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Original Communication

Lutein, a carotenoid, inhibits lipopolysaccharide-induced alveolar bone loss associated with inflammation in a mouse model of periodontitis

Hiroshi Takeda[1], Tsukasa Tominari[2,3], Ryota Ichimaru[2], Chiho Matsumoto[2], Kenta Watanabe[1], Michiko Hirata[2], Masaki Inada[1,2,3] and Chisato Miyaura[1,2,3,*]

[1]Cooperative Major of Advanced Health Science; [2]Department of Biotechnology and Life Science; [3]Institute of Global Innovation Research, Tokyo University of Agriculture and Technology, Tokyo, Japan.

ABSTRACT

Lutein, a member of the xanthophyll family of carotenoids, possesses antioxidant properties and regulates various cell functions. We examined the effects of lutein on inflammatory bone resorption. In calvarial organ cultures, lutein clearly suppressed lipopolysaccharide (LPS)-induced bone resorption. In osteoblasts, lutein suppressed the LPS-induced expression of cyclooxygenase (COX)-2 and membrane-bound PGE synthase (mPGES)-1 mRNAs, as well as prostaglandin E (PGE) production. LPS-induced bone resorption of mandibular alveolar bones was attenuated by lutein *in vitro*, and the loss of alveolar bone mass was restored by lutein in a mouse model of periodontal disease.

KEYWORDS: periodontal disease, PGE_2 production by osteoblasts, xanthophyll family of carotenoids

ABBREVIATIONS

RANKL : receptor activator of NF-κB ligand
LPS : lipopolysaccharide
TLR4 : toll-like receptor 4

INTRODUCTION

Lutein is a member of the xanthophyll family of carotenoids, a group of 40-carbon hydroxylated compounds that are synthesized in green leafy plants such as spinach and kale [1]. Lutein acts as a powerful antioxidant and filters high-energy blue light, and the dietary intake of lutein is mainly accumulated in the retina of the eye to protect against hypoxia-induced cell damage by removing free radicals [1, 2]. However, the role of lutein in other tissues remains to be elucidated. Recently, we have found that lutein suppressed interleukin (IL)-1-induced osteoclast differentiation, and it enhanced new bone formation [3], suggesting that lutein may be beneficial for bone health in humans. In bone tissues, bone mass is regulated by bone resorption and bone formation. It is well known that the receptor activator of NF-κB ligand (RANKL) is a pivotal factor required for osteoclast differentiation [4, 5]. 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 osteoclasts by a mechanism of RANK-RANKL interaction. PGE_2 is a typical mediator associated with inflammation, and it is one of the bone resorbing factors which induce RANKL expression in osteoblasts [6]. Osteoblasts produce PGE_2 by the induction of cyclooxygenase (COX)-2 and membrane-bound PGE synthase (mPGES)-1 [7]. We have reported that the bone resorption associated with inflammation was attenuated in mPGES-1-deficient mice due to the lack of PGE

*Corresponding author: miyaura@cc.tuat.ac.jp

production by osteoblasts [8], suggesting that PGE_2 is a critical regulator of bone metabolism.

Periodontal diseases are caused by the infection resulting from bacterial plaque accumulation in the periodontal pocket located in the junction between gingiva and tooth. In the progression of periodontal diseases, Gram-negative anaerobic bacteria are considered to be pathogen in humans [9], and LPS is a typical pathogen-associated molecule. Using our original mouse model of periodontitis, we have reported that periodontitis induces the destruction of alveolar bone by increased bone resorption [8], and that PGE_2 is closely related to the LPS-induced bone resorption in periodontitis, since we detected LPS-induced alveolar bone resorption in wild-type mice, but not in mPGES-1-null mice. Therefore, PGE_2 production may play a key role in infection-dependent and LPS-induced periodontal bone resorption.

We examined the influence of lutein on the calvarial bone resorption induced by LPS, and on the COX-2- and mPGES-1-dependent PGE synthesis in mouse osteoblasts. Lutein suppressed LPS-induced bone resorption in organ cultures of alveolar bone *in vitro*, and attenuated the inflammatory bone loss of the mouse mandibular alveolar bone in the model of experimental periodontitis.

