ARTICLE
Journal of Cellular Biochemistry 111:1445–1452 (2010)

Journal of Cellular Biochemistry

Low-Level Laser Irradiation Enhances BMP-Induced Osteoblast Differentiation by Stimulating the BMP/Smad Signaling Pathway

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ABSTRACT

Low-level laser irradiation (LLLI) has been shown to induce bone formation and osteoblast differentiation both in vivo and in vitro. However, the molecular mechanism by which LLLI stimulates osteoblast differentiation is still unclear. The aim of the present study was to examine whether Ga-Al-As laser irradiation could enhance BMP2-induced alkaline phosphatase (ALP) activity in C2C12 cells. Laser irradiation at 0.5 W for 20 min enhanced BMP2-induced ALP activity. Laser treatment alone did not affect ALP activity. To exclude the effect of pH or temperature changes during irradiation, we shortened the exposure time to 2 min, with various levels of laser power. At 2.5 W, irradiation stimulated BMP2-induced ALP activity but not cell proliferation, whereas 1 or 5 W laser power did not induce any significant effects. Irradiation stimulated BMP2-induced phosphorylation of Smad1/5/8 and BMP2 expression, but had no effect on the expression of inhibitory Smads 6 and 7, BMP4, or insulin-like growth factor 1. Laser irradiation enhanced Smad-induced Id1 reporter activity as well as expression of bone morphogenetic protein (BMP)-induced transcription factors such as Id1, Osterix, and Runx2. Laser irradiation also stimulated BMP-induced expressions of type I collagen, osteonectin, and osteocalcin mRNA, markers of osteoblasts. This enhancement of BMP2-induced ALP activity and Smad phosphorylation by laser irradiation was also observed in primary osteoblasts. These results suggest that LLLI accelerates the differentiation of BMP-induced osteoblasts by stimulating the BMP/Smad signaling pathway. J. Cell. Biochem. 111: 1445–1452, 2010.

KEY WORDS: LOW-LEVEL LASER; OSTEOBLASTS; BMP; SMAD PHOSPHORYLATION,

Low-level laser irradiation (LLLI) has been widely investigated for over 30 years [Moshkovska and Mayberry, 2005; Gavish et al., 2006]. LLLI using visible light around 650 nm has been reported to offer numerous benefits in clinical practice, including pain relief [Walker, 1983], regeneration of severed nerves [Rochkind et al., 1986; Anders et al., 1992], anti-inflammation [Honmura et al., 1992; Shimizu et al., 1995], and wound healing [Mester et al., 1985; Conlan et al., 1996], and is used in many medical fields, including orthopedics and dentistry [Ross and Ross, 2008]. The recently developed diode laser, which delivers near infrared wavelengths between 750 and 950 nm, provides better tissue penetration than the helium–neon (HeNe) visible laser and offers new potential targets for

Grant sponsor: Kyushu Dental College Internal Grants; Grant sponsor: Kyuchiren Grants.

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Received 8 June 2010; Accepted 30 August 2010 • DOI 10.1002/jcb.22872 • © 2010 Wiley-Liss, Inc.
Published online 9 September 2010 in Wiley Online Library (wileyonlinelibrary.com).

LLLI, such as soft tissue healing and bone metabolism [Ozawa et al., 1998; Schubert et al., 2007]. LLLI has proven to be a safe and effective therapeutic option in clinical and histologic trials; however, a great deal of skepticism remains regarding the efficacy of this treatment when applied at the clinical level [Ross and Ross, 2008; De Felice, 2010].

The stimulatory effect of LLLI has been confirmed in vitro in numerous cell lines. In osteoblast-like cells isolated from fetal rat calvariae, LLLI stimulated proliferation and differentiation, inducing expression of alkaline phosphatase (ALP), osteopontin (OP), and bone sialoprotein [Ueda and Shimizu, 2003]. Similar results were obtained in cultured human SaOS-2 cells, where early induction of ALP, type I collagen (ColI), and OP was observed in cells irradiated with a 670 nm diode laser [Stein et al., 2005, 2008]. Moreover, LLLI treatment of human osteoblast-like cells cultured on titanium induced both proliferation and differentiation in a dose-dependent manner through increased osteocalcin and transforming growth factor (TGF)-β1 production [Khadra et al., 2005]. LLLI also promotes bone repair in vivo [Barushka et al., 1995] and stimulates bone formation by increasing osteoblast activity and decreasing osteoclast number [Ninomiya et al., 2007]. However, the molecular mechanism by which laser irradiation stimulates osteoblast differentiation is not well understood.

Bone morphogenetic proteins (BMPs), members of the TGF-β superfamily, are known to be essential for the stimulation of bone formation processes such as osteoblastogenesis and osteoblast secretion of bone matrix protein. These activities are mediated by morphogen interactions with two types of serine/threonine kinase receptors [Massagué et al., 2005; Katagiri et al., 2008] and the activation of the Smad signaling pathway. Of the BMPs, BMP2, 4, 6, 7, and 9 have been shown to be the most potent inducers of osteoblast differentiation in mesenchymal stem cells. Importantly, the combination of BMP2 with an absorbent, USA, collagen sponge was recently approved by the US Food and Drug Administration for clinical use in spine fusion, repair of orthopedic trauma, and oral maxillofacial surgery [McKay et al., 2007]. However, the available clinical data supporting the efficacy of this treatment are not all robust [Kusumoto et al., 2002; Vaidya et al., 2008], and further studies will be needed to develop a method to accelerate bone formation and osteoblastogenesis as well as to understand the variable effects of BMP2 on different cell types, including adult stem cells, osteoblasts, and osteocytes.

The purpose of this study was to examine the effects of irradiation by a Ga-Al-As laser on BMP2-induced ALP activity in C2C12 cells, which differentiate into osteoblasts upon treatment with BMP2 [Katagiri et al., 1994], as well as primary osteoblasts (POBs), and to develop a method to enhance BMP-induced osteoblast differentiation by laser irradiation. We also assessed whether irradiation induced changes in the BMP signaling pathway in these cells.

