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ORIGINAL ARTICLE

Increased cell proliferation and differential protein expression induced by low-level Er:YAG laser irradiation in human gingival fibroblasts: proteomic analysis

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Abstract Erbium-doped yttrium aluminum garnet (Er:YAG) laser treatment has demonstrated favorable wound healing effect after periodontal therapy. One of the reasons may be the positive biological effect of the low-level laser on the irradiated tissues, although the mechanism remains unclear. The aim of this study was to investigate the effect of low-level Er:YAG laser irradiation on cell proliferation and laser-induced differential expression of proteins in human gingival fibroblasts (HGFs) by proteomic analysis. In the first experiment, HGFs were exposed to low-level Er:YAG laser irradiation and the laser-induced cell proliferation and damage were evaluated on day 3. In the second experiment, proteomic analysis was performed on day 1 after irradiation. The peptides prepared from HGFs were analyzed by a hybrid ion trap-Fourier transform mass spectrometer, Mascot search engine, and UniProtKB database. A significant increase in cell proliferation without cell damage after irradiation was observed. Among the total identified 377 proteins, 59 proteins, including galectin-7, which was associated with the process of wound healing, were upregulated and 15 proteins were downregulated in laser-treated HGFs. In the third experiment, the increase in messenger RNA (mRNA) and protein expression of galectin-7 in the irradiated HGFs was validated by various analytical techniques. In addition, the effect of recombinant human galectin-7 on the modulation of HGFs proliferation was confirmed. The results indicate that low-level Er:YAG laser irradiation can promote HGF proliferation and induce a significant change in protein expression and the upregulation of galectin-7 expression may partly contribute to the increase in cell proliferation.

Keywords Er:YAG laser · Galectin-7 · Gingival fibroblasts · Low-level laser therapy · Proteomics

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Introduction

Periodontitis is a chronic inflammatory disease caused by bacterial infection and affects the tissues around the teeth, eventually resulting in tooth loss [1]. In conventional periodontal treatment, mechanical tools such as hand curettes and power-driven scalers are mainly used for subgingival debridement in periodontal pockets to arrest the progress of the disease. However, the mechanical therapy cannot always completely remove the bacterial and calcified deposits from the diseased root surfaces and thus may cause the recurrence

of inflammatory periodontal diseases in the presence of remaining bacteria [2].

Recently, various types of lasers, e.g., erbium-doped: yttrium aluminum garnet (Er:YAG) laser, have been applied as an adjunct therapy or alternative to the mechanical treatment to remove subgingival biofilms from periodontal pockets [3]. Among those dental lasers, Er:YAG laser is considered as one of the safest and most promising laser devices in periodontal therapy [4, 5]. So far, favorable results of periodontal wound healing following Er:YAG laser treatment have been reported [3, 6, 7]. Several previous clinical studies indicated that compared with conventional treatments, Er:YAG laser irradiation applied to periodontal therapy led to significant short- or long-term improvement in periodontal tissue healing [3, 6].

This favorable result may be attributed to the advantageous properties of the Er:YAG laser over the conventional mechanical methods in terms of complete ablation of diseased tissues and decontamination and detoxification of the diseased sites [4, 5]. Furthermore, the biostimulation effect of lasers may also contribute to the improved wound healing [8, 9]. During Er:YAG laser ablation of tissues, the low-level laser energy may simultaneously penetrate and stimulate the surrounding tissues and cells during irradiation and facilitate wound healing, as proposed by Ohshiro et al. [10] for low-level laser therapy (LLLT) achieved during high-level laser therapy. However, only few studies have addressed the biological effects of the low-level Er:YAG laser on periodontal wound healing [7, 11, 12].

Several groups have previously demonstrated the biostimulation effects of various low-level lasers on periodontal cells in vitro at the messenger RNA (mRNA) and protein levels [13]. To better understand the associated biological mechanisms, it is essential to comprehensively explore the changes in gene and protein expression levels in laser-irradiated cells. Zhang et al. [14] used complementary DNA (cDNA) microarrays to investigate the gene expression profiles of human skin fibroblasts irradiated by a low-intensity red light. However, both the production rate and stability of the mRNAs were lower than those of proteins [15].

Recently, proteomic analysis has been gaining increasing usage in medical realms for the detection of novel biomarkers in various diseases. In the periodontal field, proteomic analysis has been employed for investigating the biomarkers of periodontal diseases using gingival crevicular fluids (GCF) and gingival tissues and has been used to identify the expression of various proteins in periodontal cells [16–18]. Proteomic analysis may be useful for providing a precise and comprehensive understanding of the mechanisms of photobiomodulatory effects of lasers on periodontal tissues at the protein level.

Human gingival fibroblasts (HGFs) are important in periodontal wound healing, as HGF are the most abundant cell type in human periodontal tissue and can influence tissue repair and sustain inflammation caused by periodontal diseases [19]. Activation of HGF cell proliferation is important for promoting the wound healing process.

Hence, the aim of this study was to reveal the effect of the low-level Er:YAG laser irradiation on the proliferation of HGFs and to investigate irradiation-induced changes in protein expression of HGFs by gel-free proteomic analysis. Furthermore, we analyzed the expression of galectin-7, which has been reported to be associated with wound healing and the tissue repair process [20–24]; moreover, its expression following laser treatments has recently been reported [20, 24].