MATERIALS AND METHODS

Animals and experimental reagents

Newborn and 6-week-old *ddy* mice were obtained from Japan SLC Inc. All procedures were performed in accordance with the institutional guidelines for animal research, and the experimental protocol was approved by the Animal Care and Use Committee of the Tokyo University of Agriculture and Technology. LPS was obtained from Sigma Aldrich Co. LLC., MO, USA. Highly purified lutein (purity: >94%) was obtained from CaroteNature Co., Ltd. Lutein was dissolved in dimethyl sulfoxide (DMSO) and added to cultures (0.1%).

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 minimum essential medium (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 lutein, and further cultured for 24 h for measurement of PGE_2.

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%.

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 lutein 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.

Quantitative polymerase chain reaction (q-PCR) analysis

Total RNA was extracted from cultured osteoblasts, and cDNA was synthesized by reverse transcriptase, and amplified via PCR. The q-PCR was performed with iQ SYBR Green Supermix (Bio-Rad). The primers used for the mouse RANKL, mPGES-1, and COX-2 were constructed from the sequence of the respective genes.

Bone-resorbing activity of mouse mandibular alveolar bone in organ cultures

Mouse mandibular alveolar bone was collected from the molar region and three molars were removed under a microscope. The isolated alveolar bone was cultured for 24 h in BGJb containing 1 mg/ml BSA. After 24 h in the organ cultures, the alveolar bone was transferred to new media,

with or without LPS and with or without lutein, and was cultured for another five days. The bone-resorbing activity was determined by the increase in medium calcium compared to control culture [7].

Model of experimental periodontitis using the mouse alveolar bone mass

In the model of experimental periodontitis, LPS (25 μg/mouse) was dissolved in 50 μL of phosphate buffered saline (PBS) and injected into the outside of the lower gingiva of the mice on days 0, 2 and 4. As a control, PBS was injected into the lower gingiva at the same time points. After seven days of the first injection, the mandibular alveolar bones were collected from mouse molar region, and three molars were removed. The bone mineral density (BMD) of the total area of alveolar bone was measured by dual-energy X-ray absorptiometry (DEXA) [7].

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's test for *post hoc* analysis, and expressed as the means ± standard error of the mean (SEM). Statistical analyses were performed using IBM SPSS Statistics Ver. 23 software.

RESULTS AND DISCUSSION

Lutein is a member of the xanthophyll family of carotenoids (Fig. 1A). Using *ex vivo* cultures of mouse calvariae, we examined the effects of lutein on LPS-induced bone-resorbing activity. LPS markedly induced bone-resorbing activity, while lutein (3-30 μM) suppressed bone resorption in a concentration-dependent manner (Fig. 1B). Lutein did not show any effects on bone-resorption in the absence of LPS (data not shown). We have reported that LPS recognizes toll-like receptor 4 (TLR4) and induces PGE_2 production by osteoblasts, and that PGE_2-dependent RANKL expression is essential for osteoclastic bone resorption induced by LPS [8]. In the cultures of primary mouse osteoblasts, the addition of lutein suppressed the mRNA expression of mPGES-1, COX-2 and RANKL that were induced by LPS in a q-PCR (Fig. 2A). The production of PGE_2 was greatly induced by LPS in osteoblasts, and the addition of lutein clearly suppressed the PGE_2 production by osteoblasts (Fig. 2B). In IκB kinase (IKK) assay, lutein suppressed the IKK activity (Fig. 2C), indicating that lutein suppresses NF-κB activation to regulate the expression of mPGES-1, COX-2 and RANKL mRNA. These results indicate that lutein acts on osteoblasts to suppress the PGE_2 production, and to negatively regulate LPS-induced bone resorption.

