Gene Expression in Early Stages of Low-Intensity Pulsed Ultrasound Exposure on Bone Marrow Cells

Daisuke Yamaguchi1, Kazuo Takeuchi1, Atsuko Ueno1, Masataka Yamaguchi2, Hiroshi Murakami1 and Suguru Kimoto1

1) Department of Gerodontology and Home Care Dentistry, School of Dentistry, Aichi Gakuin University, Nagoya, Japan
2) Department of Endodontics, School of Dentistry, Aichi Gakuin University, Nagoya, Japan

Abstract: The aim of this study was to identify genes that are prominently expressed in rat bone marrow cell-derived osteoblasts during the initial phase of low-intensity pulsed ultrasound (LIPUS) exposure. Bone marrow cells were obtained from three Sprague-Dawley rats (8-week-old, male), and cell cultures were prepared by suspension in osteogenic medium. After cultures were established, test cultures were exposed to LIPUS from the bottom of the cell culture plate for 15 min/d on days 1–4 (LIPUS group). LIPUS signals were transmitted at a frequency of 3 MHz and a spatial average intensity of 40 mW/cm². The control group was not exposed to LIPUS. On day 14, alizarin red S staining was performed to detect calcification. On day 4, total RNA was extracted from both cultures, hybridized to microarray slides, and the resulting data set was analyzed. Genes exhibiting a fold-change ≥2 and a p-value <0.05 (LIPUS vs. control) were identified as differentially expressed genes. Pathway analysis was performed on genes whose expression increased in the LIPUS group. The cellular areas stained with alizarin red S were significantly larger in the LIPUS group than in the control group on day 14. LIPUS exposure increased the expression of genes related to type II interferon signaling, and endochondral ossification was observed after 4 d of culture. The results demonstrated that LIPUS exposure activated the immune response and promoted osteoblast differentiation.

Key words: Bone marrow cells, LIPUS, Microarray analysis, Mineralization

Introduction

Dental implants, which are used to treat tooth loss, contribute to the improvement and long-term stability of masticatory functions1–3 and psychometric conditions4–6. Although dental implants have been recognized as an important treatment option for prosthetic procedures for partial or full edentulous patients, they still require further improvements. The average recovery time for a dental implant procedure is 3–6 months; however, the recovery period can be longer depending on the problem that initially caused the need for dental implants and any unique surgical additions such as bone grafts. The prolonged treatment period for implant procedures is a burden for patients, especially in the elderly.

Several researchers have investigated methods to shorten the healing period and reported new strategies for accelerating bone formation after implant placement, with the simplest being immediate implant placement and restoration12–13. However, only patients with sufficient bone mass and strength are eligible for this treatment, and few patients are able to receive it. Other attempts have been made to enhance cell adhesion and protein adsorption at the implant-bone interface. To enhance cell adhesion and protein adsorption at the implant-bone tissue interface, implant materials and implant surface treatments have been developed14–16. Ogawa reported that the UV-treated titanium surface generates a unique electrostatic state that directly attracts cells and effectively shortens the osseointegration period11. In addition to these local meth-
Bone marrow cells were obtained from the femora of five Sprague-Dawley rats (8-week-old, male; Japan SLC, Inc., Shizuoka, Japan) and suspended in α-Minimum Essential medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 15% fetal bovine serum (Equitech-Bio, Kerrville, TX, USA), 10 nM dexamethasone (Sigma-Aldrich, St. Louis, MO, USA), 50 mg/ml L-ascorbic acid 2-phosphate (Sigma-Aldrich), 10 mM Na-β-glycerophosphate (Sigma-Aldrich), and an antibiotic-antimycotic solution (Thermo Fisher Scientific). Cell suspensions (5 × 10⁶ cells/ml) were seeded into 12-well cell culture plates (Corning, Corning, NY, USA), cultured at 37°C in a 5% CO₂ atmosphere, and the culture medium was changed once every 3 d.