Materials and methods

Cell preparation

Healthy gingival tissue samples were obtained after informed consent was given by patients who presented to the periodontics clinic of the Tokyo Medical and Dental University (TMDU) Dental Hospital for periodontal surgery. The study protocol was approved by the ethical committee of the Faculty of Dentistry, TMDU (#740). Primary HGFs were isolated from the gingival tissues by the outgrowth method. HGFs were cultured in 10-cm tissue culture dishes with Dulbecco's modified Eagle's medium (DMEM; phenol red-free DMEM; Wako, Osaka, Japan) supplemented with 10 % fetal bovine serum (FBS; Bioserum, Melbourne, Victoria, Australia) and 1 % antibiotic-antimycotic mixture (Invitrogen Corporation, Carlsbad, CA, USA) and incubated at 37 °C under a humidified atmosphere of 95 % air and 5 % CO_2. The cells were then seeded into 35-mm dishes at 2.5×10^4 cells per dish for subculture and applied after two to six passages from the primary culture. Twenty-four hours after the subculture, the cells were serum-starved by replacing the medium with DMEM containing 0.5 % FBS for additional 24 h prior to Er:YAG laser irradiation.

Experiment 1: the effects of low-level Er: YAG laser irradiation on the proliferative activity of human gingival fibroblasts

Laser irradiation

An Er:YAG laser device (DELight; HOYA ConBio, Fremont, CA, USA) with an emitting wavelength of 2.94 μm was employed in this study. The energy output of the device was monitored by a power meter (Field Master and detector LM-P10i; Coherent, OH, USA). Immediately after the culture medium was removed from the culture dish, laser irradiation was performed perpendicularly to the bottom of the culture dish (in the absence of culture medium) at a distance of 20 cm

for 30 s. To ensure the exposure of entire bottom surface, mounting neither cover sleeve nor contact tip was used for the hand piece. The output energy was set at 30, 40, and 50 mJ/pulse, which corresponded to an energy density of 1.84, 2.35, and 2.90 mJ/cm^2 per pulse, respectively, resulting in actual total energy density (ED) at the dish surface to be 1.65, 2.11, and 2.61 J/cm^2, respectively. Immediately after irradiation, the dish was re-filled with the previous culture medium to resume cell culture. The cells receiving sham laser irradiation in the absence of culture medium under the same aforementioned conditions were used as control.

Figure 1 schematically illustrates the design of the study including cell culture, laser irradiation, and various postirradiation analyses performed on a real-time basis.

Cell proliferation assay (water-soluble tetrazolium salt-8 assay)

The effects of laser irradiation on the proliferative activity of HGFs were determined by water-soluble tetrazolium salt-8 (WST-8) colorimetric assay, a novel tetrazolium salt method, using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, 3 days after irradiation, each dish was incubated with 750 μl of the mixture containing kit solution and DMEM (1:10) at 37 °C for 30 min. Optical absorbance was measured on a microplate reader (Vmax, Molecular Devices, Sunnyvale, CA, USA) at 450 nm. Relative cell proliferative activity was expressed as the ratio of the proliferative activity of the irradiated cells to that of control cells.

Lactate dehydrogenase assay

Additionally, the cytotoxic effect of laser irradiation on HGFs was evaluated 3 days after irradiation. The cell supernatants were collected for measuring the level of lactate dehydrogenase (LDH) released from the cytosol of the damaged cells using a Cytotoxicity Detection Kit (Roche, Mannheim, Germany) as per the manufacturer's instructions.

Experiment 2: proteomic analysis of the human gingival fibroblasts following low-level Er: YAG laser irradiation

After irradiation at 2.11 J/cm^2 in the same manner as in experiment 1, the samples were prepared for comprehensive protein expression analysis according to the following procedures.

In-solution digestion of proteins

Twenty-four hours after irradiation, the cells were washed three times with PBS and spiked with 4 M urea and 100 mM ammonium bicarbonate. The lysate was vortexed for 10 min and centrifuged at 20,000×*g* for 15 min. Subsequently, 2 μl of 200 mM DTT was added to the samples, which were then incubated at 57 °C for 30 min. After that, 2 μl of 600 mM iodoacetoamide was added to the samples, which were incubated at room temperature for 30 min in the dark. After addition of 5 μl of trypsin and 26 μl of distilled water, the samples were incubated at 37 °C for another 6 h. Finally, the resulting peptides were added to 5 μl of 5 % trifluoroacetic acid or 5 % formic acid for analysis.

Protein identification—liquid chromatography-tandem mass spectrometry analysis

In-solution digested peptides were injected into a trap column (C_{18}, 0.3×5 mm, Dionex, CA, USA) and an analytical column (C_{18}, 0.075×120 mm, Nikkyo Technos, Tokyo, Japan) attached to an Ultimate 3000 HPLC system (DIONEX, CA, USA). The mobile phase was passed through the column at a flow rate of 300 nl/min. The mobile phase composition was programmed to change over 120-min cycles, and within each cycle, the mixing ratio between solvent A (2 %*v*/*v* CH_3CN and 0.1 %*v*/*v* HCOOH) and solvent B (90 %*v*/*v* CH_3CN and 0.1 %*v*/*v* HCOOH) varied linearly: 5–10 % solvent B in 5 min, 10–13.5 % solvent B in 35 min, 13.5–35 % solvent B in 65 min, 35–90 % solvent B in 4 min, 90 % solvent B in 0.5 min, 90–5 % solvent B in 0.5 min, and finally 5 % solvent B in 10 min. The purified peptides were introduced from HPLC system to an LTQ Orbitrap XL hybrid ion trap-Fourier transform mass spectrometer (liquid chromatography-tandem mass spectrometry (LC-MS/MS); Thermo Scientific, San Jose, CA). Mascot search engine (version 2.2.6, Matrix Science, London, UK) was used to identify the proteins from the mass and tandem mass spectra of peptides. The peptide mass data were matched with those from the UniProtKB database (SwissProt 2010x, November 2010, 9590 entries) using parameters listed as follows: peptide mass tolerance of 1.2 Da, fragment tolerance of 0.6 Da, enzyme set as trypsin, one missed cleavage allowed, and methionine oxidation set as variable modification. False discovery rate (FDR) was estimated by searching against a randomized decoy database created by a Mascot Perl program provided by Matrix Science (London, UK), and FDR <1 % was determined as the minimum criterion for protein identification.

