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RESEARCH ARTICLE

Anti-Oral Microbial Activity and Anti-Inflammatory Effects of Rosmarinic Acid in Lipopolysaccharide-Stimulated MC3T3-E1 Osteoblastic Cells on a Titanium Surface

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Background: The purpose of this study was to investigate the anti-oral microbial activity and anti-inflammatory effects of rosmarinic acid (RA) in lipopolysaccharide (LPS)-stimulated MC3T3-E1 osteoblastic cells on a titanium (Ti) surface during osseointegration, and to confirm the possibility of using RA as a safe natural substance for the control of peri-implantitis (PI) in Ti-based dental implants.

Methods: A disk diffusion test was conducted to confirm the antimicrobial activity of RA against oral microorganisms. In order to confirm the anti-inflammatory effects of RA, inflammatory conditions were induced with 100 ng/ml of LPS in MC3T3-E1 osteoblastic cells on the Ti surface treated with or without 14 μg/ml of RA. The production of nitric oxide (NO) and prostaglandin E2 (PGE_2) in LPS-stimulated MC3T3-E1 osteoblastic cells on the Ti surface was confirmed using an NO assay kit and PGE_2 enzyme-linked immunosorbent assay kit. Reverse transcription polymerase chain reaction and western blot analysis were performed to confirm the expression of interleukin (IL)-1β and tumor necrosis factor (TNF)-α in total RNA and protein.

Results: RA showed weak antimicrobial effects against *Streptococcus mutans* and *Escherichia coli*, but no antimicrobial activity against the bacteria *Aggregatibacter actinomycetemcomitans* and the fungus *Candida albicans*. RA reduced the production of pro-inflammatory mediators, NO and PGE_2, and proinflammatory cytokines, TNF-α and IL-1β, in LPS-stimulated MC3T3-E1 osteoblastic cells on the Ti surface at the protein and mRNA levels.

Conclusion: RA not only has anti-oral microbial activity, but also anti-inflammatory effects in LPS-stimulated MC3T3-E1 osteoblasts on the Ti surface, therefore, it can be used as a safe functional substance derived from plants for the prevention and control of PI for successful Ti-based implants.

Key Words: Anti-inflammatory effect, Anti-microbial activity, MC3T3-E1 osteoblastic cell, Rosmarinic acid, Titanium

Introduction

Titanium (Ti) and some of its alloys are strong and have high biocompatibility, thus, they are widely used in dentistry, in dental implants[1)]. Although the success rate of dental implants is high, failure may occur due to the cytotoxicity of ions from implant materials, periodontitis and peri-implantitis (PI) caused by oral microorganisms, local bone volume reduction, and delayed wound healing[1,2)]. Various studies have indicated that in successful dental implants there is an increase in the osseointegration between Ti and living bone tissue through adhesion, differentiation, and mineralization of osteoblasts on the Ti surface, and a reduction the differentiation and activity of osteoclasts. These studies have also examined fluoride treatment[3)], biological material coatings, such as transforming

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growth factor-β1 and type I collagen[1], and the formation of nanoscale roughness on the Ti surface[4]. However, for a successful dental implant, it is necessary to not only control and alleviate the inflammatory reaction around the implant also control the oral microorganisms that cause inflammation. A variety of microorganisms inhibits a dental plaque, and many bacterial species and fungi that cause dental caries and periodontal diseases have been isolated from it[5]. Colonized periodontal disease-causing bacteria such as *Prevotella intermedia*, *Porphylomonas gingivalis*, and *Aggregatibacter actinomycetemcomitans* which can cause PI, depending on the immunity of the host[5,6], Have been observed in a healthy peri-implant sulcus. When dental implants are present in the oral cavity, there is a risk of infection from these bacteria, leading to a failure of the dental implant[6]. Peri-implant mucositis (PIM) and PI frequently occur on the surface and areas surrounding the dental implants[7]. PIM appears only in gingival tissues around dental implants, whereas PI is characterized by periodontal tissue destruction and alveolar bone resorption[7]. PI is an inflammatory disease caused by infections induced by an imbalance between the host's immune response and an increase in pathogenic periodontal disease-causing bacteria after the osseointegration of dental implants[5,7,8]. *Streptococcus* spp., *Aggregatibacter* spp., *Actinomyces* spp., and *Fusobacterium* spp. Have been isolated at sites where PI occurs[9,10]. Many clinical studies have also reported that PI is one of the major causes of implant loss[7,11].

