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ORIGINAL ARTICLE

Low-level laser therapy induces the expressions of BMP-2, osteocalcin, and TGF-β1 in hypoxic-cultured human osteoblasts

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Abstract The aim of this study was to examine the effect of low-level laser therapy (LLLT) on the cell viability and the expression of hypoxia-inducible factor-1s (HIF-1s), bone morphogenic protein-2 (BMP-2), osteocalcin, type I collagen, transforming growth factor-β1 (TGF-β1), and Akt in hypoxic-cultured human osteoblasts. Human fetal osteoblast cells (cell line 1.19) were cultured under 1 % oxygen tension for 72 h. Cell cultures were divided into two groups. At the experimental side, low-level laser (808 nm, GaAlAs diode) was applied at 0, 24, and 48 h. After irradiation, each cell culture was incubated 24 h more under hypoxia. Total energy was 1.2, 2.4, and 3.6 J/cm^2, respectively. Non-irradiated cultures served as controls. Comparisons between the two groups were analyzed by *t* test; a *p* value <0.05 was considered statistically significant. Hypoxia resulted in a decrease in the expression of type I collagen, osteocalcin, and TGF-β1 ($p<0.001$, $p<0.001$, and $p<0.01$, respectively). Cell viability and BMP-2 expression were not decreased by hypoxic condition. On the other hand, LLLT on hypoxic-cultured osteoblast promoted the expression of BMP-2, osteocalcin, and TGF-β1 ($p<0.05$, $p<0.01$, and $p<0.001$, respectively). Cell proliferation was also increased time-dependently. However, hypoxia decreased in type I collagen expression ($p<0.001$), and LLLT did not affect type I collagen expression in hypoxic-cultured osteoblasts. Furthermore, LLLT inhibited HIF-1 and Akt expression in hypoxic conditioned osteoblasts. We concluded that LLLT induces the expression of BMP-2, osteocalcin, and TGF- β1 in 1 % hypoxic-cultured human osteoblasts.

Keywords Hypoxia · HIF-1 · Oxygen tension · Cell viability · Type 1 collagen

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Introduction

O_2 tension in the environment surrounding bone cells can vary under certain circumstances. Normally, O_2 tension is around 13 % (100 mmHg) in arterial blood, approximately 6 % (45 mmHg) in mixed venous blood, and considerably lower than 6 % in most tissues including bone tissues [1, 2]. Measurements of bone marrow aspirates from normal human volunteer donors yielded mean O_2 tension values of 6.6 % [3].

Lack of oxygen can result in a failure to generate sufficient ATP to maintain essential cellular functions, whereas excess oxygen (hyperoxia) results in the generation of damaging reactive oxygen intermediates [4]. Therefore, regulating constant O_2 tension constantly is an important factor for tissue homeostasis including bone remodeling. However, in the context of a lack of oxygen, there are many circumstances causing hypoxic condition on bone. Hypoxia occurs when the blood supply to tissues is reduced or disrupted and can occur in situations such as: aging [5], inflammation [6],

fractures [7], diabetes [8], and possibly also at bone graft site. Bones are not static but are rather dynamic, and therefore, bone remodeling continuously occurs in these conditions.

Although the data are inconclusive, hypoxia has been shown to have significant effects on bone cell physiology. Some studies have shown that hypoxia increases osteoblast vascular endothelial growth factor (VEGF) [9, 10]. In addition, hypoxia enhances bone morphogenic protein-2 (BMP-2) expression in osteoblasts by hypoxia-inducible factor-1α (HIF-1α) [11], while others have demonstrated decreased osteogenic differentiation [12], cell proliferation [4, 13], and osteocalcin [14].

Hypoxia activates a distinct transcription factor called hypoxia-inducible factor-1 (HIF-1) [15]. Under normoxic condition, HIF-1α undergoes rapid ubiquidation and proteosomal degradation. Under hypoxic conditions, this degradation is inhibited, HIF-1α translocates to the nucleus, dimerizes with HIF-1β, and binds to its consensus sequence within the hypoxia-responsive element, and regulates transcription of VEGF, erythropoietin, and glycolytic enzymes that enhance cellular adaptation to hypoxia [16–18].

