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COMP-Ang1 prevents periodontitic damages and enhances mandible bone growth in an experimental animal model

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ABSTRACT

COMP-Ang1, a chimera of angiopoietin-1 (Ang1) and a short coiled-coil domain of cartilage oligomeric matrix protein (COMP), is under consideration as a therapeutic agent enhancing tissue regeneration with increased angiogenesis. However, the effect of COMP-Ang1 on periodontitic tissue damages and the related mechanisms are not yet investigated. We initially explored whether a local delivery of COMP-Ang1 protects lipopolysaccharide (LPS)/ligature-induced periodontal destruction in rats. As the results, μCT and histological analyses revealed that COMP-Ang1 inhibits LPS-mediated degradation of periodontium. COMP-Ang1 also suppressed osteoclast number and the expression of osteoclast-specific and inflammation-related molecules in the inflamed region of periodontitis rats. Implanting a COMP-Ang1-impregnated scaffold into critical-sized mandible bone defects enhanced the amount of bone in the defects with increased expression of bone-specific markers. The addition of COMP-Ang1 prevented significantly osteoclast differentiation and activation in LPS-stimulated RAW264.7 macrophages and inhibited the phosphorylation of c-Jun, mitogen-activated protein kinases, and cAMP response element-binding protein in the cells. On contrary, COMP-Ang1 increased the level of phosphatidylinositol 3-kinase (PI3K) in LPS-exposed macrophages and a pharmacological PI3K inhibitor diminished the anti-osteoclastogenic effect of COMP-Ang1. Similarly, COMP-Ang1 blocked the expression of inflammation-related molecules in LPS-stimulated human periodontal ligament fibroblasts (hPLFs). Further, the COMP-Ang1 enhanced differentiation of hPLFs into osteoblasts by stimulating the expression of bone-specific markers, Tie2, and activator protein-1 subfamily. Collectively, our findings may support the therapeutic potentials of COMP-Ang1 in preventing inflammatory periodontal damages and in stimulating new bone growth.

1. Introduction

Periodontitis is an immune-inflammatory disorder that leads to the destruction of connective tissues such as the subgingivae, periodontal ligament (PDL), and alveolar bone, eventually causing tooth loss [1]. Massive bone defects referred as critical size defects occurred from severe trauma or persistent periodontitis and these often requires external treatment for reconstruction. Thus, multiple invasive surgeries and bone grafting are essential for patients with trauma-related injury and congenital or malignant disease [2]. Various therapies such as mechanical and chemical root conditioning, implantation of autografts, allografts, and alloplastic materials, guided tissue regeneration, and various combinations of these approaches have been used in clinical practice with the aim of accomplishing periodontal regeneration [3,4]. However, the clinical resultants vary from the cases applied and are often unpredictable. In order to improve the healing processes in reconstructive surgical treatment, many researchers have focused their efforts on the development of biomaterials capable of stimulating reconstruction of large bone defects [5–8]. To date, combined treatment with biomaterials and growth factors has been used to synergistically stimulate the processes involved in bone healing [9–11].

Interest has been currently focused on the combined application of recombinant protein with a barrier in the development of regenerative medicine [12]. Angiopoietin (Ang)-Tie plays an important role in regulation of angiogenesis and vascular homeostasis. Ang1 has been described as a secreted protein ligand of tyrosine kinase with Ig and epidermal growth factor homology domain 2 (Tie2) [13]. However, Ang1 tends to aggregate and is in insoluble form, which limits its output

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in clinical application. To get over these disadvantages, a chimera of Ang1 with a short coiled-coil domain of cartilage oligomeric matrix protein (COMP-Ang1) was developed. The N-terminal portion of COMP-Ang1 is substituted with a non-collagenous extracellular matrix protein comprised of five identical glycoprotein subunits [14]. COMP-Ang1 showed soluble, stable, and potent Tie2 phosphorylation more than did native Ang1 [14]. COMP-Ang1 also revealed multiple potentials to promote angiogenesis and to induce osteogenesis in rat model of spinal fusions [15], to stimulate wound recovery in diabetic mice [16], and to enhance osteoblast differentiation and bone formation during distraction osteogenesis [17,18]. In addition, COMP-Ang1 inhibited lipopolysaccharide (LPS)-induced acute kidney injury by inhibiting the induction of inflammatory molecules [19]. We recently found that a local delivery of COMP-Ang1 accelerates new bone formation in rat calvarial defects by increasing the expression of angiogenic and osteogenic factors [20]. Our findings with other reports suggest that COMP-Ang1 exerts its biological potentials by stimulating angiogenesis and osteogenesis as well as by inhibiting inflammatory responses. This leads us to hypothesize that COMP-Ang1 could be clinically used to prevent inflammatory periodontal destruction, as well as to improve bone healing. However, the therapeutic potential of COMP-Ang1 on periodontitis-mediated tissue damages and mandible bone defect has not been investigated.

In this study, we explored whether a local treatment of COMP-Ang1 prevents LPS/ligature-induced degradation of periodontal tissues in rats. We also examined whether a local delivery of the COMP-Ang1 using a barrier enhances bone regeneration using a rat model of critical-sized mandible defects. In addition, we investigated the regulatory effects of COMP-Ang1 on LPS-stimulated osteoclast formation and inflammatory responses, as well as on osteoblast differentiation and mineralization by using RAW 264.7 macrophages and human PDL fibroblasts (hPLFs).

