〈Original Article〉

Humoral response to therapeutic low-intensity pulsed ultrasound (LIPUS) treatment of rat maxillary socket after the removal of a molar tooth

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Summary LIPUS treatment of the socket of removed first molar tooth from the upper jaws of retired breeder rats induced neovascularization at the wound site. In addition, LIPUS treatment transiently but significantly increased the blood flow rate at remote sites while transiently reducing the socket flow rate. *In vivo* preincubation with the EP4 receptor antagonist abrogated the LIPUS-induced reduction in socket flow rate, whereas the EP3 antagonist did not. Topical application of PGE_2 to the socket also transiently reduced socket flow rate, whereas topical PGE_2 to intact gingival epithelium did not. Moreover, increased CXCR4-positive tibia bone marrow cells without increased CXCL12 staining suggested the possibility that PGE_2 released from the socket was responsible, at least in part, for the effects of LIPUS through the circulation directly affecting bone marrow cells. LIPUS exerts humoral effects on remote tissues through factor(s) released by mechanically stimulated cells in the healing oral tissues.

Key words: LIPUS, Prostaglandin E_2, Socket healing, CXCR4, Angiogenesis

1. Introduction

Low-intensity pulsed ultrasound (LIPUS) has been found to be a potent therapeutic tool that accelerates many steps of fracture healing[1-3]. These effects are mediated in part through COX2, as we previously showed in COX2 KO mice, in which the eliminated anabolic LIPUS effect was restored by injection of EP2 and EP4 agonists[4]. These effects have also been associated with alterations in the expression of

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vascular endothelial growth factor (VEGF) via a mechanism mediated by prostaglandin E_2 (PGE_2), accelerating the healing of fractures in mouse and rat femurs[4, 5].

LIPUS was shown to have site specific effects on mechanotransduction of bone cells, in that the behavior of mandibular osteoblasts differed from the behavior of osteoblasts derived from long bones and calvaria[6]. Mandibular bone remodeling has also shown site specificity, likely due to different functional demands that reflect occlusion[7]. The effects of LIPUS in oral environments, in which the immune system remains constitutively active to protect cells from oral microbiomes, however, have not been well explored. This *in vivo* study was designed to determine the responses to LIPUS treatment of tooth sockets following the removal of first molar teeth and some surrounding tissue from aged female rats.

2. Materials and methods

The study protocol was approved by the institutional Animal Care Committee of Kanagawa Dental University, Yokosuka, Japan. All procedures involving animals were in accordance with the guidelines for the Care and Use of Laboratory Animals (ministerial notification No. 71 by the Minister of Education, Culture, Sports, Science and Technology of Japan, 2006).

1. Reagents

The EP antagonists L-161,982 and L-798,106 were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). PGE_2 was from Wako Chemical Company (Tokyo, Japan). Anti-CXCR4 antibody AB2074 was from Abcam PLC (Cambridge, UK), anti-CXCL12 antibody was from Affimetrix Inc (Santa Clara, CA), anti-VEGF (147) and anti-CD34 (C-18) antibodies were from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Alexa Fluor 488 and 546 conjugated to F(ab')2 secondary antibodies were from Molecular Probes (Eugene, OR, USA).

2. Removal of rat maxillary first molar teeth

Right maxillary first molars (M1) were removed from 10 female retired breeder Wistar rats (Japan SLC Inc., Hamamatsu, Japan), in 2 groups, LIPUS treated rats and mock treated rats. Because the excessive cementum apposition on the roots in these aged rats deters extraction, the M1 molars were removed using a dental air-turbine (Portable Unit-7G; Osada Inc., Japan) and a diamond point (MaryDia F-010XL; Hinatawada Seimitsu MFG, Co. Ltd., Tokyo, Japan. Operations were performed under chloral hydrate anesthesia. Sufficient water was provided to cool the diamond point and excess water was vacuumed.