**LIPUS application**

LIPUS exposure was performed for 15 min/d on days 1–4 after the establishment of cell cultures (LIPUS group). LIPUS signals were transmitted at a frequency of 3 MHz, with a spatial-average intensity of 40 mW/cm² and 2 ms-burst sine waves at 100 kHz, using a clinical LIPUS device (BR-Sonic Pro, ITO Co., Ltd., Tokyo, Japan). LIPUS was applied to cells through the bottom of cell culture plates via an ultrasound coupling gel (ITO ultrasound gel, ITO Co., Ltd.) applied to LIPUS probes (effective radiating area: 4.5 cm²). Control cultures were not exposed to LIPUS, yet were treated identical to the test group.

**Mineralization assay**

Alizarin red S staining was performed on day 14 to detect calcification in cultured cell samples. Alizarin red S is a negatively charged dye that specifically binds metal ions and is used to analyze calcium deposits. Alizarin red S (Thermo Fisher Scientific) was dissolved in 100 ml distilled water, and the pH was adjusted to 4.1–4.3 using 0.1% NH₄OH. Cultures were washed with phosphate buffered saline, fixed with 10% neutral buffered formalin (Wako Pure Chemical Industries, Osaka, Japan) for 10 min, stained for 10 min, and subsequently washed four times with distilled water. Stained areas were detected with an imaging scanner and quantified using ImageJ imaging software (NIH, Bethesda, MD, USA).

**Total RNA extraction**

Total RNA was extracted from LIPUS-treated and control cultures on day 4 using the guanidinium thiocyanate-phenol-chloroform extraction method (TRIzol, Thermo Fisher Scientific) and purification columns (RNeasy, QIAGEN, Venlo, Nederland) in accordance with the manufacturer’s instructions. Acquired samples were treated with DNase I (RNase-Free DNase Set, QIAGEN) to remove contaminating DNA. The concentration of extracted RNA was measured using a spectrophotometer (ND-1000, Thermo Fisher Scientific).

**Microarray analysis**

Total RNA (250 ng/sample) was converted to complementary DNA (cDNA), amplified, and labeled using the GeneChip™ WT PLUS Reagent Kit (Thermo Fisher Scientific). Amplified RNA was quantified using a spectrophotometer (ND-1000) and hybridized to Clariom™ S Assay (Thermo Fisher Scientific). Thereafter, slides were washed using a washing solution (GeneChip™ Fluidics Station 450, Thermo Fisher Scientific), fluorescence images of hybridized arrays were scanned using a microarray scanner (GeneChip™ Scanner 3000 7G, Thermo Fisher Scientific), and data were extracted using the Expression Console™ Software system (Thermo Fisher Scientific). The resulting files were imported into Microarray Data Analysis Tool Ver3.2 (Filgen Inc., Nagoya, Japan) for statistical analysis. Genes exhibiting a fold-change ≥2 and a p-value <0.05 (LIPUS vs. control) were identified as differentially expressed genes. Pathway analysis was performed on extracted genes whose expression increased in the LIPUS-treated group.

**Statistical analysis**

Differences in calcification were assessed using Student’s t-test. cDNA microarray data were analyzed using Student’s t-test followed by Benjamini-Hochberg corrections.
Results
Evaluation of calcification
Histological images of cultured osteoblast-like cells stained with alizarin red S are shown in Fig. 2. The number of pixels increased >6.57-fold in the LIPUS-stimulated group compared to that in the control group (p<0.05) (Fig. 3).

gene expression
Scatter plots of gene expression in the LIPUS group and the control group on the fourth day of culture are shown in Fig. 4. Fig. 4A shows the scatter plot before data mining, and Fig. 4B shows the scatter plot of genes whose expression increased >2-fold in the LIPUS group (334 genes). Classification results by pathway are shown in Table 1.

discussion
In this study, we cultured bone marrow cells in a medium supplemented with dexamethasone, L-ascorbic acid diphosphate, and β-glycerophosphate, which are known to promote the differentiation of bone marrow cells into osteoblast-like cells. Bone marrow contains mesenchymal stem cells, which have multiple differentiation potential, and the added reagents have the ability to differentiate mesenchymal stem cells into osteoblast-like cells. After 4 d of LIPUS exposure under these conditions, calcified nodule formation increased on day 14 of culture. In addition, the expression of genes related to type II interferon (IFN) signaling and endochondral ossification were observed after 4 d of culture.

