Effect of sonicates of *Treponema denticola* on osteoblast differentiation

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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 Ca^{2+} from the shaft of the radii and ulnae

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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℃ 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℃ 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 ㎖ 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℃ with vigorous shaking, and then centrifuged at 1,500 ×g for 5 min. The first supernatant was discarded and another 10㎖ 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 ㎍/ml of streptomycin, 25 ㎍/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 8×10^5 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 ㎍/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-

brane. The lysate was centrifuged at 5,000 ×g for 10 min and the supernatant was used for the ALpase assay. The ALpase activity in the lysate was measured by the hydrolysis of p-nitrophenyl phosphate to p-nitrophenol. An aliquot (20㎕) of the lysate was mixed with 1㎖ of an ALP-10 solution (Sigma, St. Louis, MO, USA) and incubated for 2 min at 30℃. The p-nitrophenol concentration of the mixture was determined by a spectrophotometer at a wavelength of 405 nm.

5. Von Kossa staining

The isolated calvarial cells were seeded onto 6 cm culture dish (Nunc, Rochester, NY, USA) at an initial cell density of 8×10^5 cells. When calvarial cells reached confluence, the cells were treated with 10 mM GP and 50 ㎍/ml AA in the absence or presence of *T. denticola* sonicates for 21 days. The medium was changed every 2 days. The formation of mineralized nodules in cultured cells was measured by Von Kossa staining as follows. The cells cultured for 21 days were fixed in 10% formaldehyde, stained with silver nitrate, treated with sodium carbonate formaldehyde and washed with distilled water. The dishes with mineralized nodules were photographed.

6. Measurements of PGE_2

The cells (8×10^5 cells/well) were inoculated onto 24-well plates. After the cells were grown to confluence, they were treated with either *T. denticola* sonicates solely or with a mixture of the sonicates and indomethacin in the presence of 10 mM GP and 50 ㎍/ml AA for 24 hrs. The medium was changed every 3 days. The amount of PGE_2 in the media and cells was assessed using commercially available PGE_2 immunoassay system (Amersham Pharmacia Biotech, Buckinghamshire, England).

7. Cytotoxicity assays

The calvarial cells were seeded onto 24-well plates at an initial cell density of 8×10^5 cells/well and cultured in α- MEM containing 10 % FBS for 3 days. After various concentrations of *T. denticola* sonicates were added to each well, the cells were cultured for an additional 10 days. The viability of the cells was determined by a MTT (3-[4,5-dimethylthiazol-2-yl]- 2,5-diphenyltetrazolium bromide; Sigma, St.Louis, MO, USA) assay. After 10 days of culture, 100 ㎕ of the MTT solution (5㎎/㎖) was added to each well and incubated for 4 h at 37 ℃. The MTT solutions in the wells were removed and 1 ml of dimethyl sulfoxide was added to dissolve the formazan crystals. The optical density of the formazan solution was measured at 570 nm.

8. Statistical analyses

Statistical differences were determined by the Mann-Whitney U test. A p value < 0.05 was considered significant.

III. Results

1. Effects of bacterial sonicates on ALPase activity and the formation of mineralized nodules in calvarial cells

The effects of *T. denticola* sonicates on osteoblast differentiation were estimated by measuring ALPase activity and mineralized nodule forming ability of calvarial cells treated with sonicates. The calvarial cells were cultured in media supplemented with either 0.25 or 2.5 ㎍/ml of the sonicates. Inhibition of ALPase activity and mineralized nodule formation

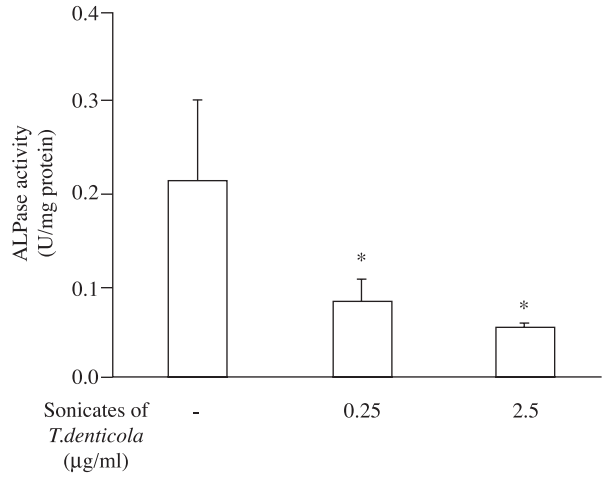

Figure 1. The inhibitory effects of *T. denticola* sonicates on osteoblast differentiation.

