Epigallocatechin gallate suppresses expression of receptor activator of NF- κ B ligand (RANKL) in *Staphylococcus aureus* infection in osteoblast-like NRG cells

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Catechin, a constituent of tea, possesses various bioactivities. In particular, the most abundant catechin in tea is epigallocatechin gallate (EGCg), which has an anti-inflammatory effect. In the present study, the usability of EGCg for osteomyelitis treatment was examined. Osteomyelitis is a difficult disease to cure, partly due to bone lysis caused by infected osteoblasts. Since bone lysis is promoted by proinflammatory cytokines and the receptor activator of NF- κ B ligand (RANKL), osteoblasts were infected with *Staphylococcus aureus* and the effect of EGCg on the production of cytokines was examined. It was found that the production of interleukin 6 and RANKL was suppressed in the osteoblasts treated with EGCg, which indicated an inflammation suppression effect of EGCg in osteomyelitis treatment.

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INTRODUCTION

Osteomyelitis causes progressive inflammatory destruction of bone (Sax & Lew, 1999). The number of infectious diseases of bones and joints has increased because of new drug-resistant bacteria, the development of artificial joint replacement techniques, and an increase in the number of compromised patients and elderly people (Morris *et al.*, 2006). Due to its intractability, recurrence and chronicity of osteomyelitis are a major problem. Chronic osteomyelitis is intractable because sequestrum, pus or diseased granulation tissue inside bone marrow lesions causes poor blood circulation in the diseased part and hence lowers the effective concentration of antibiotics against pathogens. Another obstacle to treatment is inflammatory bone resorption by infected osteoblasts (Wright & Friedland, 2004). Tumour necrosis factor alpha (TNF- α) acts on marrow-derived macrophages to promote the induction of differentiation to osteoclast cells (Kobayashi *et al.*, 2000; Azuma *et al.*, 2000), and interleukin 1 (IL-1) and the receptor activator of NF- κ B ligand (RANKL) acts on mature osteoclast cells to induce bone resorption activity (Jimi *et al.*, 1999). If IL-1 or IL-6 affects osteoblasts, the expression of RANKL, an osteoclast differentiation factor, is induced causing bone resorption (Suda *et al.*, 1999;

Yasuda *et al.*, 1998). In addition to conventional treatment, controlling the reproduction and absorption of bone is important (Deutschmann *et al.*, 2005).

Epigallocatechin gallate (EGCg) is a constituent of green tea that has various bioactivities such as antibacterial activity (Toda *et al.*, 1990), antioxidative activity (Lin & Lin, 1997), anticancer activity (carcinoma inhibition) (Brown, 1999), inhibition activity of blood glucose and blood pressure (Strobel *et al.*, 2005; Ahn *et al.*, 1999), and antiallergic activity (Maeda-Yamamoto *et al.*, 2004). In particular, EGCg has anti-inflammatory effects, such as suppression of the activities of TNF and IL-1 β (Wheeler *et al.*, 2004; Yang *et al.*, 2001). We examined the effect of EGCg on osteoblasts to help determine the usefulness of EGCg for controlling inflammatory bone resorption.

Staphylococcus aureus is a major causative agent of human osteomyelitis (Lew & Waldvogel, 1999). It is a capable bone pathogen with adhesion molecules that facilitate its binding to the bone matrix (Boden & Flock, 1994; McDevitt *et al.*, 1994) and toxin secretion that could stimulate bone resorption (Nair *et al.*, 1995). Proinflammatory cytokines such as IL-1, IL-6 or TNF α are produced in *S. aureus*-induced osteomyelitis (Yoshii *et al.*, 2002). Cytokines such as IL-6 or IL-12 were induced when osteoblasts were stimulated with *S. aureus* (Bost *et al.*, 1999). Thus, we infected osteoblasts with *S. aureus in vitro*, and examined the effect of EGCg on the proinflammatory cytokines

Abbreviations: EGCg, epigallocatechin gallate; IL, interleukin; RANKL, receptor activator of NF- κ B ligand; TLR, Toll-like receptor; TNF, tumour necrosis factor.

induced there. We also studied the suppression of RANKL expression.