Experiment 3: validation of galectin-7 expression in human gingival fibroblasts following low-level Er: YAG laser irradiation

Quantitative real-time PCR

Total RNA was isolated from HGFs after 2 and 4 h after irradiation at 2.11 J/cm^2 using an RNeasy Mini Kit (Qiagen

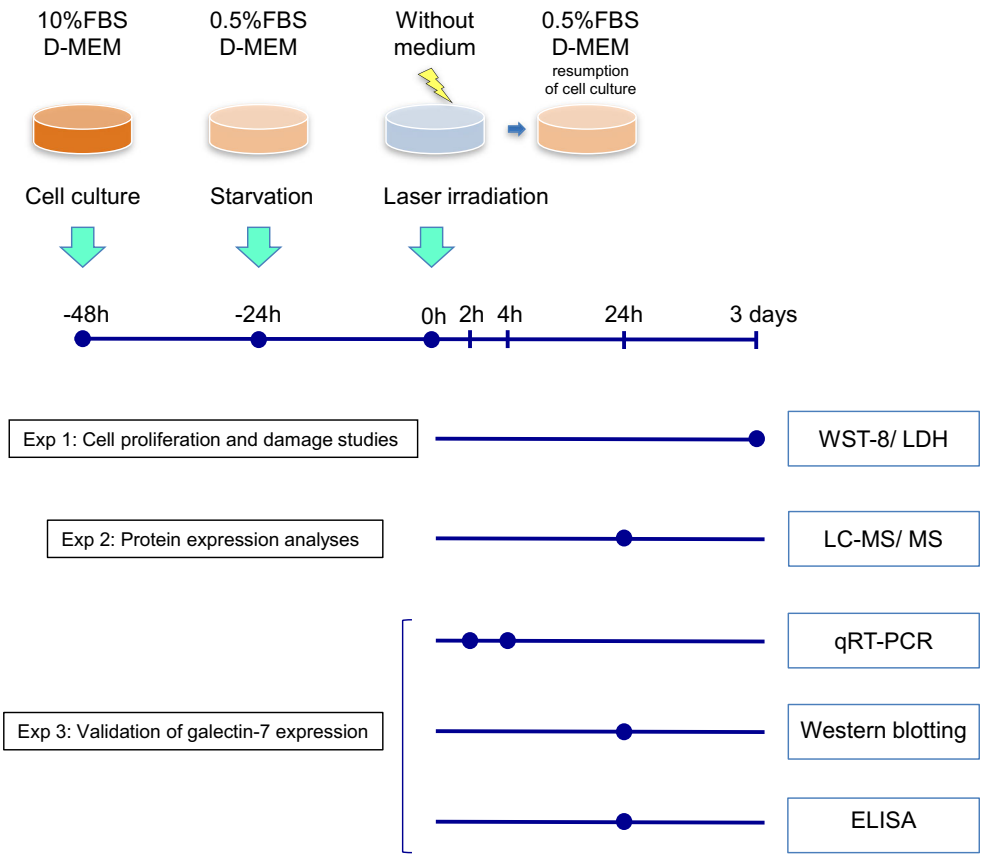

Fig. 1 Schematic illustration of the study design

Inc., Valencia, CA, USA), and cDNA was synthesized by a QuantiTect Reverse Transcription Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. *Galectin-7* transcripts were detected with sense primer 5′-TTG CTC CTT GCT GTT GAA GAC CAC-3′ and antisense primer 5′-AGG TTC CAT GTA AAC CTG CTG TGC-3′. *Glyceradehyde-3-phosphate dehydrogenase* (GAPDH) cDNA was amplified with sense primer 5′-CGG AGT CAA CGG ATT TGG TCG TAT-3′ and antisense primer 5′-AGC CTT CTC CAT GGT GGT GAA GAC-3′. Quantitative real-time polymerase chain reaction (PCR) using SYBR Premix Ex Taq II (TaKaRa Bio, Shiga, Japan) was performed in a Thermal Cycler Dice Real-Time System II (TaKaRa Bio, Shiga, Japan). The results were presented as the relative expression of galectin-7 mRNA normalized against to GAPDH expression.

Sodium dodecyl sulfate-PAGE and Western blot analysis

Twenty-four hours after irradiation at 2.11 J/cm^2, HGFs were lysed with radio immunoprecipitation assay (RIPA) lysis buffer (1 % Nonidet P-40, 0.5 % sodium deoxycholate, 0.1 % sodium dodecyl sulfate (SDS), and protease inhibitor cocktail) and treated with ultrasound waves for preparing the whole cell extracts. Cell lysate samples (6 μg) were separated by SDS-polyacrylamide gel electrophoresis on a 10–20 % polyacrylamide gradient gel and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA) in a Bio-Rad Trans-Blot apparatus (Bio-Rad, Hercules, CA, USA). The membranes were blocked in phosphate-buffered saline (PBS, pH 7.5) containing 5 % skim milk and 0.05 % Tween 20 at room temperature. The membranes were incubated with anti-galectin-7 rabbit polyclonal antibody (ab10482; Abcam, Cambridge, MA, USA) diluted 1:1000 and anti-β-actin goat polyclonal antibody (C-11; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) diluted 1:500 in the same buffer at 4 °C overnight. Subsequently, the membranes were washed three times with PBS containing 0.05 % Tween 20 and incubated for 1 h with HRP-conjugated goat anti-rabbit IgG antibodies (Bio-Rad Laboratories, Hercules, CA, USA) used as secondary antibodies diluted 1:2000 in blocking buffer. The bands were visualized by using an enhanced chemiluminescence kit (GE Healthcare UK Ltd., Buckinghamshire, UK).