Confluent calvarial cells grown in 24-well plates were cultured in α-MEM supplemented with 10% FBS and GP/AA for 10 days in the presence or absence of sonicates (0.25 ㎍/ml, 2.5 ㎍/ml). ALPase activity was measured using p-nitrophenylphosphate as a substrate. Results are expressed as the means ± SE of 4 cultures. * $p<0.05$ significantly different from non-treated cells (A). Von Kossa staining of mouse calvarial cells treated with GP/AA for 21 days (B) in the absence (a) or presence (b) of T. denticola sonicates (2.5 ㎍/ml). Stained mineralized nodules were seen as small spots.

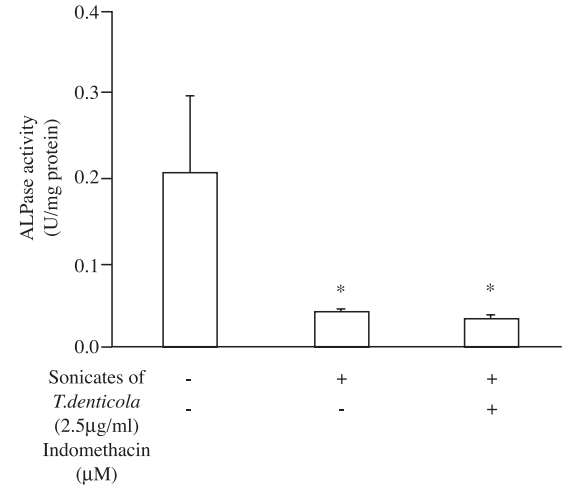

Figure 2. The inhibitory effects of heat-treated *T. denticola* sonicates on ALPase activity in mouse calvarial cells.

Confluent calvarial cells grown in 24-well plates were cultured in α-MEM supplemented with 10% FBS and GP/AA for 10 days in the presence of the sonicates (2.5 ㎍/ml), which were either not heated or heated at 80℃ for 30 min. The ALPase activity was measured using p-nitrophenylphosphate as a substrate. Results are expressed as the means ± SE of 4 cultures. ** $p<0.05$ significantly different from the non-treated cells.

was observed in the treated-cells when compared with the non-treated cells (Figure 1). ALPase activity was not altered when the calvarial cells were treated with the heat-denatured *T. denticola* sonicates (80 ℃, 30 min) (Figure 2).

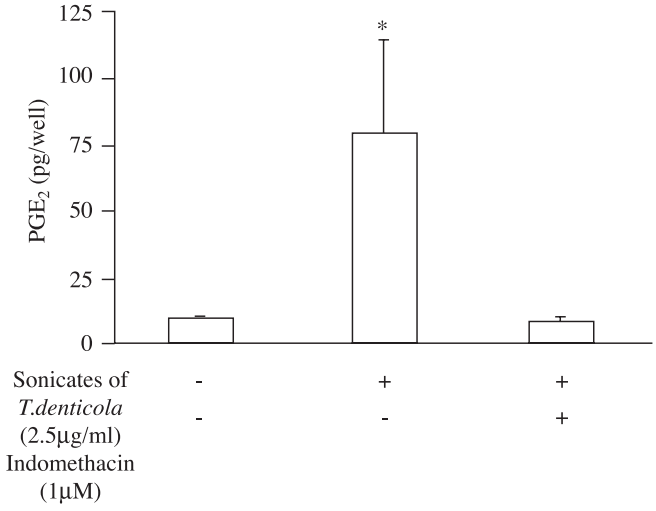

Figure 3. The effects of *T. denticola* sonicates on PGE_2 formation in mouse calvarial cells.