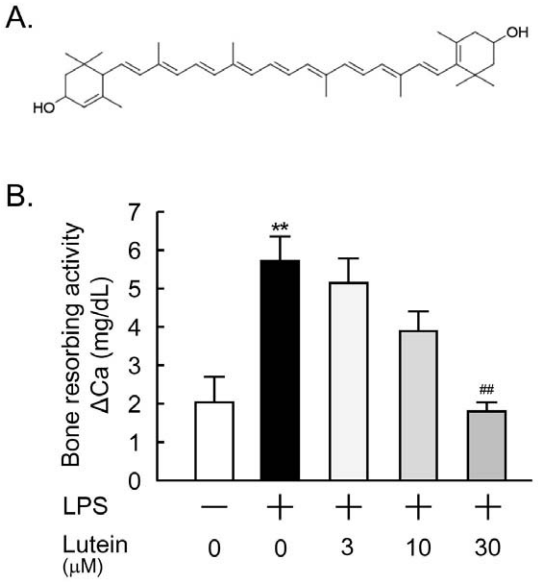

Fig. 1. The effects of lutein on LPS-induced bone resorption. (A) The chemical structure of lutein. (B) Mouse calvariae were cultured for 24 h in BGJb medium containing 1 mg/ml of BSA, and transferred to new media and cultured for 5 days, with or without LPS (1 μg/ml) and with or without lutein (3, 10 or 30 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 independent wells. Asterisks and hashes indicate a significant difference: $^{**}p < 0.01$ *vs. control,* $^{\#\#}p < 0.01$ *vs. LPS.*

Using an organ culture system of mouse mandibular alveolar bone, we examined the effects of lutein on bone resorption induced by LPS. The alveolar bones were collected from mouse lower mandibles (Fig. 3A), and were cultured with or without LPS. LPS markedly induced the bone resorbing activity in the organ cultures of alveolar bone, and adding lutein clearly suppressed the resorption of alveolar bone (Fig. 3B). Lutein did not influence the alveolar bone resorption in cultures without LPS (data not shown). To define the effects of lutein

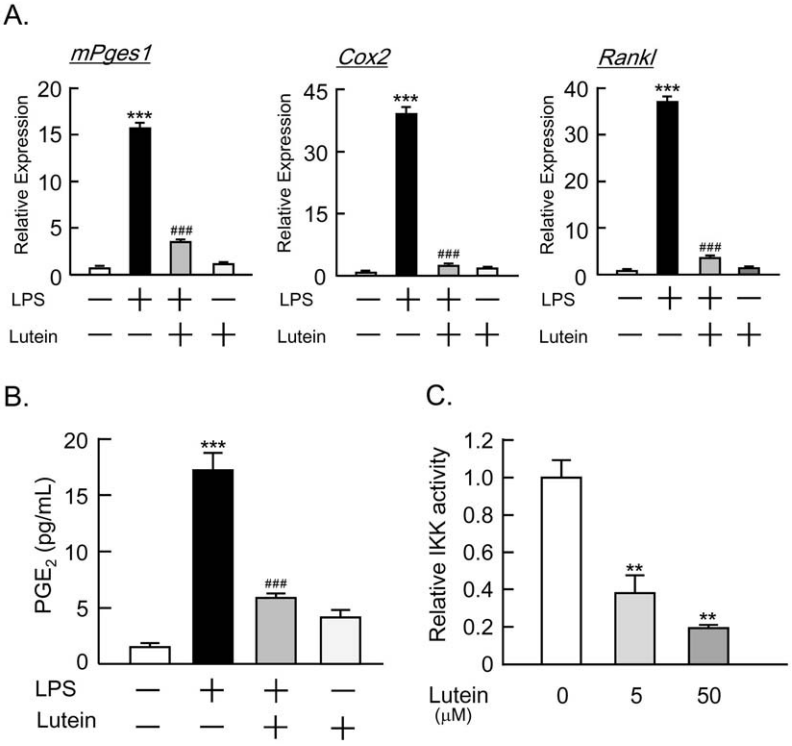

Fig. 2. Lutein acts on osteoblasts and suppresses PGE_2 production. (A) Mouse osteoblasts were pretreated with or without lutein (30 μM) for 24 h and then treated with LPS (1 ng/ml) and with or without lutein (30 μM) for 24 h, and the total RNA was extracted. The levels of mRNA expression of mPGES-1, COX-2 and RANKL were measured by a q-PCR. The data are expressed as the means ± SEM of three independent wells. (B) To examine the effects of lutein on PGE_2 production by osteoblasts, mouse osteoblasts were pretreated with or without lutein (30 μM) for 24 h, and then treated with LPS (1 ng/ml) and with or without lutein (30 μM) for 24 h. The levels of PGE_2 in the culture supernatant were measured by EIA. The data are expressed as the means ± SEM of six independent wells. Asterisks and hashes indicate a significant difference: $^{***}p < 0.001$ *vs. control*, $^{###}p < 0.001$ *vs. LPS.* (C) Effects of lutein on IKK activity were examined using IKK assay. Using purified IKK, the IKK activity was measured in conditions with or without lutein (5 and 50 μM). The data are expressed as the means ± SEM of three independent reactions. Asterisks and hashes indicate a significant difference: $^{**}p < 0.01$ *vs. control.*

