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Epigallocatechin gallate (EGCG) suppresses lipopolysaccharide-induced inflammatory bone resorption, and protects against alveolar bone loss in mice

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ABSTRACT

Epigallocatechin gallate (EGCG), a major polyphenol in green tea, possesses antioxidant properties and regulates various cell functions. Here, we examined the function of EGCG in inflammatory bone resorption. In calvarial organ cultures, lipopolysaccharide (LPS)-induced bone resorption was clearly suppressed by EGCG. In osteoblasts, EGCG suppressed the LPS-induced expression of COX-2 and mPGES-1 mRNAs, as well as prostaglandin E2 production, and also suppressed RANKL expression, which is essential for osteoclast differentiation. LPS-induced bone resorption of mandibular alveolar bones was attenuated by EGCG *in vitro*, and the loss of mouse alveolar bone mass was inhibited by the catechin *in vivo*.

1. Introduction

In bone tissues, bone mass is regulated by bone resorption and bone formation, and bone resorption is elicited by osteoclasts differentiated from the macrophage lineage cells. Previous studies have identified the receptor activator of NF-κB ligand (RANKL), also known as the osteoprotegerin (OPG) ligand [1–4], as a pivotal factor required for osteoclast differentiation. Osteoblasts express RANKL in response to bone-resorbing factors such as lipopolysaccharide (LPS) and IL-1, and interact with osteoclast precursors expressing RANK, inducing their differentiation into mature osteoclasts by a mechanism involving the RANK-RANKL interaction [3,4].

PGE_2 is a typical mediator associated with inflammation, and is produced by various types of cells. In bone tissue, PGE_2 is mainly produced by osteoblasts, and is one of the major inducers of RANK-dependent osteoclast differentiation and osteoclastic bone resorption [5]. Previous studies have shown that cyclo-oxygenases (COX)-2 and membrane-bound PGE synthase (mPGES)-1 are inducible enzymes that initiate PGE biosynthesis in various cells, including osteoblasts, after inflammatory stimuli [6,7]. We have reported that the bone resorption associated with inflammation was attenuated in mPGES-1-deficient mice (*mPges1*$^{-/-}$) due to the lack of PGE production by osteoblasts [7]. Therefore, the PGE_2 produced by osteoblasts is a critical regulator of bone metabolism.

Previous studies have shown that compounds in fruits and vegetables are beneficial to our health. Among these, polyphenols, including flavonoids, are known to exhibit physiological and pharmacological properties such as anti-oxidative, anti-bacterial and anti-tumor activities [8,9]. Isoflavones have been reported to show beneficial effects on the bone mass *in vivo* [10]. Catechins are major

Abbreviations: BMN, bone mineral density; COX, cyclo-oxygenase; EGCG, (−)-epigallocatechin-3-gallate; LPS, lipopolysaccharide; mPGES, membrane-bound PGE synthase; OCPC, o-cresolphthalein complexon; OPG, osteoprotegerin; PGE_2, prostaglandin E_2; PSD, polymicrobial synergy and dysbiosis; RANKL, receptor activator of NF-kB ligand

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flavonoids naturally present in certain species of plants, including tea. (−)-Epigallocatechin-3-gallate (EGCG), a major component of green tea catechins, has been reported to show the strongest effects among the catechins on various cell functions, and exhibits anti-oxidant and anti-tumor activity [8,9]. However, there have been only a few studies of the effects of EGCG on bone metabolism and skeletal health. Catechin is reported to enhance alkaline phosphatase activity in osteoblasts and osteogenic differentiation of mesenchymal stem cells [11,12], and EGCG inhibits osteoclastic differentiation from macrophage [13], but the effects of EGCG on inflammatory bone resorption are not known.

It is known that periodontitis is initiated by a synergistic and dysbiotic microbial community, and the recent pathological concept for reasoning of periodontal diseases is based on the polymicrobial synergy and dysbiosis (PSD) model [14], Periodontal plaque from the disease site induces inflammatory responses through Toll-like receptor (TLR) activation [15], and the simultaneous infection with *P. gingivalis* is also required. Our model for inflammatory bone resorption of alveolar bone is focused and mimicked on the periodontal bone resorption which is based on the TLR4-induced osteoclastgenesis in mice [7]. Using our model of the destruction of alveolar bone *in vivo*, we have reported that PGE_2 is closely related to the LPS-induced alveolar bone resorption, since we detected LPS-induced alveolar bone resorption in wild-type mice, but not in mPGES-1-null mice [7]. Therefore, PGE_2 production may play a key role in LPS-induced alveolar bone resorption via TLR4 in mice [7].

We examined the influence of EGCG on the calvarial bone resorption induced by LPS, and on the COX-2- and mPGES-1-dependent PGE synthesis in mouse osteoblasts. EGCG inhibited LPS-induced osteoclastic bone resorption by suppressing the PGE_2 production by osteoblasts *in vitro*, and attenuated the inflammatory bone loss of the mouse mandibular alveolar bone *in vivo*.