Plants have long been used in traditional medicines for the control of various diseases and the development of new drugs. Plant-derived therapeutic substances have been used for the prevention and treatment of diseases because they have fewer side effects and various biological activities[12]. Rosmarinic acid (RA, α-o-caffeoyl-3,4-dihydroxyphenyl-lactic acid) is a natural polyphenolic compound that is extracted from *Perilla frutescens* which is widely cultivated for food in Korea, and Lamiaceae herbs, such as sage, basil, and mint[13]. RA j=has been shown to not only have anti-inflammatory activity through the inhibition of nuclear factor kappa B (NF-κB) production in various cells[13], but also anti-oxidative, anti-inflammatory, anti-mutagen, anti-bacterial and anti-viral activities[14]. On the other hand, unlike other polyphenolic compounds that show weak antimicrobial activity against oral bacteria, RA has been reported to have no microbial activity in oral microorganism[8]. The various studies on the antimicrobial activities of RA against oral micro-organisms causing oral diseases, including PI, are in-sufficient, and there are no studies on the anti-inflammatory effect of RA in the lipopolysaccharide (LPS)-stimulated osteoblasts on the Ti surface to enable the elucidation of its potential for the regulation of PI.

The purpose of this study was to investigate the antimicrobial activity of RA against oral microorganisms and the anti-inflammatory effect of RA on LPS-stimulated MC3T3-E1 osteoblastic cells on the Ti surface during osseointegration, and to confirm the possibility of using RA as a safe natural substance for the control of PI in Ti-based dental implants.

Materials and Methods

1. Microbial strains

Microbial strains to confirm the antimicrobial activity of RA against oral microorganisms were purchased from the Korea Microbial Conservation Center (KCCM) and the Gene Bank (KCTC) and used in the experiment (Table 1). *S. mutans* was cultured in Brain Heart Infusion (MB cell Ltd., Seoul, Korea) agar and broth, *A. actinomycetemcomitans* in MRS (MB cell Ltd.) agar and broth, and *E. coli* in Luria Bertani (MB cell Ltd.) agar and

Table 1. Microbial Strains for the Disk Diffusion Test

Microorganism	Strain	Aero condition	Kind
Streptococcus mutans	KCCM 40105	Facultative anaerobic	Bacteria
Aggregatibacter actinomycetemcomitans	KCTC 2581	Microaerophilic	Bacteria
Escherichia coli	KCTC 1039	Aerobic	Bacteria
Candida albicans	KCCM 11282	Aerobic	Fungus

broth. The fungus, *C. albicans* was cultured in potato dextrose agar (MB cell Ltd.) and broth (MB cell Ltd.) and used in the experiment.

2. Disk diffusion test

According to the method standardized by Bauer et al.[15], microbial strains were incubated from colonies for 24 hours, diluted with sterilized saline solution to 5×10^6 CFU/ml, and then coated with 100 μl on agar medium prepared in a petri dish. Distilled water and 20 μl of RA of each concentration were absorbed onto sterilized paper discs (ϕ6 mm; Advantec Toyo Kaisha Ltd., Tokyo, Japan), and the dried paper discs were placed on agar plates coated with microbial strains, and incubated for 24 hours in an incubator at 36.5°C, the clear zones were then measured. Ampicillin (10 IU; Oxoid Ltd., Hampshire, United Kingdom) and penicillin G (10 μg; Oxoid Ltd.) antibiotic discs were used as positive controls for RA.

3. Cell culture and Ti disc

The MC3T3 E1 osteoblastic cell line derived from mouse calvaria was maintained in Alpha-modified Eagle's medium (α-MEM) containing 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) antibiotic antimycotic solution (WelGENE Inc., Daegu, Korea). The cells were transferred onto the Ti disc surface and replaced with α-MEM medium containing 5% (v/v) FBS, 10 mM β-glycerol phosphate, and 50 μg/ml ascorbic acid, and cultured in a CO2 incubator at 37°C. According to the method of Jeong et al.[16], polished pure Ti discs with diameters of 15, 20, and 48 mm of 2 mm were used in the experiment.