MATERIALS AND METHODS

REAGENTS

Purified recombinant human BMP2 was kindly provided by Wyeth Pharmaceuticals (Madison, NJ). Anti-phosphorylated Smad1/5/8 (#9511), Smad1 (#9743), and Smad4 (#9514) antibodies were obtained from Cell Signaling (Beverly, MA) and anti-β-actin (AC-15) was purchased from Sigma–Aldrich (St. Louis, MO).

CELL CULTURE

C2C12 cells, a mouse myoblast cell line, and POBs from 1-day-old *ddY* mouse calvariae were, respectively, maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma–Aldrich) containing 15% FBS and antibiotics (100 units/ml of penicillin and 100 mg/ml of streptomycin) (Sigma–Aldrich) and α-minimal essential medium (α-MEM) containing 5% FBS and antibiotics in a humidified atmosphere of 5% CO_2 at 37°C.

LASER IRRADIATION

A low-energy Ga-Al-As laser apparatus (P-LASER; Matsushita, Inc., Osaka, Japan) with a continuous wavelength (CW) of 805 nm was used in this study. The total energy density of the spot was expected to be 2.0–12.0 J/cm^2 as measured by a laser power meter (Ophir Optronics Ltd. Jerusalem, Israel), according to previous reports [Ozawa et al., 1998; Hamajima et al., 2003].

DETECTION OF ALP ACTIVITY

C2C12 cells were seeded at a density of 1.0×10^4 cells/well in 6-well plates and cultured in DMEM containing 5% FBS for 1 day before treatment. The cells were then continuously irradiated with a Ga-Al-As laser at either (i) 0.5 W, duty 20%, for 20 min, (ii) various levels of laser power (W) at CW for 2 min, or (iii) left unirradiated. Afterwards, the cells were cultured for an additional 3 days in the presence or absence of BMP2 (100 ng/ml). The cells were fixed with an acetone/ethanol mixture (50:50, v/v) and incubated with a substrate solution (0.1 M diethanolamine, 1 mM $MgCl_2$, and 10 mg/ml *p*-nitrophenyl phosphate). The reaction was terminated by the addition of 5 M NaOH, and the absorbance was measured at 405 nm using a microplate reader (Bio-Rad, Hercules, CA). All experiments were performed in triplicate. Histochemical analysis of ALP activity was also carried out. Irradiated cells with or without BMP2 were stained using a mixture of 0.1 mg/ml naphthol AS-MX phosphate and 0.6 mg/ml Fast Blue BB salt and observed by light microscopy.

PROLIFERATION ASSAY

Proliferation of C2C12 cells was measured using the Cell Counting Kit 8 (Dojin, Kumamoto, Japan). C2C12 cells were left unirradiated or irradiated with a Ga-Al-As laser at various levels of laser power (W) at CW for 2 min, then cultured for an additional 3 days in the presence or absence of BMP2 (100 ng/ml). At the end of this incubation period, sodium 2-(4-iodophenyl)-3-(4-nitrophenyl)-5 -(2,4-disulfophenyl)-*2H*-tetrazolium was added to the culture medium, and the plates were incubated for an additional hour at 37°C. The amount of reduced tetrazolium was determined by measuring the absorbance at 450 nm in a microplate reader (iMark; Bio-Rad Laboratories, Tokyo, Japan).

LUCIFERASE ASSAY

The IdWT4F(Id1)-luciferase reporter plasmid expresses a luciferase protein under the control of a BMP-responsive element (4× 5′-CATGGCGACCGCCCGCGCGGCGCCAGCCT-3′) from the human *Id1* gene [Katagiri et al., 2002]. The Id1-luciferase reporter was

transfected into C2C12 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total amounts of transfected plasmids in each group were equalized by the addition of empty vector. After irradiation in the presence or absence of BMP2, luciferase activity was measured using a dual luciferase reporter assay system (Promega, Madison, WI).

WESTERN BLOT ANALYSIS

The cells were lysed in buffer containing 1% Triton X-100 and a mixture of 1× protease inhibitor and phosphatase inhibitor (Roche Diagnostics) in 25 mM Tris–HCl and 150 mM NaCl, pH 7.5. Western blot analysis was performed using whole-cell lysates. Equal amounts of protein from each lysate were separated by 10% SDS–polyacrylamide gel electrophoresis and blotted onto an Immobilon-P Transfer membrane (Millipore Corp., Bedford, MA). Blotted membranes were blocked in PBS containing 5% bovine serum albumin and incubated with antibodies. The membranes were then incubated with the secondary antibodies. The immunoreactive proteins were visualized using ECL (Amersham, Piscataway, NJ).

REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR)

C2C12 cells (1.0×10^4 cells/well) were incubated in 6-well plates for 24 h and then either irradiated with laser or left unirradiated in the presence or absence of BMP2 for the indicated periods. Total RNA from the cells was prepared with Trizol (Invitrogen) and amplified by Superscript II and Taq polymerase (Invitrogen). Primer sequences were as follows: Id1: 5′-TCCTGCAGCATGTAATCGAC-3′ (forward) and 5′-GAGAGGGTGAGGCTCTGTTG-3′ (reverse); ALP: 5′-ACTG-CTGATCATTCCCACGTT-3′ (forward) and 5′-GAACAGGGTGCGTA-GGGAGA-3′ (reverse); glyceraldehyde 3-phosphate dehydrogenase (GAPDH): 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′ (forward) and 5′-CATGTAGGCCATGAGGTCCACCAC-3′ (reverse). The PCR program was as follows: 30 cycles at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min for ALP, and Id-1; 25 cycles at 94°C for 40 s, annealing at 60°C for 40 s, and extension at 72°C for 1 min for GAPDH. The PCR products were separated by electrophoresis on 1.0% agarose gels and visualized by ethidium bromide staining under ultraviolet light illumination. The relative intensities of ALP, Id1, osteocalcin, and GAPDH mRNA expression levels were calculated using an NIH image analyzer.