Some studies have investigated the effects of low-level laser therapy (LLLT) on osteoblasts or osteoblastic cells. These studies have demonstrated that LLLT promotes proliferation and maturation of human osteoblasts [19], acts as a proliferative stimulus on osteoblast [20], stimulates mineralization, and increases BMP expression [21]. However, these studies that show the effects of LLLT on osteoblasts and osteoblastic cells have been conducted under normoxic condition. In fact, hypoxic condition is readily encountered in clinical practice as previously described. Therefore, our study focuses on the effect of LLLT on osteoblasts under hypoxic condition.

There are many cytokines that are involved in osteoblast differentiation and proliferation. BMP-2 plays an important role in the process of bone formation and remodeling as well as bone development and osteoblast differentiation [22, 23]. Osteocalcin is a marker of mature osteoblasts. Type I collagen is the major organic component of bone matrix and a primary product of the differentiated osteoblast. Additionally, transforming growth factor-βs (TGF-βs) are potent growth regulatory molecules that stimulate osteoblast proliferation and induce the synthesis of collagen, osteocalcin, and other extracellular matrix proteins [24–28]. It has been reported that Akt, a serine–threonine protein kinase, related signaling pathways are involved in osteogenic process [29–31]. We postulated that LLLT affects these cytokines and genes of osteoblast of hypoxic condition.

To test this hypothesis, we investigated the effect of LLLT on human osteoblast cell line in which human fetal osteoblast (hFOB) cells were exposed to 1 % oxygen tension at different time intervals. We first examined whether hypoxia would affect cell viability. Additionally, using real-time PCR and Western blot analysis, we analyzed the effect of hypoxia and LLLT on the expression of BMP-2, TGF-β1, type I collagen, osteocalcin, HIF-1s, and Akt.

Materials and methods

Cell culture

A hFOB 1.19 human osteoblast cell line was purchased from the ATCC (Rockville, MD, USA). This cell line was maintained at 34°C with 5 % CO_2 in air atmosphere in D-MEM/F-12 medium with 4 mM L-glutamine, 1.5 μg/L sodium bicarbonate, 4.5 g/L glucose, and 1.0 mM sodium pyruvate supplemented with 10 % fetal bovine serum.

Hypoxia of cultured osteoblasts

The cells were cultured under 1 % oxygen tension. Cells were seeded on six-well plate (1×10^5 cells) before exposure to hypoxia. Cells were gassed with 95 % N_2 and 5 % CO_2 (Anaerobic System PROOX model 110; BioSpherix) and incubated at 34°C within the chamber for different time intervals (0, 24, 48 and 72 h).

Low-level laser irradiation

Cultures were irradiated with a low-level GaAlAs laser (λ=808±3 nm, 1,000 mW, 80 mA, NDLux, Seoul, Korea). Laser energy was delivered to the culture in continuous mode, with the laser positioned vertically above each well. Irradiation time for each well was 15 s. Cell cultures were divided into two groups. Laser irradiation was applied at 0, 24, and 48 h. After irradiation, each cell culture was incubated 24 h more under hypoxic condition. For example, 72-h cultured samples were irradiated three times (0, 24, and 48 h). Total energy was 1.2, 2.4, and 3.6 J/cm^2, respectively. Control non-irradiated cells were incubated at 0, 24, 48, and 72 h under hypoxia without laser irradiation.

MTT assay

Cells were cultured in a 96-well plate and incubated for 24 h. The cells were exposed to low-level laser irradiation and hypoxic condition for different time intervals. After treatment (laser irradiation), 100 μl of MTT (0.5 mg/ml final concentration) was added and incubated in the dark for an additional 4 h to induce the production of formazan crystals at 37°C, and the supernatants were discarded. The medium was aspirated, and formed formazan crystals were dissolved in DMSO. Cell viability was monitored on an ELISA reader (Sunrise Remote Control, Tecan, Austria) at 570 nm excitatory emission wavelength.

