Low-Intensity Pulsed Ultrasound Accelerates Differentiation of Osteoblastic Cells on Roughened Titanium Surface

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Abstract: Use of low-intensity pulsed ultrasound (LIPUS) as a clinical tool is expected to accelerate bone-titanium integration in dental implant therapy. This study aimed to evaluate the effects of LIPUS treatment on bone marrow cells cultured under osteogenic conditions on the roughened surface of titanium disks in vitro. Bone marrow cells, obtained from the femora of 8-wk-old rats, were suspended in osteogenic-inducing medium. Cells were cultured on acid-etched titanium disks and exposed to LIPUS of 3.0 MHz sine wave frequency, repeated at 100 Hz with a spatial average intensity of 40 mW/cm² for 15 min/d from day 3 after primary seeding (LIPUS group). The control group was cultivated in the same manner. Cell proliferation, expression of osteoblastic genes, synthesis of collagen, and mineralization were compared between the 2 groups. No significant difference was observed in the cell number between the LIPUS and the control groups on days 5 and 7. The expression of osteocalcin and osteopontin genes were upregulated in the LIPUS group compared with that in the control group. The production of collagen, assessed by Sirius Red staining on day 14, and mineralization, assessed with Alizarin Red S staining on day 14 and 21, were both increased in the LIPUS group relative to the control group. Treatment with LIPUS did not affect the proliferation of osteoblastic cells cultured on roughened titanium disks, whereas it affected the acceleration of osteoblastic differentiation, synthesis of collagen, and calcification.

Key words: Cell culture, Low-intensity pulsed ultrasound, Osteoblastic cell, Titanium

Introduction

Dental implant therapy is a commonplace treatment for replacing missing teeth nowadays3, although a healing period of 3 and 6 mo for the mandible and maxilla, respectively, is required between the primary and secondary surgeries in the conventional 2-stage implant protocol4. This healing time is the main reason for the discomfort of patients and the long duration of the total implant therapy5. To reduce the healing period after implantation, many strategies were performed or proposed, such as reducing the period of placing the superstructures after implantation as immediate6 or early loading7, implant design8, and surface modification of the implant9.

The application of low-intensity pulsed ultrasound (LIPUS) has been reported to reduce the healing period after bone fractures in the orthopedic field10. The U.S. Food and Drug Administration (FDA) approved the LIPUS equipment for healing fresh bone fractures in 1994 and for non-unions in 200011. High healing success rates were reported for fractures, including delayed unions and non-unions12, with the time of third cortical bridging being earlier after LIPUS therapy in fresh fractures13. In implant-related studies, noninvasive low-intensity ultrasound was demonstrated to significantly affect bone ingrowth into porous titanium implants in an in vivo animal model14 and bone formation was reported to be stimulated around titanium implants in dog mandibles15. We demonstrated that the application of LIPUS enhanced differentiation and mineralization of osteoblastic cells cultured in polystyrene cell-culture dishes16 and promoted the expression of genes in the extracellular matrix and the differentiation of osteoblast-like cells into osteocytes in an in vitro cell culture model17. In in vivo studies, LIPUS was shown to accelerate bone formation around titanium implants and enhance the strength of bone-titanium integration in rat femora18. In this study, we hypothesized that culturing osteoblastic cells on rough titanium surface with LIPUS treatment would enhance osteogenic differentiation. To test this hypothesis, we examined the effects of exposing osteoblastic cells cultured on the surface of roughened titanium disks to LIPUS.