REAL-TIME PCR ANALYSIS

Real-time PCR was performed using SYBR Green PCR master mix with the 7300 Real-time PCR system (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. Samples were matched to a standard curve generated by amplifying serially diluted products under the same PCR conditions. GAPDH expression served as an internal control. Primer sequences are shown in Table I.

STATISTICAL ANALYSIS

Statistical significance was determined using the Student's *t*-test. A *P*-value of less than 0.05 is considered significant. The data are expressed as mean ± SEM.

RESULTS

LASER IRRADIATION ENHANCED BMP2-INDUCED ALP ACTIVITY IN C2C12 CELLS

We first examined the effect of laser irradiation on BMP2-induced ALP activity in C2C12 cells. Based on previous reports [Ozawa et al., 1998; Hamajima et al., 2003], cells were exposed to laser at 0.5 W for various time periods (0–30 min). There was no difference in ALP activity between non-irradiated cells and irradiated cells in the absence of BMP2 treatment (Fig. 1A). BMP2 induced an approximately twofold increase in ALP activity compared to non-treated cells. When the cells were treated with laser irradiation at 0.5 W for 20 min together with BMP2, ALP activity was significantly increased compared to BMP2-treated cells that had not undergone laser irradiation, with the maximal activity occurring after a 20-min exposure (Fig. 1A). However, to exclude the effects of pH and temperature changes during exposure, we shortened the exposure time to 2 min, with various levels of laser power. Laser irradiation at 2.5 W and CWs for 2 min (5.90 J/cm^2) enhanced BMP2-induced ALP activity in a fashion similar to laser irradiation at 0.5 W, duty 20%, for 20 min, whereas 1 (2.36 J/cm^2) or 5 W (11.79 J/cm^2) laser power did not induce any significant effects (Fig. 1A–C). Laser irradiation did not affect proliferation of C2C12 cells (Fig. 1D), suggesting that single exposures to different energy densities did not cause significant cellular damage. Cells were exposed at 2.5 W, CW for 2 min in the subsequent experiments.

To determine whether laser irradiation enhances BMP-2-induced ALP activity through changes at the transcriptional level or in the kinase activity itself, we performed RT-PCR analysis on treated cells using mouse ALP primers. Laser irradiation in combination with

TABLE I. Mouse Primer Sequences for Real-Time PCR

Target gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Nucleotide
Runx2	gccgggaatgatgagaacta	ggaccgtccactgtcacttt	451–651
Osterix	ggaggcacaaagaagccata	gggaagggtgggtagtcatt	124–279
BMP2	gctccacaaacgagaaaagc	agcaaggggaaaaggacact	819–997
BMP4	cctggtaaccgaatgctgat	agccggtaaagatccctcat	7–267
IGF-1	tggatgctcttcagttcgtg	cttcagtggggcacagtaca	128–292
Smad6	gaccagtacaagccactactgga	cctactcaacaaccacagat	858–1,467
Smad7	tgttgctgtgaatcttacgggaag	atggacgggtttcaccgtgca	532–1,167
Type I collagen	ccaacaagcatgtctggttaggag	gcaatgctgttcttgcagtggta	3,826–3,991
Osteonectin	gcatgcgtgactggctcaa	aagtctcgggccaacagctc	800–962
Osteocalcin	aagcaggagggcaataaggt	tttgtaggcggtcttcaagc	158–313
GAPDH	aactttggcattgtggaagg	acacattgggggtaggaaca	493–716

Runx2, runt-related transcription factor 2; BMP, bone morphogenetic protein; IGF-1, insulin-like growth factor 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