Quantitative reverse transcriptional PCR

Total RNA was extracted from the hFOB cells using Trizol reagent (Invitrogen, Life Technologies, Carlsbad, NM, USA) according to manufacturer's instructions. Total RNA (2 μg) was reverse transcribed using a RevertAid™ First Strand cDNA synthesis kit (Thermo, Fremont, CA, USA) according to the manufacturer's protocols. Real-time PCR was performed on ABI 7500 Fast Real-Time PCR System (Applied Biosystems 7500 System Sequence Detection System (SDS) software version 2.0.1) using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The primers for BMP-2 (forward: 5′-ACC AGG TTG GTG AAT CAG AA-3′ and reverse: 5′-TTT GGC TTG ACG TTT TTC TC-3′), type I collagen (forward: 5′-CGT GGT GTA ACT GGT CCT TC-3′ and reverse: 5′-ACC GGG CTC TCC CTT ATC -3′), osteocalcin (forward: 5′- ATG AGA GCC CTC ACA CTC CT-3′ and reverse: 5′-GGA TTG AGC TCA CAC ACC TC-3′), and TGF- β1 (forward: 5′-TCT TTT GAT GTC ACC GGA GT-3′ and reverse: 5′-CGT GGA GCT GAA GCA ATA GT-3′) were used. GAPDH (forward: 5′-GGA AGG ACT CAT GAC CAC AG-3′ and reverse: 5′-TTG GCA GGT TTT TCT AGA CG-3′) was used as an internal control. The conditions for the PCR were as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s, 60°C for 1 min, and 72°C for 30 s. Real-time PCR data were analyzed by the SDS 2.0.1 software package (Applied Biosystems, Foster, CA, USA).

Western blot assay

Cells were plated at a density of 1×10^5 cells in six-well plates. Cells were washed twice with ice-cold PBS and centrifuged at 2,000 rpm for 10 min. Total cell proteins were lysed with a RIPA buffer [300 mM NaCl, 50 mM Tris–HCl (pH 7.6), 0.5 % Triton X-100, 2 mM PMSF, 2 μg/ml aprotinin, and 2 μg/ml leupeptin] and incubated at 4°C for 1 h. The lysates were centrifuged at 14,000 revolutions per min for 15 min at 4°C, and sodium dodecyl sulfate (SDS) and sodium deoxycholic acid (0.2 % final concentration) were added. Protein concentrations of cell lysates were determined with Bradford protein assay (Bio-Rad, Richmond, CA, USA), and BSA was used as a protein standard. A sample of 50 μg protein from each well was separated and loaded onto 7.5–10 % SDS/PAGE. The gels were transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, UK) and reacted with each antibody. Immunostaining with antibodies was performed using SuperSignal West Pico enhanced chemiluminescence substrate and detected with Alpha Imager HP (Alpha Innotech, San Leandro, USA). Equivalent protein loading was confirmed by Ponceau S staining.

Statistical analysis

Experiments were repeated four to six times. Comparisons between the two groups were analyzed by *t* test. The data were expressed as the mean±standard deviation. Values of $p<0.05$ were considered significant.

Results

Hypoxia does not alter cell viability and LLLT stimulate cell proliferation

The proliferation assay was performed at 0 (immediately), 24, 48, and 72 h; the results are presented in Table 1. Control non-irradiated group has shown that there are increases in cell proliferation time-dependently (48 h, $p=0.0002$ and 72 h, $p=0.0003$). Therefore, hypoxia does not seem to reduce cell viability. In order to assess the effect of LLLT on hypoxic-cultured osteoblast, cells were irradiated at different time intervals (0, 24, and 48 h). At 24 and 72 h, there are significant increases in cell proliferation compared to control non-irradiated group ($p<0.05$ and $p<0.01$, respectively).

BMP-2 expression is increased by LLLT in hypoxic-cultured osteoblasts

To examine the effects of hypoxia and laser irradiation on BMP-2 expression, hFOB cells were exposed to hypoxia and laser irradiation at different time intervals. The results are presented in Table 2 and Fig. 1. Hypoxic condition did not ($p>0.05$) alter BMP-2 expression time-dependently in the non-irradiated group except at the 72-h exposure that showed a slight increase (1.05-fold, $p=0.0440$). However, BMP-2 expression was significantly increased throughout the experiment in the irradiated group compared to the non-irradiated group (0 h, $p<0.05$; 24 h, $p<0.05$; 48 h, $p<0.001$; and 72 h, $p<0.001$, and peak BMP-2 expression was noted at 72 h (2.8-fold).