4. LPS and RA treatment

The medium for cells on the Ti disc was replaced with fresh medium with or without 14 μg/ml of RA before LPS treatment. After 1 hour, 100 ng/ml of LPS (E. coli serotype 055:B5; cat. No. L2880; Sigma-Aldrich, Chemical Co., St. Louis, MO, USA) was added to the medium. Cells treated with LPS or LPS/RA were cultured according to the set time for the experiment and used in the experiment, and the control was maintained under the same culture conditions.

5. Measurement of NO and PGE_2

NO was extracted by processing according to the manufacturer's method using a commercial NO assay kit (R&D Systems, Mineapolis, MN, USA), and measured at 540 nm absorbance using an enzyme-linked immunosorbent assay (ELISA) reader (Molecular Devices; Sunnyvale, CA, USA). PGE_2 concentration was measured with an ELISA reader at 490 nm absorbance after treatment according to the manufacturer's method using a PGE_2 ELISA kit (R&D Systems).

6. RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted by processing according to the manufacturer's method using RiboEXTM reagent (GeneAll, Seoul, Korea). Complementary DNA (cDNA) was synthesized using 1 μg of isolated total RNA using RT Premix (GeNet Bio, Daejeon, Korea). PCR was performed using a thermocycler (Takara Bio Inc., Shiga, Japan) after adding 1 μl of cDNA and the gene-specific primers to the PCR premix (GeneAll) to amplify tumor necrosis factor (TNF-α) and interleukin (IL)-1β genes from cDNA. The PCR products were electrophoresed on 1.5% agarose gel (Takara Bio Inc.) buffered with 0.5×Tris-borate-ethylenediaminetetraacetic acid, stained with ethidium bromide (Sigma-Aldrich), and then visualized with a Gel-Doc System (BioRad Laboratories, Inc., Hercules, CA, USA). The intensity of the band was measured using a Science Lab Image Gauge (FUJI FILM, Tokyo, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control. The PCR primers were as follows: TNF-α forward, 5'-TCT CAT CAG TTC TAT GGC CC-3' and reverse, 5-GGG AGT AGA CAA GGT ACA AC-3'; IL-1β forward: 5'-TCT GTG ACT CGT GGG ATG AT-3' and reverse, 5'-TGT CGT TGC TTG TCT CTC TCC T-3'; GAPDH forward, 5'-CCA TGG AGA AGG CTG GG-3 and reverse: 5'-CAA AGT TGT CAT GGA TGA CC-3' (Bioneer Corp., Ltd., Daejeon, Korea) The annealing temperature for each primer and number of cycles were as follows: TNF-α, 58°C and 35 cycles; IL-1β, 59°C and 36 cycles; and GAPDH, 60°C and 30 cycles.

7. Protein extraction and western blot analysis

Total protein was extracted from MC3T3-E1 cells using an NP-40 lysis buffer, and protein concentration was determined using the Bradford Protein assay kit (Bio-Rad Laboratories, Inc.). The protein samples (30 μg/lane) were electrophoresed on 10% SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride membranes (Merck Millipore, Darmstadt, Germany). The membranes were blotted with primary antibodies at 4°C overnight, i.e., 1:1,000 of anti-rabbit TNF-α (Abcam, Inc., Cambridge, MA, USA), IL-1β antibody (Abcam, Inc.), and 1:2,500 anti-mouse β-actin antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). After washing, the membrane was incubated with 1:5,000 horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit or mouse-IgG, Santa Cruz Biotechnology Inc.) for 1 hour. The development was performed using an X-ray film (FUJI FILM, Tokyo, Japan) after detection using an ECL solution (Merck Millipore). The intensity of bands was measured using a Science Lab Image Gauge (FUJI FILM, Tokyo, Japan). β-Actin was used as a control.

8. Statistical analysis

All the experiments were carried out in triplicate. All the data were expressed as means±standard deviations. The statistical analysis was performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Statistically significant differences were determined using the Student's t-test. The significance level for determining statistical significance was set at 0.05.

