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(Degree)

博士 (保健学)

(Date of Degree)

2011-03-25

(Date of Publication)

2011-09-08

(Resource Type)

doctoral thesis

(Report Number)

甲5155

(URL)

<https://hdl.handle.net/20.500.14094/D1005155>

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Effect of Ultrasound Irradiation on α -SMA and TGF- β 1 Expression in Human Dermal Fibroblasts

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Received 9 December 2010/ Accepted 22 December 2010

Key Words: Ultrasound, α -SMA, TGF- β 1, fibroblasts

Ultrasound therapy is used to promote pressure ulcer healing as an adjunctive therapy. However, the efficacy and the scientific basis of this treatment are unclear. We investigated the effect of ultrasound irradiation on alpha-smooth muscle actin (α -SMA) and transforming growth factor-beta1 (TGF- β 1) expression in human dermal fibroblasts. These are important factors for acceleration of wound closure. We used pulsed ultrasound of 0, 0.1, 0.5, and 1.0 W/cm². TGF- β 1 and α -SMA mRNA was measured by quantitative real-time polymerase chain reaction, α -SMA protein was examined by western blot, and localization of α -SMA was evaluated by immunofluorescence staining. Expression of α -SMA and TGF- β 1 mRNA was increased at 24 h but not at 48 h after ultrasound irradiation. There were significant differences between controls of 0 W/cm² and 0.1 W/cm² with a 1.34 ± 0.26 fold increase in α -SMA ($P < 0.05$) and a 1.78 ± 0.57 fold increase in TGF- β 1 ($P < 0.05$). Protein levels of α -SMA were also increased and detected in ultrasound irradiated fibroblasts at 24 h. Ultrasound irradiation promotes α -SMA expression in human dermal fibroblasts and this suggests the biological mechanism of ultrasound efficacy on chronic wound treatment.

Ultrasound (US) therapy is used to promote pressure ulcer (PU) healing as an adjunctive therapy. However, there is no evidence that US therapy promotes healing of chronic wounds, including PUs.^{1,2} To evaluate the efficacy of US therapy for PUs, we conducted a clinical study with the following method of US, which has not been applied in previous clinical studies.³⁻⁶ As a moist environment promotes healing, wounds being treated with US need to be covered with a dressing.⁷ Therefore, we irradiated US to PUs by determining the intensity based on the US permeability of the wound dressings.⁸ As a result, we observed that US promoted wound contraction of PUs.⁹

Undesirable wound contraction can occur, resulting in cosmetic and functional problems in burn and trauma patients.^{10,11} However, wound contraction can be beneficial to overall wound healing by decreasing the wound area and forming a mechanically strong granulation,^{12,13} and wounds that remain open heal mainly by contraction.¹⁴ These findings indicate that promotion of wound contraction contributes to healing of PU in chronic wounds. Therefore, to find a more effective US intensity that promotes this process, the mechanism of wound healing promotion by US irradiation needs to be investigated.

Altomare M et al. reported the effect of US irradiation on ischemic ulcer healing *in vivo*.¹⁵ They reported that US irradiation is not effective for such ulcer healing, and α -smooth muscle actin (α -SMA) expression, which is reported to correlate with granulation tissue contractility,¹⁶ is less in the granulation of US irradiated ulcers than that of controls. This is not in accordance with our previous clinical report.⁹ In Altomare et al.'s study, however, US treatment was begun just after creating ulcers, while we applied US for the ulcers at the proliferation and remodeling phase in our clinical study. To resolve this controversy, molecular biological investigation during this healing phase is required.

It has been reported that α -SMA expression promotes contractile activity of fibroblasts or fibroblast-populated collagen lattices *in vitro*.^{17,18} Autocrine production of transforming growth factor- β 1 (TGF- β 1) by fibroblasts is important for preserving fibrogenic activity once the inflammatory stimulus has ceased.^{19,20} Many experiments and clinical observations have shown that TGF- β 1 plays a key role in stimulating characteristics of differentiated myofibroblasts including α -SMA expression.²¹ Therefore, to determine the mechanism of wound contraction, evaluation of α -SMA and TGF- β 1 expression in fibroblasts is required.