The cells at confluency were cultured in α-MEM containing 10% FBS and GP/AA or 10% FBS, GP/AA and bacterial sonicates in the presence or absence of indomethacin for 24 hrs. The PGE_2 concentration in the media and cells was analyzed using a PGE_2 enzymeimmunoassay system. Results are expressed as the means ± SE of 4 cultures. * $p<0.05$ significantly different from non-treated cells.

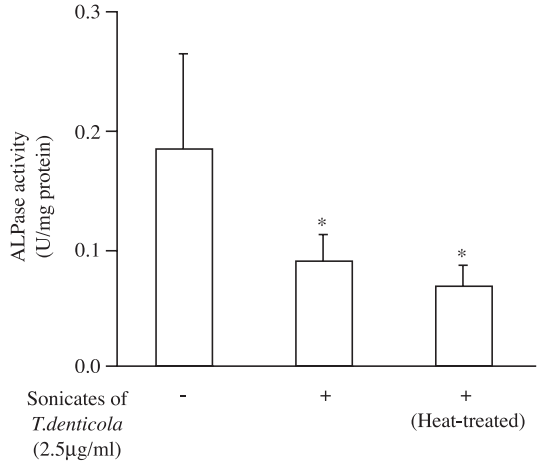

Figure 4. The effects of indomethacin on the ALPase inhibitory ability of *T. denticola* sonicates in mouse calvarial cells.

The calvarial cells were cultured to confluence, then treated with GP/AA and the sonicates (2.5 ㎍/ml) in the presence or absence of indomethacin (1 ㎛M) for 10 days. ALPase activity was estimated using p-nitrophenylphosphate as a substrate. Results are expressed as the means ± SE of 5 cultures. * $p<0.05$ significantly different from GP/AA-treated cells.

2. Effects of bacterial sonicates on PGE2 production in calvarial cells

The PGE_2 production and ALPase activity of the calvarial cells cultured in the sonicates with/without indomethacin for 10 days were measured in order to confirm whether PGE_2 was responsible for *T. denticola*-induced inhibition of ALPase activity. Bacterial sonicates (2.5 ㎍/ml) caused an increase in PGE2 formation in cells, whereas the non-treated cells

produced only low levels. Indomethacin (1 μM) abolished sonicate-induced PGE_2 production (Figure 3). However, indomethacin did not affect sonicate-induced inhibtion of ALPase activity (Figure 4).

3. Effects of bacterial sonicates on cell viability

To determine whether *T. denticola* could affect cell viability, the viability of cells cultured in the presence or absence of sonicates (0.25 and 2.5 μg /ml) for 10 days was compared by a MTT assay. At concentrations of 0.25 μg/ml and 2.5 μg/ml, *T. denticola* sonicates did not affect calvarial cell viability, when compared with non-treated cells (data not shown).

IV. Discussion

This report provides the first evidence showing that T. denticola sonicates inhibited osteoblast differentiation in vitro. In this study we used mouse calvarial cells as osteoblastic cells. Rodent calvarial cells contain a subpopulation of osteoprogenitor cells, which proliferate and differentiate in osteoblastic cells and finally form mineralized nodules over the course of the culture period in the presence of GP/AA (Nefussi et al., 1985; Aubin et al., 1995). The stages in osteogenic differentiation correlate with the bone matrix protein expression in vitro (Lian and Stein, 1992; Yao et al., 1994). Alkaline phosphatase (ALP) is expressed early in bone cell differentiation and is considered to characterize mature osteoblasts and to be associated with mineralization of the bone matrix (Yao et al., 1994, Aubin et al., 1995). Therefore, the effects of T. denticola on osteoblast differentiation was estimated by the ALPase activity and the mineralized nodule forming ability of mouse calvarial cells cultured in the presence of GP/AA and sonicates of this bacterium. ALPase activity of GP/AA-treated mouse calvarial cells was reduced by the presence of T. denticola sonicates and the inhibition of mineralized nodules formation was also observed in calvarial cells after treatment with a combination of GP/AA and sonicates. This means that T. denticola inhibits osteoblast differentiation.