Results

1. Antimicrobial activity of RA against oral microorganisms

The results of the disk diffusion test to confirm the antimicrobial activity of RA against oral microorganisms are presented in Table 2 and illustrated in Fig. 1. RA showed a weak antimicrobial effect on *S. mutans* and *E. coli*, and the antimicrobial activity of RA increased with increasing concentration. RA did not show any antibacterial activity against the bacterium *A. actinomycetemcomitans* and the fungus *C. albicans*. These results show that RA has the potential to control some oral bacteria.

Table 2. Anti-Microbial Activity of Rosmarinic Acid (RA) and Antibiotics by the Disk Diffusion Test

Microorganism	RA (μg)						Ampicillin (10 IU)	Penicillin G (10 mcg)
	0	18	36	54	72	90		
Streptococcus mutans	-	-	-	-	+	+	+++	++
Aggregatibacter actinomycetemcomitans	-	-	-	-	-	-	++	++
Escherichia coli	-	-	-	-	-	+	+	+
Candida albicans	-	-	-	-	-	-	-	-

-: resistrant (＜5 mm), +: susceptible (5～14 mm), ++: more susceptible (15～24 mm), +++: most susceptible (＞25 mm).

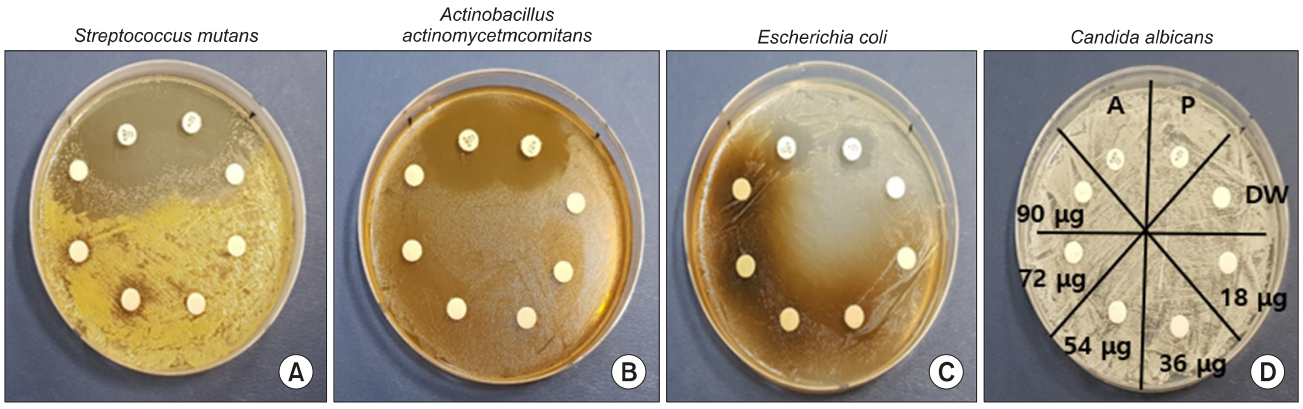

Fig. 1. Anti-microbial activity of rosmarinic acid against oral microorganisms. A: ampicillin, P: penicillin G, DW: distilled water.

2. Effect of RA on LPS-induced production of pro-inflammatory mediators in MC3T3-E1 osteoblastic cells on the Ti disc surface

The amount of NO production was compared between the group treated with LPS alone (LPS/MC3T3-E1) and the group treated with LPS and RA (LPS/RA/MC3T3-E1). The amount of NO produced in LPS/RA/MC3T3-E1 at all time points was decreased compared to that in LPS/MC3T3-E1, and the amount of NO at 12 and 24 hours decreased significantly (Fig. 2A). The amount of PGE_2 produced was significantly lower in LPS/RA/MC3T3-E1 than in LPS/MC3T3-E1, except at 2 hours (Fig. 2B). In