Mechanical tension causes fibroblasts to differentiate into proto-myofibroblasts with cytoplasmic actin and fibronectin. Similarly, mechanical tension causes proto-myofibroblasts to differentiate into myofibroblasts with α -SMA.²¹ Therefore, modulation of mechanical tension has a profound effect on α -SMA expression in fibroblasts.

Several studies have reported the effect of mechanical stimulation on α -SMA expression. Wang JH et al. reported an up-regulatory effect by dynamic tensile force in human tendon fibroblasts,²² and Wang J et al. reported the same effect by static tensile force in rat cardiac fibroblasts.²³ Since US can be regarded as a mechanical vibratory form of energy causing mechanical stress to culture cells through medium,²⁴ it might induce α -SMA expression in fibroblasts. However, the effect of US irradiation has not been reported in human dermal fibroblasts. In rat tendon cells, Tsai WC et al. reported that a single 20% pulsed US of 0, 0.1, 0.5, and 1.0 W/cm² spatial average temporal peak (SATP) for 5 min promoted migration, but did not promote α -SMA expression.²⁵ In their study, US waves below the dish were not controlled, and therefore, reflected US waves could have had an effect. With US irradiation, cells were cultured in medium with fetal bovine serum (FBS), which is regarded as chemically undefined medium.²⁶ Therefore, the effect of direct US irradiation on human dermal fibroblasts, where the reflected waves are controlled under serum-free conditions, needs to be investigated.

The up-regulatory effect of US irradiation on TGF- β 1 expression has been reported in several cell types. Tsai WC et al. reported an up-regulatory effect in human tendon cells cultured in medium with FBS by pulsed US of intensities as described above,²⁷ and Mukai S et al. reported the same effect in aggregated rat chondrocytes with or without FBS by multiple low-intensity 20% pulsed US of 30 mW/cm² spatial average temporal average (SATA), as well as 0.15 W/cm² SATP for 20 min.²⁸ Hiyama A et al. reported the same effect

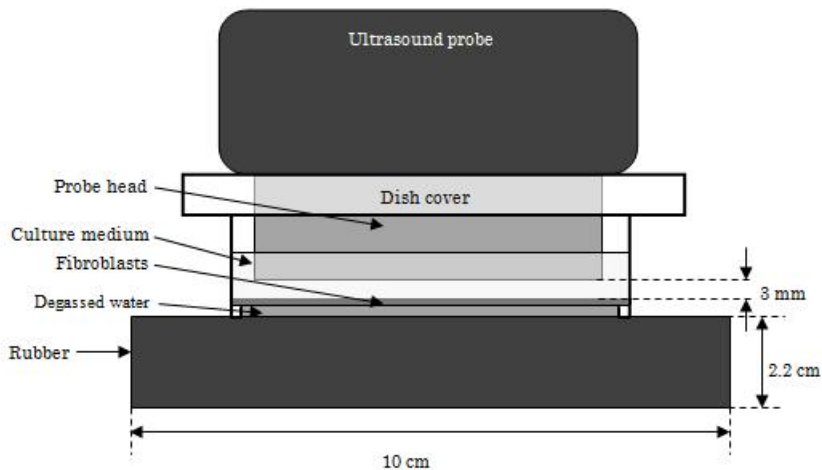


Figure 1. Procedure of US irradiation. Degassed water was dropped on the rubber plate (size: 10 cm × 10 cm, thickness: 2.2 cm) and the tissue culture dish was put on the degassed water, filling the space between the dish bottom and the rubber plate with degassed water. A dish cover with a hole made of glass was created to prevent bacterial contamination. The tissue culture dish was covered with the dish cover, and the transducer head was inserted into the culture medium through the dish cover hole.

in beagle nucleus pulposus cells with FBS by the same method as Mukai.²⁹ However, this effect has not been reported in human dermal fibroblasts.