Materials and Methods

Preparation of experimental titanium disks

Commercial pure titanium rods and disks were prepared using a lathe. Three commercial pure titanium rods (1.0 mm in diameter and 1.7 mm length, JIS grade 2, Nishimura Metal Co., Fukui, Japan), welded up equidistantly around each commercial pure titanium disks (20 mm in diameter and 1 mm in thickness, JIS grade 2, Nishimura Metal Co., Fukui, Japan), were used to prepare the 3-legged titanium disks. Disks were acid-etched in 48% sulfuric acid (Kanto Chemical Co., Tokyo, Japan) at 60°C for 10 min19, washed in ultra-pure water 3 times, and sterilized at 200°C in a drying oven (DV400, Yamato Scientific Co., Tokyo, Japan) for 30 min (Fig. 1A). All disks were etched and sherardized 1 d before seeding of primary cultures. The morphology of the surface was examined using a scanning electron microscopy (SEM) (EVO 40, Carl Zeiss, Cambridge, UK) and 3D microstructures were investigated using atomic force microscopy (AFM) (SPM-9500J3, Shimadzu Corporation, Kyoto, Japan), force microscopy (AFM) (SPM-9500J3, Shimadzu Corporation, Kyoto, Japan).
Japan) at a contact mode over square areas of $5.0 \times 5.0 \mu m^2$. The obtained AFM data were analyzed using the software for topographical parameters: the arithmetic average height (Ra), the largest peak to valley height (Ry), and ten points of height (Rz).

Bone marrow cell culture

The study protocol was approved by the Institutional Committee for Animal Care, Aichi Gakuin University (AGUD 069). The experimental design and analytical time points are shown in Fig. 2. Bone marrow cells were obtained from the femora of five rats (8-wk-old male Sprague-Dawley rats; Japan SLC, Hamamatsu, Japan) and suspended at a concentration of $5.6 \times 10^6$ cell/ml in alpha-modified Eagle’s medium (41061-029, Gibco, CA, USA) supplemented with 15% fetal bovine serum (SFBM31, Equitech-Bio, TX, USA), $10^{-8}$ M dexamethasone (D2915, Sigma-Aldrich Inc., MO, USA), 10 mM Na-β-glycerophosphate (G9422, Sigma-Aldrich Inc., MO, USA), 50 µg/ml L-ascorbic acid 2-phosphate (A8960, Sigma-Aldrich Inc., MO, USA), and an antibiotic-antimycotic solution including 100 units/ml of Penicillin G sodium, 100 µg/ml of Streptomycin sulfate, and 250 ng/ml of Amphotericin B (15240-062, Gibco, CA, USA). The suspension was directly poured onto the titanium disks placed in 12-multiwell cell culture plates (3513, Corning, AZ, USA) and cultured at 37ºC in an atmosphere with 5% CO2. The culture medium was changed every 3 d.

Low-intensity pulsed ultrasound (LIPUS) treatment

LIPUS was generated using a therapeutic ultrasound device with 3 connected piezoelectric transducers (BR-sonic Pro, ITO Co., Tokyo, Japan). Each transducer can generate a pulsed ultrasound (2 msec burst and 8 msec off) of 3.0 MHz sine wave frequency, repeated at 100 Hz with a spatial average intensity of 40 mW/cm². These transducers were placed in a mini incubator and cell cultures were exposed to LIPUS from the bottom of the cell culture plates via an ultrasound coupling gel (Ultrasound Gel, ITO Co., Tokyo, Japan). The transducers were 36 mm in diameter and their effective radiating area was 4.5 cm². The attenuation of the ultrasound power through the cell culture plate was under 35%, which was confirmed using a hydrophone (Specialty Engineering Associates, CA, USA). The control group was exposed to LIPUS for 15 min/d from day 3 after primary seeding (LIPUS group). Titanium disks were placed upside-down during the period of exposure to LIPUS (Fig. 3). The control group without treatment with LIPUS was also cultivated in the same manner.