3. LIPUS treatment

The custom-built sonic-accelerated fracture-healing system for animals (Teijin Pharma Limited, Japan) included transducers 13-mm in diameter, providing US at a frequency of 1.5 MHz and a repetition frequency of 1 kHz, with pulse burst width of 200 μs and intensity of 30 mW/cm^2. Starting the day after M1 removal, the rats were anesthetized daily with isoflurane and a LIPUS probe was attached to the smooth shaven cheek of each rat using high viscosity gel, with clay used to direct the probe toward the socket. The rats were placed onto a warm plate set at 37℃, and the M1 sockets of five rats were exposed to LIPUS for 20 min every 24 hours. Five control rats received mock treatment of daily anesthesia for the same duration but without LIPUS probes.

4. Blood flow measurements

Blood flow on days 3, 7, 11 and 14 after surgery was measured before and after LIPUS or mock treatment. A TBF-LN1 laser Doppler flowmeter (Unique Medical Co., Ltd., Tokyo, Japan) with a 2.0 mm probe was placed at the surfaces of the M1 socket, the center of the tail and the dorsum of the foot. In a separate set of experiments, three rats were injected 20 min before LIPUS treatment with a sufficient amount of the EP4 receptor antagonist L-161,982 or the EP3 antagonist, L-798,106 to yield a concentration of 1 μM, assuming a blood volume of 15 mL through the tail vein. As controls, either 100 μL 1.0% DMSO was injected or 2 nmol PGE_2 was topically applied.

5. Immunohistochemical analysis

After LIPUS treatment for 2-weeks, isolated tissue pieces were trimmed and fixed with 4% PFA for two days, placed in 10% sucrose and 20% sucrose for more than 2 days each and into Super Cryo Embedding Medium (SCEM; Leica Microsystems Japan; Tokyo, Japan), and frozen at -103℃ in a UT-2000F cooling unit (Tokyo Rikakikai, Co., Ltd; Tokyo, Japan), which uses as coolant a solution of equal volumes of hexane and isopentane. In a cryomicrotome (CM3050S, Leica Microsystems GmbH, Wetzlar, Germany), the sample block surface was covered with an adhesive thin plastic Cryofilm Ⅱ (9) (Leica Microsystems) and serial 3-μm thick frozen undecalcified sections were cut and placed on the films. The sections on the films were fixed and stored in 70% ethanol at -20℃. Except for VEGF staining, samples were decalcified with 10% formic acid for 10 min prior to immunostaining. The sections were washed three times with phosphate-buffered saline (PBS), treated with 0.1% Triton X-100/PBS for 10 min, washed another 3 times with PBS, and incubated for 2 h at room temperature with primary antibodies against VEGF (1:50), CD34 (1:50), CXCL12 (1:100) and CXCR4 (1:1,000), all diluted in blocking PBS solution containing 1.5% normal goat serum and 0.05% Triton X-100. The sections were washed with PBS and subsequently incubated for 1 h with

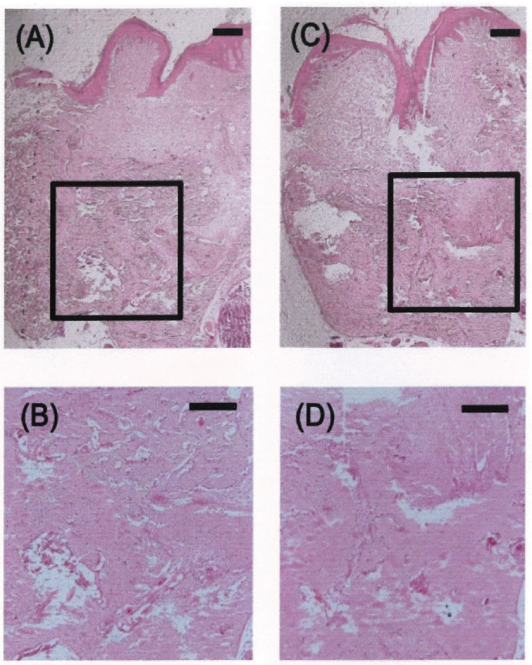

Fig. 1 Histologic morphology of healing sockets with regenerated gingival epithelium after LIPUS treatment for 2 weeks. A: LIPUS treated rat socket at the original magnification of x4. B: LIPUS treated rat socket at the original magnification of x10. C: mock treated rat socket at the original magnification of x4. D: mock treated rat socket at the original magnification of x10. Hematoxylin and eosin staining was performed with 3- μm thick sections after decalcification with 10% formic acid. Squared areas in (A) and (C) were magnified in (B) and (D). Scale bars = 300 μm.