In this study, we investigated the effect of US on α -SMA and TGF- β 1 expression under serum free conditions in human dermal fibroblasts. According to previous molecular biological studies and our clinical studies, 20% pulsed US for 10 min was used.^{9,15,25,27,28,29} To cover whole intensities used previously, 0, 0.1, 0.5 and 1.0 W/cm² SATP were applied. Furthermore, to evaluate the effective period of these US parameters, we used single US irradiation and evaluated that effect with time.

MATERIALS AND METHODS

Cell culture

Normal human dermal fibroblasts (CC-2511; Clonetics, San Diego, CA) were grown at 37°C in a CO₂ incubator in 100-mm tissue culture dishes (Iwaki, Tokyo, Japan) using Dulbecco's modified Eagle medium (Dainippon Sumitomo Pharma, Osaka, Japan) supplemented with 10% FBS (Nichirei, Tokyo, Japan), penicillin (50 U/ml), and streptomycin (50 µg/ml) (MP Biomedicals, Illkirch, France). For the experiments, fibroblasts (60×10^4 cells) were seeded into 60-mm tissue culture dishes (Iwaki), and cultured in serum-free medium to eliminate the effect of TGF- β 1 in FBS.³⁰ US irradiation was conducted 24 h later. To analyze the US-induced changes of cell morphology, cells were observed with an Axiovert 25 microscope using 50× magnification (Carl Zeiss, Oberkochen, Germany), and photographs were taken at 24 h after US irradiation with a digital camera (Camedia c-5050 zoom; Olympus, Tokyo, Japan).

Ultrasound irradiation

The US device was ItoUS750 (beam non-uniformity ratio 3.2; effective radiating area 6.0 cm²) (Ito, Tokyo, Japan), which is used in a wide range of medical conditions. To irradiate US directly to attached fibroblasts and to absorb the US energy below the dish, we designed a US irradiation model by modifying previous methods (Fig. 1).^{25,28,31,32} Degassed water of 2

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ml was dropped on the US absorptive rubber plate, with a size of 10 cm $\times$ 10 cm and the thickness was 2.2 cm. The tissue culture dish was put on the degassed water, filling the space between the dish bottom and the rubber plate with degassed water. To prevent bacterial contamination, a dish cover with a hole made of glass was created. The US transducer and the dish cover were sterilized by 75% alcohol and ultraviolet light. Just before US irradiation, culture medium was refreshed to 7 ml of serum-free medium to immerse the transducer head. The dish cover and transducer head were then washed with sterile phosphate buffered saline, and the tissue culture dish was covered with the dish cover, and the transducer head was inserted into the culture medium through the hole of the dish cover. The whole procedure was conducted in a sterile air flow cabinet. We selected the frequency of 3MHz of which energy is absorbed at a more shallow level than 1MHz, because fibroblasts of US targets are on the surface of tissue culture plates.^{33,34} A 20% pulse mode was used to eliminate the thermal effects.^{25,33} Fibroblasts were treated with US once at exposure dosages of 0 (control), 0.1, 0.5, or 1.0 W/cm² (SATP) for 10 min. After 24 h and 48 h, cell-free supernatant was collected and fibroblasts were processed for total RNA and protein isolation using ISOGEN reagents (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions.

Quantitative real-time PCR analysis

First-strand cDNAs were synthesized from the isolated RNAs with the First-Strand cDNA Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). The cDNAs were used for subsequent quantitative real-time PCR analysis using SYBR Premix Ex Taq II (Takara Bio, Otsu, Japan) with the following primers: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward, 50-catcaagaagggtgaagc-30; GAPDH reverse, 50-cctccccagcaagaatgtct-30; α -SMA forward, 50-cgtgggtgacgaagcacag-30; α -SMA reverse, 50-ggtgggatgctcttcagg-30; TGF- β 1 forward, 50-gggactatccactgcaaga-30; TGF- β 1 reverse, 50-cctccttggcgtagtagtcg-30. The PCR reactions were run on iCycler IQ (Bio-Rad, Hercules, CA, USA) for 40 cycles at 95°C for 30 s and 62.5°C for 30 s and 72.0°C for 30 s. Post-PCR melting curves were confirmed by the specificities of single-target amplification and the relative expressions of each gene were calculated based on GAPDH expression in duplicate.

