Effect of sonicates of *Treponema denticola* on osteoblast differentiation

Bong-Kyu Choi¹,³, Jung-Hwa Kang¹, Seung-Wook Jin¹, Seung-Ho Ohk¹,³, Syung-IL Lee¹,²,³, Yun-Jung Yoo¹,²

Department of Oral Biology¹ and Oral Science Research Center², College of Dentistry, Yonsei University, Brain Korea 21 Project for Medical Sciences³, Yonsei University

I. Introduction

Periodontitis is an inflammatory disease often accompanied by extensive alveolar bone loss, which surrounds the root of the teeth (Schwartz et al., 1997). The destruction of bone in this disease is considered to be a direct effect of pathogens or a result of enhanced osteoclastic resorption as a consequence of cytokine and prostaglandin production by the activated inflammatory cells. Various bacteria have been isolated from the subgingival pockets of periodontitis patients and spirochaetes are one of the most common bacteria in subgingival plaque (Loesche, 1988; Kigure et al., 1995; Simonson et al., 1988). Oral spirochetes fall into the genus Treponema and at least 25 species have been detected (Choi et al., 1994). However, only eight species have been cultured so far (Moter et al., 1998; Wyss et al., 1999; Dewirst et al., 2000). Among these, Treponema denticola has been most intensively studied (Fenno and McBride, 1998; Chan and McLaughlin, 2000). In this species, the pathogenic properties that have been identified include adhesion (Haapasalo et al., 1996), proteolytic enzyme production (Mäkinen et al., 1995), cytopathic activity (Greiner, 1991; Mathers et al., 1996; Fenno et al., 1998) and immunomodulation (Shenker et al., 1984; Ding et al., 1996).

Physiological bone remodeling is controlled by a balance between bone formation and resorption. This balance requires the coupled activities of both osteoblasts and osteoclasts and is controlled by a wide variety of systemic factors including hormones, steroids, and local factors such as prostaglandins, cytokines and growth factors (Nair et al., 1996; Hill, 1998). Although it was reported that LPS-like materials in the outer membrane of *T. denticola* may be responsible for bone resorption by measuring the release of Ca²⁺ from the shaft of the radii and ulnae.

Correspondence and reprints: Yun-Jung Yoo, DDS, Ph.D, Dept. of Oral Biology, College of Dentistry, Yonsei University 134 Shinchon-Dong, Seodaemun-Gu, Seoul, Korea, Tel: +82-2-361-8042, Fax: +82-2-364-1085, e-mail: yu618@yumc.yonsei.ac.kr

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of rats (Gopalsami et al., 1993) and that sonicates of *T. denticola* stimulate osteoclastogenesis via prostaglandin-dependent mechanism (Choi et al., 1999), its effects on osteoblast differentiation are not known. In this study, we investigated the effects of *T. denticola* sonicates on the differentiation of osteoblasts. Effect on osteoblast differentiation was estimated by alkaline phosphatase (ALPase) activity and formation of mineralized nodules in mouse calvarial cells.

**II. Materials and Methods**

1. **Materials**

   The mice (ICR strain) were obtained from Bio Korea Co, (Seoul, Korea), α-Minimum essential medium (α-MEM) and heat-inactivated fetal bovine serum (FBS) were purchased from GIBCO BRL (Grand Island, NY, USA), Ascorbic acid (AA), β-glycerophosphate (GP), indomethacin, and ALPase activity assay kit were obtained from Sigma (St, Louis, MO, USA).

2. **Preparation of sonicates**

   *T. denticola* (ATCC 33521) was cultured anaerobically in an OMIZ-PAT broth for 3-5 days, as described previously (Wyss et al., 1996). The bacterial cells were harvested by centrifugation at 5,000 × g for 10 min at 4°C and washed 3 times with a phosphate buffered saline (PBS). The bacterial cells were then disrupted for 5 min using an ultrasonic processor (Sonic Dismembrator, Fisher Scientific, Pittsburg, PA, USA) at an output power of 8 watts with 20 sec intervals. The sonicates were centrifuged at 15,000 × g for 5 min at 4°C and the supernatant was then collected. The protein concentrations were determined by a Comassie brilliant protein assay reagent (Pierce, IL, USA).

3. **Preparation of primary calvarial cells**

   The osteoblastic cells were isolated from the calvariae of 1-2-day-old ICR mice using a slight modification of the method described by Suda et al., (1997). The calvariae (10 - 20 of mice) were digested in 10 ml of α-MEM containing 0.2 % collagenase (Wako Pure Chemicals, Osaka, Japan) and 0.1% dispase (GIBCO BRL, Grand Island, NY, USA) for 20 min at 37°C with vigorous shaking, and then centrifuged at 1,500 × g for 5 min. The first supernatant was discarded and another 10ml of collagenase/dispase enzyme solution were added and incubated for 20 min. The digestion was repeated four times and the cells isolated by the last three digestions were combined as an osteoblastic cell population. They were cultured in an α-MEM containing 10% FBS, antibiotics and antimycotics solution (100 U/ml of penicillin, 100 μg/ml of streptomycin, 25 μg/ml of amphotericin B) and used for the assay of ALPase activity.

4. **ALPase activity assays**

   The isolated calvarial cells were seeded onto 24 - well plates (Nunc, Rochester, NY, USA) at an initial cell density of 8 × 10⁴ cells/well. When calvarial cells reached confluence, the cells were treated with the sonicates of *T. denticola* or a combination of sonicates and indomethacin in the presence of 10 mM GP and 50 μg/ml AA for 10 days. The medium was changed every 3 days. The cultured cells were detached from the plates using an enzyme solution containing 0.2% collagenase and 0.1% dispase, and washed twice with PBS. After adding triton X-100 (0.2%, Sigma, St, Louis, MO, USA), the cells were incubated on ice for 30 min to break the cell mem-