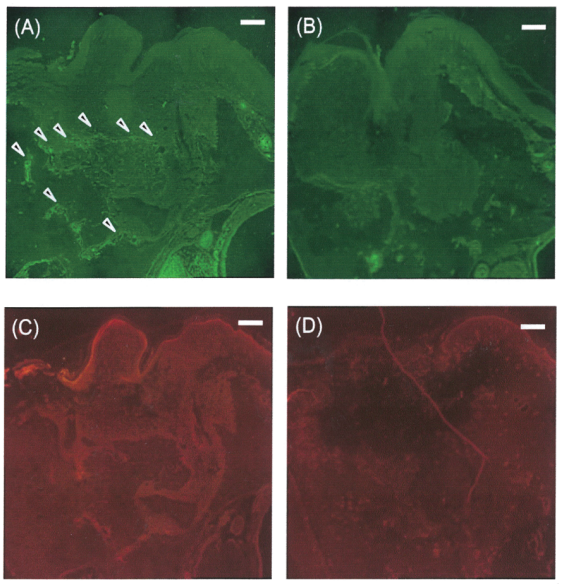

Fig. 2 Profiles of CD34 and VEGF immunohistochemical staining of healing sockets after LIPUS treatment for two weeks. A: Socket tissue sections from a LIPUS-treated rat to detect endothelial cells, and B: from a mock-treated rat. C: Socket tissue sections from a LIPUS-treated rat to detect secreted VEGF, and D: from a mock-treated rat. Sections were decalcified and incubated with anti-CD34 primary antibody and Alexa 488 conjugated secondary antibody in (A) and (B) or anti-VEGF primary antibody and Alexa 546 conjugated secondary antibody without decalcification in (C) and (D). Arrowheads indicate the areas of intense staining. Scale bars = 300 μm.

fluorochrome-conjugates (Alexa 488 and 546) of the respective secondary antibodies, diluted 1:1,000 in blocking PBS solution. The samples were viewed using an AxioImager Z1 microscope (Carl Zeiss AG, Oberkochen, Germany) at the appropriate excitation wavelength depending on the fluorochrome. As negative controls, sections were prepared without primary antibodies.

6. Statistical analysis

Baseline characteristics and changes over time after LIPUS exposure were compared using Mann Whitney U-test. All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA), with p values < 0.05 defined as statistically significant. Data are presented as the mean±SD.

3. Results

1. Histological evaluation of LIPUS effect on peripheral blood vessel formation.

Hematoxylin and eosin stained sections of M1 tooth sockets showed granulation tissue after exposure to LIPUS for 2-weeks (Fig. 1). Areas particularly rich in blood vessels were observed between the granulation tissue and alveolar bone, areas in the healing socket can be reached by LIPUS pulses of 30 mW/cm^2. LIPUS increased the density of vessels possibly the width as well (Fig. 1B). LIPUS-associated neovascularization was also observed by fluorescence with anti-CD34 antibody (Fig. 2A). A similar area, as well as the granulation tissue adjoining the area of CD34 positivity, was highly stained by anti-VEGF antibody (Fig. 2C). Little staining was observed when primary antibodies were omitted, followed by staining with Alexa488 and 546 conjugated secondary antibodies against goat or rabbit IgG (data not shown).

2. LIPUS exposure increased blood flow rate at the wound site

Baseline flow rates were measured at three anatomic sites prior to the daily LIPUS/mock treatment (Fig. 3). Baseline flow rates in M1 sockets were significantly higher on days 7 and 14 in LIPUS-exposed than in control rats. A similar trend was seen on day 11, but not on day 3. Baseline flow rates in the intact gingiva before tooth removal (day 0) near the M1 tooth were significantly higher than after tooth removal on days 3, 7, 11 and 14.