Western blotting

Seven samples with equal protein quantity were collected into one pooled sample. The pooled sample was mixed with an equal volume of 2 $\times$ sodium dodecylsulfate–polyacrylamide gel electrophoresis reducing sample buffer, followed by boiling for 5 min. A total of 1.5 μ g of protein of cell extracts was separated by 12.5% sodium dodecylsulfate–polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred to nitrocellulose membranes using a mini VE vertical electrophoresis system (GE Healthcare, Buckinghamshire, UK). Membranes were washed in Tris-buffered saline (TBS; 5 mM Tris-HCl and 250 mM NaCl, pH 7.5) containing 0.1% (w/v) Tween 20 (TBS-T) and then blocked with 5% (w/v) dried skimmed milk in TBS-T for 1 h at room temperature. After 3 $\times$ brief washes with TBS-T, the membranes were incubated with mouse monoclonal antibody against α -SMA (Sigma, St. Louis, MO, USA) (1:1000) or mouse monoclonal antibody against β -actin (Sigma, St. Louis, MO, USA) (1:5000) diluted in Can Get Signal solution 1 (Toyobo, Tokyo, Japan) overnight at 4°C. The membranes were then rinsed with TBS-T 3 $\times$ for 5 min before incubation with horseradish peroxidase–conjugated anti-mouse immunoglobulin G antibody (GE Healthcare) (1:10000) diluted in Can Get Signal solution 2

(Toyobo) for 1 h at room temperature. Antibody binding was detected using enhanced chemiluminescence plus reagents (GE Healthcare) and developed on film.

Enzyme-linked immunosorbent assay

The concentration of TGF- β 1 contained in the cell-free supernatant of each dish from each time point was analyzed by enzyme-linked immunosorbent assay (ELISA). The ELISA kit was the Quantikine ELISA kit for TGF- β 1 (R&D system, Minneapolis, MN). The sensitivity of the TGF- β 1 assay kit was 31.2 pg/ml. The ELISA plate was read at 450/595 nm using a plate reader (Model 680; Bio-Rad, Hercules, CA, USA).

Immunofluorescence staining

Localization of α -SMA in fibroblasts was analyzed by immunofluorescence staining. Fibroblasts treated with 0.5 W/cm² US on the tissue culture dish were fixed in 4% paraformaldehyde for 30 min and then immersed for 10 min in blocking solution that contained 1% FBS in TBS at room temperature. After washing in TBS, the cells were incubated for 1 h with mouse monoclonal antibody against α -SMA (Sigma) (1:200) at room temperature, followed by incubation with Alexa Fluor 594 goat anti-mouse IgG (Invitrogen, Carlsbad, CA, USA) (1:500) for 1 h at room temperature. Finally, nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (1:1000). Immunofluorescent staining patterns were observed with a BX50 fluorescence microscope using 100 $\times$ magnification (Olympus), and recorded with a digital camera (Camedia c-5050 zoom; Olympus).

Statistical analysis

The data are expressed as mean $\pm$ standard deviation. Differences were considered significant if $P < 0.05$ as determined by the Tukey-Kramer post hoc test.