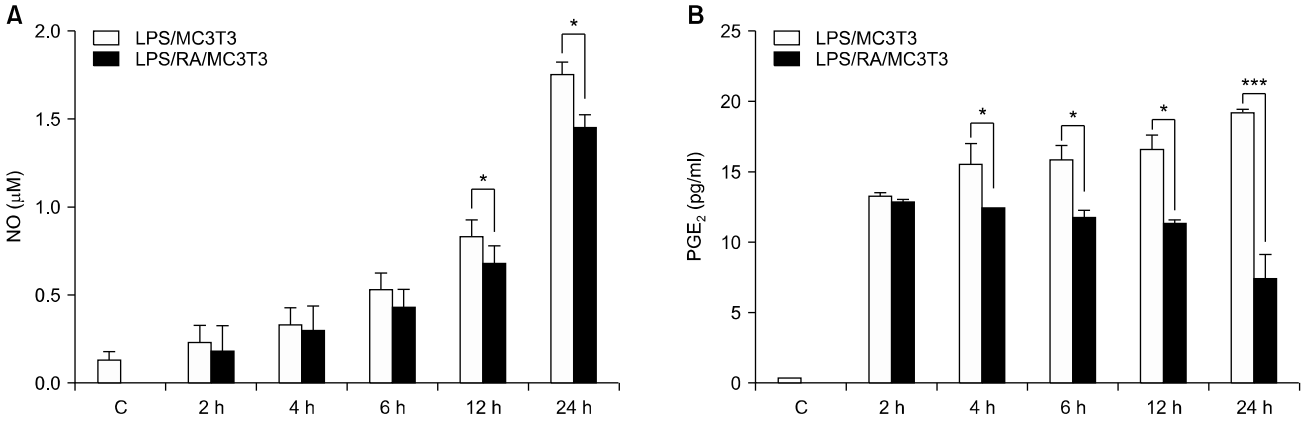

Fig. 2. Effects of rosmarinic acid (RA) on production of pro-inflammatory mediators in MC3T3-E1 osteoblastic cells on a titanium (Ti) surface. RA significantly reduced production of NO (A) and PGE_2 (B) in LPS-stimulated MC3T3-E1 osteoblastic cells on a Ti surface (*$p<0.05$; ***$p<0.001$). NO: nitric oxide, LPS: lipopolysaccharide, C: control.