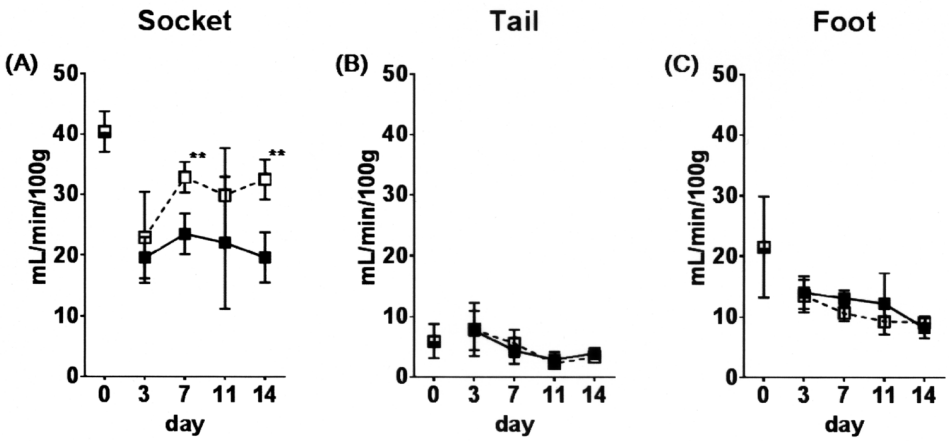

Fig. 3 Effect of daily LIPUS treatment for two weeks on baseline blood flow rates. A: Socket baseline flow rates in the LIPUS (open squares) and mock treated (filled squares) groups. A half-filled square represents the blood flow rate at the epithelium near the first molar gingiva prior to removal of the tooth. Blood flow rates were significantly higher in the LIPUS group rats after 7 but not 3 days. B: Baseline blood flow rates in rat tails and C: Blood flow rates in rat feet similarly presented as in the sockets (A). All data are presented as the mean ± SD. Double asterisks represent p <0.01 by Mann Whitney U-test.

3. Transient effect of LIPUS exposure on blood flow rates

Flow rates over time in the M1 socket immediately after LIPUS treatment for 20 min on days 7, 11 and 14 are shown in Fig. 4A. Five min after the end of LIPUS treatment, flow rates were slightly lower than at baseline. The flow rate further declined until 15 to 20 min after treatment, returning to near-basal levels after about 1 h. Mean actual flow rates 20 min after treatment are shown in Fig. 4B. Starting on day 7, but not on day 3, blood flow rate was significantly lower in the socket after than before treatment. The blood flow rate in the foot became significantly higher from day 3 on. Significance was seen on days 11 and 14.