RESULTS

α -SMA and TGF- β 1 mRNA expression in ultrasound irradiated fibroblasts

In preliminary experiments, mRNA expression of α -SMA and TGF- β 1 was not increased at any of the intensities compared with controls in the culture medium with 10% FBS. Furthermore, mRNA expression of α -SMA and TGF- β 1 was not increased under serum-free conditions at 3 h, 6 h, and 12 h after US irradiation (data not shown). Therefore, we evaluated mRNA expression at 24 h and 48 h. We found that mRNA expression of α -SMA was increased 24 h after US irradiation. The increase in expression was comparable among all US intensities, although it was only statistically significant at 0.1 W/cm² (1, 1.34 ± 0.26 , 1.24 ± 0.29 , 1.22 ± 0.16 for controls, 0.1 W/cm², 0.5 W/cm², and 1.0 W/cm², respectively; Fig. 2-a). TGF- β 1 mRNA expression was also significantly increased by US irradiation at 0.1 W/cm² (1, 1.78 ± 0.57 , 1.39 ± 0.44 , 1.60 ± 0.67 for control, 0.1 W/cm², 0.5 W/cm², and 1.0 W/cm², respectively; Fig. 3-a). These changes were not observed at 48 h for α -SMA (1, 1.01 ± 0.31 , 0.89 ± 0.25 , 1.09 ± 0.64 for control, 0.1 W/cm², 0.5 W/cm², and 1.0 W/cm², respectively; Fig. 2-b) and TGF- β 1 (1, 0.95 ± 0.25 , 1.01 ± 0.32 , 0.97 ± 0.48 for control, 0.1 W/cm², 0.5 W/cm², and 1.0 W/cm², respectively; Fig. 3-b).

The expression of α -SMA protein in ultrasound irradiated fibroblasts

We evaluated protein expression of α -SMA 24 h after US irradiation, and found that α -SMA protein was increased by US irradiation. Although there was peak expression of α -SMA at 1.0 W/cm², an increase was also observed with the other intensities (1, 1.42, 1.38, 1.72 for control, 0.1 W/cm², 0.5 W/cm², and 1.0 W/cm², respectively; Fig. 4).

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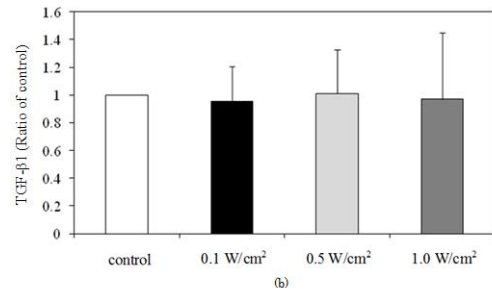
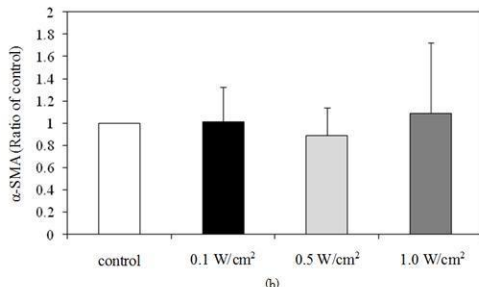
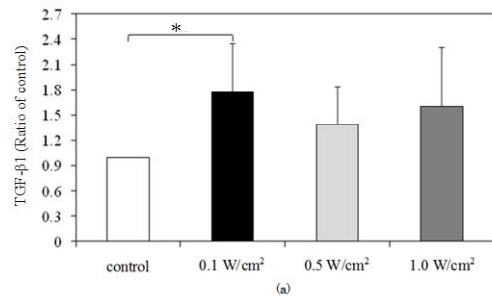
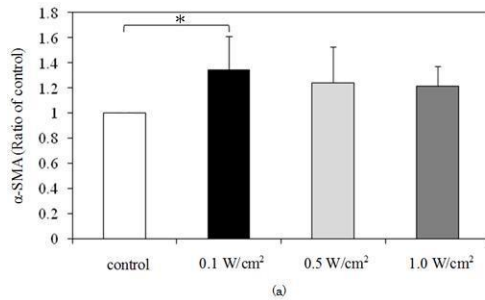


Figure 2. Effect of US irradiation with intensities of 0, 0.1, 0.5, and 1.0 W/cm² on α -SMA mRNA expression. (a) Twenty-four h after US irradiation. (b) Forty-eight h after US irradiation. Data are presented as ratios of the control. Each column represents mean $\pm$ SD (N=7, *P < 0.05, Tukey-Kramer post hoc test).