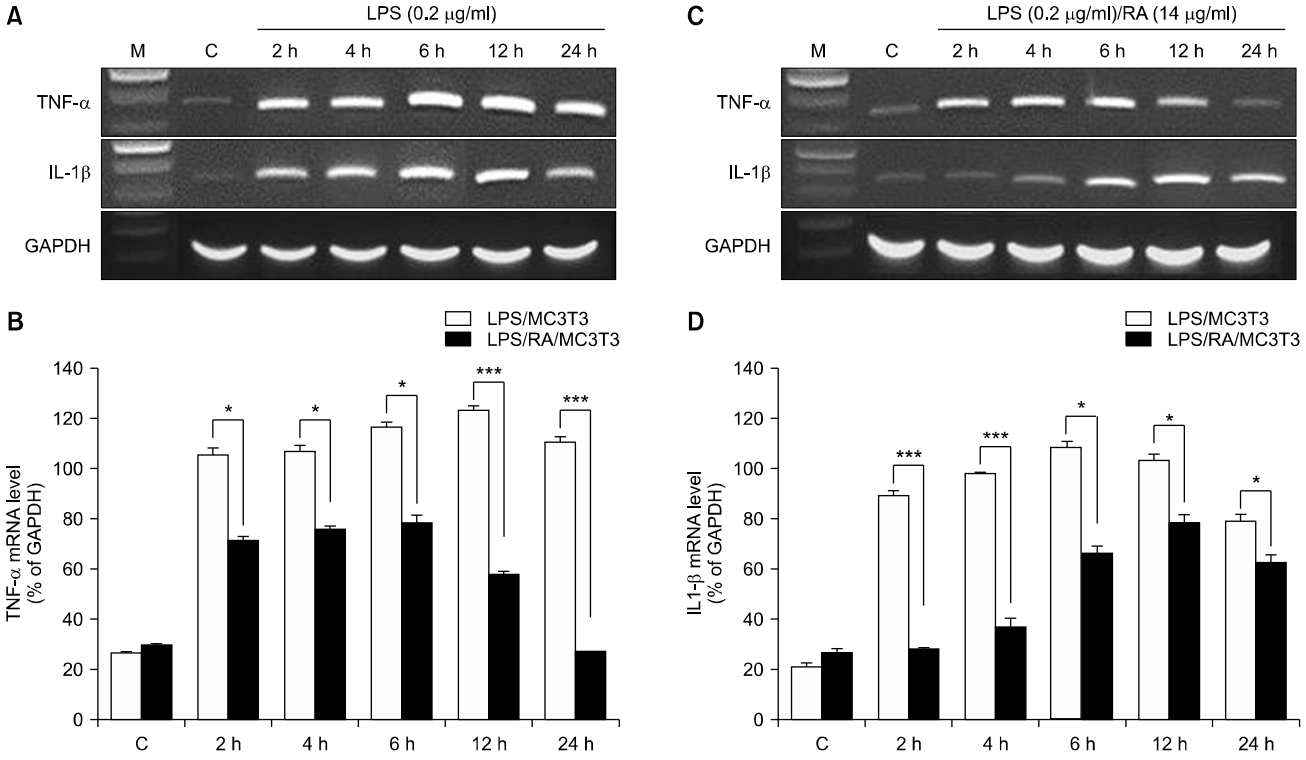

Fig. 3. Effects of rosmarinic acid (RA) on TNF-α and IL1-β mRNA expression in MC3T3-E1 osteoblastic cells on a titanium (Ti) surface. RA significantly reduced the mRNA expression of TNF-α (A, B) and IL1-β (C, D) in lipopolysaccharide (LPS)-stimulated MC3T3-E1 osteoblastic cells on a Ti surface (*$p<0.05$; ***$p<0.001$). M: maker, C: control.

particular, the production of PGE_2 at 24 hours in LPS/RA/MC3T3-E1 showed a large decrease of 2.6 times compared to that of LPS/MC3T3-E1. Therefore, RA significantly reduced the production of NO and PGE_2, a pro-inflammatory mediator, in LPS-stimulated MC3T3-E1 osteoblastic cells on the Ti surface, and this result shows that RA has the potential to control inflammatory conditions.

3. Effect of RA on LPS-induced productions of pro-inflammatory cytokines in MC3T3-E1 osteoblastic cells on Ti disc surface

The mRNA and protein expression of TNF-α and IL1-β in LPS/MC3T3-E1 and LPS/RA/MC3T3-E1 on the Ti surface are shown in Fig. 3 and 4. The mRNA expression of TNF-α on the Ti surface was significantly decreased in LPS/RA/MC3T3-E1 at all time points than in LPS/MC3T3-E1, and decreased by 2 and 4 times at 12 and 24 hours, respectively (Fig. 3A, 3B). The protein expression of TNF-α was also significantly decreased at all time points of LPS/RA/MC3T3-E1 than in LPS/MC3T3-E1 cells (Fig. 4A, 4B). In comparison with LPS/MC3T3-E1 cells, the IL1-β mRNA expression of LPS/RA/MC3T3-E1 cells was significantly decreased at all time points, and 3.2 and 2.7 times decreased at 2 and 4 hours, respectively (Fig. 3C, 3D). IL1-β protein expression was also significantly decreased in LPS/RA/MC3T3-E1 cells (Fig. 4C, 4D). From the above results, in LPS-stimulated MC3T3-E1 osteoblastic cells on the Ti surface, RA significantly reduced the mRNA and protein expression of TNF-α and IL1-β, pro-inflammatory cytokines that play an important role in the initial inflammatory response. This indicates that RA is effective in relieving LPS-induced inflammation.

Discussion

Periodontal disease is an inflammatory disease caused by oral microbial infection, which induces destruction of periodontal tissue and alveolar bone, resulting in tooth loss[9]. PI that occurs after dental implant treatment is also an inflammatory disease caused by infection with oral