METHODS

Cells and strain under test. We used NRG cells (provided by RIKEN cell bank) as the osteoblasts. NRG cells are a mouse marrow-derived fibroblast-like cell strain and have osteoblast-like features (Umezawa *et al.*, 1992). They were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) with 10 % fetal bovine serum and 5 µg gentamicin ml⁻¹ in 5 % CO₂ at 37 °C.

We used *S. aureus* 209P as the test strain for the experiment. The bacteria were cultured in tryptic soy broth (EIKEN).

Intracellular growth assay. NRG cells were distributed onto 48-well flat-bottom plates at 1×10^5 cells per well. The cells were stimulated with EGCg (50, 100 and 200 µg ml⁻¹) for 2 h before use in the intracellular growth assay. EGCg was provided by Dr Yukihiko Hara (Mitsui Norin Co., Ltd, Tokyo, Japan). After culturing for 2 h, the medium was replaced with EGCg-free, antibiotic-free culture medium with 10 % serum concentration.

Then we inoculated the cells with *S. aureus* at a m.o.i.=100 and centrifuged them at 450 g for 5 min to enhance the attachment of the bacteria to the NRG cells, and we incubated them at 37 °C for 2 h to facilitate the ingestion of the bacteria. Extracellular bacteria were removed by washing three times with PBS, and gentamicin was then added to the culture medium to achieve a concentration of 5 µg ml⁻¹ (we reset the time at this point). After 0, 24 and 48 h culturing, we measured c.f.u. of viable intracellular bacteria using tryptic soy agar plates (Eiken).

Inflammatory cytokine assay. We detected proinflammatory cytokines using a partly modified method based on that reported by Wright & Friedland (2004). Namely, NRG cells were stimulated with EGCg (125 µg ml⁻¹) for 2 h before the experiment. After replacing the media with antibiotic-free media, we inoculated the cells with *S. aureus* at a m.o.i.=100. Two hours later, we replaced the media with DMEM of 10 % serum concentration with 5 µg gentamicin ml⁻¹ added. After 24 h culture, we measured the concentration of IL-1α, IL-1β, IL-6 and TNFα in the culture supernatant. The measurement was conducted with Duo Set (R&D Systems) for IL-1α and OptEIA (BD Biosciences) for the others, based on its instruction manual.

Detection of RANKL. Pretreatment of osteoblasts with EGCg was conducted just as for the method of measuring proinflammatory cytokines. After the pretreatment, we detected the expression of RANKL over time with Western blotting. After 0, 2, 4, and 6 h culture, we removed the culture supernatant and homogenized the cells with lysis buffer (0.125 M Tris/HCl, 10 % 2-mercaptoethanol, 4 % SDS, 10 % sucrose and 0.004 % bromophenol blue). Each sample was analysed by SDS-PAGE using 10 % acrylamide gels and transferred to polyvinylidene difluoride membranes (Amersham Biosciences). After being blocked, the membranes were incubated with goat anti-rabbit RANKL antibody (R&D Systems). After being washed, the membranes were incubated with horseradish-peroxidase-conjugated rabbit anti-goat IgG (Zymed). The immunoreactive band was visualized with a chemiluminescence kit (GE Healthcare).

Statistical analysis. All assays for intracellular growth of *S. aureus*, measurement of inflammatory cytokines and detection of RANKL were independently repeated three times or more, and similar results were obtained. Representative data were expressed as means ± SD of triplicate determinations. Student's *t*-test was used to determine the

statistical significance. A *P* value <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Intracellular growth of *S. aureus*

The MIC of EGCg against *S. aureus* 209P was 250 µg ml⁻¹ as we reported previously (Takahashi *et al.*, 1995). In osteoblasts processed with 50, 100 or 200 µg ml⁻¹, the intracellular growth of *S. aureus* progressed almost in the same way as in the unprocessed type (Fig. 1). Namely, the processing of NRG cells at an EGCg concentration less than the MIC had no effect on the intracellular growth of *S. aureus*, and hence we used 125 µg ml⁻¹ to process the osteoblasts in the following experiments.