Enzyme-linked immunosorbent assay

Laser-irradiated HGFs and control cells were prepared by the procedure described above. Twenty-four hours after irradiation at 2.11 J/cm^2, the cells were washed three times with PBS and lysed with 1 mM phenylmethylsulfonyl fluoride (Wako, Osaka, Japan) RIPA buffer (Cell Signaling Technology, Beverly, MA, USA) followed by ultrasonication. The

expression level of galectin-7 in HGFs was determined by a commercial enzyme-linked immunosorbent assay (ELISA) kit, (Abcam, Cambridge, MA, USA) according to the manufacturer's instructions.

Cell proliferation activity after recombinant human galectin-7 treatment

Furthermore, the effect of exogenous galectin-7 treatment on HGFs was determined. HGFs were seeded in 96-well plates at a cell density of 3×10^3 cells per well filled with 100 μl of culture medium. Twenty-four hours after incubation, the cells were serum-starved by replacing the medium with DMEM containing 0.5 % FBS for additional 24 h and then stimulated with recombinant human galectin-7 at a concentration of 0, 3.75, and 7.5 μg/ml separately (R&D systems, Minneapolis, MN, USA). Six and twelve hours after stimulation, cell proliferative activity was examined by WST-8 assay. Relative cell proliferation rate was expressed as the ratio of proliferative activity of the treated cells to that of control.

Statistical analysis

Continuous variables were expressed as mean±standard deviation (SD). A one-way analysis of variance was used for all group comparisons, followed by Dunnett test to compare the difference between each group and control. A p value <0.05 was considered significant. All statistical analyses were performed using the StatView software ver. 5.0 (SAS Co Ltd., Cary, NC, USA).

Results

Experiment 1: the effects of low-level Er: YAG laser irradiation on the proliferative activity of human gingival fibroblasts

The proliferative activity of HGFs at two to four passages from different human subjects increased at all energy levels 3 days after laser irradiation (Fig. 2a). Laser irradiation at 2.11 and/or 2.61 J/cm^2 significantly enhanced cell proliferation 1.13- to 1.22-fold ($p<0.05$) in six independent experiments using HGFs from two different subjects. No significant change in LDH level of any irradiated groups was observed in these experiments (Fig. 2b).

Experiment 2: proteomic analysis of the human gingival fibroblasts following low-level Er: YAG laser irradiation

According to the results of cell proliferative activity measurement following laser irradiation, we selected the ED of 2.11 J/cm^2, which was the lower energy level between the two showing positive results, for proteomics and the following experiments.

Twenty-four hours after irradiation, 377 proteins were identified in the irradiated and/or control HGFs by LC-MS/MS analysis (Suppl. Table 1).

Among them, 59 proteins demonstrated increased numbers of unique peptides in the laser-treated HGFs: 36 proteins were exclusively expressed in the irradiated samples, and 23 proteins expressed a larger number of peptides in the irradiated samples than that in the control samples (i.e., two or more peptides were expressed in the irradiated samples, whereas only one peptide was identified in the control samples). All of the upregulated proteins are listed in Table 1.

Upregulated proteins included 14-3-3 proteins (14-3-3 protein sigma, 14-3-3 protein epsilon, and 14-3-3 protein zeta/delta), various enzymes (arginase-1, glutathione *S*-transferase P, cathepsin D, protein-glutamine gamma-glutamyltransferase E, L-lactate dehydrogenase A chain, transitional endoplasmic reticulum ATPase, ADP-ribosylation factor 1, and ATP synthase subunit alpha, mitochondrial), calcium binding proteins (calmodulin-like protein 5 and 3), translation elongation factors (elongation factor 2 and 1-gamma), an adhesive protein (desmocollin-1), ribosomal proteins (40S and 60S ribosomal proteins), lipid mobilizing factor (zinc-alpha-2-glycoprotein), cytoskeletal proteins (keratins, type II cytoskeletal 80, plakophilin-1, and tubulin beta-2C chain), a stress protein (heat shock 70-kDa protein 1A/1B), and a cysteine-rich protein (CYR61).

Forty-seven proteins showed the same unique peptide numbers between control and irradiated samples. The unregulated proteins included protein disulfide isomerase, plectin, cofilin-2, and calreticulin, which have been reported to be associated with the cytoskeletal structure.

In total, 15 proteins showed increased numbers of unique peptides in the control HGFs; three proteins were exclusively expressed in the nonirradiated control samples, and 12 proteins presented a larger number of peptides in the control samples than in the irradiated samples (i.e., two or more peptides were expressed in the control samples, whereas only one peptide was identified in the irradiated samples). Among the downregulated proteins, the detected proteins were apolipoprotein C-I, lamin-B2, tenascin (MS/MS unique peptide number of these proteins: control 2 vs. laser 0), activated RNA polymerase II transcriptional coactivator p15, coronin 1C, cysteine and glycine-rich protein 1, eukaryotic translation initiation factor 5A-1, heterogeneous nuclear ribonucleoprotein G, heterogeneous nuclear ribonucleoprotein M, Hsc70-interacting protein, protein AHNAK2, stathmin, tumor protein D54, 28-kDa heat- and acid-stable phosphoprotein, and 40S ribosomal protein S28 (control 2 vs. laser 1).

Thus, compared to the control HGFs, the irradiated HGFs showed a significant change of upregulation and downregulation in protein expression.

Experiment 3: validation of galectin-7 expression in human gingival fibroblasts following low-level Er: YAG laser irradiation

Quantitative real-time PCR

The expression level of galectin-7 mRNA in HGFs significantly increased 4 h after irradiation, as compared with control ($p<0.05$) (Fig. 3a) and was confirmed by three independent experiments using HGFs from three different subjects.