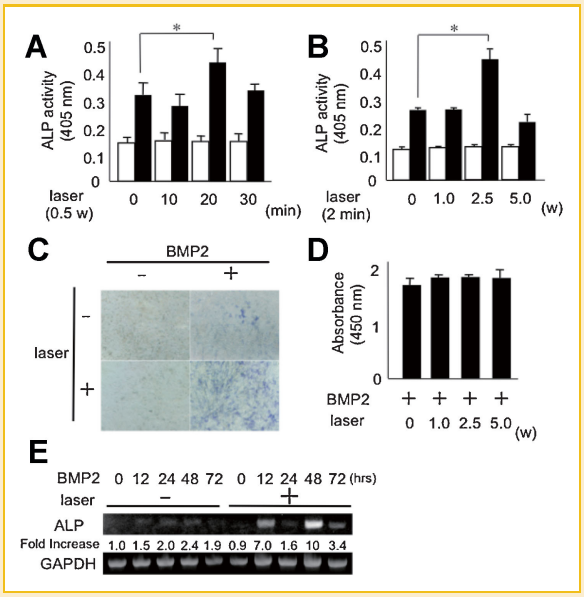

Fig. 1. Laser irradiation enhanced BMP2-induced ALP activity in C2C12 cells. A: C2C12 cells were cultured in DMEM containing 5% FBS for 1 day before treatment then continuously irradiated with a Ga-Al-As laser at 0.5 W, duty 20%, for the indicated periods or left unirradiated. Cells were then cultured for an additional 3 days in the presence (closed column) or absence (open column) of BMP2 (100 ng/ml). The cells were fixed with an acetone/ethanol mixture (50:50, v/v) and incubated with a substrate solution (0.1 M diethanolamine, 1 mM $MgCl_2$, 10 mg/ml *p*-nitrophenyl phosphate). ALP activity was then determined. Data are means ± SEM (n = 3). $^*P < 0.05$ (B) C2C12 cells were irradiated with a Ga-Al-As laser at various levels of laser power (W), CW for 2 min or left unirradiated, then cultured for an additional 3 days in the presence (closed column) or absence (open column) of BMP2 (100 ng/ml). ALP activity was then determined. Data are means ± SEM (n = 3). $^*P < 0.01$ (C) C2C12 cells were irradiated with a Ga-Al-As laser at 2.5 W, CW for 2 min or left unirradiated, then cultured for an additional 3 days in the presence or absence of BMP2 (100 ng/ml). Cells were stained for ALP activity. D: C2C12 cells were irradiated with a Ga-Al-As laser at various levels of laser power (W), CW for 2 min, then cultured for an additional 3 days in the presence of BMP2 (100 ng/ml). Cell proliferation was assessed. Data are means ± SEM (n = 3). E: C2C12 cells were irradiated at 2.5 W, CW for 2 min with the Ga-Al-As laser or left unirradiated in the presence of BMP2 for the indicated periods. Total RNA was isolated, and expression levels of ALP and GAPDH mRNA were measured by RT-PCR analysis. Numbers below the gels represent n-fold increases in the intensity of ALP relative to the corresponding GAPDH signals. Similar results were obtained in three independent experiments.

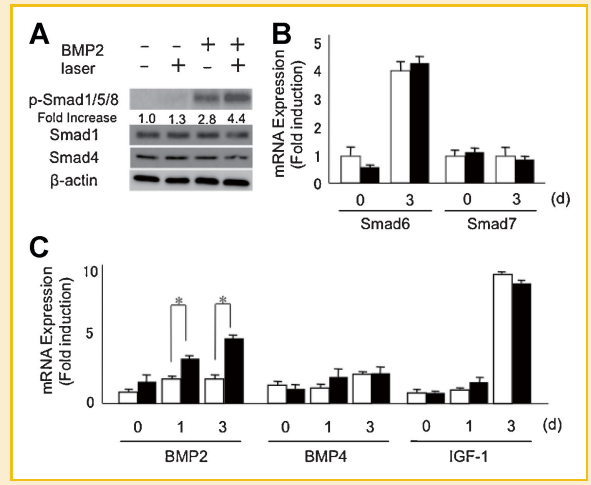

Fig. 2. Laser irradiation enhanced BMP2-induced phosphorylation of Smad1/5/8 and BMP2 expression, but not expression of Smad6, Smad7, BMP-4, or IGF-1. A: C2C12 cells were irradiated with a Ga-Al-As laser at 2.5 W, CW for 2 min, or left unirradiated, then cultured for an additional 30 min in the presence or absence of BMP2 (100 ng/ml). Total cell lysates were immunoblotted with anti-phosphorylated Smad1/5/8, Smad1, or Smad4 antibodies, and anti-β-actin was used as a loading control. Numbers below the gels represent n-fold increases in the intensity of phosphorylated Smad1/5/8 relative to the corresponding Smad1 signals. $^*P < 0.01$ (B) C2C12 cells were irradiated with the Ga-Al-As laser at 2.5 W, CW for 2 min (closed column), or left unirradiated (open column), then cultured for an additional 3 days in the presence of BMP2 (100 ng/ml). Total RNA was isolated and the expression levels of Smad6 and Smad7 relative to GAPDH were measured by quantitative real-time PCR analysis. Data are means ± SEM of Smad6,7/GAPDH (n = 3). Similar results were obtained in three independent experiments. C: C2C12 cells were irradiated with the Ga-Al-As laser at 2.5 W, CW for 2 min (closed column), or left unirradiated (open column), then cultured in the presence of BMP2 (100 ng/ml) for the indicated periods. Total RNA was isolated and the expression levels of BMP2, BMP4, and IGF-1 relative to GAPDH were measured by quantitative real-time PCR analysis. Data are means ± SEM of osteogenic genes/GAPDH (n = 3).

BMP2 strongly induced ALP expression, suggesting that laser irradiation modulates BMP2-induced ALP mRNA up-regulation at the transcriptional level (Fig. 1E).

LASER IRRADIATION ENHANCED BMP2-INDUCED PHOSPHORYLATION OF SMAD 1/5/8 AND BMP2 EXPRESSION, BUT NOT EXPRESSION OF SMAD6, SMAD7, BMP4, OR IGF-1

Phosphorylation of Smad1/5/8 upon BMP stimulation is a key step in BMP signal transduction [Massagué et al., 2005; Katagiri et al., 2008]. As shown in Figure 2A (upper panel), Smad1/5/8 phosphorylation was significantly higher in the cells irradiated at 2.5 W, CW for 2 min in the presence of BMP2 than in non-irradiated BMP2-treated cells. Expression of Smad1 and Smad4 was not affected (Fig. 2A second and third panels). To exclude the possibility that laser irradiation suppresses the expression of inhibitory Smads, we examined Smad6 and Smad7 expression levels after BMP2 treatment with or without laser irradiation. Laser irradiation did not affect the expression of either Smad6 or Smad7 in the absence of BMP2 treatment (Fig. 2B). BMP2 induced approximately fourfold increase in Smad6 but not Smad7 expression compared to non-treated cells, but laser irradiation did not affect BMP2-induced Smad6 expression (Fig. 2B).

Certain reports have suggested that laser irradiation stimulates bone formation by inducing the expression of not only insulin-like growth factor 1 (IGF-1) [Shimizu et al., 2007] but also BMP ligands and Smad proteins [Matsui et al., 2008]. Therefore, we examined the expression levels of BMP2, BMP4, and IGF-1. Laser irradiation in combination with BMP2 enhanced BMP2-induced BMP2 expression in comparison with BMP2 treatment alone at day 1 and day 3 (Fig. 2C). BMP2 treatment resulted in a significant up-regulation of IGF-1 expression by day 3, but this effect was not enhanced by laser irradiation. Neither BMP2 nor BMP2 together with laser irradiation affected BMP4 expression during the culture period (Fig. 2C).