Cell Proliferation Assay

We measured the number of cells on day 5 and 7 after seeding using the WST-8 cell proliferation reagent (CCK-8, Dojindo Laboratories, Kumamoto, Japan). Briefly, cell cultures were rinsed with phosphate-buffered saline (PBS) without calcium ions (D8537, Sigma-Aldrich Inc., MO, USA) and 100 µl of WST-8 solution and 900 µl of cell culture medium were added into each well and incubated at 37ºC for a further 20 min. Then, 200 µl of each supernatant was transferred into a 98-multiwell plate and quantified through measurement of the optical density (OD) at 460 nm using a multi-well plate reader (Model 680, BIORAD, CA, USA). The number of cells was estimated using a linear regression equation made from a serial dilution of cell suspensions and their respective OD numbers. Three independent cultures were analyzed at each time point.

Reverse-transcription / Polymerase chain reaction (RT-PCR)

We performed RT-PCR analysis to examine the expressions of osteoblastic genes. Total RNA was extracted from each cell culture using a hybrid method of TRizol (Invitrogen, CA, USA) and purification column (RNaseasy, QIAGEN, MD, USA). Contaminating DNA was removed by treatment with DNase (TURBO DNA-free, Invitrogen, CA, USA). The reverse transcription reaction was performed at 42ºC for 50 min us-
Collagen synthesis colorimetry

The Sirius Red staining-based colorimetric assay was used to quantify the total collagen deposition. This method specifically detects type I and III collagen molecules without identifying other components of the bone extracellular matrix. Cell cultures were gently rinsed with PBS and fixed with 1 ml of Bouin’s fluid (Polysciences Inc., PA, USA) for 1 h. Cultures were washed by running tap water for 15 min before staining with 1 ml Sirius Red dye (Polysciences Inc., PA, USA) dissolved in saturated aqueous picric acid (Kanto Chemical Co., Inc., Tokyo, Japan) at a concentration of 100 mg/100 ml for 1 h with mild shaking. Cultures were washed with 0.01 M HCl (Kanto Chemical Co., Inc., Tokyo, Japan) to remove the unbound dye, and the staining was dissolved in 0.6 ml 0.1 M NaOH (Kanto Chemical Co., Inc., Tokyo, Japan) using a microplate shaker for 30 min at 25°C. The OD of the dye solution was measured using a spectrophotometer (Model 680, BIORAD, CA, USA) equipped with a 550 nm optical filter against a 0.1 N NaOH solution equipped as blank in 3 independent cultures. Results were reported as OD data were expressed as mean ± SD. Differences in cell proliferation, collagen synthesis, and calcification were statistically evaluated by means of two-way analysis of variance (ANOVA) performed using the factors “culture day” and “with or without LIPUS”. When two-way ANOVA showed a significant interaction between 2 factors, a one-way ANOVA was performed for each level of the factor “culture day”. Statistical significance was accepted at p < 0.05. All analyses were performed using JMP Pro 15 (SAS Institute Japan, Tokyo, Japan).

Statistical analysis

For each assay, all samples were cultured in disks in triplicate and data were expressed as mean ± SD. Differences in cell proliferation, collagen synthesis, and calcification were statistically evaluated by means of two-way analysis of variance (ANOVA) performed using the factors “culture day” and “with or without LIPUS”. When two-way ANOVA showed a significant interaction between 2 factors, a one-way ANOVA was performed for each level of the factor “culture day”. Statistical significance was accepted at p < 0.05. All analyses were performed using JMP Pro 15 (SAS Institute Japan, Tokyo, Japan).

Table 1. Primers and conditions for RT-PCR analysis

| Genes          | Acc #    | Upstream primer                | Downstream primer               | Annealing Temperature | Number of Cycles | Size of PCR Products (bp) |
|----------------|----------|--------------------------------|---------------------------------|-----------------------|------------------|--------------------------|
| Osteocalcin (Rat) | M23637   | 5-GTCCCAACAGCAACTCGG-3         | 5-CCAAAGGTGAAGCTGCCG-3          | 61                    | 25               | 287                      |
| Osteopontin (Rat) | M14656   | 5-GCTGCTCTACTACAATG-3          | 5-GGATACATGTATGGAG-3            | 43                    | 19               | 380                      |
| GAPDH          | M17701   | 5-TGAGGTGGGTGCAACGGATTTTGC-3   | 5-CATAGGCGCATAGGTACCAC-3        | 67                    | 27               | 983                      |
for the expression levels of these genes with the factors of cultivation duration with/without LIPUS treatment.