on experimental periodontitis, we injected LPS with or without lutein into the gingiva of the lower mandibles of mice. After seven days of the first injection, alveolar bone was collected from mouse, and the BMD was measured by DEXA. In the experimental model of periodontitis, LPS markedly decreased BMD of the mandibular alveolar bone, while the simultaneous injection of lutein significantly restored the LPS-induced loss of BMD in mice (Fig. 3C).

In the present study, we showed that lutein suppresses LPS-induced bone resorption in *ex vivo* cultures of mouse calvaria and alveolar bone. Since NF-κB activation is critical for LPS-induced expression of mPGES-1, COX-2 and RANKL mRNAs, it is possible that NF-κB is one of the molecular targets of lutein in bone resorption. Indeed, the IKK enzyme activity was suppressed by adding lutein. Recently we have reported that lutein acts on macrophages, osteoclast precursors, and suppresses osteoclast differentiation induced by soluble RANKL in cultures [3]. Therefore, lutein may act on both osteoblasts and osteoclast precursor cells to suppress osteoclastic bone resorption.

It is well known that TLRs, TLR1-TLR13, play key roles in innate immunity [10]. The ligands for the respective TLRs stimulate signal transduction in the target cells and regulate the host defense system against pathogens. We previously reported

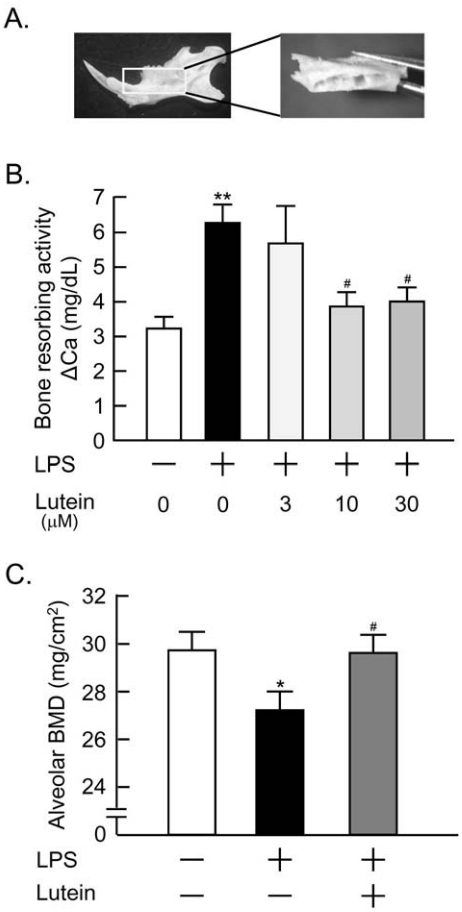

Fig. 3. The administration of lutein restored the LPS-induced loss of mandibular alveolar bone in mice. (A) Mandibular alveolar bone specimens were collected from the molar region of the lower jaw under a microscope, and three molars were removed. (B) The collected mandibular alveolar bones were cultured for five days with LPS (1 μg/ml), and with or without lutein (3, 10 or 30 μM). The concentration of calcium in the medium was measured, and the increase in calcium in the medium was calculated as the bone-resorbing activity. The data are expressed as the means ± SEM of four independent wells. Significant differences are indicated by: $^{**}p < 0.01$ *vs. control.* $^{\#}p < 0.05$, *vs. LPS.* (C) As a model of experimental periodontitis, LPS (25 μg/mouse) was injected into the mouse lower gingiva on days 0, 2 and 4. As a control, PBS was injected into the lower gingiva at each time point. Lutein (0.3 mg/mouse) was injected into the mouse lower gingiva with LPS in some animals. The mandibular alveolar bone was collected seven days after the first injection, and the BMD of the mandibular alveolar bones was measured. The data are expressed as the means ± SEM of seven mice. A significant difference between the two groups is indicated by: $^{*}p < 0.05$ *vs. control,* $^{\#}P < 0.05$, *vs. LPS.*

