS to total points of intersection were calculated.

Immunohistological staining and quantification

The third and fourth subsections from each group were used for immunohistological staining of IL-1β- and RANKL-positive cells as previously described (31,36). Serial subsections were deparaffinized and incubated with normal rabbit or goat serum for 30 min at room temperature. They were then immersed in primary rabbit anti-mouse IL-1β (R&D Systems, Minneapolis, MN, USA) or anti-mouse RANKL (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibody at 4°C overnight. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide, followed by incubation with a secondary antibody (biotinylated goat anti-rabbit immunoglobulin; Dako, Glostrup, Denmark). Finally, these subsections were incubated with peroxidase-conjugated streptoavidin (Dako), followed by diaminobenzidine tetraoxide solution, and were then counterstained with haematoxylin.

The evaluated field fell within 250 μm from the medial side of the alveolar bone surface of the first molar of the left mandible. Unit area was defined as a square measuring 250 $\mu m^2 \times 250\ \mu m^2$. The numbers of IL-1β- and RANKL-positive cells per unit area were calculated for each field.

In vitro osteoclastogenesis

Osteoclasts were generated from mouse bone marrow macrophages according to our previously described method (34). Briefly, BALB/c mice were killed by cervical dislocation, and the femora and tibiae were harvested. After the removal of excess tissues, they were placed into α-minimal essential medium (α-MEM) containing 10% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, USA), 100 μg/mL streptomycin and 100 IU/mL penicillin G. Bone marrow cells were eluted from bone marrow by flushing with culture medium using a 26 gauge needle. Bone marrow cells were cultured in 10 cm diameter dishes at a concentration of 1.5×10^7 to 2×10^7 cells per well in 10 mL of α-MEM containing 10% FBS with 5 ng/mL human macrophage colony-stimulating factor (M-CSF; R&D Systems; cross-reactive with mouse M-CSF in equimolar doses) for 12 h to separate adherent cells from non-adherent cells. Then the non-adherent cells were harvested and cultured in a 96-well plate at a concentration of $2{\times}10^5$ cells per well in 0.2 mL of α-MEM containing 10% FBS with 25 ng/mL M-CSF for 12 h. Adherent cells were used as bone marrow macrophages after washing out the non-adherent cells, including lymphocytes. Bone marrow macrophages were cultured with Sunphenon BG (2, 5 or 10 μg/mL) in a medium containing 50 ng/mL human RANKL (Peprotech, London, UK; cross-reactive with mouse RANKL in equimolar doses) and 25 ng/mL M-CSF for 4 days. On day 3, the medium was replaced with a fresh batch. Adherent cells were washed with PBS and fixed with 4% paraformaldehyde at 4°C for 30 min. After washing with PBS, the cells were treated with 0.2% Triton X-100/PBS for 5 min. The cells were rinsed with PBS and incubated with the TRAP staining solution at room temperature for 10 min. We defined TRAP-positive multinucleated (more than three nuclei) cells as mature osteoclasts. The mature osteoclasts were counted under a light microscope.

Flow cytometric analysis of NF-κB activation by LPS

The engineering of the CD14-expressing Chinese hamster ovary (CHO) reporter cell line, CHO/CD14.elam.tac, has been previously described in detail (39). The cell line, which was constructed by cotransfection with a human CD14-expressing plasmid and an NF-κB-dependent reporter plasmid, can express CD25 antigen on the cell surface through NF-κB activation induced by LPS.

The CHO/CD14 cells were plated in 24-well tissue culture dishes at a density of 10^5 cells per well. After overnight incubation, confluent monolayers of all cells were stimulated with 10 ng/mL ultrapure *E. coli* LPS (InvivoGen, San Diego, CA, USA) in the presence or absence of Sunphenon BG (1 or 10 μg/mL). Following incubation for 18 h, the cells were treated with 30 mM EDTA (Gibco BRL, Rockville, MD, USA) for 1 min, and detached cells were stained with fluorescein isothiocyanate-labeled anti-CD25 monoclonal antibody (Becton Dickinson, Bedford, MA, USA) and subjected to flow cytometric analysis for the expression of the NF-κB-dependent transgene (CD25).