Western blotting analysis and enzyme-linked immunosorbent assay

Western blotting analysis demonstrated a pronounced increase in protein expression of galectin-7 in the HGFs 24 h after irradiation (Fig. 3b).

In addition, ELISA analysis revealed a significant increase in the protein level of galectin-7 24 h after irradiation ($p<0.05$) (Fig. 3c) and the result was confirmed by three independent experiments using HGFs from two different subjects.

Proliferation activity of human gingival fibroblasts after recombinant galectin-7 treatment

The cell proliferative activity increased in a dose-dependent manner 6 and 12 h after the exogenous galectin-7 treatment (Fig. 4a, b). The proliferative activity of HGFs treated with 7.5 μg/ml recombinant galectin-7 significantly increased 1.20- to 2.56-fold 6 and/or 12 h after the stimulation in four independent experiments using HGFs from three different subjects ($p<0.05$).

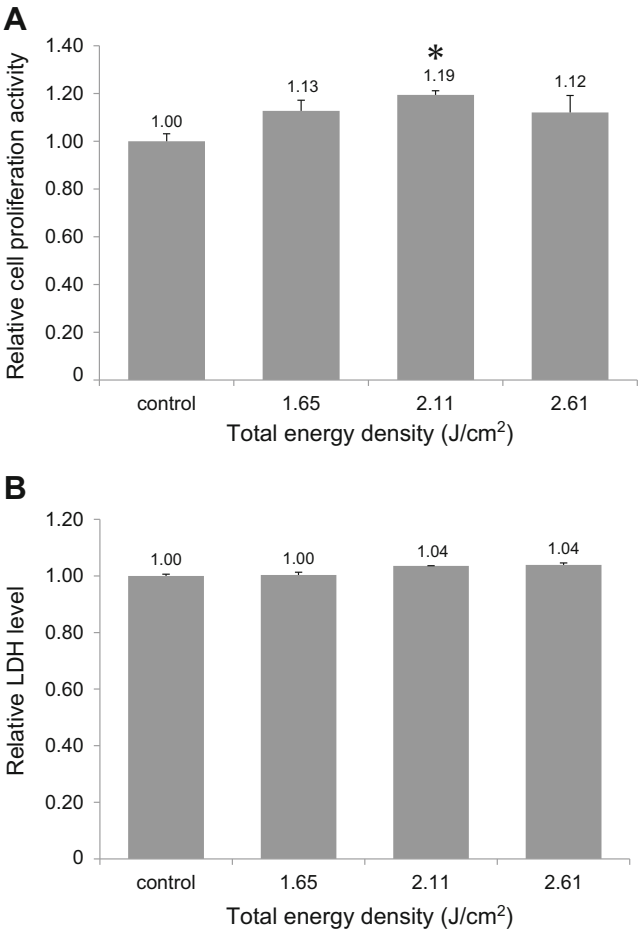

Fig. 2 Cell proliferative activity after low-level Er:YAG laser irradiation. **a** HGF proliferation was examined 3 days after irradiation using WST-8 assay. As compared with the control (i.e., without irradiation), the cell proliferative activity was significantly increased by irradiation at the total energy density of 2.11 J/cm^2. **b** Cell damage was also examined 3 days after irradiation using the LDH assay. No significant change in the LDH activity was observed in any irradiated group. Data are presented as mean±SD, * $p<0.05$

Discussion

In the present study, we demonstrated that low-level Er:YAG laser irradiation increased proliferative activity of HGFs on day 3 by 13–22 % without causing cell damage, which was in good agreement with the results of previous studies, where Pourzarandian et al. [11] and Aleksic et al. [12] reported that low-level Er:YAG laser irradiation induced a small to medium increase in the proliferative activity of both HGFs and osteoblasts. Furthermore, proteomics technology was employed for HGFs irradiated under conditions that increased proliferative activity.

Consequently, LC-MS/MS analysis identified 59 upregulated proteins in HGFs at 24 h after irradiation. Among them, expressions of 14-3-3 protein sigma and arginase-1 (unique peptide number: control 0 vs. laser 6), desmocolin-1 (control 0 vs. laser 4), and galectin-7 and CYR61 (control 1 vs. laser 2), which were previously reported to be associated with cell proliferation or wound healing, were noted.

In particular, 14-3-3 protein sigma was highly expressed in the irradiated HGFs. The 14-3-3 protein family has been reported to play crucial roles in a diverse range of cellular responses including cell cycle progression, DNA damage checkpoints, and apoptosis [25]. The gene encoding 14-3-3 protein sigma was originally characterized as a target gene induced by p53 in cell cycle checkpoint signaling [26]. Recently, Fukuhara et al. [27] performed low-level diode laser irradiation on osteoblasts and demonstrated increased cell proliferation and bone nodule formation after irradiation. They employed 14-3-3 sigma as the marker for G2/M arrest of the cell cycle, which may cause wound healing similar to tissue repair [28] and reported that the laser irradiation induced

Table 1 LC-MS/MS-identified 59 proteins and their increased numbers of unique peptides in the low-level Er:YAG laser-irradiated HGFs