that mouse osteoblasts express TLR4 and LPS-TLR4 signaling induces severe bone resorption associated with inflammation [8], and that mPGES-1-null mice are resistant to both LPS-induced osteoclast formation and LPS-induced PGE_2 production in osteoblasts [8]. Therefore, TLR4-mediated PGE biosynthesis is essential for the effects of LPS on bone tissues, but it is unknown whether lutein interacts with TLR4. Further studies are needed to define the mechanism of action of lutein in LPS-induced and TLR4-mediated PGE biosynthesis.

Using an *in vivo* model of experimental periodontitis, we reported that polymethoxy flavonoids such as nobiletin, and green tea catechin EGCG prevented LPS-induced inflammatory bone loss, and restored the alveolar bone loss induced by LPS *in vivo* [11, 12]. The intake of natural components derived from fruits and vegetables may be beneficial for bone mass. In the present study, lutein suppressed bone resorption by inhibiting LPS-induced bone resorption, and restored the alveolar bone loss induced by LPS in a mouse model of experimental periodontitis. Although our studies suggest that lutein is effective for preventing periodontitis, further studies are needed to define the role of lutein in the pathogenesis of human periodontitis.

CONCLUSION

Lutein suppressed LPS-induced bone resorption in *ex vivo* cultures of mouse calvaria and alveolar bone. In the culture of osteoblasts, lutein suppressed the LPS-induced expression of COX-2 and mPGES-1 mRNAs, as well as PGE_2 production. In a mouse model of experimental periodontitis, lutein restored the alveolar bone loss induced by LPS. Since lutein is a major xanthophyll carotenoid in green leafy plants such as spinach, it might be useful to perform a human study examining the effects of intake of green leafy plants on periodontal disease in order to further understand the possible roles of lutein for the prevention and/or treatment of periodontitis.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest in association with this study.

REFERENCES

1. Krinsky, N. I., Landrum, J. T. and Bone, R. A. 2003, Annu. Rev. Nutr., 23, 171.
2. Dilsiz, N., Sahaboglu, A., Yildiz, M. Z. and Reichenbach, A. 2006, Graefes Arch. Clin. Exp. Ophthalmol., 244, 627.
3. Tominari, T., Matsumoto, C., Watanabe, K., Hirata, M., Grundler, F. M., Inada, M. and Miyaura, C. 2016, Biosci. Biotechnol. Biochem., in press. doi:10.1080/09168451. 2016.1243983.
4. Lacey, D. L., Boyle, W. J., Simonet, W. S., Kostenuik, P. J., Dougall, W. C., Sullivan, J. K., San Martin, J. and Dansey, R. 2012, Nat. Rev. Drug Discov., 11, 401.
5. Boyle, W. J., Simonet, W. S. and Lacey, D. L. 2003, Nature, 423, 337.
6. Blackwell, K. A., Raisz, L. G. and Pilbeam, C. C. 2010, Trends Endocrinol. Metab., 21, 294.
7. Miyaura, C., Inada, M., Suzawa, T., Sugimoto, Y., Ushikubi, F., Ichikawa, A., Narumiya, S. and Suda, T. 2000, J. Biol. Chem., 275, 19819.
8. Inada, M., Matsumoto, C., Uematsu, S., Akira, S. and Miyaura, C. 2006, J. Immunol., 177, 1879.
9. Lerner, U. H. 2006, J. Dent. Res., 85, 596.
10. Kawai, T. and Akira, S. 2010, Nat. Immunol., 11, 373.
11. Tominari, T., Hirata, M., Matsumoto, C., Inada, M. and Miyaura, C. 2012, J. Pharmacol. Sci., 119, 390.
12. Tominari, T., Matsumoto, C., Watanabe, K., Hirata, M., Grundler, F. M., Miyaura, C. and Inada, M. 2015, FEBS Open Bio., 5, 522.