Figure 3. Effect of US irradiation with intensities of 0, 0.1, 0.5, and 1.0 W/cm² on TGF- β 1 mRNA expression. (a) Twenty-four h after US irradiation. (b) Forty-eight h after US irradiation. Data are presented as ratios of the control. Each column represents mean $\pm$ SD (N=7, *P < 0.05, Tukey-Kramer post hoc test).

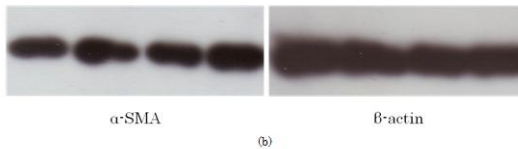
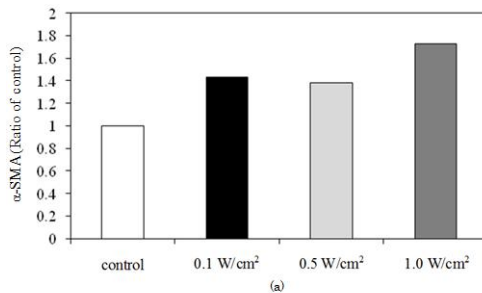


Figure 4. Effect of US irradiation with intensities of 0, 0.1, 0.5, and 1.0 W/cm² on α -SMA protein expression at 24 h. (a) Protein expression of α -SMA and β -actin (one pooled sample of 7 trials). (b) α -SMA/ β -actin ratio, which was evaluated using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

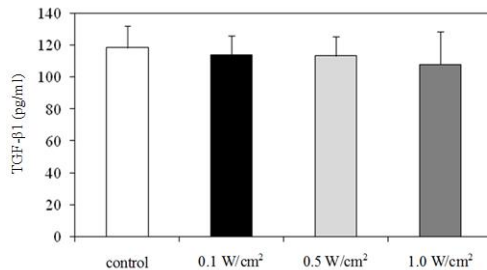


Figure 5. TGF- β 1 concentration in the fibroblast culture medium 48 h after US irradiation with intensities of 0, 0.1, 0.5, and 1.0 W/cm². Each column represents mean $\pm$ SD (N=4).

TGF- β 1 concentrations in the fibroblast culture medium

TGF- β 1 concentrations in serum-free medium 24 h after US irradiation were under the detectable level (31.2 pg/ml). Therefore, we assayed the concentrations at 48 h. TGF- β 1 concentrations were not increased at any of the intensities compared with controls (118.2 ± 13.6 , 113.9 ± 12.0 , 113.1 ± 12.0 , 107.7 ± 20.5 pg/ml for control, 0.1 W/cm², 0.5 W/cm², and 1.0 W/cm², respectively; Fig. 5).

Morphology and α -SMA localization in US irradiated fibroblasts

Fibroblasts did not grow up to confluence throughout this study. Morphologically, US irradiated fibroblasts had the same features as controls (Fig. 6). Furthermore, immunofluorescence staining was conducted to determine the localization of α -SMA. Double staining with DAPI and α -SMA showed that US irradiated fibroblast α -SMA was localized in the cytoplasm around nuclei (Fig. 7)

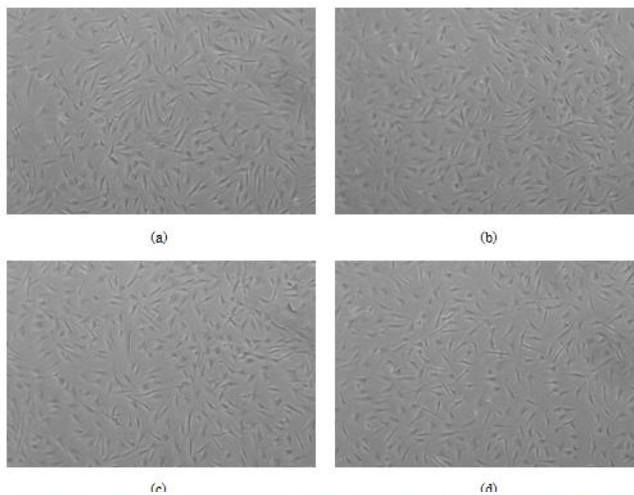


Figure 6. Morphological features of fibroblasts 24 h after US irradiation and control experiments. (a) Control. (b) 0.1 W/cm². (c) 0.5 W/cm². (d) 1.0 W/cm². US irradiated fibroblasts had morphologically similar features to those of controls.