Table 1 Comparisons between the control group and LLLT group in MTT assay

Cell viability	Control	LLLT	*p* value (control vs LLLT)
0 h	100.2±1.99	99.3±3.98	0.2977
24 h	106.2±8.20	114.0±3.36	0.0294**
48 h	121.4±6.10*	126.2±4.20	0.0743
72 h	137.4±11.96*	162.5±9.04	0.0011***

*$p<0.001$, statistically significant compared with 0-h control; **$p<0.05$, statistically significant compared with hypoxia-only treated group; ***$p<0.01$, statistically significant compared with hypoxia-only treated group

Table 2 Comparisons between the control group and LLLT group in BMP-2 expression

BMP-2	Control	LLLT	*p* value (control vs LLLT)
0 h	1	1.12±0.08	0.0101**
24 h	0.86±0.12	1.13±0.17	0.0216**
48 h	1.11±0.17	1.73±0.15	0.0008***
72 h	1.05±0.04*	2.83±0.11	0.0000***

*$p<0.05$, statistically significant compared with 0-h control; **$p<0.05$, statistically significant compared with hypoxia-only treated group; ***$p<0.001$, statistically significant compared with hypoxia-only treated group

TGF- β1 expression is increased by LLLT in hypoxic-cultured osteoblasts

In order to assess the effect of hypoxia and laser irradiation on TGF-β1 expression in hFOB cells, cells were exposed to hypoxia at 0, 24, 48, and 72 h. The results are presented in Table 3 and Fig. 1. Hypoxia resulted in a marked decrease in TGF-β1 expression at 24, 48, and 72 h compared to 0 h in non-irradiated group ($p=0.0003$, $p=0.0010$, and $p=0.0028$, respectively). TGF-β1 expression remained below baseline levels at 24, 48, and 72 h (46, 54, and 67 %, respectively). In the irradiated group, however, TGF-β1 expression was markedly increased at 24, 48, and 72 h (2.9-, 2.6-, 2.5-fold, respectively) compared to the hypoxic-cultured group at the same time intervals ($p<0.001$, $p<0.001$, and $p<0.001$, respectively).

Hypoxia decreases type I collagen expression, and laser irradiation does not affect on type I collagen expression in hypoxic-cultured osteoblasts

To determine the effects of hypoxia and laser irradiation on type I collagen, hFOB cells were cultured under hypoxic condition. In the irradiated group, laser irradiation was done as scheduled. The results are presented in Table 4 and Fig. 1. Hypoxia resulted in a marked decrease in type I collagen expression in both groups throughout the experimental period ($p<0.001$). Type I collagen expression remained below baseline levels at 24, 48, and 72 h (32, 32, and 29 %, respectively).

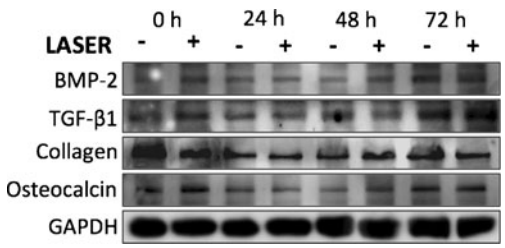

Fig. 1 Immunoblotting for BMP-2, TGF-β1, type I collagen, and osteocalcin in both groups. GAPDH was used as internal control

Table 3 Comparisons between the control group and LLLT group in TGF-β1 expression

TGF-β1	Control	LLLT	*p* value (control vs LLLT)
0 h	1	1.13±0.17	0.0951
24 h	0.45±0.07**	1.31±016	0.0000***
48 h	0.54±0.09*	1.41±0.11	0.0000***
72 h	0.67±0.09*	1.70±0.13	0.0000***

*$p<0.01$, statistically significant compared with 0-h control; **$p<0.001$, statistically significant compared with 0-h control; ***$p<0.001$, statistically significant compared with hypoxia-only treated group

In addition, laser irradiation had little or negative effect on hypoxic-cultured osteoblasts. Although the expression of type I collagen was higher at 24 h in the irradiated group compared to the non-irradiated group ($p<0.01$), type I collagen expression remained lower than in the non-irradiated group for the remainder of the experimental period, at 48 ($p<0.001$) and 72 h ($p<0.001$).