Effect of EGCg on the production of proinflammatory cytokines with osteoblasts infected by *S. aureus*

No proinflammatory cytokines were produced in NRG cells stimulated only with EGCg (Fig. 2). When NRG cells were inoculated with *S. aureus*, IL-1α and IL-6 were produced but not IL-1β or TNFα (Fig. 2). IL-1β and TNFα were also not produced when the cells were stimulated with LPS (data not shown). When NRG cells were processed with EGCg before being inoculated with *S. aureus*, the amount of IL-6 production after 24 h was 0 pg ml⁻¹, which indicated a clear inhibition of production, in comparison to the IL-6 production (11.9 pg ml⁻¹). In contrast, the production of IL-1α was slightly suppressed but no significant differences were shown. These results indicate that EGCg affects the osteoblast function in respect to production of IL-6.

Fig. 1. Effect of EGCg on intracellular growth of *S. aureus* in NRG cells. The concentrations of EGCg used were 0 (○), 50 (●), 100 (□) and 200 (■) µg ml⁻¹. The number of bacteria was counted 0, 24 and 48 h after the start of the culture. The values are the means ± SD for triplicate assays.

Fig. 2. Production of inflammatory cytokines (a) IL-1 α , (b) IL-1 β , (c) IL-6 and (d) TNF α in EGCg-processed and unprocessed NRG cells stimulated with *S. aureus* 209P (m.o.i. = 100). The values are the means $\pm$ SD for triplicate assays. An asterisk indicates that the *P* value was <0.05 for a comparison with the cytokine level in normal osteoblasts stimulated with *S. aureus*.

Detection of RANKL

Fig. 3 shows the expression of RANKL when NRG cells were infected with *S. aureus*. We did not observe expression of RANKL before the cells were inoculated with *S. aureus*, but found a strong expression of RANKL after the inoculation irrespective of whether EGCg processing was performed or not. The expression of RANKL was observed in EGCg-processed NRG cells in 2, 4 or 6 h after they were inoculated with *S. aureus*, but was clearly less significant than that observed in NRG cells with no EGCg

Fig. 3. Expression of RANKL in EGCg-processed and unprocessed NRG cells stimulated with *S. aureus* 209P. RANKL was detected by Western blotting using anti-RANKL antibody. Lane A, normal NRG cells stimulated with *S. aureus* (m.o.i. = 100); lane B, EGCg-treated ($125 \mu\text{g ml}^{-1}$) NRG cells stimulated with *S. aureus* (m.o.i. = 100); lane C, EGCg-treated ($125 \mu\text{g ml}^{-1}$) NRG cells, not stimulated. Clear suppression of the RANKL expression was not found in the 2 h culture, but was found in the 4 h culture.

processing performed. It was thus demonstrated that EGCg-processed NRG cells inhibited the expression of RANKL.

Few studies have been reported on the pathological condition of bacterial infection causing osteomyelitis; however, it has recently been clarified that *S. aureus* invades not only the surface of bones, but also into cells to induce its pathogenicity. *S. aureus* not only colonizes bone matrix, but also is internalized by osteoblasts. This has been demonstrated *in vitro* (Ellington *et al.*, 1999) and *in vivo* (Reilly *et al.*, 2000). Extensive work has delineated the mechanisms of *S. aureus* invasion, and the immune response resulting from its infection of osteoblasts (Ellington *et al.*, 2003). The ability of *S. aureus* to invade and survive within osteoblasts may be critical to the observation that 80 % of all cases of chronic osteomyelitis are caused by *S. aureus*. Intracellular invasion provides protection from the humoral immune response and several classes of antibiotics. This could explain the persistence of the disease despite what has been considered adequate surgical and antibiotic management. Also in the present experiment, we noted the intracellular growth when NRG cells were inoculated with *S. aureus* (Fig. 1) and the promotion of the production of IL-6, IL-1 α and RANKL, the important cytokines that contribute to bone resorption (Fig. 2). In bone marrow inflammation caused by infection with *S. aureus*, the production of various cytokines is locally induced. There are some reports on osteoblast cells, such as that IL-6 and IL-12 were produced in mouse osteoblast or MC3T3E-1 cells inoculated with *S. aureus* (Bost *et al.*, 1999), and that IL-1 β or TNF α was not produced but IL-6 and RANKL were produced in mouse