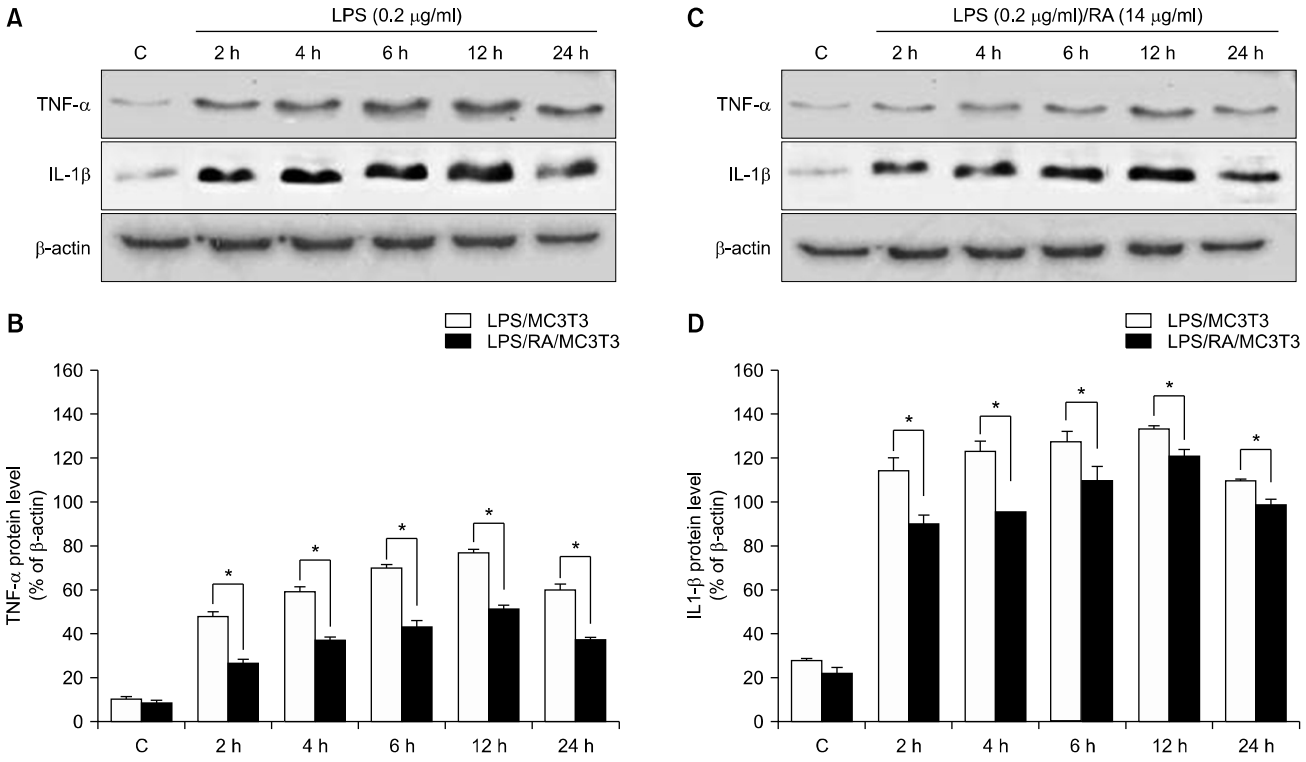

Fig. 4. Effects of rosmarinic acid (RA) on TNF-α and IL1-β protein expression in MC3T3-E1 osteoblastic cells on a titanium (Ti) surface. RA significantly reduced the protein expression of TNF-α (A, B) and IL1-β (C, D) in lipopolysaccharide (LPS)-stimulated MC3T3-E1 osteoblastic cells on a Ti surface (*$p<0.05$).

bacteria, initiated by an imbalance between an increase in oral pathogenic bacteria and the host's response[6-8], and is characterized by the destruction of periodontal tissue and alveolar bone resorption[7]. Pain and loss of physical function due to PI eventually induces the removal of the implant, leading to implant failure[7,11]. LPS, which is present in the outer membrane of gram-negative bacteria, is a pathogenic endotoxin that induces periodontal tissue destruction and bone resorption, and is commonly used to induce inflammatory conditions to evaluate the effects of drugs[17]. *S. mutans* causes dental caries by synthesizing glucan from sucrose by producing glucosyltransferrase[18,19]. *E. coli* is known to account for 15% of the oral flora, is a gram-negative bacillus that lives in the intestine and is an important contamination indicator for hygiene[20]. *E. coli* is a major causative bacterium of hospital infection and causes gastrointestinal diseases showing symptoms of food poisoning and diarrhea[21,22]. Most of them live transiently in the oral cavity, but their presence increases with age and affects saliva secretion, leading to dry mouth[20]. *A. actinomycetemcomitans* causes aggressive periodontitis and is a highly permeable bacterium that can penetrate the epithelial and vascular endothelial cells[23]. Their strong penetratve ability can act as an important virulence factor in the spread of bacteria to periodontal tissues and induce inflammation, as well as have a relationship with alveolar bone absorption[23,24]. *C. albicans*, a representative fungus living in the oral cavity, is generally non-pathogenic, but attaches to the oral cavity and dentures of patients with weakened immunity or poor oral hygiene, causing oral candidiasis or opportunistic infections[25,26]. In previous studies, RA did not show antibacterial activity against oral pathogenic bacteria[8], however, in this study, RA showed a weak anti-bacterial effect on some of the oral bacteria, but not on the fungus, *C. albicans* (Table 2, Fig. 1). These results were similar as those for other polyphenolic compounds[8]. Therefore, RA has a weak antibacterial effect on some of the bacteria living in the oral cavity and has the potential to be utilized as a safe substance derived from natural products for the prevention and control of periodontitis and PI.