Statistics

Statistical analyses for comparison of the experimental groups were performed using the Kruskal–Wallis test and the Mann–Whitney *U*-test at a significance level of $p < 0.05$.

Results

GTC inhibits LPS-induced alveolar bone resorption in mice

To examine the effect of GTC on LPS-induced bone resorption, GTC was injected into the mouse gingiva with *E. coli* LPS. A slight increase in inflammatory cell infiltration but no remarkable change and no TRAP-positive cells were seen on the alveolar bone surface in the control group (Fig. 1A). In the LPS group, infiltration of inflammatory cells mainly, of neutrophils and macrophages, was seen within the gingival connective tissue near the anterior edge of alveolar bone. The osteoblast lining on the alveolar bone surface was partly interrupted by resoption lacunae with many TRAP-positive osteoclasts (Fig. 1B). There was little difference among the indomethacin group, catechin group and catechin-drinking group; they showed slight inflammatory cell infiltration of the gingival connective tissue near the anterior edge of alveolar bone compared with the LPS group. However, the numbers of TRAP-positive multinuclear cells were decreased in these groups compared with the number in the LPS group (Fig. 1C–E). The percentage of ARS in each group is shown in Fig. 1F. The percentages of ARS were significantly lower in the catechin group and the

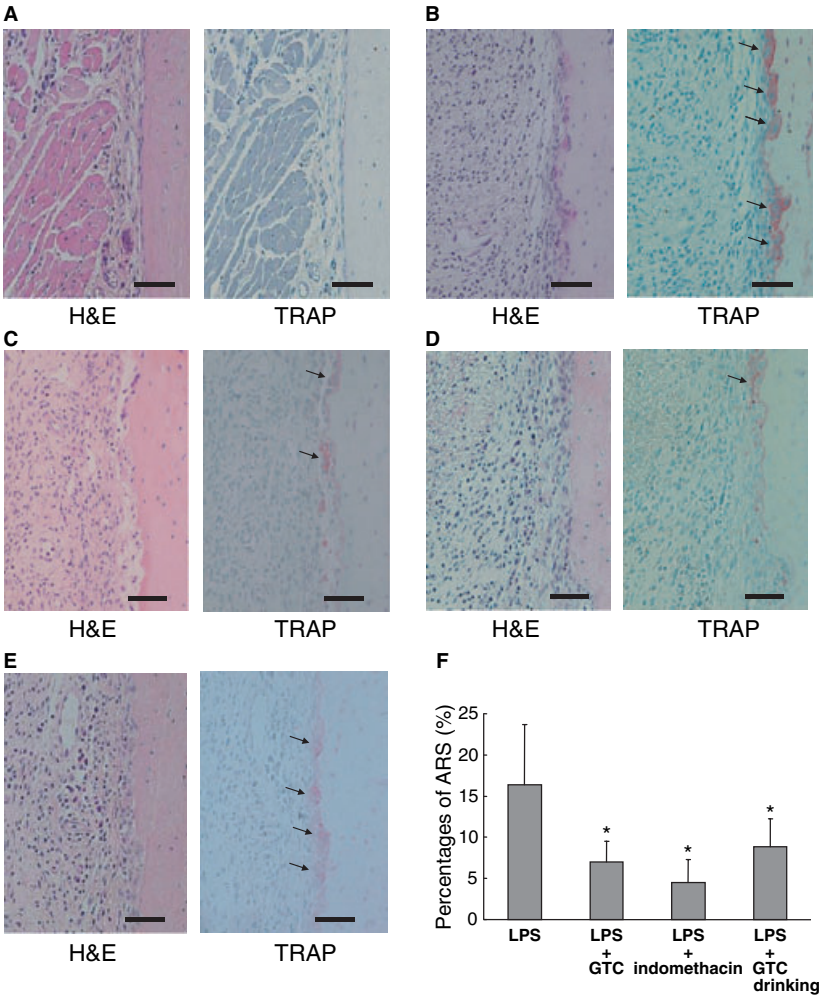

Fig. 1. Histopathological findings in alveolar bone and connective tissue in the control group (A), LPS group (B), catechin group (C), indomethacin group (D) and catechin-drinking group (E). The sections were stained with H&E or TRAP. Arrows indicate TRAP-positive cells. Scale bars represent 50 μm. (F) Percentage of ARS in the LPS, catechin, indomethacin and catechin-drinking groups. *$p < 0.05$ with respect to the LPS group. Bars represent means + SD.