LASER IRRADIATION ENHANCED BMP2-INDUCED EXPRESSION OF BMP/SMAD-RESPONSIVE TRANSCRIPTION FACTORS AND OSTEOBLAST DIFFERENTIATION MARKERS

Id1 is known to be a BMP-responsive gene, and, accordingly, its promoter region contains a BMP-responsive element [Katagiri et al., 2002]. C2C12 cells transfected with an Id1-luciferase plasmid were treated with laser irradiation or left unirradiated in the presence or absence of BMP2. Although Id1-luciferase activity was increased in BMP2-treated cells relative to controls, laser irradiation together with BMP2 further stimulated Id1-luciferase activity compared with BMP2 alone (Fig. 3A). Consistent with these results, laser irradiation enhanced BMP2-induced Id1 mRNA expression (Fig. 3B). We next examined whether laser irradiation affected the expression of BMP/Smad-induced transcription factors Osterix and Runx2 [Lee et al., 2000; Nakashima et al., 2002]. The combined treatment significantly increased Osterix expression compared with BMP2 alone (Fig. 3C). At day 1, Runx2 expression levels were similar in cells that received BMP2 alone or in combination with irradiation, but the combination group showed a significant increase in expression by day 3 (Fig. 3D). Although Osterix has been shown to be downstream of Runx2 in osteoblasts, Osterix expression was induced before Runx2 induction. These results suggest that laser irradiation enhances Osterix expression through the activation of preexisting Runx2 in C2C12 cells.

Upon differentiation into osteoblasts, the cells express proteins related to their stage of differentiation at high levels, such as ALP, ColI, osteonectin, parathyroid hormone receptor, bone sioloproteins, and osteocalcin [Aubin, 2001]. We therefore measured the mRNA levels of ColI, osteonectin, and osteocalcin. The expressions of ColI, osteonectin, and osteocalcin were significantly higher in cells treated with BMP2 and irradiated compared to cells treated with BMP2 alone (Fig. 3E).

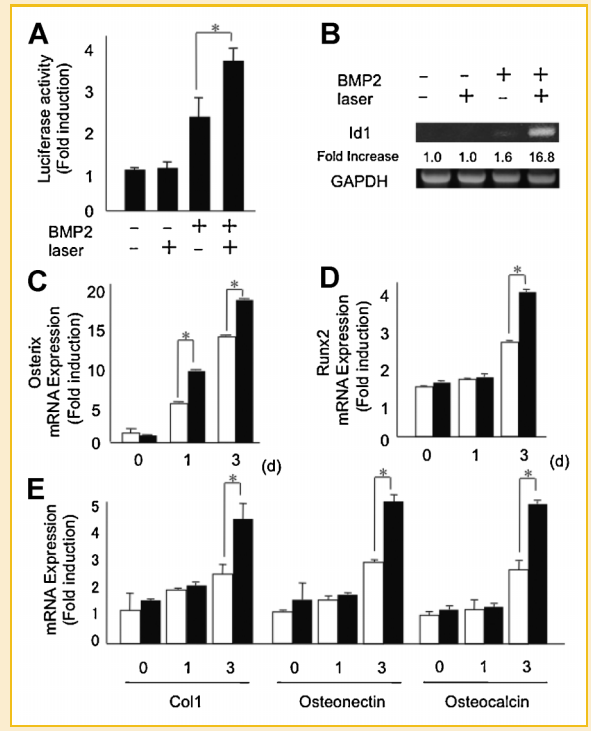

Fig. 3. Laser irradiation enhanced BMP2-induced expression of BMP/Smad-responsive transcription factors and osteogenesis-related genes. A: C2C12 cells were transiently transfected with an Id1-luc reporter and then continuously irradiated with a Ga-Al-As laser at 2.5 W, CW for 2 min, or left unirradiated, then cultured for an additional 24 h in the presence or absence of BMP2 (100 ng/ml). Cells were assayed for luciferase activity after 24 h. $^{*}P < 0.01$ (B) C2C12 cells were irradiated with the Ga-Al-As laser at 2.5 W, CW for 2 min or left unirradiated, then cultured in the presence or absence of BMP2 for 12 h. Total RNA was isolated, and mRNA levels of Id1 and GAPDH were measured by RT-PCR analysis. Numbers below the gels represent n-fold increases in the intensity of Id1 relative to the corresponding GAPDH signals. Similar results were obtained in three independent experiments. C and D: C2C12 cells were irradiated with the Ga-Al-As laser at 2.5 W, CW for 2 min (closed column), or left unirradiated (open column), then cultured in the presence of BMP2 (100 ng/ml) for the indicated periods. Total RNA was isolated, and the mRNA levels of Osterix (C), Runx2 (D), and GAPDH were measured by quantitative real-time PCR analysis. Data are means ± SEM of Osterix or Runx2 relative to GAPDH ($n = 3$). Similar results were obtained in three independent experiments. E: C2C12 cells were irradiated with the Ga-Al-As laser at 2.5 W, CW for 2 min (closed column), or left unirradiated (open column), then cultured in the presence of BMP2 (100 ng/ml) for the indicated periods. Total RNA was isolated and the expression levels of ColI, osteonectin, and osteocalcin relative to GAPDH were measured by quantitative real-time PCR analysis. Data are means ± SEM of osteogenic genes/GAPDH ($n = 3$). Similar results were obtained in three independent experiments.

LASER IRRADIATION STIMULATED BMP2-INDUCED ALP ACTIVITY AND PHOSPHORYLATION OF SMAD1/5/8 IN PRIMARY OSTEOBLASTS

Laser treatments can have different stimulatory effects in different cell types [Walsh, 1997]. To determine whether the stimulatory effect of laser irradiation could be generalized to cell types other than C2C12 cells, we examined the effect of laser irradiation on BMP2-induced osteoblast differentiation using POBs instead of C2C12 cells. The laser irradiation without any stimuli (e.g., β-glycerophosphate and ascorbic acid) did not induce ALP activity even in POBs within 3 days (Fig. 4A). In contrast, laser irradiation enhanced BMP2-induced ALP activity in POBs (Fig. 4A). Therefore, it is likely that POBs are also affected by laser irradiation. In support of this notion, laser irradiation stimulated BMP2-induced phosphorylation of Smad1/5/8 in POBs as well as C2C12 cells (Fig. 4B).