Porphyromonas gingivalis and Actinobacillus actinomycetemcomitans have been also implicated in the etiology of periodontitis (Slots and Ting, 1999). In P. gingivalis, LPS inhibited ALPase activity in rat calvarial cells (Kadono et al., 1999). In A. actinomycetemcomitans, it has been demonstrated that the surface-associated protein (SAP) is a potent growth inhibitor of the human osteoblast-like cell line MG63. The characterization of this antiproliferative activity of SAP demonstrated that it was not cytotoxic (White et al., 1995). Some groups (Morimoto et al., 1999; Yamamoto et al., 1999) have found that the proteinaceous components of *A. actinomycetemcomitans sonicates* and its capsular-like polysaccharide antigen induced apoptotic cell death in human osteoblastic MG63 cells and mouse osteoblastic MC3T3 - E1 cells, respectively. On the other hand, it was reported that sonicates of this bacterium inhibited ALPase activity in MC3T3-E1 cells (Murata et al., 1997).

They showed that the alkaline phosphatase inhibitory activity occurred at a concentration 100 times lower than the concentration which showed the apoptosis-inducing property and that the ALPase inhibitory substance was not a protein. These results suggest that various bacterial components are involved in inhibiting osteoblast differentiation by inducing apoptosis, inhibiting proliferation or inhibiting the expression of ALPase activity.

Although it has been shown that *T. denticola* components, such as the Msp (major surface protein) and chymotypsin-like protease, have potent cytotoxic effects on some types of cells (Mathers et

al. 1996; Fenno et al., 1998), *T. denticola* sonicates did not affect the viability of mouse calvarial cells at a concentration which showed ALPase inhibitory activity. We have also found that heating the sonicates at 80℃ for 30 min prior to addition did not alter the effect on ALPase activity. These findings suggest that the inhibition of the osteoblast differentiation was not due to cytotoxicity and that the inhibitory factor of osteoblast differentiation in the sonicates was heat-stable. *T. denticola* contains an outer membrane lipid, which is chemically close to lipoteichoic acid, but with properties similar to lipopolysaccahride (LPS; Schultz et al., 1998; Ishihara and Okuda, 1999). Although the components of *T. denticola* that mediate the inhibition of ALPase activity are not known, possible bacterial components may include a heat-stable LPS like material.

In vitro studies showed that a higher PGE_2 concentration suppressed ALPase activity in osteoblastic MC3T3-E1 cells, while low PGE_2 concentrations stimulated ALPase activity (Suda et al., 1996; Kajii et al., 1999). In our study, PGE_2 biosynthesis in mouse calvarial cells was enhanced by bacterial sonicates. Therefore, to examine whether the increased production of PGE_2 is associated with ALPase inhibitory ability of the sonicates, the effect of the sonicates on ALPase activity of cells was observed in the condition in which the PGE_2 response was suppressed. Indomethacin, an inhibitor of prostagalndin biosynthesis, completely inhibited bacteria-induced PGE_2 production. However, treatment of the cells with this inhibitor did not affect the capacity of the bacterial sonicates to inhibit ALPase activity. This result demonstrates that the inhibitory effect on ALPase activity is not linked to bacteria-induced PGE_2 production.

Physiological bone remodeling is controlled by a balance between bone formation and resorption. This balance requires the coupled activities of both osteoblast and osteoclast. In our previous study (Choi et al., 1999), at a concentration (2.5 ㎍/ml) which nhibited the differentiation of osteoblast, *T. denticola* sonicates stimulated osteoclast formation, in part via PGE_2. Taken together, these results suggest that *T. denticola* may be involved in bone destruction by causing the stimulation of bone resorption an the inhibition of bone formation.

In conclusion, our data indicate that sonicates of *T. denticola* inhibit osteoblast differentiation. The inhibition of osteoblast differentiation may be induced by a PGE_2-independent pathway and heat-stable components of the bacterium may be involved in osteoblast differentiation. Our results indicate that *T. denticola* may play an important role in bone destruction by inhibiting of bone formation.