Calcified nodule formation
After 14 d of culture, calcified nodule areas were significantly larger in the LIPUS group than in the control group. The difference in these areas in stained cultures of the two groups on each sampling day was evident to the naked eye. Increases in collagen production and calcified nodule formation can be explained as follows: during the early phase of calcification, osteoblast-like cells secrete matrix vesicles, extracellular matrices such as collagen are produced around the cells, and hydroxyapatite crystals are precipitated within matrix vesicles in parallel. When hydroxyapatite crystals develop, they are released from matrix vesicles, infiltrate intermolecular spaces of surrounding collagen, and become crystalline nuclei, resulting in calcification along with col-

Figure 4. Scatter plots of gene expression in the low-intensity pulsed ultrasound (LIPUS) and control groups on day 4. (A) Scatter plot before data mining. (B) Scatter plots of genes whose signal intensities in the LIPUS and control groups were statistically significant (p<0.05) and whose signal intensity ratio was >2-fold in the LIPUS group.

Table 1. Pathway analysis results of genes whose expression increased more than 2-fold in the LIPUS group

| PathwayName                  | P-value        | Expression value Ratio | Gene Accession | Gene Symbol | Gene Description               |
|------------------------------|----------------|------------------------|----------------|-------------|--------------------------------|
| Rn_Type_II_interferon_signaling_(IFNG)_WP1289_86846 | 0.00331395 | 2488.407               | NM_001024753   | Itif2       | interferon-induced protein with tetratricopeptide repeats 2 |
|                              | 0.00331395 | 36.87297               | NM_001106700   | Isg15       | ISG15 ubiquitin-like modifier  |
|                              | 0.00331395 | 310.6771               | NM_138913      | Oas1a       | 2-5 oligoadenylate synthetase 1A |
|                              | 0.00331395 | 922.2733               | NM_139089      | Cxcl10      | chemokine (C-X-C motif) ligand 10 |

| PathwayName                  | P-value        | Expression value Ratio | Gene Accession | Gene Symbol | Gene Description               |
|------------------------------|----------------|------------------------|----------------|-------------|--------------------------------|
| Rn_Endochondral_Ossification_WP1308_107233 | 0.003314163 | 270.9018               | NM_012862      | Mgp         | matrix Gla protein              |
|                              | 0.003314163 | 3049.659               | NM_012886      | Timp3       | TIMP metallopeptidase inhibitor 3 |
|                              | 0.003314163 | 7723.367               | NM_00110333    | Vegfa       | vascular endothelial growth factor A |
|                              | 0.003314163 | 6950.681               | NM_024400      | Adamts1     | ADAM metallopeptidase with thrombospondin type 1 motif, 1 |
|                              | 0.003314163 | 267.8435               | NM_013107      | Bmp6        | bone morphogenetic protein 6    |
lager fibers\textsuperscript{35}. These calcification processes are likely enhanced by LIPUS exposure.

**Comprehensive analysis of gene expression**

Rat bone marrow cells were cultured in osteoblast differentiation medium, and gene expression was analyzed using a cDNA microarray. Pathway analysis was performed to extract related genes from the annotations in the Entity List.