Effect of hypoxia and laser irradiation on osteocalcin expression in osteoblasts

The expressions of osteocalcin under hypoxia and laser irradiation are summarized in Table 5 and Fig. 1. In hypoxia-only condition, osteocalcin expression had decreased time-dependently ($p=0.0000$, respectively). The expression of osteocalcin at 24, 48, and 72 h was 53, 47, and 26 %, respectively, compared to 0 h. However, laser irradiation induced an increase in osteocalcin expression immediately after irradiation (0 h, 1.09-fold, $p<0.05$), and this result remained as irradiation frequency was increased (24 h, 2.4-fold, $p<0.001$; 48 h, 1.5-fold, $p<0.001$; and 72 h, 2.7-fold, $p<0.001$, respectively). Peak expression was noted at 24 h (1.3-fold compared to normoxic condition). Although the expression of osteocalcin was higher in the irradiated group than in the non-irradiated group at 48 and 72 h, absolute expression levels at these intervals were lower than in normoxic control (71 %, respectively).

Table 4 Comparisons between the control group and LLLT group in type I collagen expression

Type I collagen	Control	LLLT	*p* value (control vs LLLT)
0 h	1	1.02±0.07	0.2643
24 h	0.32±0.01*	0.46±0.06	0.0031**
48 h	0.32±0.01*	0.21±0.01	0.0000***
72 h	0.29±0.01*	0.21±0.01	0.0000***

*$p<0.001$, statistically significant compared with 0-h control; **$p<0.01$, statistically significant compared with hypoxia-only treated group; ***$p<0.001$, statistically significant compared with hypoxia-only treated group

Table 5 Comparisons between the control group and LLLT group in osteocalcin expression

Osteocalcin	Control	LLLT	*p* value (control vs LLLT)
0 h	1	1.09±0.05	0.0049**
24 h	0.53±0.02*	1.27±0.04	0.0000***
48 h	0.47±0.01*	0.71±0.06	0.0002***
72 h	0.26±0.01*	0.71±0.05	0.0000***

*$p<0.001$, statistically significant compared with 0-h control; **$p<0.01$, statistically significant compared with hypoxia-only treated group; ***$p<0.001$, statistically significant compared with hypoxia-only treated group

LLLT inhibits HIF-1 expression in hypoxic conditioned osteoblasts

To determine the effect of hypoxia on HIF-1 activation, hFOB cells were cultured under hypoxia for 0, 24, 48, and 72 h. Furthermore, to assess the effect of LLLT on protein levels of HIF-1 in hFOB cells under hypoxic condition, low-level laser irradiated at different time intervals. Our results on the effect of LLLT on HIF-1 expression in osteoblasts that are cultured under hypoxia are shown in Fig. 2. Western blot analysis indicated that hypoxia induced a marked increase in protein levels of HIF-1α time-dependently. In addition, HIF-1β protein levels at 24 h were slightly increased, and markedly increased at 48 and 72 h in the hypoxia-only cultured group. However, in the irradiated group, HIF-1α was not expressed throughout the entire experimental period. Furthermore, like HIF-1α, HIF-1β expression was not increased in the irradiated group.

LLLT inhibits phosphorylation of Akt in hypoxic conditioned osteoblasts

Our results on the effect of LLLT on Akt activation in osteoblasts that are cultured under hypoxia are shown in Fig. 2. Both groups were cultured under hypoxia for 0, 24, 48, and 72 h; one group was irradiated with low-level laser (LLL) at different time intervals. In non-irradiated group, expression of phosphorylated Akt (pAkt) was increased at 48 and 72 h. However, in the irradiated group, pAkt protein levels were not changed time-dependently.

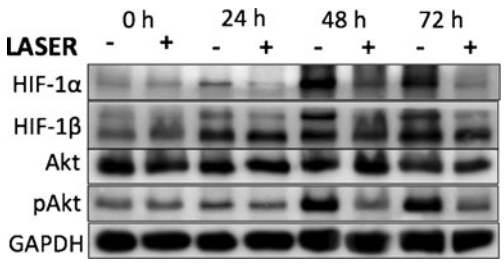

Fig. 2 Immunoblotting for HIF-1α, HIF-1β, Akt, and phosphorylated Akt (pAkt) in both groups. GAPDH was used as internal control

Discussion

Several studies have investigated the effect of hypoxia on osteoblasts and osteoblastic cells [9–14]. However, to our knowledge, the effect of LLLT on osteoblasts under hypoxia has not been described until this paper.