V. Conclusion

Periodontal disease is an infectious disorder that leads to the destruction of alveolar bone. *Treponema (T) denticola* is predominant in lesions of adult periodontitis, but its effects on osteoblasts have not been reported.

This study was undertaken to determine the effects of *T. denticola* sonicates on the differentiation of osteoblasts.

Osteoblast differentiation was analyzed by the alkaline phosphatase (ALPase) activity and the formation of mineralized nodules in mouse calvarial cells. Prostaglandin E_2 (PGE_2) production in mouse calvarial cells treated with *T. denticola* was measured by an enzyme immunoassay system.

1. *T. denticola* sonicates (2.5 ㎍/ml) reduced ALPase activity and the formation of mineralized nodules in mouse calvarial cells.
2. *T. denticola* sonicates (2.5 ㎍/ml) increased PGE_2 production of mouse calvarial cells.

3. Although indomethacin, an inhibitor of prostaglandin synthesis, reduced PGE_2 production in the sonicate-treated cells, it had no effect on ALPase inhibitory ability of *T. denticola*.
4. Heating the sonicates did not affect the inhibitory activity on ALPase activity. Taken together, this study indicates that *T. denticola* has the ability to inhibit osteoblast differentiation via a prostaglandin-independent mechanism. Heat-stable components of the bacterium may be involved in the inhibition of osteoblast differentiation. These results suggest that *T. denticola* may be involved in the loss of alveolar bone by decreasing bone formation.

VI. References

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-국문초록-

Treponema denticola 분쇄액에 의한 조골세포분화 억제효과

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치주질환은 세균감염에 의해 치조골이 파괴되는 염증성질환으로서 치아상실의 주된 원인이다.

*Treponema denticola*는 성인성 치주염의 병소에서 자주 발견되는 세균으로서 부착능 및 단백분해효소생성능과 같은 독성 인자가 밝혀져 치주조직 파괴에 있어서 중요성이 강조되어 왔다. 골개조는 조골세포의 골형성 및 파골세포에 의한 골흡수의 균형에 의하여 유지되며 치주염시 야기되는 치조골파괴는 조골세포 및 파골세포 기능의 불균형에 의하여 야기되는 것으로 설명되고 있다. 골세포에 대한 영향으로서 *T. denticola*는 파골세포의 형성을 촉진시키는 것으로 보고되었으나 조골세포에 대한 영향은 아직 밝혀져 있지 않다. 따라서 본 연구에서는 *T. denticola*가 골형성에 미치는 영향을 알아보고자 마우스의 두개골세포로부터 조골세포를 분리한 후 *T. denticola* 분쇄액으로 처리하여 본 세균이 조골세포의 alkaline phosphatase(ALPase) 활성, 석회화결절 형성 및 Prostaglandin E_2 (PGE_2) 생성에 미치는 영향을 평가하였다. ALPase활성은 p-nitrophenylphosphate분해능, 석회화결절형성은 Von Kossa 염색법, 그리고 PGE_2의 농도는 효소면역측정법으로 측정하였다. *T. denticola*분쇄액 (2.5 ug/ml)은 마우스 두개골세포의 ALPase활성을 억제하였으며 석회화결절의 형성을 감소시켰다. 또한 동일한 농도의 균분쇄액은 마우스 두개골세포의 PGE_2 생산을 증가시켰다. 균분쇄액과 prostaglandin의 합성억제제인 indomethacin으로 세포를 동시에 처리한 경우 *T .denticola*분쇄액에 의한 PGE_2의 생산은 감소되었으나, ALPase의 활성억제에는 변화가 없었다. 균분쇄액을 열처리하여 마우스 두개골세포에 처리하였을 때에도 ALPase의 활성이 억제되는 것에는 변함이 없었다. 이러한 결과는 *T. denticola*의 구성성분 중 열에 안정한 물질이 prostaglandin과 무관한 경로를 통해 조골세포의 분화를 억제함을 시사하며 이와 같은 *T. denticola*에 의한 골형성억제가 치주염시 야기되는 치조골 파괴에 관여할 수 있을 것으로 생각된다.

주요어: 치주염, Treponema denticola, 조골세포, 골형성