Genes related to type II IFN signaling in this study were IFN-stimulated gene 15 kDa (ISG15), IFN-induced protein with tetratricopeptide repeats 2 (IFIT2), 2,5-Oligoadenylate synthase 1A (OAS1A), and C-X-C motif chemokine ligand 10 (CXCL10) and were upregulated in the LIPUS group. There are three types of IFNs, type 1–3\textsuperscript{17}, of which type II is known as interferon gamma (IFN-\gamma)\textsuperscript{18} and plays an important role in host defense by activating macrophages, increasing the expression of antigen-presenting molecules, activating Th1 cells, activating natural killer (NK) cells, and regulating B cell function\textsuperscript{19,20}. ISG15 is a ubiquitin-like molecule and also the gene most strongly induced by IFN\textsuperscript{21}. ISG15 was found to be induced in response to various stresses\textsuperscript{22,23}, however, there are no reports of its induction by LIPUS as mechanical stress. IFIT2 is one of the most responsive IFN-stimulated genes and responds to viruses and various drugs in cultured human and rodent cell lines\textsuperscript{24,25}. OASIA is a synthetic enzyme with antiviral activity induced by IFN and activated by double-stranded RNA\textsuperscript{26}. Furthermore, induction by IFN has been shown to be a signal transducer and activator of transcription (STAT)-1 and STAT2-dependent\textsuperscript{27}. CXCL10 is secreted from monocytes, endothelial cells, and fibroblasts in response to IFN-\gamma\textsuperscript{28} and is expressed on monocytes/macrophages, T cells, and NK cells\textsuperscript{29,30}. Nakao et al. reported that MC3T3-E1 cells treated with lipopolysaccharide and exposed to LIPUS suppressed the increase in CXCL10 expression and had an anti-inflammatory effect\textsuperscript{31}. Although the culture system and the time of analysis were different from those in the present study, the result that LIPUS exposure increased the gene expression of CXCL10 is different from the results of this study. These gene changes indicate that LIPUS exposure promotes differentiation of bone marrow into osteoblast-like cells, which consequently creates an immune response by generating blood cells and blood stem cells other than mesenchymal stem cells.

Among the genes related to endochondral ossification, matrix Gla protein (MGP), tissue inhibitor of metalloproteinase 3 (TIMP3), vascular endothelial growth factor A (VEGFA), a disintegrin and metalloproteinase with thrombospondin motif 1 (ADAMTS1), and bone morphogenetic protein 6 (BMP6) were identified in the LIPUS group of this experiment. Endochondral ossification is one of the mechanisms of osteogenesis in which mesenchymal cells condense and differentiate into chondrocytes, forming a chondrogenic matrix that is later replaced by bone matrix\textsuperscript{32}. MGP is a vitamin K-dependent protein. It is synthesized in mesenchymal cells and expressed in vascular smooth muscle cells and chondrocytes\textsuperscript{33}. ADAMTS1, like matrix metalloproteinases, is an extracellular matrix degrading enzyme. The degradation of extracellular matrix components is effectively controlled by the common endogenous inhibitor TIMP\textsuperscript{34}. In this study, TIMP3 was upregulated in LIPUS-irradiated cells, and the expression of TIMP3 was 1.72 times higher than that of ADAMTS1 in LIPUS-exposed cells, suggesting that TIMP3 inhibits extracellular matrix degradation. VEGFA shows proliferative activity on vascular endothelial cells, increases vascular permeability, and is involved in matrix metabolism in cartilage tissue. VEGFA is also reported to be induced by mechanical stress\textsuperscript{35}, and in this study, was expressed by LIPUS, which is considered a mechanical stress. BMP is a member of the secreted signaling molecule family that induces ectopic bone growth and belongs to the transforming growth factor-beta superfamily\textsuperscript{36}. BMP6 is closely related to BMP5 and BMP7 and has an osteoinductive effect. Yang et al. reported the expression of BMP6 by LIPUS in periodontal ligament cells\textsuperscript{37}. Although the cell type\textsuperscript{37} was different from that in the present study, upregulation of BMP6 gene expression at the same time point was consistent in both studies. To obtain a chondrogenic phenotype by culturing bone marrow-derived cells, conditions such as the addition of TGF-\beta to the culture medium (used in this study), three-dimensional culture, and hypoxia are required. However, the expression of genes related to endochondral ossification varied, suggesting that LIPUS-induced mechanical stress may also be involved in the promotion of chondrogenesis.

In summary, we cultured bone marrow cells under osteogenic conditions with LIPUS and were able to capture the trend of gene changes in the early stage of LIPUS exposure. In future research, it is necessary to quantify the expression levels of mRNA used in the real-time polymerase chain reaction analysis.

**Acknowledgements**

This work was supported by JSPS KAKENHI (Grant Number 20K18585). We thank Editage for English language editing.

**Conflict of Interest**

The authors have declared that no COI exists.