Identified proteins	Accession number[a]	Molecular weight[b] (kDa)	MS/MS unique peptide	
			Control	Laser
14-3-3 protein sigma	1433S_HUMAN	28	0	6
Arginase-1	ARGI1_HUMAN	35	0	6
Calmodulin-like protein 5	CALL5_HUMAN	16	0	5
Glutathione S-transferase P	GSTP1_HUMAN	23	0	5
Protein-glutamine gamma-glutamyltransferase E	TGM3_HUMAN	77	0	5
Elongation factor 2	EF2_HUMAN	95	0	5
L-Lactate dehydrogenase A chain	LDHA_HUMAN	37	0	4
Cathepsin D	CATD_HUMAN	45	0	4
Desmocollin-1	DSC1_HUMAN	100	0	4
40S ribosomal protein S3	RS3_HUMAN	27	0	3
Zinc-alpha-2-glycoprotein	ZA2G_HUMAN	34	0	3
Elongation factor 1-gamma	EF1G_HUMAN	50	0	3
Keratin, type II cytoskeletal 80	K2C80_HUMAN	51	0	3
Heat shock 70-kDa protein 1A/1B	HSP71_HUMAN	70	0	3
Plakophilin-1	PKP1_HUMAN	83	0	3
Transitional endoplasmic reticulum ATPase	TERA_HUMAN	89	0	3
Cystatin-A	CYTA_HUMAN	11	0	2
Calmodulin-like protein 3	CALL3_HUMAN	17	0	2
Nucleoside diphosphate kinase B	NDKB_HUMAN	17	0	2
ADP-ribosylation factor 1	ARF1_HUMAN (+1)	21	0	2
Gamma-glutamylcyclotransferase	GGCT_HUMAN	21	0	2
Peroxiredoxin-6	PRDX6_HUMAN	25	0	2
14-3-3 protein epsilon	1433E_HUMAN	29	0	2
Phosphoglycerate mutase 1	PGAM1_HUMAN	29	0	2
40S ribosomal protein S4, X isoform	RS4X_HUMAN	30	0	2
60S ribosomal protein L7a	RL7A_HUMAN	30	0	2
Ig gamma-1 chain C region	IGHG1_HUMAN	36	0	2
Serpin B12	SPB12_HUMAN	46	0	2
Protein disulfide-isomerase A6	PDIA6_HUMAN	48	0	2
Tubulin beta-2C chain	TBB2C_HUMAN	50	0	2
Bleomycin hydrolase	BLMH_HUMAN	53	0	2
RNA-binding protein FUS	FUS_HUMAN	53	0	2
ATP synthase subunit alpha, mitochondrial	ATPA_HUMAN	60	0	2
Vinculin	VINC_HUMAN	124	0	2
Clathrin heavy chain 1	CLH1_HUMAN	192	0	2
Hornerin	HORN_HUMAN	282	0	2
Eukaryotic initiation factor 4A-I	IF4A1_HUMAN	46	1	6
Serpin B4	SPB4_HUMAN	45	1	5
Histone H2A type 1-B/E	H2A1B_HUMAN (+9)	14	1	3
Thioredoxin	THIO_HUMAN	12	1	3
Rho GDP-dissociation inhibitor 1	GDIR1_HUMAN	23	1	3
Guanine nucleotide-binding protein subunit beta-2-like 1	GBLP_HUMAN	35	1	3
Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	60	1	3
Ig kappa chain C region	IGKC_HUMAN	12	1	2
Eukaryotic translation initiation factor 1b	EIF1B_HUMAN (+1)	13	1	2
Galectin-7	LEG7_HUMAN	15	1	2
Ubiquitin-60S ribosomal protein L40	RL40_HUMAN (+3)	15	1	2

Table 1 (continued)

Identified proteins	Accession number[a]	Molecular weight[b] (kDa)	MS/MS unique peptide	
			Control	Laser
60S ribosomal protein L22	RL22_HUMAN	15	1	2
60S ribosomal protein L17	RL17_HUMAN	21	1	2
Ras-related protein Rab-7a	RAB7A_HUMAN	23	1	2
14-3-3 protein zeta/delta	1433Z_HUMAN	28	1	2
Tropomyosin alpha-3 chain	TPM3_HUMAN	33	1	2
Protein CYR61	CYR61_HUMAN	42	1	2
Heterogeneous nuclear ribonucleoprotein D-like	HNRDL_HUMAN	46	1	2
Drebrin-like protein	DBNL_HUMAN	48	1	2
60S ribosomal protein L4	RL4_HUMAN	48	1	2
CD44 antigen	CD44_HUMAN	82	1	2
Heterogeneous nuclear ribonucleoprotein U	HNRPU_HUMAN	91	1	2
Filaggrin-2	FILA2_HUMAN	248	1	2

A total of 59 proteins demonstrated increased numbers of unique peptides in the low-level Er:YAG laser-treated HGFs, of which 36 proteins were exclusively expressed in the irradiated samples and 23 proteins expressed larger numbers of peptides in the irradiated samples than those of control samples. The upregulated proteins of the laser-treated cells are listed in the descending order according to their unique peptide numbers

[a] Reference for protein identification

[b] Theoretical molecular mass based on Swiss-Prot database

the initial G2/M arrest of the osteoblasts by confirming increased expression of 14-3-3 sigma after irradiation. Thus, the upregulation of 14-3-3 sigma in the Er:YAG laser-treated HGFs may also indicate the promotion of cell proliferation.

Arginase-1 was also highly detected, and it is reportedly expressed across a range of cell types involved in wound healing [29]. Arginase-1 metabolizes L-arginine into L-ornithine and urea. L-Ornithine is a precursor of proline and polyamines, which promote collagen synthesis and cell proliferation, respectively—the key aspects of tissue regeneration [30]. Campbell et al. [31] reported that local arginase-1 activity is a required role for cutaneous wound healing. Arginase-1 may be an important protein related to cell proliferation and wound healing promoted by low-level Er:YAG laser irradiation. The desmocolin-1 gene has been reported to be expressed in HGFs [32] and also to be downregulated in periodontitis-affected gingival tissues [33]. The expression of desmocollin-1 plays an important role in intercellular adhesion and barrier maintenance in the epidermis [34]. Thus, in gingival tissue treatment using the Er:YAG laser, desmocollin-1 expression following irradiation may contribute to the epithelialization of the exposed connective tissues.