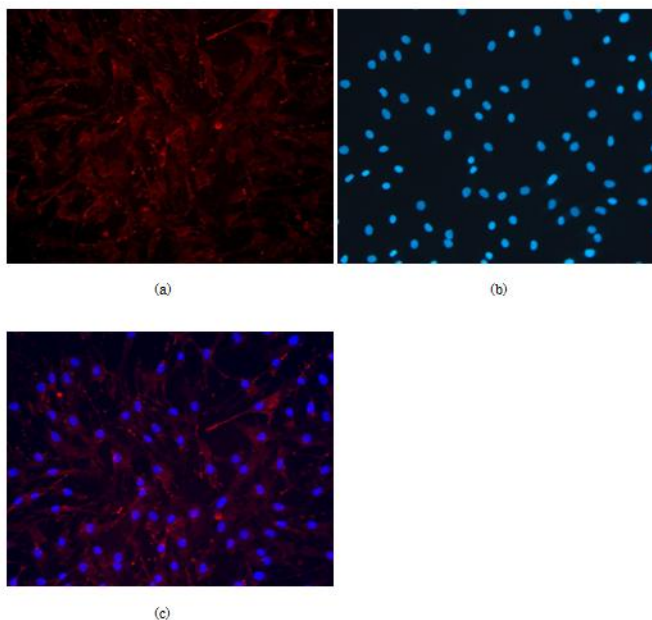


Figure 7. Immunofluorescent staining patterns of α -SMA in US irradiated fibroblasts. Fibroblasts were stained for α -SMA (a, red) and nuclei (b, blue). An image overlay (c, merge) that was edited using ImageJ (National Institutes of Health) demonstrated that α -SMA is expressed around the nuclei in each fibroblast. Micrographs were obtained with an Olympus BX50 fluorescence microscope using 10 $\times$ magnification (Olympus).

DISCUSSION

This study presents two novel findings. First, we showed that US irradiation increased α -SMA expression of mRNA and protein in human dermal fibroblasts and α -SMA expression in the cytoplasm around nuclei in US irradiated fibroblasts. Second, US irradiation increased TGF- β 1 mRNA but did not increase its protein concentration in culture medium. These results support the hypothesis that mechanical stimulation of US promotes α -SMA expression in human dermal fibroblasts. This finding suggests that US therapy accelerates wound closure of chronic wounds at the proliferation and remodeling phase.

Our immunofluorescent double-staining analysis of α -SMA and nuclei showed that the location of α -SMA in US irradiated fibroblasts is in the cytoplasm around nuclei. This localization is the same as that of stress fibers observed in US irradiated fibroblasts in a study by Zhou et al.³⁵ It has been reported that α -SMA is recruited to stress fibers, which are defined as intracellular axial bundles of filamentous-actin.^{21,36} Although these observations suggest the appropriate localization of α -SMA, our results indicating up-regulation of α -SMA mRNA and protein 24 h after US irradiation is not in agreement with Tsai et al.'s report that US irradiation promotes tendon cells migration, but not α -SMA expression.²⁵ It is unclear whether this contradiction is due to modification of US irradiation methods and/or elimination of FBS and/or a difference in the cells used. However, our results in serum-free conditions with modified US irradiation suggest a direct up-regulatory effect of US irradiation on fibroblasts. Our finding of 1.4-1.7 fold higher protein expression is consistent with previous reports that fibroblasts that received mechanical tension had an approximately 1.6 fold increase in α -SMA protein compared with controls.^{22,23} However, our intervention duration was much less with 10 min of US irradiation compared with that of an 8 h dynamic tensile force²² and a 4 h static tensile force²³. This difference between studies suggests the efficiency of US irradiation on α -SMA expression. Additionally, a negative result at 48 h suggests that to maintain the US efficacy for α -SMA expression, multiple irradiations would be required.