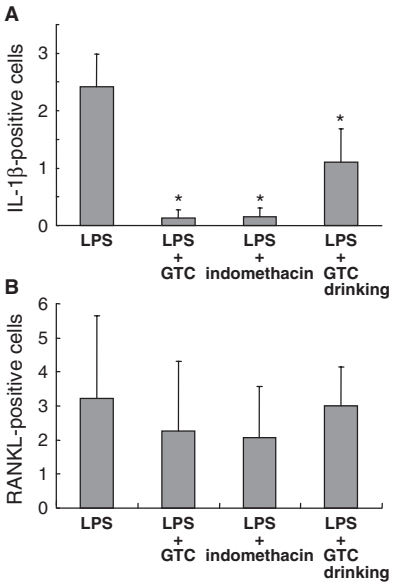

Fig. 2. (A) The number of IL-1β-positive cells per unit area was calculated in the LPS, catechin, indomethacin and catechin-drinking groups. The numbers of IL-1β-positive cells were significantly decreased in the catechin group, indomethacin group and catechin-drinking group compared with the number in the LPS group; *$p < 0.05$ with respect to the LPS group. Bars represent means + SD. (B) The number of RANKL-positive cells per unit area was calculated in the LPS, catechin, indomethacin and catechin-drinking groups. No significant difference was found in the number of RANKL-positive cells among the experimental groups. Bars represent means + SD.

indomethacin group than in the LPS group. A significant decrease of the percentages of ARS was also observed even in the catechin-drinking group.

GTC decreases IL-1β expression in gingival tissue in response to LPS injection but does not decrease RANKL expression

The numbers of IL-1β- or RANKL-positive cells per unit area in each group are shown in Fig. 2. No IL-1β- or RANKL-positive cells were observed in the gingival connective tissue in the control group (data not shown). Many IL-1β-positive cells were present within the gingival connective tissue in the LPS group, and they seemed to be macrophages, neutrophils and fibroblasts based on their histological features. In contrast, a few IL-1β-positive cells were observed in the catechin group, indomethacin group and catechin-drinking group (Fig. 2A). However, there was no histological difference in IL-1β-expressing cells observed in the experimental groups. The RANKL expression levels were similar in all experimental groups, although the control group showed no expression of RANKL. The numbers of RANKL-positive cells in each experimental group were increased, but there was no significant difference among the four groups (Fig. 2B).

GTC inhibits RANKL-induced osteoclastogenesis

When bone marrow macrophages were cultured with both RANKL and M-CSF for 4 days, a large number of multinucleated TRAP-positive cells were generated. The number of multinucleated TRAP-positive cells was decreased in a dose-dependent manner when GTC (2, 5 or 10 μg/mL) was added to the culture medium (Fig. 3). There was no cytotoxicity at the concentrations of GTC tested (2–10 μg/mL) as assessed by the trypan blue dye exclusion assay (data not shown).

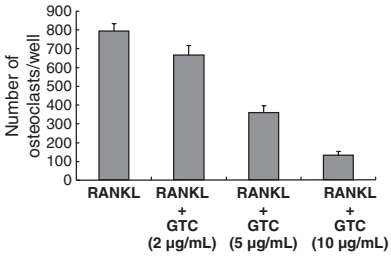

Fig. 3. The number of osteoclasts. Bone marrow macrophages were cultured with GTC (2, 5, or 10 μg/mL) in a medium containing 50 ng/mL human RANKL and 25 ng/mL M-CSF for 4 days. Cells that were TRAP positive with numerous ($n > 3$) nuclei were considered as mature osteoclasts. Bars represent means + SD of triplicate wells from two trials. Significant differences were observed among all of the experimental groups at a significance level of $p < 0.05$.