Galectins can be found in cell nuclei, cytoplasms, cytoplasmic membranes, and extracellular spaces [35]. Galectin-7, which is a prototype galectin, can control epidermal homeostasis and can accelerate wound healing process in injured corneal, dermal, and kidney epithelia [21–23]. Interestingly, Bertoldi et al. demonstrated that galectin-7 was underexpressed in pathological periodontal tissues [36]. CYR61 is a member of the connective tissue growth factor (CTGF), CYR61, and nephroblastoma overexpressed gene (CCN) family, which stimulates mitosis, adhesion, apoptosis, extracellular matrix production, and migration of multiple cell types [37]. The upregulation of these proteins may also be associated with cell proliferation and wound healing.

In addition, increased expression of heat shock 70 may indicate the presence of thermal stress caused by irradiation; therefore, not only a photochemical effect but also a photothermal effect could have influenced the HGFs in the case of low-level Er:YAG laser treatment.

Among the unregulated proteins, protein disulfide isomerase is a member of a large family of dithiol/disulfide oxidoreductases, the thioredoxin superfamily, and is essential for protein folding in the cell [38]. Plectin is a major linker and scaffolding protein of the cytoskeleton as an focal adhesion constituent protein [39]. Cofilin is a widely distributed intracellular actin-modulating protein and a central component of actin dynamics in migrating cells [40]. These proteins are cytoskeletal proteins, and their expression remained unchanged following irradiation. With regard to growth factors, in previous studies, the expression of several growth factors, e.g., transforming growth factor-beta and insulin-like growth factor, has been demonstrated at the gene and protein levels after LLLT [13]. However, in the present study, a few growth factors, e.g., transforming growth factor-beta-induced protein ig-h3 and CTGF, were detected but unregulated in the irradiated HGFs.

In contrast, among the downregulated proteins, apolipoprotein C-I and tenascin, which mediate the inflammatory response were noted. Apolipoprotein C-I has classically been recognized as an inhibiting factor for lipoprotein clearance. A recent study

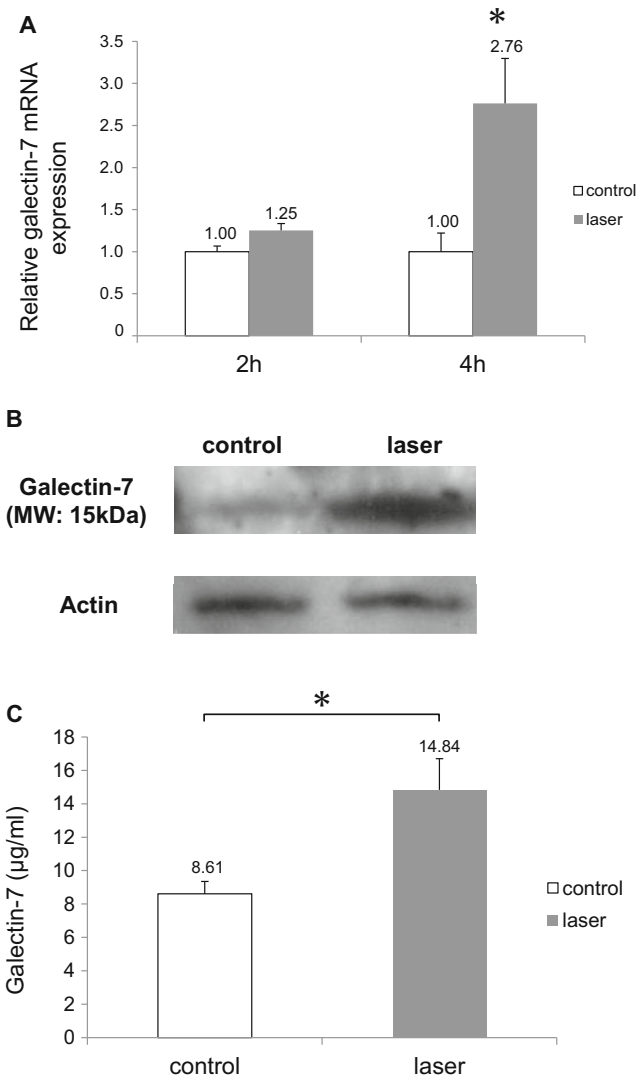

Fig. 3 Galectin-7 mRNA and protein expression after low-level Er:YAG laser irradiation. **a** mRNA expression of galectin-7 was significantly increased 4 h after irradiation at 2.11 J/cm^2. **b** Western blotting analysis of the whole cell extract conducted 24 h after irradiation showed clearly increased protein expression of galectin-7. **c** ELISA of the whole cell extract conducted 24 h after irradiation demonstrated a significantly high level of galectin-7 protein. Data are presented as mean±SD, *$p<0.05$