DISCUSSION

In this study, we used C2C12 cells and POBs to examine whether LLLI stimulates BMP2-induced osteoblast differentiation. Our results clearly show that LLLI at 2.5 W, CW for 2 min enhanced BMP2-induced ALP activity in C2C12 cells without affecting proliferation. Interestingly, LLLI stimulated BMP2-induced Smad1/5/8 phosphorylation and BMP2 expression, but not expression of BMP4, IGF-1, or inhibitory Smads. Furthermore, LLLI enhanced the expression of BMP/Smad target genes such as Id1, Osterix, and Runx2 as well as osteogenic genes such as ALP, ColI, osteonectin, and osteocalcin. We also observed enhancement of BMP2-induced ALP activity and Smad phosphorylation by laser irradiation in POBs.

Several lines of evidence indicate that LLLI can induce bone formation and remodeling in an in vivo model of bone defects,

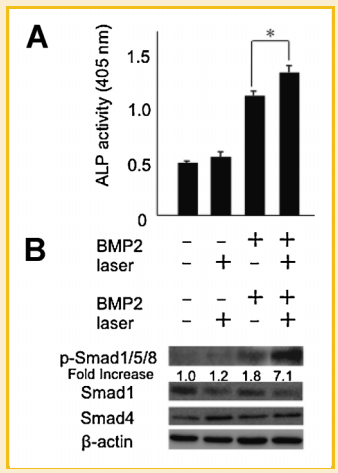

Fig. 4. Laser irradiation stimulated BMP2-induced ALP activity and phosphorylation of Smad1/5/8 in primary osteoblasts. A: Primary osteoblasts were cultured in α-MEM containing 5% FBS for 1 day before the treatment, then continuously irradiated with the Ga-Al-As laser at 2.5 W, CW for 2 min, or left unirradiated. Cells were then cultured for an additional 3 days in the presence or absence of BMP2 (100 ng/ml). The cells were fixed, and ALP activity was determined. B: Primary osteoblasts were irradiated at 2.5 W, CW for 2 min with the Ga-Al-As laser or left unirradiated, then cultured for an additional 1 h in the presence or absence of BMP2 (100 ng/ml). Total cell lysates were immunoblotted with anti-phosphorylated Smad1/5/8, Smad1, or Smad4 antibodies, and anti-β-actin was used as a loading control. Numbers below the gels represent n-fold increases in the intensity of phosphorylated Smad1/5/8 relative to the corresponding Smad1 signals. Similar results were obtained in three independent experiments.

suggesting that LLLI facilitates bone metabolism and accelerates bone formation at defective sites such as bone graft areas and the furcation area in periodontitis [de Almeida et al., 2008]. Furthermore, in vitro studies have also demonstrated that LLLI using a Ga-Al-As laser enhanced β-glycerophosphate and ascorbic acid-induced ALP activity as well as nodule formation by modifying the proliferation [Fukuhara et al., 2006], the expression of IGF-1 [Shimizu et al., 2007], osteocalcin [Saracino et al., 2009], and TGF-β [Khadra et al., 2005], and promoting the synthesis of bone matrix proteins such as osteoglycin [Hamajima et al., 2003]. As induction of ALP activity in primary culture without BMP takes a long time (~7 days) and osteoblastic differentiation occurs sequentially in the culture system via the effects of β-glycerophosphate and ascorbic acid, it is difficult to examine the molecular events that occur during this process. Thus, we examined the role of LLLI in BMP-induced osteoblast differentiation using C2C12 cells. The advantages of this system are that C2C12 cells do not spontaneously differentiate into osteoblasts without BMP stimulation and that the Smad signaling pathway activated by BMP has been thoroughly investigated. We also used lower dose of BMP2 (100 ng/ml) compared with normally used (300 ng/ml) to examine the effect of LLLI. Using this culture system, we found that laser irradiation enhances BMP2-induced osteoblast differentiation by stimulating Smad phosphorylationon.

It seems that the present study contradicts previous work demonstrating increased cell proliferation of osteoprogenitors in response to various LLLI laser powers [Fukuhara et al., 2006; Tuby et al., 2007]. Based on previous works using C2C12 cells, we changed differentiation media (10% FBS) form growth media (15% FBS) before BMP2 treatment to induce osteoblast differentiation form C2C12 cells and BMPs strongly induce osteoblast differentiation rather than cell proliferation [Katagiri et al., 1994; Nakashima et al., 2002]. Thus, LLLI enhances BMP2-induced osteoblast differentiation without affecting cell proliferation.

The design of the LLLI protocol used in this study was based on an extensive review of previous data obtained in vitro in bone cells and in vivo in bone tissue repair [Ninomiya et al., 2007; Shimizu et al., 2007]. Despite the accumulated information about the effects of total energy dose, laser spectrum, energy density, and irradiation phase, it is still unclear which of these parameters has the greatest effect on therapeutic efficacy, even in LLLI, which has shown diverse bio-stimulatory effects [Walsh, 1997]. Although the present study did not compare all of these parameters, the bio-stimulatory effect of laser irradiation at 2.5W, CW for 2 min most effectively enhanced BMP-induced ALP activity compared with power at 1 or 5 W. The reason why laser power at 2.5 W has the greatest effect remains unclear. Several studies have also suggested that bio-stimulatory effects depend on the delivered energy density, which seems to be restricted to a very narrow therapeutic window. Using a 905 nm wavelength on rat fetal calvaria cells, Fukuhara et al. [2006] showed that a single laser exposure of 3.75 J/cm^2 stimulated cell proliferation, whereas energy densities of 1.25 or 6.25 J/cm^2 did not induce any significant effects. LLLI at the energy density of 0.5 J/cm^2 had a stronger stimulatory effect on proliferation of bone marrow stem cells compared with 1, 2, or 5 J/cm^2, without any cytotoxicity [Tuby et al., 2007]. These results support the postulation of Karu [1990], who argued for the existence of "window-specificity" at certain wavelengths and energy dosages.