**References**

1. Carlsson GE and Lindquist LW. Ten-year longitudinal study of masticatory function in edentulous patients treated with fixed complete dentures on osseointegrated implants. Int J Prosthodont 7: 448-453, 1994
2. Kent G. Effects of osseointegrated implants on psychological and social well-being: a literature review. J Prosth Dent 68: 515-518, 1992
3. Roccuzzo M, Aglietta M and Cording L. Implant loading protocols for partially edentulous maxillary posterior sites. Int J Oral Maxillofac Implants 24 Suppl: 147-157, 2009
4. Cordaro L, Torsello F and Roccuzzo M. Implant loading protocols for the partially edentulous posterior mandible. Int J Oral Maxillofac Implants 24 Suppl: 158-168, 2009
5. Shibata Y and Tanimoto Y. A review of improved fixation methods for dental implants. Part I: Surface optimization for rapid osseointegration. J Prosthodont Res 59: 20-33, 2015
6. Shibata Y, Tanimoto Y, Maruyama N and Nagakura M. A review of improved fixation methods for dental implants. Part II: biomechanical integrity at bone-implant interface. J Prosthodont Res 59: 84-95, 2015
7. Ogawa T. Ultraviolet photofunctionalization of titanium implants. Int J Oral Maxillofac Implants 29: e85-102, 2014
8. Watanabe T, Nakada H, Takahashi T, Fujita K, Tanimoto Y, Sakae T, Kimoto S and Kawai Y. Potential for acceleration of bone formation after implant surgery by using a dietary supplement: an animal study. J Oral Rehabil 42: 447-453, 2015
9. Takahashi T, Watanabe T, Nakada H, Tanimoto Y, Kimoto S, Miyares DQ, Zhang Y and Kawai Y. Effect of a dietary supplement on peri-implant bone strength in a rat model of osteoporosis. J Prosthodont Res 60: 131-137, 2016
10. Jiang X, Savchenko O, Li Y, Qi S, Yang T, Zhang W and Chen J. A review of low-intensity pulsed ultrasound for therapeutic applica-
11. Tanzer M, Harvey E, Kay A, Morton P and Bobyn JD. Effect of noninvasive low intensity ultrasound on bone growth into porous-coated implants. J Orthop Res 14: 901-906, 1996

12. Kokubu T, Matsu N, Fujioka H, Tsunoda M and Mizuno K. Low intensity pulsed ultrasound exposure increases prostaglandin E2 production via the induction of cyclooxygenase-2 mRNA in mouse osteoblasts. Biochem Biophys Res Commun 256: 284-287, 1999

13. Yamaguchi D, Takeuchi K, Furuta H, Miyamae S, Murakami H and Hattori M. Gene expression in response to low frequency pulsed ultrasound treatment of bone marrow cells under osteogenic conditions in vitro. J Hard Tissue Biol 25: 137-148, 2016

14. Ohgushi H, Doi H, Katada T, Tamai S, Tabata S and Suwa Y. In vitro bone formation by rat marrow cell culture. J Biomed Mater Res 32: 333-340, 1996

15. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S and Marshak DR. Multilineage potential of adult human mesenchymal stem cells. Science 284: 143-147, 1999

16. Jones EA, Kinsey SE, English A, Jones RA, Strasznyski L, Meredith DM, Markham AF, Jack A, Emery P and McGonagle D. Isolation and characterization of bone marrow multipotential mesenchymal progenitor cells. Arthritis Rheum 46: 3349-3360, 2002

17. Pestka S, Krause CD and Walter MR. Interferons, interferon-like cytokines, and their receptors. Immunol Rev 202: 8-32, 2004

18. Adams DO. Molecular interactions in macrophage activation. Immuno Today 10: 33-35, 1989

19. Schroder K, Hertzog J, Ravasi T and Hume DA. Interferon-gamma: an overview of signals, mechanisms and functions. J Leukoc Biol 75: 163-189, 2004

20. Duque G, Huang DC, Dion N, Maccarone M, Rivas D, Li W, Yang XF, Li J, Lian J, Marino FT, Barralet J, Lascau V, Deschênes C, Ste-Marie LG and Kremer R. Interferon-γ plays a role in bone formation in vivo and rescues osteoporosis in ovariectomized mice. J Bone Miner Res 26: 1472-1483, 2011