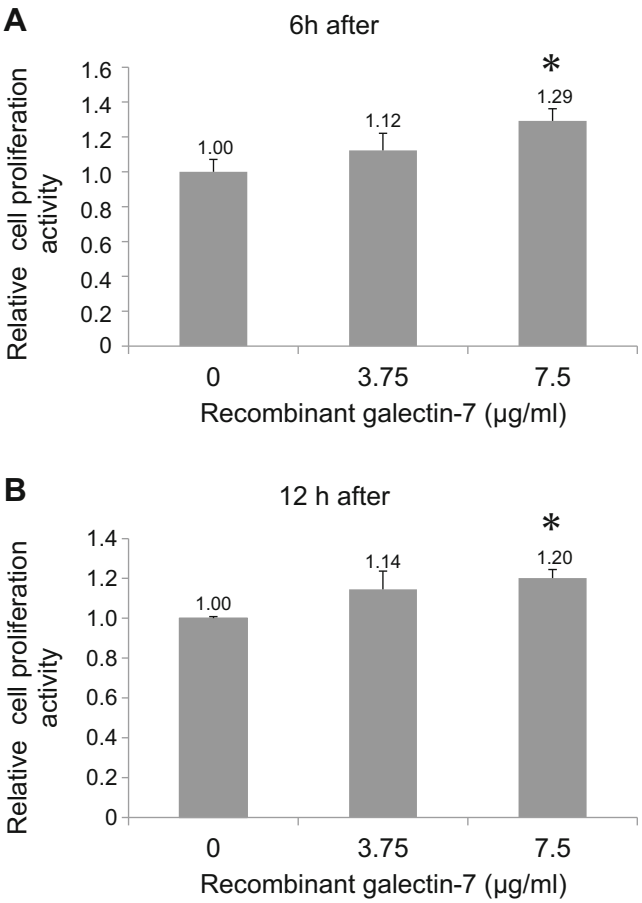

Fig. 4 Cell proliferative activity after recombinant galectin-7 treatment. The proliferative activity of HGFs significantly increased 6 and 12 h after treatment at 7.5 μg/ml. Data are presented as mean±SD, *$p<0.05$

identified apolipoprotein C-I as a lipopolysaccharide (LPS)-binding protein, with an apparent LPS-binding motif in its C-terminal helix and activity as a biological enhancer of the proinflammatory response toward LPS [41]. Tenascin-C is an extracellular matrix glycoprotein associated with tissue injury and repair. It is not normally expressed in most adult tissues but is specifically and transiently upregulated during acute inflammation and persistently expressed in chronic inflammation [42]. Thus, these proteins are associated with the inflammatory response and their downregulation by low-level Er:YAG laser irradiation may reduce the inflammation of diseased sites, which would be therapeutically useful. Further detailed studies are necessary to validate this hypothesis.

Recently, in wound healing studies following laser irradiation, Cho et al. [24] immunohistochemically revealed galectin-7 expression in the epidermis and the underlying connective tissue after CO_2 laser irradiation and demonstrated that exogenous galectin-7 might reduce the appearance of hypertrophic scars and keloids. Furthermore, they [20] reported that the excimer laser irradiation produced a marked upregulation of galectin-7 expression in the corneal epithelium of mouse and that galectin-7 showed potential in the mediation of corneal epithelial cell migration and re-epithelialization of wounds. Based on these results, in the present study, we focused on galectin-7 among the various proteins showing upregulation following low-level Er:YAG laser irradiation and validated the mRNA and protein expression of galectin-7 in HGFs. Finally, we examined the potential relationship between galectin-7 expression and increased cell proliferative activity because the effect of galectin-7 on periodontal cells has not been reported thus far. We demonstrated that exogenous galectin-7 could significantly increase in the proliferative activity of HGFs, leading to our assumption that the upregulation of galectin-7 after low-level Er:YAG laser irradiation may be associated with increased cell proliferation. In fact, if

galectin-7 expression could be demonstrated to be upregulated in the gingival tissues treated by Er:YAG laser, it could be possibly proven to improve the repair of injured connective tissues as well as re-epithelialization of the exposed connective tissues.

With regard to the influence of laser irradiation on cell signaling pathways, a few previous studies have already reported the association between cell proliferation and mitogen-activated protein kinase (MAPK) activation. Shefer et al. [43] showed that low-level He-Ne laser irradiation induced proliferation of skeletal muscle cells by specifically activating MAPK/extracellular signal-regulated kinase (ERK). Ejiri et al. [44] have reported that the low-level diode laser irradiation specifically activates MAPK/ERK in the gingival epithelial cells. In addition, Aleksic et al. [12] have reported that low-level Er:YAG laser irradiation promotes the proliferation of osteoblasts through the activation of MAPK/ERK. The 14-3-3 proteins detected in the present study have the ability to bind a multitude of functionally diverse signaling proteins and are reportedly associated with the regulation of RAF activity in the upstream of MAPK [45]. Also, recently, ADP-ribosylation factor 1 (ARF1) has been reported to have a novel function in regulating the MAPK signaling pathway [46]. Therefore, the effect of 14-3-3 proteins and ARF1 upregulated by laser irradiation on MAPK activation needs to be clarified by further studies. Some studies have found that low-level diode lasers can influence mitochondrial respiration [47], ATP synthesis [48], and the cellular redox state [49], leading to a photobiostimulative effect on wound healing [8, 9]. In the present study, upregulation of ATPases such as transitional endoplasmic reticulum ATPase and ATP synthase subunit alpha, mitochondrial was detected, and these may affect ATP synthesis. Thus, several laser-induced cell phenomena have been identified, although the signaling pathways to induce protein expressions remain unclear. Further studies are required to clarify the molecular mechanism of the upregulation of proteins, including galectin-7 following laser irradiation.

In the present study, we focused on galectin-7 expression as the first step of the validation of the upregulation of various proteins. The contribution of other upregulated and downregulated proteins to the cell proliferation and their association with other cell phenomena induced by the biostimulating effects of the Er:YAG laser will be investigated in our future studies. The information obtained from this proteomic analysis may be important for explaining various wound healing phenomena attributed to laser therapy and for developing new therapeutic strategies.

Conclusion

In conclusion, we demonstrated that low-level Er:YAG laser irradiation significantly stimulated the proliferative activity of HGFs in vitro. Proteomic analysis of the laser-irradiated HGFs showed a significant change in protein expression 24 h after irradiation and that the upregulation of galectin-7 may be partly associated with the increase in cell proliferation.

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