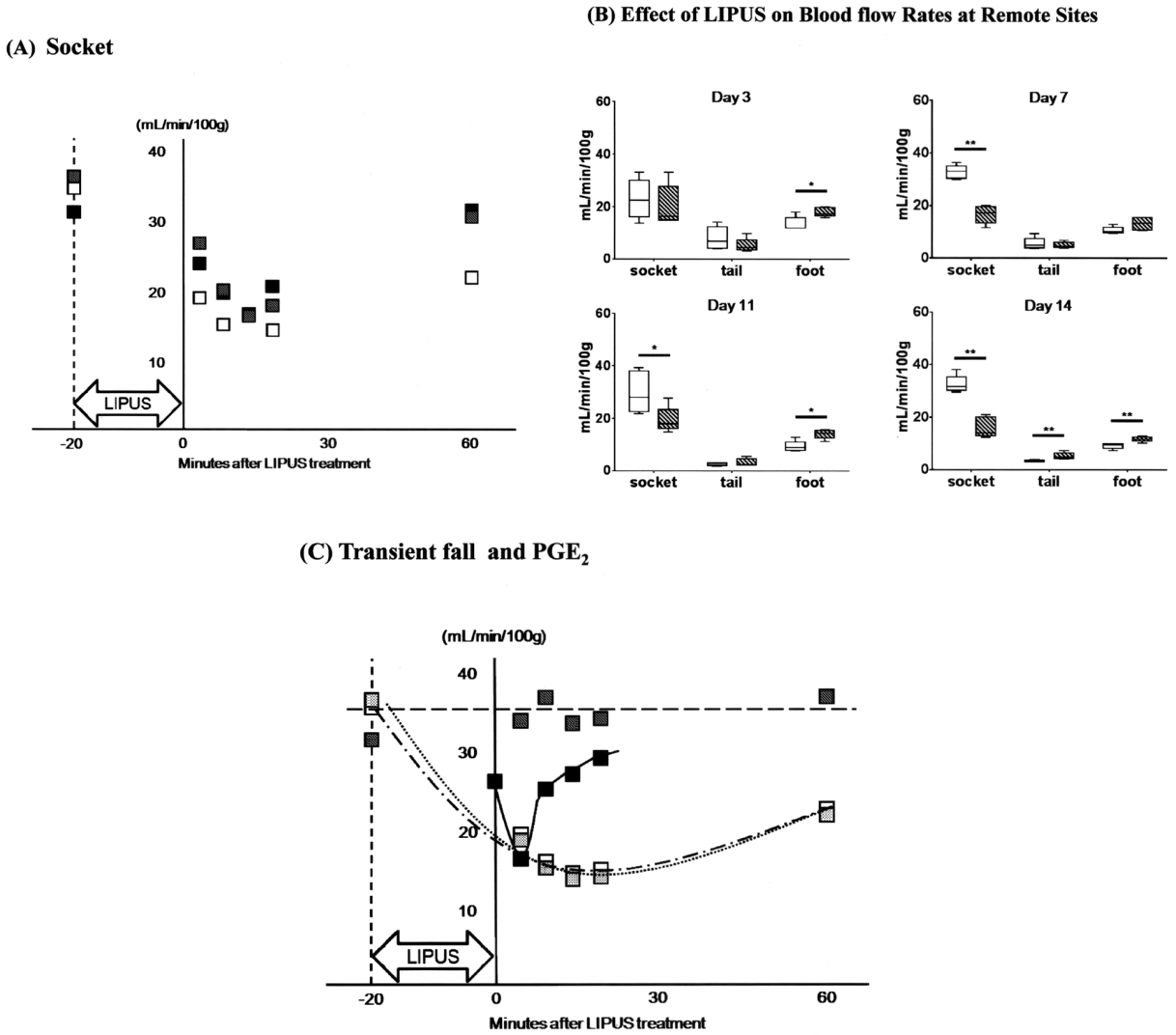

Fig. 4 Transient effect of LIPUS on blood flow rates after LIPUS treatment for 20 min. A: Typical time course profiles of LIPUS treated rats on days 7 (filled squares, #1 rat), 11 (lined squares, #2 rat) and 14 (open squares, #6 rat). Baseline values were measured before LIPUS treatment. B: Effect of LIPUS treatment on blood flow rates 20 min after LIPUS exposure for 20 min on post-operational days 3, 7, 11 and 14. Flow rates in the socket, tail, and foot 20 min after treatment (lined squares) were compared with baseline rates just prior to treatment (open squares). Data are presented as the mean ± SD. Asterisks represent $*p < 0.05$ and $**p < 0.01$ by Mann Whitney U-test. C: Effects of LIPUS on socket blood flow rate on day 14 in rats administered the EP3 antagonist L-798,106 (dotted squares with dotted line) or the EP4 antagonist L-161,982 (lined squares with dashed line) 20 min before LIPUS treatment relative to untreated rats (open squares and dashed dotted line), and effect of topical application of 2 μL 1 mM PGE_2 (2 nmol) on socket epithelium of a LIPUS group rat on day 14 (filled squares, solid lines).