Laser irradiation stimulated BMP2-induced phosphorylation of Smad1/5/8 in both C2C12 cells and POBs. However, the molecular mechanism by which laser irradiation enhances BMP2-induced phosphorylation of Smad1/5/8 remains unclear. BMP signaling could be modified by laser irradiation in either the extracellular or the intracellular compartment. It is well known that BMP signaling is negatively regulated by the inhibitory Smads (Smad6 and Smad7), the E3 ubiquitin ligases (Smurf1 and Smurf2), and transcriptional corepressors such as c-Ski, SnoN, and Tob [Katagiri et al., 2008]. Among these, we examined the effect of laser irradiation on the expression of Smad6 and Smad7, which directly inhibit Smad1/5/8 phosphorylation by preventing the association between these Smads and BMP receptors. We found that laser irradiation did not affect Smad6 or Smad7 expression levels. Therefore, laser irradiation might modify the affinity and/or avidity of BMPs for BMP receptors or directly modulate the kinase activity of BMP receptors. Further studies will be required to reveal the details of the mechanism by which laser irradiation promotes BMP-induced phosphorylation of Smad.

Some reports have suggested that laser irradiation can stimulate bone formation by inducing the expression of not only IGF-1 [Shimizu et al., 2007] and TGF-β [Khadra et al., 2005], but also BMP ligands and Smad proteins [Matsui et al., 2008]. We observed that LLLI enhanced BMP2-induced levels of BMP2 but not BMP4 or IGF-1 1 and 3 days after irradiation. LLLI also enhanced Id1, Osterix, and Runx2 expression. Although the role of Id1 in osteoblast

differentiation has not yet been clarified, both Osterix and Runx2 are known to be important regulators of osteoblastogenesis [Komori et al., 1997; Nakashima et al., 2002]. Both BMP2-induced BMP2 and Osterix expression might be involved in the biphasic induction of ALP expression. These results suggest that LLLI affects osteoblast differentiation by stimulating multiple steps that are required for the differentiation of osteoblast progenitors into mature osteoblasts. Smad1/5/8 signaling is now generally accepted to be a downstream effector of the BMP signaling pathway and is thus fundamental for osteoblastic differentiation in response to BMP [Katagiri et al., 2008; Jimi et al., 2010]. Therefore, increasing BMP2-induced Smad phosphorylation by laser irradiation might contribute to the phenomenon of increased BMP2-induced osteoblast differentiation after laser treatment.

Taken together, our results indicate that low-level irradiation with a Ga-Al-As laser accelerates BMP2-induced osteoblast differentiation in both C2C12 cells and POBs through the activation of Smad signaling. Our results also suggest that optimized stimulation by LLLI in a clinical setting may facilitate autogenous or BMP2-induced bone formation in areas of defective bone.

ACKNOWLEDGMENTS

We thank Mr. Hiroaki Tokikawa (Panasonic Shikoku Electronics Co., Ltd.) for technical assistance for laser irradiation. This work was supported by a grant-in-aid from Kyushu Dental College Internal Grants (to C. K., H. F., M. T., and E. J.) and a grant-in-aid from Kyuchiren Grants (to H. F.).

REFERENCES

Anders JJ, Borke RC, Woolery SK, Van de Merwe WP. 1992. Low power laser irradiation alters the rate of regeneration of the rat facial nerve. Lasers Surg Med 13:72–82.

Aubin JE. 2001. Regulation of osteoblast formation and function. Rev Endocr Metab Disord 2:81–94.

Barushka O, Yaakobi T, Oron U. 1995. Effect of low-energy laser (He–Ne) irradiation on the process of bone repair in the rat tibia. Bone 16:47–55.

Conlan MJ, Rapley JW, Cobb CM. 1996. Biostimulation of wound healing by low-energy laser irradiation. J Clin Periodontol 23:492–496.

de Almeida JM, Theodoro LH, Bosco AF, Nagata MJ, Oshiiwa M, Garcia VG. 2008. In vivo effect of photodynamic therapy on periodontal bone loss in dental furcations. J Periodontol 79:1081–1088.

de Felice E. 2010. Shedding light: Laser physics and mechanism of action. Phlebology 251:11–28.

Fukuhara E, Goto T, Matayoshi T, Kobayashi S, Takahashi T. 2006. Optimal low-energy laser irradiation causes temporal G2/M arrest on rat calvarial osteoblasts. Calcif Tissue Int 79:443–450.

Gavish L, Perez L, Gertz SD. 2006. Low-level laser irradiation modulates matrix metalloproteinase activity and gene expression in porcine aortic smooth muscle cells. Lasers Surg Med 38:779–786.

Hamajima S, Hiratsuka K, Kiyama-Kishikawa M, Tagawa T, Kawahara M, Ohta M, Sasahara H, Abiko Y. 2003. Effect of low-level laser irradiation on osteoglycin gene expression in osteoblasts. Lasers Med Sci 18:78–82.

Honmura A, Yanase M, Obata J, Haruki E. 1992. Therapeutic effect of Ga-Al-As diode laser irradiation on experimentally induced inflammation in rats. Lasers Surg Med 12:441–449.

Jimi E, Hirata S, Shin M, Yamazaki M, Fukushima H. 2010. Molecular mechanisms of BMP-induced bone formation: Cross-talk between BMP and NF-κB signaling pathways in osteoblastogenesis. Jpn Dent Sci Rev 46:33–42.

Karu TI. 1990. Effects of visible radiation on cultured cells. Photochem Photobiol 52:1089–1098.