The transcription factor, NF-κB is translocated to the nucleus by inflammatory stimuli such as LPS, and regulates DNA transcription and production of various pro-inflammatory genes such as inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2), IL-1β, TNF-α[27,28]. iNOS and COX-2 are responsible for the production of NO and PGE_2, respectively. Bone cells stimulated by LPS increase iNOS gene expression and induce the release of overproduced NO[27]. NO not only inhibits osteoblast growth and increases apoptosis, but also regulates osteoclast activity and recruitment[27]. PGE_2, catalyzed by COX-2 during the inflammatory reaction, causes a decrease in the bone alkaline phosphatase activity and induces osteoclast differentiation in the stem cells[17,27]. TNF-α and IL-1 are both osteoclastogenic factors and bone resorption factors[29]. TNF-α and IL-1β, produced through NF-κB signaling inhibit osteoblastic bone formation and induce an increase in the expression of the receptor activator of NF-κB ligand (RANKL), and the secreted RANKL induces the formation of osteoclasts and plays an important role in the initiation and acceleration of alveolar bone resorption and periodontal disease[23,24]. This means that the control of pro-inflammatory mediators and cytokines can also control osteoclast formation and bone resorption. This study showed that RA reduced the

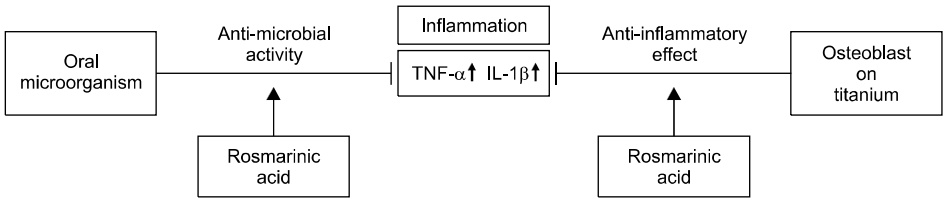

Fig. 5. Schematic diagram of anti-oral microbial activity and anti-inflammatory effects of rosmarinic acid in MC3T3-E1 osteoblastic cells on a titanium (Ti) surface.

production of pro-inflammatory mediators, NO and PGE_2 and pro-inflammatory cytokines, TNF-α and IL-1β in LPS-stimulated MC3T3-E1 osteoblastic cells on the Ti surface at the protein and mRNA levels (Fig. 2～4). This implies that RA plays a role at the transcriptional level.

Therefore, RA not only has anti-oral microbial activity, but also anti-inflammatory effects in LPS-stimulated MC3T3-E1 osteoblasts (Fig. 5). It can be used as a safe functional substance derived from natural products for the prevention and control of PI for successful Ti-based implants.

Notes

Conflict of interest

No potential conflict of interest relevant to this article was reported.

Ethical approval

This article is not necessary for IRB screening.

Author contributions

Conceptualization: Moon-Jin Jeong, Soon-Jeong Jeong. Data acquisition: Do-Seon Lim, Kyungwon Heo. Formal analysis: Do-Seon Lim, Kyungwon Heo. Funding: Soon-Jeong Jeong. Supervision: Soon-Jeong Jeong. Writing-original draft: Soon-Jeong Jeong. Writing-review & editing: Moon-Jin Jeong, Soon-Jeong Jeong.

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