GTC inhibits activation of NF-κB in response to LPS

When CHO/CD14 reporter cells were stimulated with ultrapure *E. coli* LPS (10 ng/mL), NF-κB-driven CD25 expression was induced (Fig. 4A). To confirm the effect of GTC on LPS-induced activation of CHO/CD14 cells, 1 or 10 μg/mL catechin was added to the medium, and the cells were stimulated for 18 h (Fig. 4B,C). Although the addition of 1 μg/mL GTC to the medium had no significant effect on LPS-induced CD25 expression in CHO/CD14 reporter cells (Fig. 4B), the addition of 10 μg/mL GTC clearly decreased the level of CD25 expression, indicating that GTC inhibited LPS-induced NF-κB activation (Fig. 4C).

Discussion

A number of studies have demonstrated the availability of EGCG, a major ingredient of green tea catechin, for anti-inflammatory, anticarcinogenic, anti-oxidant and antimicrobial properties (21–25). Sunphenon BG is a highly purified extract of green tea, and has been reported to have equivalent activities to EGCG in several studies (40–42). In particular, Hirasawa *et al*. (43) reported the antibacterial effects of Sunphenon BG on pathogenic gram-negative anaerobic rods in periodontal pockets *in vivo*. However, the effect of Sunphenon BG on LPS-induced bone resorption *in vivo* has never been demonstrated. In the present experiments, we aimed to investigate whether daily intake of green tea would influence periodontal disease. We used Sunphenon BG, which contains several components, although most of the biological effects of green tea extract are mediated by EGCG. Our results showed the inhibitory effect of Sunphenon BG on LPS-induced bone resorption and osteoclastogenesis.

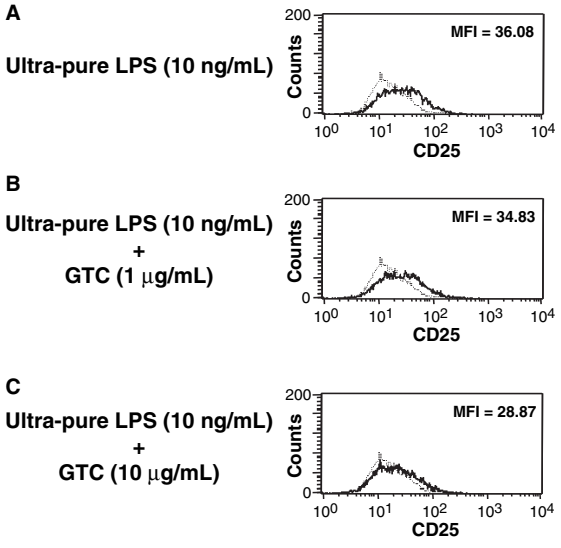

Fig. 4. Inhibition of LPS-induced activation of CHO/CD14 cells by GTC. The CHO/CD14 reporter cells were stimulated with (continuous line) or without ultrapure *E. coli* LPS (10 ng/mL; dotted line) in the presence or absence of GTC (1 or 10 μg/mL) for 18 h. The cells were stained with fluorescein isothiocyanate-labeled anti-CD25 monoclonal antibody and subjected to flow cytometric analysis for the expression of the NF-κB-dependent transgene (CD25). The results shown are representative of four independent experiments.

In this study, the percentage of ARS and the number of IL-1β-positive cells within the gingival connective tissue in the catechin group were significantly decreased compared with those in the LPS group. These inhibitory effects of GTC were equal to those of indomethacin. Lipopolysaccharide stimulates inflammatory cells, such as neutrophils, macrophages and fibroblasts, to secrete IL-1, IL-6, TNF-α and prostaglandin E_2 (2–4,44). Interleukin-1β has been reported to accelerate osteoclast formation and bone resorption potently *in vivo* and *in vitro* (5,7,8). In our previous study, we observed an increase of IL-1β-positive cells together with an increase in LPS-induced bone resorption (31). This observation suggests that suppression of IL-1β production is involved in the inhibitory effect of GTC on LPS-induced bone resorption. Lipopolysaccharide activates NF-κB thorough CD14 and toll-like receptor 4 (TLR4; 45,46). Nuclear factor-κB activation is important for the synthesis of inflammatory cytokines, such as IL-1β (47). To examine the mechanism by which GTC inhibits LPS-induced IL-1β production, we stimulated CHO/CD14 reporter cells with LPS in the presence or absence of GTC. When GTC was added to the culture, expression of CD25 on CHO/CD14 cells was decreased, indicating that GTC inhibited NF-κB activation induced by LPS stimulation. Our results suggest that the decrease in IL-1β production observed in the catechin group was concomitant with inhibited activation of NF-κB by GTC.