**Expression of osteocalcin gene**

The expression levels of the osteocalcin gene were found to differ significantly depending on the time points (p < 0.01) and treatments with LIPUS (p < 0.01). In addition, a significant interaction was noted for the combination of these factors (time × treatment) (p < 0.05). Results of one-way ANOVA on independent time points revealed that the expression levels of the osteocalcin gene in the LIPUS group on days 7 and 14 were significantly higher compared with those in the control group (p < 0.01), whereas no significant difference was found on day 21 (Fig. 5).

**Expression of osteopontin gene**

Similarly, the expression levels of the osteopontin gene were found to be significantly different depending on the time points (p < 0.01) and treatments with LIPUS (p < 0.01), as well as the combination of the factors of time and treatment (p < 0.05). One-way ANOVA of independent time points revealed that the expression levels of the osteopontin gene in the LIPUS group on days 7 and 14 were significantly higher compared with those in the control group, whereas no significant difference was found on day 21 (Fig. 6).

**Synthesis of collagen**

Two-way ANOVA revealed that there were no significant differences observed in the duration of cultivation, whereas a significant difference...
was only noted on day 14 between the LIPIUS and control groups (Scheffé’s multiple comparison, p<0.01). In addition, we did not observe any significant interaction between the 2 factors (Fig. 7).

Deposition of calcium

We found that the calcification in the LIPIUS group was increased on days 14 and 21 compared with that in the control group (Fig. 8). The Alizarin Red-S stained area was found to be significantly larger on days 14 and 21 in the LIPIUS treated group (p<0.01). Moreover, we observed a significant factorial interaction between the 2 factors (p<0.01). Alizarin Red S stained experimental disks are demonstrated in Fig. 8.

Discussion

Bone marrow cell culture as a tool for implant study

Osseointegration is a unique phenomenon, which was observed and identified using a light microscope in an animal study, and defined as the direct contact between living bone and the surface of titanium without the interposition of nonbone tissue19. The establishment of osseointegration involves complex biological processes20,21. Although bone formation around titanium implants has been reported to occur via both contact and distance osteogenesis, the importance of the former is crucial in osseointegration. Contact osteogenesis relies upon osteoconductance, or the recruitment and migration of differentiating osteogenic cells to the surface of the implant, together with de novo bone formation by those cells on the surface of the implant20. To investigate the mechanisms of osseointegration, in vitro studies have been conducted, attempting to elucidate the biological and molecular roles of osteoblastic cells to titanium22,23. In this study, we seeded bone marrow cells in a cell culture medium with osteogenic supplements24, then poured the mixture of bone marrow cells and osteogenic medium directly onto experimental titanium disks. Bone marrow cells are known to contain mesenchymal stem cells (MSCs) and thus, the addition of osteogenic supplements in the cell culture medium would initiate the differentiation of MSCs into osteoblastic cells25.

Preparation of titanium disks

The topography of the titanium surface is a significant property for controlling cellular and tissue responses around titanium implants. Roughened titanium surface is known to promote stiffer and harder biomechanical characteristics of osteoblastic mineralized cultures21,23. Titanium is an osteoconductive material, but the time-dependent biological degradation of its surface from bioactive to bioinert state was observed via both in vivo and in vitro experiments20. To prevent the degradation of the osteoconductivity of titanium disks, we performed an acid-etched treatment of the disks on the day before seeding the mixture of bone marrow cells. The topography of the experimental disk surface resembled commercially available acid-etched titanium implants26 (Fig. 1), indicating that the titanium disks in this study had potential osteoconductivity. Thus, we could assess the effect of the LIPIUS treatment without biological degradation of the titanium surface.