osteoblast cells inoculated with the Gram-positive coccus *Streptococcus pyogenes* (Okahashi *et al.*, 2003). Our experimental results are consistent with these findings, and it is now clear that in osteoblast cells the production of cytokines, such as IL-1 α and IL-6, is promoted not only by the stimulation on the cellular surface, but also by the stimulation inside the cells.

Catechin is an astringent constituent of green tea and its functions have been actively studied since the late 1980s. The catechin from green tea and the flavonoids of tea have bacteriostatic activity, antiviral activity, an antioxidant effect, antitumour activity, antiallergic activity, an immuno-enhancing effect, etc. In particular EGCg, which comprises 50–60 % of green tea catechin, is found to have anti-inflammatory effects inhibiting the expression of TNF and IL-1 β . In the present study, we examined the effect of EGCg on the induction of proinflammatory cytokine production in osteoblast cells infected with *S. aureus*, and found that it had no significant effect on the production of IL-1 α but had certain effects on the inhibition of the production of IL-6 (Fig. 2) and of RANKL (Fig. 3).

Four signal cascades play roles in the induction of RANKL in osteoblasts: a signal cascade of activated vitamin D via VDR (Takasu *et al.*, 2006), that of IL-6 and IL-11 via STAT 3 (O'Brien *et al.*, 1999), that of PTH and PGE via cAMP/PKA for promoting the expression of RANKL genes (Kondo *et al.*, 2002), and that of LPS and IL-1 via PKC-MEK/ERK (Kikuchi *et al.*, 2001). In bacterial infection, pathogen-associated molecular patterns (PAMP) that are composed of bacteria are detected via Toll-like receptors (TLRs) and then the expression of RANKL genes is promoted through MEK/ERK, in the same way as that of IL-1 (Kikuchi *et al.*, 2001). There should also be a system in our experiment that induces RANKL production via PKC-MEK/ERK, but we consider that the induction of RANKL production by this signal cascade was not significant because the production of IL-1 was small and because *S. aureus*-derived PAMP were not detected by TLR since the bacterial body resided inside the cells. Actually, it is reported that in macrophages and other cells, TLR develops on the surface of the cell and detects PAMP, and that fungus bodies such as LPS inside the cell cytoplasm are usually detected by Nod (Philpott & Girardin, 2004).

The inhibitory effect clarified in this study is a new finding on the bioactivity of EGCg. In our earlier reports we pointed out various antibacterial effects of EGCg, such as the antibacterial action of the simultaneous use of antibiotics and EGCg against methicillin-resistant *S. aureus* (Hu *et al.*, 2002), and the antitoxic property of EGCg against staphylococcal enterotoxin B (Hisano *et al.*, 2003). Others also reported the EGCg antibacterial effect on methicillin-resistant *S. aureus* (Kono *et al.*, 1994) and on *Pseudomonas aeruginosa* and *Escherichia coli*, the causative bacteria of bone marrow inflammation (Yoda *et al.*, 2004). Since EGCg can be used for bone cement for the treatment of bone marrow inflammation because of its property of

stability against heat, our study indicates its additional effectiveness for the treatment in that EGCg suppresses bone resorption and has antibacterial effects.

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