The results of our study indicating an up-regulatory effect of TGF- β 1 mRNA expression is consistent with that in Tsai et al.'s report with rat tendon cells, Mukai et al.'s report with rat chondrocytes, and Hiyama et al.'s report with beagle nucleus pulposus cells.²⁷⁻²⁹ However, our protein results are not in agreement with their studies, which showed detectable concentrations of TGF- β 1 at 24 h and an increase of TGF- β 1 by US under higher baseline concentrations of 255 pg/ml in Tsai et al.'s study and approximately 650 pg/ml in Mukai et al.'s studies^{27,28}, and no increase under lower baseline concentrations of 179 pg/ml by Hiyama et al.²⁹ The lack of TGF- β 1 protein at 24 h in the control and treatment groups in our study could be due to the absence of TGF- β 1 included in FBS^{27,29} or not using aggregated cells;²⁸ however, these methods should not be applied to evaluate the direct effect of US irradiation. Negative results of TGF- β 1 protein at 48 h under low baseline concentrations of 118 pg/ml in our study and the results of previous reports described above suggest that baseline TGF- β 1 concentrations affect the additive release of TGF- β 1 by US irradiation. Therefore, the direct effect of US irradiation on TGF- β 1 protein release might be unexpectedly small. It has been reported that TGF- β 1 has a key role in stimulating characteristics of differentiated myofibroblasts including α -SMA expression.²¹ However, our finding of α -SMA up-regulation at 24 h with no change in TGF- β 1 protein is in contrast to reports by Serini et al.³⁷ and Desmoulière et al.³⁸ who found that with TGF- β 1 administration without mechanical stimulation, α -SMA up-regulation required a much higher TGF- β 1 concentration of 5 or 10 ng/ml and a longer incubation with TGF- β 1 for 3 or 7 days. From this contradiction, it is speculated that α -SMA expression by US irradiation might be

independent from TGF- β 1 release. To determine this issue, more detailed studies using TGF- β 1 inhibitors are required. In our study, the detection of TGF- β 1 in the control group at 48 h is in accordance with Fan *et al.*'s study demonstrating that non heparin stimulated human dermal fibroblasts release TGF- β 1 in serum free conditions.³⁹ This result suggests that human dermal fibroblasts incubated on a dish release TGF- β 1 without any stimulation and serum. Further studies are required to determine the standard release of TGF- β 1 on fibroblasts.

With regard to US intensity, all intensities increased each gene expression almost equivalently. This finding suggests that direct irradiation of 0.1 W/cm² (SATP) was sufficient to induce the gene expression of TGF- β 1 and α -SMA. Our findings showed the same morphological features of fibroblasts at 24 h after US irradiation among the control and treatment groups as Tsai *et al.*'s study of tendon cells with the same US intensities.²⁵ However, de Oliveira RF *et al.* showed that a higher intensity, especially above 0.8 W/cm² (SATP), could cause cell damage.⁴⁰ Although *in vitro* results are not always available for clinical application, low-intensity US would be suitable for therapeutic application. Clinical treatments and previous clinical trials treating chronic wounds have rarely used low-intensity US such as 0.1 W/cm² (SATP).^{3-6,9,34} Therefore, the efficacy of such intensity with a pulse or continuous mode also needs to be investigated in further *in vivo* and clinical studies.

This study showed the up-regulatory effect of US irradiation on α -SMA expression in human dermal fibroblasts. Effective parameters and procedures of US irradiation for each healing phase need to be investigated in future studies based on the finding of this study, which could result in improvement of adjunctive therapy of chronic wound treatments.

ACKNOWLEDGMENTS

The authors thank Mr. Katsuhito Shuno, Mr. Norihito Iizuka and Ms. Mari Watanabe for their encouragement and help given during the course of this work, Dr. Shunsuke Sakakibara and Dr. Shingo Kamoshida for their help in immunofluorescence staining, and Dr. Gojiro Nakagami for his help in preparing the manuscript.

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