Katagiri T, Yamaguchi A, Komaki M, Abe E, Takahashi N, Ikeda T, Rosen V, Wozney JM, Fujisawa-Sehara A, Suda T. 1994. Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. J Cell Biol 127:1755–1766.

Katagiri T, Imada M, Yanai T, Suda T, Takahashi N, Kamijo R. 2002. Identification of a BMP-responsive element in Id1, the gene for inhibition of myogenesis. Genes Cells 7:949–960.

Katagiri T, Suda T, Miyazono K. 2008. The bone morphogenetic proteins. In: Miyazono K, Derynck R, editors. The TGF-β family. New York: Cold Spring Harbor Press. pp 121–149.

Khadra M, Lyngstadaas SP, Haanaes HR, Mustafa K. 2005. Effect of laser therapy on attachment, proliferation and differentiation of human osteoblast-like cells cultured on titanium implant material. Biomaterials 26:3503–3509.

Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T. 1997. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 89:755–764.

Kusumoto K, Bessho K, Fujimura K, Akioka J, Okubo Y, Wang Y, Iizuka T, Ogawa Y. 2002. Osteoinduction by recombinant human bone morphogenetic protein-2 in muscles of non-human primates. J Int Med Res 30:251–259.

Lee KS, Kim HJ, Li QL, Chi XZ, Ueta C, Komori T, Wozney JM, Kim EG, Choi JY, Ryoo HM, Bae SC. 2000. Runx2 is a common target of transforming growth factor beta1 and bone morphogenetic protein 2, and cooperation between Runx2 and Smad5 induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2C12. Mol Cell Biol. 20:8783–8792.

Massagué J, Seoane J, Wotton D. 2005. Smad transcription factors. Genes Dev 19:2783–2810.

Matsui S, Takeuchi H, Tsujimoto Y, Matsushima K. 2008. Effects of Smads and BMPs induced by Ga-Al-As laser irradiation on calcification ability of human dental pulp cells. J Oral Sci 50:75–81.

McKay WF, Peckham SM, Badura JM. 2007. A comprehensive clinical review of recombinant human bone morphogenetic protein-2 (INFUSE Bone Graft). Int Orthop 31:729–734.

Mester E, Mester AF, Mester A. 1985. The biomedical effects of laser application. Laser Surg Med 5:31–39.

Moshkovska T, Mayberry J. 2005. It is time to test low level laser therapy in Great Britain. Postgrad Med J 81:436–441.

Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, de Crombrugghe B. 2002. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 108:17–29.

Ninomiya T, Hosoya A, Nakamura H, Sano K, Nishisaka T, Ozawa H. 2007. Increase of bone volume by a nanosecond pulsed laser irradiation is caused by a decreased osteoclast number and an activated osteoblasts. Bone 40:140–148.

Ozawa Y, Shimizu N, Kariya G, Abiko Y. 1998. Low-energy laser irradiation stimulates bone nodule formation at early stages of cell culture in rat calvarial cells. Bone 22:347–354.

Rochkind S, Nissan M, Razon N, Schwartz M, Bartal A. 1986. Electorophysical effect in He–Ne laser on normal and injured sciatic nerve in the rat. Acta Neurochir 83:125–130.

Ross G, Ross A. 2008. Low level lasers in dentistry. Gen Dent 56:629–634.

Saracino S, Mozzati M, Martinasso G, Pol R, Canuto RA, Muzio G. 2009. Superpulsed laser irradiation increases osteoblast activity via modulation of bone morphogenetic factors. Lasers Surg Med 41:298–304.

Schubert MM, Eduardo FP, Guthrie KA, Franquin JC, Bensadoun RJ, Migliorati CA, Lloid CM, Eduardo CP, Walter NF, Marques MM, Hamdi M. 2007. A phase III randomized double-blind placebo-controlled clinical trial to determine the efficacy of low level laser therapy for the prevention of oral mucositis in patients undergoing hematopoietic cell transplantation. Support Care Cancer 15:1145–1154.

Shimizu N, Yamaguchi M, Goseki T, Shibata Y, Takiguchi H, Iwasawa T, Abiko Y. 1995. Inhibition of prostaglandin E_2 and interleukin 1-β production by low-power laser irradiation in stretched human periodontal ligament cells. J Dent Res 74:1382–1388.

Shimizu N, Mayahara K, Kiyosaki T, Yamaguchi A, Ozawa Y, Abiko Y. 2007. Low-intensity laser irradiation stimulates bone nodule formation via insulin-like growth factor-I expression in rat calvarial cells. Lasers Surg Med 39:551–559.

Stein A, Benayahu D, Maltz L, Oron U. 2005. Low-level laser irradiation promotes proliferation and differentiation of human osteoblasts in vitro. Photomed Laser Surg 23:161–166.

Stein E, Koehn J, Sutter W, Wendtlandt G, Wanschitz F, Thurnher D, Baghestanian M, Turhani D. 2008. Initial effects of low-level laser therapy on growth and differentiation of human osteoblast-like cells. Wien Klin Wochenschr 120:112–117.

Tuby H, Maltz L, Oron U. 2007. Low-level laser irradiation (LLLI) promotes proliferation of mesenchymal and cardiac stem cells in culture. Lasers Surg Med 39:373–378.

Ueda Y, Shimizu N. 2003. Effects of pulse frequency of low-level laser therapy (LLLT) on bone nodule formation in rat calvarial cells. J Clin Laser Med Surg 21:271–277.

Vaidya R, Sethi A, Bartol S, Jacobson M, Coe C, Craig JG. 2008. Complications in the use of rhBMP-2 in PEEK cages for interbody spinal fusions. J Spinal Disord Tech 21:557–562.

Walker J. 1983. Relief from chronic pain by low power laser irradiation. Neurosci Lett 43:339–344.

Walsh LJ. 1997. The current status of low level laser therapy in dentistry. Part 2. Hard tissue applications. Aust Dent J 42:302–306.