The blood flow rate in the tail, which is also remote from the wound/LIPUS exposure site, tended to be elevated and was significant on day 14. Injection of the EP4 receptor antagonist L-161,982 prior to LIPUS treatment completely abrogated the effects of LIPUS treatment, whereas the EP3 receptor antagonist L-798,106 had no effect, suggesting that the effects of LIPUS are dependent on PGE_2 (Fig. 4C). In contrast, topical application of PGE_2 to socket epithelium mimicked the effects of LIPUS by reducing the blood flow rate in the socket.

4. Effect of LIPUS on the CXCL12-CXCR4 axis of cell recruitment from tibial bone marrow

Levels of CXCR4 and CXCL12 proteins were assessed immunochemically in bone marrow cells (BMCs) of the sagittal sections of tibias derived from LIPUS-treated and control rats as in Fig. 5. In Fig. 5A, the equivalent area depicted in Fig. 5B, C, D and E was framed in a film section of proximal half of the whole tibia. With CXCR4 antibody, more intense staining was detected in LIPUS-treated rat bone marrow (B vs. C). No significant difference was seen with CXCL12 antibody (D vs. E).

4. Discussion

Results show that LIPUS induced neovascularization and increased the density and possibly the width of peripheral blood vessels in the wound site of sockets from which teeth had been removed. Immunohistochemical analysis showed increased CD34 and VEGF staining, indicating that LIPUS had angiogenic effects in the socket. LIPUS exposure increased the baseline blood flow rate in the socket, suggesting that LIPUS increased the width of peripheral blood vessels in the socket. Elevated blood flow was not observed in the tail or the foot, indicating that the effects of socket LIPUS exposure did not alter the blood vessels at distant sites. LIPUS exposure also transiently reduced blood flow rate in the socket, beginning after 7 days of LIPUS exposure, accompanied by increased blood flow rates at remote sites such as the feet. Pretreatment with EP receptor antag-

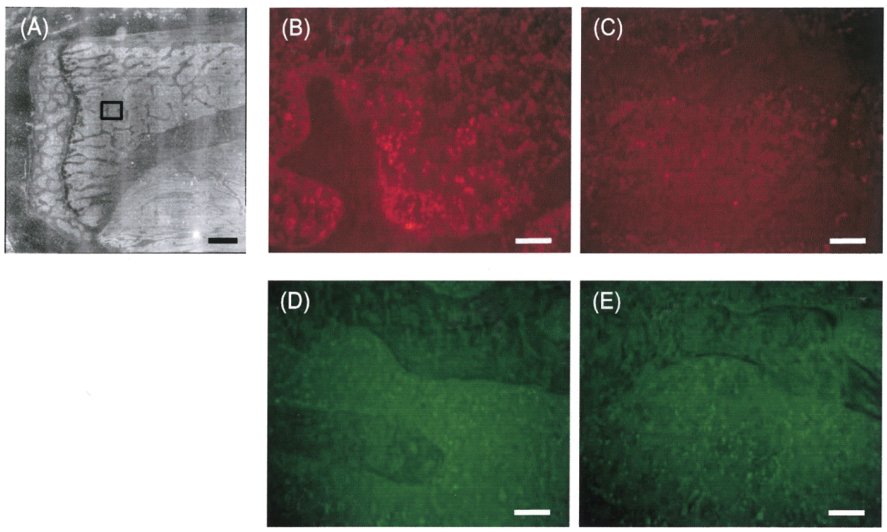

Fig. 5 Profiles of CXCR4 immunohistochemical staining of tibial bone marrow after LIPUS treatment for two weeks. A: sagittal section of the proximal end of tibia prepared on a film as described in Materials and Methods. B: Equivalent area to that in the box in (A) was tested for BMC recruitment in the LIPUS-treated rat. C: Equivalent area to that in the box in (A) was tested for BMC recruitment in the control rat. Tissue sections were stained with anti-CXCR4 primary antibody and Alexa 546 conjugated secondary antibody after decalcification. Scale bar = 1 mm in (A) and 100 μm in (B) to (E). Note that more cells with intense staining were seen in (B) than in (C). On the other hand, sections stained with anti-CXCL12 primary antibody and Alexa 488 conjugated secondary antibody after decalcification in (D) and (E) did not give any significant difference.