The percentage of ARS and the number of IL-1β-positive cells were also significantly decreased in the catechin-drinking group compared with those in the LPS group. A few *in vivo* studies have confirmed the inhibitory effect of orally administrated GTC on

cytokine production. Yang *et al*. (27) reported that mice fed an extract of green tea polyphenols had a decreased serum level of TNF-α in response to an intraperitoneal injection of *E. coli* LPS, suggesting that systemic application of green tea polyphenols affects TNF-α production. Our experiments showed that orally administrated catechin affects local inflammatory cells and inflammatory bone destruction.

In the present study, there was no significant difference between the numbers of RANKL-positive cells in the catechin group, catechin-drinking group and LPS group, although the percentages of ARS were significantly different. Ishida *et al*. (48) reported that EGCG had an inhibitory effect on the production of RANKL in osteoblast-like cells infected with *Staphylococcus aureus*. In the present experiments, we could not detect an effect of GTC on RANKL production. This discrepancy may be due to the difference in experimental conditions. In LPS-induced inflammatory bone resorption, GTC may mainly affect the production of bone-resorptive cytokines, such as IL-1, but not RANKL *in vivo*. Another possibility is that GTC may induce an increase in OPG production that inhibits osteoclast formation by neutralizing RANKL. The effect of GTC on OPG production should be examined in the future.

In our study, GTC inhibited the generation of osteoclasts from mouse bone marrow macrophages in a dose-dependent manner in the presence of M-CSF and RANKL. Yun *et al*. (49) reported that EGCG induces apoptosis in osteoclasts generated from RAW 264.7 cells (mouse leukaemic monocyte macrophage cell line) cultured with RANKL. They also demonstrated an inhibitory effect of EGCG on osteoclast formation in a co-culture system of mouse bone marrow cells and calvarial primary osteoblasts. Interaction of RANK with RANKL activates TRAF6 to induce nuclear translocation of NF-κB, resulting in osteoclastogenesis (12,13). There has been no experimental study to date to demonstrate that GTC may inhibit the RANK/RANKL signaling pathway in osteoclastogenesis; however, several studies have shown that EGCG inhibits its activation of the NF-κB pathway (50–53). Recently, Morinobu *et al*. (54) reported that EGCG inhibited osteoclastogenesis generated from human peripheral blood-derived CD14-positive mononuclear cells in the presence of M-CSF and RANKL by down-regulating expression of nuclear factor of activated T cells c1 (NFATc1), a master regulator of terminal differentiation of osteoclasts. Although we could not reveal the mechanism by which osteoclastogenesis is inhibited by GTC in our culture system, it is thought that GTC might directly block NF-κB activation through the RANK/RANKL signaling pathway in osteoclast precursor cells. In addition, a recent study has shown that RANKL-stimulated endogenous IL-1 production was to some extent involved in osteoclastogenesis in mice bone marrow macrophage culture (55). In our similar system, endogenous IL-1 may also have a role in osteoclastogenesis, since GTC may inhibit IL-1 production to decrease osteoclast formation indirectly.

In conclusion, we demonstrated that GTC had an inhibitory effect on osteoclastic bone resorption and osteoclast formation in the presence of RANKL both *in vivo* and *in vitro*. It is thought that GTC suppressed LPS-induced alveolar bone resorption by inhibiting osteoclast formation or by blocking IL-1β production through suppression of the activation of NF-κB. These inhibitory effects of GTC may have utility as a prophylactic and therapeutic agent for inflammatory bone diseases, such as periodontitis. Further studies are needed to confirm the pathway by which GTC inhibits LPS-induced alveolar bone resorption and osteoclastogenesis.

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