Treatment with LIPIUS

Ultrasound is an acoustic wave of frequencies above the range of human audition. The thermal and nonthermal effects of ultrasound in living tissues are known to be the basis of therapeutic applications27,28. Briefly, LIPIUS is a very low-power pulsed ultrasound minimizing the thermal effect and providing a nonthermal effect, which has been widely used in the medical field for diagnostic29 and therapeutic purposes30. In the orthopedic field, LIPIUS has been used to accelerate the process of bone fractures healing31,32. Although, the biological processes involved in accelerating the repair of the bone fracture when treated with LIPIUS are not still fully understood30, it was reported that the production of cyclooxygenase 2 (COX-2) in cells was enhanced; this in turn stimulated molecules for enhanced fracture repair33 through not only increasing the levels of osteogenic molecules, such as bone morphogenetic proteins (BMPs)32,34, insulin-like growth factor 1 (IGF-1)35, stromal cell-derived factor 1 (SDF-1)36, and Prostaglandin E2 (PGE2)37, but also through promoting the apoptosis of osteoclasts38.

Cell proliferation

Cell proliferation was shown to be suppressed on the roughened titanium disk compared with the machined surfaced disk39. The cell number on the surface of polystyrene culture plates on day 7 was observed to be decreased in the LIPIUS group compared with the control group40. In this study, the cell number on days 5 and 7 did not show any statistical difference between the LIPIUS and control groups. Akagi et al. also reported that LIPIUS had no effect on cell proliferation41. In contrast, LIPIUS was shown to have a positive effect in the proliferation of osteoblastic cells42,43. As such, the effect of the treatment with LIPIUS in the proliferation of osteoblasts has been controversial.

Expressions of bone-related genes

Osteopontin and osteocalcin are important in coordinating the organic matrix and bone mineral44. Osteocalcin, which accounts for approximately 20% of the non-collagenous protein content of the bone matrix, is produced by mature osteoblasts and deposited in the extracellular matrix (ECM) of the bone tissue42. Osteopontin is considered to bind to osteoblasts and osteoclasts localized on bone surfaces and to promote bone resorption by osteoclasts45. The expression levels of osteocalcin and osteopontin were observed to be highly upregulated in the LIPIUS group compared with the control group on days 7 and 14, with the maximal upregulation being observed on day 14 in both genes. These results suggested that stimulation with LIPIUS enhanced the osteoblastic differentiation from day 7 to 14 in this study (Figs. 5 and 6).

Synthesis of collagen

Sirius Red staining enables sensitive and quantitative detection of type I and type III collagen46. Type I collagen accounts for approximately 90% of the ECM in bone, while type III collagen is detected in preosteoblasts47. We noted that the density of Sirius Red staining was significantly higher in the LIPIUS group compared with that in control cultures on day 14. Yamaguchi et al., using a microarray analysis, concluded that exposure to LIPIUS increased the expression of collagen related gene molecules and promoted the differentiation of osteoblastic cells into osteocytes43. Collagen is a key determinant in bone for fortifying bone strength45. The production of collagen in the LIPIUS group was found to be significantly higher than that in control cultures on day 14, indicating that exposure to LIPIUS promoted the high strength of the osteoblastic cell culture on a titanium surface.

Calcification of cell culture on titanium

The areas of Alizarin Red S stained calcified nodules were shown to be significantly larger in the LIPIUS group than in the control group on days 14 and 21 of cell culture (Fig. 8). Regarding bone strength, the mineral content is mainly involved in determining bone stiffness48. These results indicated that exposure to LIPIUS might contribute to the increased stiffness of the osteoblastic cell culture.

In conclusion, treatment with LIPIUS did not affect the cell prolifera-
tion of osteoblastic cells cultured on roughened titanium surface, whereas it accelerated cell differentiation, deposition of collagen, and calcification.

**Conflict of Interest**
The authors have declared that no COI exists.

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