21. Farrell PJ, Broeze RJ and Lengyel P. Accumulation of an mRNA and protein in interferon-treated Ehrlich ascites tumour cells. Nature 279: 523-525, 1979

22. Nicholl MJ, Robinson LH and Preston CM. Activation of cellular interferon-responsive genes after infection of human cells with herpes simplex virus type 1. J Gen Virol 81: 2215-2218, 2000

23. Desai SD, Mao Y, Sun M, Li TK, Wu J and Liu LF. Ubiquitin, SUMO-1, and UCRP in camptothecin sensitivity and resistance. Ann NY Acad Sci 922: 306-308, 2000

24. Zhu H, Cong JP and Shenk T. Use of differential display analysis to assess the effect of human cytomegalovirus infection on the accumulation of cellular RNAs: induction of interferon-responsive RNAs. Proc Natl Acad Sci 94: 13985-13990, 1997

25. Bluyssen HA, Vliestra RJ, van der Made A and Trapman J. The interferon-stimulated gene 54 K promoter contains two adjacent functional interferon-stimulated response elements of different strength, which act synergistically for maximal interferon-α inducibility. Eur J Biochem 220: 395-402, 1994

26. Kakuta S, Shibata S and Iwakura Y. Genomic structure of the mouse 2',5'-oligoadenylate synthetase gene family. J Interferon Cytokine Res 22(9): 981-993, 2002

27. Pulit-Penaloza JA, Scherbik SV and Brinton MA. Activation of Oas1a gene expression by type I IFN requires both STAT1 and STAT2 while only STAT2 is required for Oas1b activation. Virology 425: 71-81, 2012

28. Luster AD, Unkeless JC and Ravetch JV. Gamma-interferon transcriptionally regulates an early-response gene containing homology to platelet proteins. Nature 315: 672-676, 1985

29. Dufour JH, Dziejman M, Liu MT, Leung JH, Lane TE and Luster AD. IFN-γamma-inducible protein 10 (IP-10; CXCL10)-deficient mice reveal a role for IP-10 in effector T cell generation and trafficking. J Immunol 168: 3195-3204, 2002

30. Angiolillo AL, Sgadari C, Taub DD, Liao F, Farber JM, Maheshwari S, Kleinman HK, Reeman GH and Tosato G. Human interferon-inducible protein 10 is a potent inhibitor of angiogenesis in vivo. J Exp Med 182: 155-162, 1995

31. Nakao J, Fujii Y, Kusuyama J, Bandow K, Kakimoto K, Ohnishi T and Matsuguchi T. Low-intensity pulsed ultrasound (LIPUS) inhibits LPS-induced inflammatory responses of osteoblasts through TLR4-MyD88 dissociation. Bone 58: 17-25, 2014

32. Erlebacher A, Filvaroff EH, Gitelman SE and Derynck R, Toward a molecular understanding of skeletal development. Cell 80: 371-378, 1995

33. Barone LM, Owen TA, Tassinari MS, Bortell R, Stein GS and Lian JB. Developmental expression and hormonal regulation of the rat matrix Gla protein (MGP) gene in chondrogenesis and osteogenesis. J Cell Biochem 46: 351-365, 1991

34. Murphy G. Tissue inhibitors of metalloproteinases. Genome Biol 12 (11): 233, 2011

35. Pufte T, Harde V, Petersen W, Goldring MB, Tillmann B and Mentlein R. Vascular endothelial growth factor (VEGF) induces matrix metalloproteinase expression in immortalized chondrocytes. J Pathol 202: 367-374, 2004

36. Morikawa M, Derynck R and Miyazono K. TGF-β and the TGF-β family: context-dependent roles in cell and tissue physiology. Cold Spring Harb Perspect Biol 8: a021873, 2016

37. Yang Z, Ren L, Deng F, Wang Z and Song J. Low-intensity pulsed ultrasound induces osteogenic differentiation of human periodontal ligament cells through activation of bone morphogenetic protein-smad signaling. J Ultrasound Med 33: 865-873, 2014