2. Materials and methods

2.1. Animals and experimental reagents

Mice of ddy strain were obtained from Japan SLC Inc. (Shizuoka, Japan), and animal experiments were performed under the institutional guidelines for animal research. LPS was obtained from Sigma Aldrich Co. LLC., MO, USA. EGCG was obtained from Nagara Science Corporation, Japan (Purity: more than 99%) for *in vitro* study, and from Taiyo Kagaku Co. Ltd. (Sunphenon EGCG-OP; Purity: more than 90%) for *in vivo* study.

2.2. Culture of primary mouse osteoblastic cells

Primary osteoblastic cells were isolated from newborn mouse calvariae after five routine sequential digestions with 0.1% collagenase (Roche Applied Science) and 0.2% dispase (Roche Diagnostics GmbH, Mannheim, Germany), as described previously [7]. Osteoblastic cells collected from fractions two to four were combined and cultured in α-modified MEM (αMEM) supplemented with 10% fetal calf serum (FCS) at 37 °C under 5% CO_2 in air. After 24 h in culture, they were treated with LPS with and without EGCG, and further cultured for 24 h for measurement of PGE_2.

2.3. Measurement of the PGE_2 content

The concentrations of PGE_2 in culture samples were calculated using an enzyme immunoassay (EIA) (GE Healthcare UK Ltd). The cross-reactivity of the antibody in the EIA was calculated as followed: PGE_2, 100%; PGE_1, 7.0%; 6-keto-$PGF_{1\alpha}$, 5.4%; $PGF_{2\alpha}$, 4.3% and PGD_2, 1.0%.

2.4. Bone-resorbing activity in organ cultures of mouse calvaria

Calvariae were collected from newborn mice, dissected in half and cultured for 24 h in BGJb containing 1 mg/ml of bovine serum albumin (BSA). After 24 h, the calvaria were transferred to new medium with or without EGCG and with or without LPS, and were cultured for another five days. The concentration of calcium in the conditioned medium was measured by the o-cresolphthalein complexon (OCPC) method. The bone-resorbing activity was expressed as the increase in the medium calcium concentration.

2.5. Quantitative PCR analysis

Mouse osteoblastic cells were cultured for 3, 6, 12 and 24 h in αMEM containing 1% FCS with or without LPS and with or without EGCG, and total RNA and cDNA were prepared as shown in previous papers [5,7], and the quantitative-PCR (q-PCR) was performed. The primer pairs used in the q-PCR for the mouse RANKL, COX-1, COX-2, mPGES-1, mPGES-2 and cPGES genes were as follows: Mouse RANKL: 5′-AGGCTGGGCCAAGATCTCTA-3′ (forward) and 5′-GTCTGTAGGTACGCTTCCCG-3′ (reverse), mouse COX-1: 5′-ACTGGTGGATGCCTTCTCTC-3′ (forward) and 5′-TCTCGG GACTCCTTGATGAC-3′ (reverse), mouse COX-2: 5′-GGGAGTCTGGA ACATTGTGAA-3′ (forward) and 5′-GTGCACATTGTAAGTAGGTGGAC T-3′ (reverse), mouse mPGES-1: 5′-GCACACTGCTGGTCATCAAG-3′ (forward) and 5′-ACGTTTCAGCGCATCCTC-3′ (reverse), mouse mPGES-2: 5′-CGTGAGAAGGACTGAGATCAAA-3′ (forward) and 5′-GAGGAGTCATTGAGCTGTTGC-3′ (reverse), mouse cPGES: 5′-CGAA TTTTGACCGTTTCTCTG-3′ (forward) and 5′-TGAATCATCATCTGCTC CATCT-3′ (reverse). The cDNA of the respective genes was

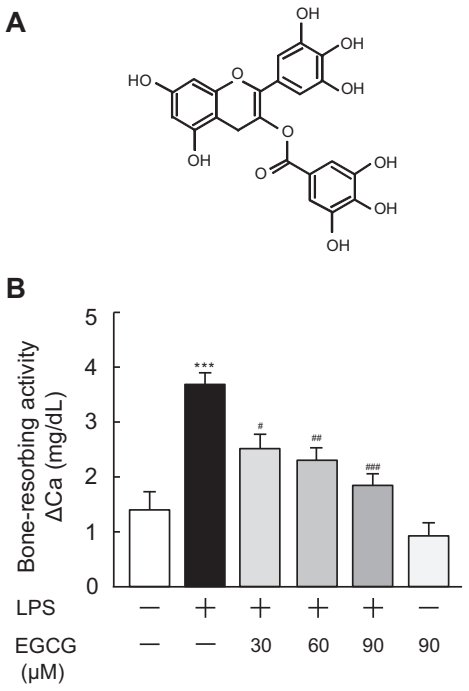

Fig. 1. The chemical structure of EGCG, and the effects of EGCG on the LPS-induced bone-resorbing activity in organ cultures of mouse calvariae. (A) The chemical structure of EGCG. (B) Mouse calvariae were cultured for 24 h in BGJb containing 1 mg/ml of BSA. After 24 h, the calvariae were transferred to new media, and were cultured for five days with or without 1 μg/ml of LPS and with or without EGCG (30, 60 or 90 μM). The concentration of calcium in the medium was measured to calculate the bone-resorbing activity. The data are expressed as the means ± SEM of four to 10 independent wells. A significant difference between the two groups was indicated by: $^{***}p < 0.001$ *vs. control*, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#}p < 0.001$ *vs. LPS*.