2. Materials and methods

2.1. Chemicals and laboratory wares

Carrier-free recombinant human COMP-Ang1 was obtained from Enzo Life Science Inc. (Farmingdale, NY, USA) and fetal bovine serum (FBS) was from HyClone Laboratories (Logan, UT, USA). LPSs produced from *Porphyromonas gingivalis* (*P. gingivalis*) and *E. coli* were purchased from InvivoGen (San Diego, CA, USA) and Sigma-Aldrich Co. LLC (St. Louis, MO, USA), respectively. Primary antibodies specific for cyclooxygenase-2 (COX-2, BS1076), matrix metalloproteinase 9 (MMP-9, BS6893), tumor necrosis factor α (TNF-α, BS1857), and osteocalcin (OCN, BS7961) were purchased from Bioworld Technology (St. Louis Park, MN, USA). Cathepsin-K (sc-48353), interleukin 8 (IL-8, sc-7922), intercellular adhesion molecule 1 (ICAM-1, sc-8439), p-c-Jun (sc-822), c-Jun (sc-1694), c-Fos (sc-52), JunD (sc-74), fos-related antigen 1 (Fra1, sc-183), phosphoinositide 3-kinase (PI3K, sc-7189), and β-actin (sc-47778) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), while osteopontin (OPN, ab8448) was from Abcam (Cambridge, UK). cAMP response element-binding protein (CREB) was obtained from Cell Signaling Technology (Danvers, MA, USA). Unless otherwise specified, other chemicals and laboratory wares were obtained from Sigma-Aldrich Co. LLC (St. Louis, MO, USA) and Falcon Labware (Becton–Dickinson, Franklin Lakes, NJ, USA), respectively.

2.2. Animals and ethics statement

Male Sprague-Dawley rats (7 weeks old) were supplied by Orient Bio (Daejeon, South Korea) and assigned randomly to each experimental group, where the mean body weights among the groups were not differed. The rats were equilibrated for seven days prior to periodontitis induction or surgical operation. Animals were housed at 22 ± 1 °C and 55 ± 5% humidity on an auto-cycling 12 h light/dark cycle with free access to food and water. This study was carried out in strict accordance with the recommendations in the Guide for the Animal Care and Use of the Chonbuk National University. The protocol was approved by the University Committee on Ethics in the Care and Use of Laboratory Animals (CBU 2014-00055). The consumption of food and water and behavior of the animals were monitored every 12 h per day during the experimental periods.

2.3. Preparation of absorbable collagen sponge (ACS)

This study used an ACS as a carrier of COMP-Ang1 for the mandibular defect model, because of its general use in a bone defect model. We prepared the ACS according to methods described previously [21]. Briefly, type I atelocollagen powder (KOKEN Corp., Osaka, Japan) was dissolved in 50 mM acetic acid at a concentration of 10 mg/ml and coprecipitated with chondroitin-6-sulfate. The resulting collagen-chondroitin-6-sulfate solution was lyophilized to yield an ACS. ACSs were incubated in 20 ml of 40% (*v*/v) ethanol containing 50 mM 2-morpholineoethane sulfonic acid (MES; Fluka Chemie, Buchs, Switzerland) (pH 5.5) for 30 min and then immersed in 20 ml of 40% (v/v) ethanol containing 50 mM MES (pH 5.5), 24 mM 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide, and 5 mM N-hydroxysuccinimide for 12 h. After serial washing with 0.1 M Na_2HPO_4 (pH 9.0), 1 M NaCl, and 2 M NaCl, ACSs were rinsed with distilled water, lyophilized, and sterilized with 10 kGy of γ-irradiation.

2.4. In vivo experimental designs for ligature/LPS-induced periodontitis and mandible defect

Ligature-induced periodontitis model was established according to the methods described in our previous report [22]. Briefly, rats were randomly divided into three groups ($n = 5$/group), including sham (no periodontitis), LPS (ligature + LPS), and COMP-Ang1 (ligature + LPS + COMP-Ang1) groups. Based on our previous finding of BV/TV in LPS rats of 45 ± 3 (Mean ± SD) and a desire to find a 20% greater value for BV/TV as significant ($\alpha = 0.05$; $\beta = 0.8$) (54 ± 4), a standard power calculation showed that a group size of 4 was sufficient [22]. General anesthesia was induced in all groups of rats with an intramuscular injection of Zoletil (0.4 ml/kg, Virbac Laboratories, Carros, France) mixed with Rompun (10 mg/kg, Bayer Korea Ltd., Seoul, South Korea). After injection, periodontitis was achieved by placing a cotton ligature (5-0) around the cervical position of the second right maxillary molars (M2). After induction of periodontitis, rats received immediately a local delivery of COMP-Ang1 solution (4.6 μl) at a concentration of 2.5 μg/kg body weight/site by injecting into the interdental spaces of distal and mesial sides of the second right maxillary molar, respectively. The injection of COMP-Ang1 was performed on day 0, 3, 6, 9, and 12 after location of ligature. The groups with LPS only or in a combination with COMP-Ang1 also received 2.6 μl of LPS (10 μg/kg/site) at the same times by injecting into the same location, whereas the sham group received the same injection protocol with phosphate buffered saline (PBS) only. All injections were carried out in anesthetic conditions using a 30-gauge needle attached to Hamilton syringe (Hamilton Company, Reno, NV, USA). During experiment periods, rats were monitored once per day and lost or loose ligatures were replaced to new one. After two weeks of periodontitis induction, rats were euthanized for further experimental analyses.

For construction of mandible defect model, right submandibular area of rats was shaved on an operating table after general anesthesia and disinfected using 10% betadine (Potadines, Sam Li Korea, Seoul, South Korea) before subcutaneous injection of 2% lidocaine containing 1:100,000 epinephrine (Lidocaine HCl Injs, Yuhan Corp., Seoul, South Korea). An incision of 1 cm was made through the skin, subcutaneous tissue, and masseter muscle perpendicular to the inferior border of the mandible. A defect in 4.0-mm external diameter in the right mandibular angle ~3 mm above the inferior border of the mandible was created