The present study showed that hypoxia reduced the expression of type I collagen, osteocalcin, and TGF-β1 in human osteoblast cells. There are some studies on the contradictory effects of hypoxia on osteoblasts and osteoblastic cells. Warren et al. [32] studied the effect of hypoxia on the gene expression in rat osteoblasts. They demonstrated that hypoxia induced TGF-β1 mRNA and type I collagen mRNA expression, in contrast to our findings. Meanwhile, Park et al. [14] showed that hypoxia reduced the expression of type I collagen and osteocalcin in human osteoblastic cells. In addition, hypoxia reduced the expression of osteocalcin, TGF-β, collagen in rat calvarial osteoblasts in a time-dependent manner [4]. Taking together our results with other investigations and considering the meaning and function of type I collagen, osteocalcin, and TGF-β, we conclude that hypoxia inhibits osteoblast differentiation, maturation, and bone-forming capacity. Interestingly, however, cell viability was not affected by hypoxia in the present study. Previous studies demonstrated that hypoxia inhibits osteoblast proliferation [4] and osteoblastic differentiation [12] and reduces the viability of osteoblastic cells [14]. On the other hand, Salim et al. [33] demonstrated that hypoxia had little effect on osteoblast differentiation. Our results showed that hypoxia does not affect osteoblast survival. Rather, although under hypoxic condition, cell counts increased time-dependently. In addition, our preliminary studies comparing the osteoblast viability between under normoxic and hypoxic condition demonstrated that there were no significant differences in cell viability between the two groups (data not shown). From these results, we conclude that 1 % oxygen tension has no cytotoxicity on human osteoblast cell line.

In contrast to other cytokines and proteins, hypoxia did not alter BMP-2 expression in osteoblasts in the present study. BMP-2 plays an important role in the process of bone formation and remodeling as well as bone development and osteoblast differentiation [22, 23]. In addition, some studies have demonstrated that Akt-related signaling pathways are involved osteoblast differentiation [29–31]. Furthermore, Akt suppresses osteoblast apoptosis [31]. Tseng et al. [11] have investigated the relationship between hypoxia and BMP-2 expression. They demonstrated that hypoxia induced BMP-2 expression via ILK/Akt/mTOR and HIF-1α

pathways in osteoblasts time-dependently. However, our study showed contradictory results. Except at 72 h, hypoxia did not alter BMP-2 expression in the present study. There are differences in culture time intervals between their experiment and our investigation. Therefore, we should consider that altered expression would have been revealed at different time intervals.

However, interestingly, phosphorylation of Akt had occurred throughout the entire experimental period in hypoxic-cultured osteoblasts in our experiment. Therefore, BMP-2 and Akt-related signaling pathways may function normally in osteoblasts exposed to 1 % hypoxia. Aditi and Peter [29] demonstrated that activation of Akt promotes BMP-2-mediated osteoblast differentiation. Taken together, our results suggest that osteoblast could differentiate via BMP-2 expression and Akt-related signaling pathways, even under hypoxia. This hypothesis may explain how osteoblasts can survive and differentiate rather than result in apoptosis in hypoxic condition. However, we also show evidence of delayed osteoblast differentiation in hypoxia through the inhibition of type I collagen, osteocalcin, and TGF-β1 expression. Thus, it seems to suggest that osteoblast can survive and proliferate although in a quiescence state and differentiated slowly in moderate ischemic condition until vascularization occurs or oxygen tension is increased.

It is proposed that biostimulation by LLLT may enhance the osteogenic potential of osteoblasts [34]. In addition, our previous studies have demonstrated that LLLT promote metabolic bone activity and bone remodeling [35, 36]. As the application of hypothesis to hypoxic-cultured osteoblast was unknown, we investigated the effect of LLLT on osteoblasts exposed to 1 % oxygen tension.

In our investigation, in the irradiated group, osteoblasts proliferated more than in the non-irradiated group, especially at 24 and 72 h. Likewise, LLL induced BMP-2 expression in hypoxic-cultured osteoblast time-dependently, and peak expression was noted at 72 h. These results were consistent with previous investigations that LLLT promotes osteoblast proliferation and increases BMP expression [19–21], even under normoxic conditions. Thus, we conclude that LLLT promotes osteoblast proliferation in hypoxic condition. However, the connection between low-level laser and increased BMP-2 expression remains unknown necessitating further study on their exact relationship.