onists indicated that LIPUS promoted the angiogenesis through PGE_2, which elevates blood flow rates[8]. Endothelial cells derived from circulating cells may be a subset of cells mobilized from bone marrow, which circulate in peripheral blood and participate in neovascularization at remote sites[9, 10]. During angiogenesis, PGE_2 binds to specific receptors on bone marrow-derived cells, stimulating these cells and significantly increasing their differentiation and migration, as well as significantly upregulating CD31 (34) and von Willebrand factor, both markers of differentiation to endothelial cells[11]. We have reported that COX2 plays an essential role in the effects of LIPUS, by upregulating VEGF during fracture healing, inasmuch as EP2 and EP4 agonists restored the effects of LIPUS, which was eliminated in COX2 KO mice[4]. PGE_2 involvement in the effects of LIPUS were also shown by the ability of LIPUS to restore delayed union of fractures in aged wild type mice[4], in good agreement with findings showing that PGE_2 treatment induced the proliferation of massive progenitor cells in aged skeletons[12]. Mechanisms responsible for age-associated impairments in angiogenesis are thought to include age-related endothelial dysfunction and reduced VEGF expression. Advanced age, however, does not preclude augmentation of collateral vessel development in response to exogenous angiogenic cytokines[13] in support of the efficacy of LIPUS.

While mechanical stimulation of cells at the wound site may also induce other as-yet-undetermined molecules which propagate effects to tissues remote from the healing oral tissues, PGE_2 has long been reported to stimulate the proliferation of hematopoietic stem cells and progenitor cells[14, 15]. Animal and human cells exposed in vitro and *ex vivo* to 16-16 dimethyl PGE_2 ($dmPGE_2$), a long-acting analog of PGE_2 showed enhanced hematopoietic colony formation and/or engraftment[16-18]. Moreover, $dmPGE_2$ treatment of hematopoietic stem and progenitor cells has been found to stabilize the transcription factor HIF1α which, in turn, increases the expression of CXCR4, a key enhancer of stem and progenitor cell homing and engraftment in the CXCL12 (SDF-1)-CXCR4 axis[19]. Moreover, HIF1α is one of the mediators induced by LIPUS[20]. Speth *et al.* showed that PGE_2 enhances hematopoietic stem and progenitor cell homing primarily by increasing CXCR4 expression through the specific hypoxia response element, HRE, which is required for CXCR4 upregulation[19]. In our result of increased CXCR4-positive cells we observed in the remote tibial bone marrow of LIPUS treated rats in Fig. 5, circulating PGE_2, which can increase blood flow in foot, may also have induced CXCR4 expression resulting in CXCR4-positive hematopoietic stem and progenitor cells in tibia bone marrow. In fact, PGE_2 concentrations in plasma drawn from rat tails 20 min after LIPUS treatment were 3 to 4 times higher than the values before tooth removal and those of mock-treated rats (data not shown). Moreover, the LIPUS effect on fracture healing in CXCL12 (SDF-1)-CXCR4 axis was conceived by Kumagai et al., as transport of osteogenic progenitor cells (CXCR4-positive) to the fracture site (CXCL12-positive) by systemic circulation[21]. In parabiotic green fluorescent protein (GFP) mouse with wild type mouse, whose femur was fractured, compared to the vehicle-treated wild type mouse, LIPUS-treated wild type mouse showed significantly more GFP cells, apparently recruited from the adjacent GFP animal. At 2 weeks post-operation, the LIPUS treated fracture site was populated with CXCR4-positive GFP cells, which were virtually absent in control fractures. CXCR4 is expressed on the surface of recruited cells and is the receptor for chemokine CXCL12 (SDF-1), which is significantly increased in the LIPUS treated fracture site[21]. A key role of CXCL12 (SDF-1) in the homing of CXCR4-positive circulating cells to sites of tissue repair was reported[22-24], but in the present study we did not detect increased CXCL12 in the bone marrow of foot where CXCR4-positive cell were increased.

Acknowledgments

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