In addition to osteoblast proliferation and BMP-2 expression, TGF-β1 expression in the irradiated group increased over time compared to the non-irradiated group, and expression levels were higher than normoxic 0-h control throughout the experiment. As previously described, BMP-2 induces osteoblast differentiation. Furthermore, TGF-β1 stimulates osteoblast proliferation, production of type I collagen and osteocalcin, a marker of mature osteoblast. From this, we can hypothesize that LLLT on hypoxic-cultured osteoblasts overcomes the inhibitory effect on osteoblasts. However, surprisingly, the peak expression level of type I collagen was noted at 24 h in the irradiated group, and the expression level decreased over time even though the frequency of laser irradiation increased. The expression of osteocalcin also showed similar results. Although expression levels were higher than the non-irradiated group and peak expression was noted at 24 h (1.3-fold) in the irradiated group, absolute expression levels at 48 and 72 h were below baseline. These observations suggest that LLLT on hypoxic-cultured osteoblasts promotes cell differentiation and proliferation via BMP-2 and TGF-β1 expressions. Furthermore, osteoblast maturation would be increased by LLLT at an early stage, and these results could be maintained to some extent compared to the hypoxia-only cultured group.

As shown in our results, hypoxia decreased significantly in type I collagen expression, and LLLT had no effect on its expression. It has been reported that hypoxia decreases collagen synthesis and type I collagen expression in osteoblastic cells and osteoblasts exposed to 1 % oxygen tension [4, 14], while Warren et al. [32] demonstrated that type I collagen expression was increased in short-term cultures. Therefore, hypoxia may have a dual effect in type I collagen expression. Nevertheless, it is interesting that type I collagen expressions were largely unaffected by LLLT in our systems. Hirata et al. [37] have demonstrated that LLLT increases type I collagen expression in osteoblastic cells. In addition, low-level laser stimulates mineralization via increased BMPs in osteoblastic cells [38]. However, in our study, although cell maturation and differentiation rate increased and increased TGF-β1 expression possibly stimulated productions of type I collagen, amounts of collagen synthesis did not increase despite the addition of laser irradiation. Thus, it is reasonable to assume that proper oxygen tension is essential to synthesize type I collagen. Further investigations are needed on hypoxia-related type I collagen synthesis and effect of low-level laser irradiation.

It is well known that hypoxia signaling is mainly affected via HIF-1 activation [15]. As previously described, under hypoxic condition, HIF-1α translocates to the nucleus, dimerizes with HIF-1β, and binds to its consensus sequence. Our data also showed that HIF-1α and 1β were expressed immediately 24 h after hypoxic incubation, and expression levels were increased as culture time was extended. In the irradiated group, however, HIF-1s were only slightly expressed at 48 h; we did not find strong HIF-1 activation during the entire experimental period. In addition to HIF-1s, phosphorylated Akt (pAkt) was not expressed in the irradiated group. It has been reported that low-level laser has a biostimulative effect. Furthermore, some laboratories have observed the formation of reactive oxygen species (ROS) in cells in vitro after LLLT [39, 40]. In studies on the relationship between Akt and HIF-1, Tseng et al. [11]

demonstrated that Akt pathway regulates HIF-1 expression and activity in osteoblasts. Likewise, Kanichai et al. [41] stated that hypoxia induces a rapid increase in Akt phosphorylation and a subsequent stabilization of HIF-1α in rat mesenchymal stromal cells.

Taken together, we hypothesize that LLLT can generate ROS even though in hypoxic-cultured osteoblast and hypoxic condition were camouflaged by generated ROS. However, how LLLT affects the interaction between HIF-1 and Akt in hypoxic-cultured osteoblast remains unknown. Thus, further investigation is needed.

In conclusion, hypoxia did not affect osteoblast viability in vitro, although it resulted in a decrease in the expression of type I collagen, osteocalcin, and TGF-β1. These findings provide evidence that hypoxia has a negative effect on differentiation and bone-forming capacity of human osteoblast. In contrast, LLLT on hypoxic-cultured osteoblast stimulates osteoblast differentiation and proliferation through increased expression of BMP-2, osteocalcin, and TGF-β1. However, the effect of LLLT on extracelluar matrix synthesis and HIF-related signaling in hypoxic osteoblast remains unclear. Further investigations are required to evaluate the possible mechanisms that may explain the response of hypoxic-cultured osteoblast to LLLT.

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