

CO₂ laser therapy accelerates the healing of ulcers in the oral mucosa by inducing the expressions of heat shock protein-70 and tenascin C

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Summary. The treatment of ulceration or stomatitis with laser therapy is known to accelerate healing and relieve pain, but the underlying biological mechanism is not fully understood. The present study used a mouse model of ulceration to investigate the molecular mechanisms by which CO₂ laser therapy accelerated the wound healing process. An ulcer was experimentally created in the palatal mucosa of the mouse and irradiated with light from a CO₂ laser. Compared with controls (no irradiation), laser irradiation induced the proliferation of epithelial cells and faster re-epithelialization of the wound area. Immunohistochemistry experiments showed that heat shock protein-70 (HSP70) was expressed mainly in the epithelium of normal palatal tissue, whereas there was little tenascin C (TnC) expression in the epithelium and mesenchyme under normal conditions. Laser irradiation induced HSP70 mRNA and protein expression in the lamina propria as well as TnC expression in the mesenchyme underlying the renewing epithelium. Epithelial cells and fibroblasts were exposed to heated culture medium or laser irradiation to establish whether hyperthermia mimicked the effect of laser irradiation. Culture of fibroblasts in heated medium increased the expressions of both TnC and TGF-β1, whereas laser irradiation induced only TnC expression. The present study indicates that CO₂ laser irradiation

exerts a photobiogenic effect to up-regulate TnC expression without inducing TGF-β1 expression. We suggest that CO₂ laser therapy has an advantage over thermal stimulation.

Key words: CO₂ laser, Wound healing, Tenascin C Heat shock protein-70, Temperature

Introduction

The carbon dioxide (CO₂) laser has been widely used in several surgical procedures and is reported to accelerate wound healing (Zand et al., 2012; Prasad and Pai, 2013). In oral and dental clinical practice, CO₂ laser irradiation therapy has become the preferred treatment method for traumatic or aphthous ulceration due to its ability to accelerate healing and rapidly relieve pain (Zand et al., 2012; Prasad and Pai, 2013). However, the mechanism by which CO₂ laser irradiation promotes wound healing remains unknown.

Wound healing involves the activation of epithelial cells, which proliferate and migrate to cover the wound. The proliferation and migration of epithelial cells are closely associated with extracellular matrix (ECM) components in the mesenchyme, and the production of ECM proteins is a key factor in tissue regeneration. Tenascin C (TnC) is an ECM and pericellular molecule that is expressed abundantly in tissues during embryogenesis (Mane et al., 2011) and regulates cell adhesion and migration during development. The expression of TnC is down-regulated in normal mature

tissue but temporarily upregulated at the invasive or migratory margin of a recovering wound (Udalova et al., 2011; Midwood and Orend, 2009). It is well known that the healing process in oral mucosa is faster than that in skin and results in minimal scar formation (Glim et al., 2013). Interestingly, oral mucosa has higher levels of TnC than the skin, and this has been investigated as a possible reason for the faster healing process and minimal scar formation in oral mucosa. The available evidence indicates that TnC may be a key factor that accelerates recovery of the oral mucosa during ulcer healing (Wong et al., 2009). However, no studies to date have reported the effect of laser therapy on TnC expression during wound healing.

During laser treatment, the temperature of the irradiated region increases. The temperature of a tissue affects its cellular biology during wound healing, in part by influencing the proliferation and/or apoptosis of cells (Xia et al., 2000; Nagarssekar et al., 2012). Heat shock proteins (HSPs) such as HSP47, HSP27 and HSP70 are molecular chaperones induced by pathophysiological stressors such as hyperthermia (Saibil, 2013). HSPs play an important role in maintaining the normal configuration of intracellular proteins. Notably, HSPs have been detected in the region of a wound and reported to inhibit fibrotic processes during wound healing (Kovalchin et al., 2006; Miyamoto et al., 2008; Vasques et al., 2013; González et al., 2013). In this way, the induction of HSP70 is thought to facilitate wound healing.

TGF- β is another factor that plays a key role in wound healing. TGF- β modulates several aspects of cellular behavior, including proliferation, migration and ECM deposition (Frank et al., 1996; Yang et al., 2001). Furthermore, TGF- β 1 plays an important role in epithelial cell regeneration (Hosokawa et al., 2005; Leydon et al., 2014). However, data are limited regarding the effects of laser irradiation on TGF- β 1 signaling in healing wounds.

The photobiogenic factor(s) mediating the actions of laser irradiation on wound healing remain uncharacterized. Furthermore, although thermal effects may play a key role in the acceleration of wound healing by laser therapy, an increase in temperature may not be the only mechanism involved. We hypothesized that the acceleration of wound healing by CO₂ laser treatment is associated with changes in the expressions of TnC, HSP70 and TGF- β 1 that are not due solely to thermal effects. Therefore, in the present study, we used *in vivo* and *in vitro* models of wound healing to compare the effects of thermal stimulation and CO₂ laser irradiation on the expressions of TnC, HSP70 and TGF- β 1 during wound healing.

Materials and methods

Creation of an ulcer in mouse oral mucosa

Eight-week-old male ICR mice (Kyudo Co., Tosu,

Japan) were used for the experiments. For creation of an ulcer, each mouse was anesthetized with 5% somnopenyl (Kyoritsu Seiyaku Corp., Tokyo, Japan), and a swab containing 50% acetic acid was applied to the palatal mucosa for one minute. Day 0 was defined as the day of ulcer creation. The mice were monitored and full recovery from anesthesia was confirmed 24 hours after ulcer creation (day 1). All animal experiments conformed to the principles of the Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of Fukuoka Dental College (Permission No. 15006, 15016).

Histology and immunohistochemistry

The wound healing process was analyzed using histology and immunohistochemistry. Mice were sacrificed on days 2, 3 or 5 (relative to ulcer creation), and ulcer-containing palatal tissue was excised. The dissected samples were fixed with 10% neutral buffered formalin solution, embedded in paraffin and sectioned (RM2125 RTS; Leica Microsystems, Tokyo, Japan). Serial horizontal sections (4–6 μ m) were mounted and stored at room temperature until use. The slides were deparaffinized by two rinses in xylene, rehydrated using a graded series of ethanol/phosphate-buffered saline (PBS) and stained with hematoxylin and eosin (H&E). Wound size was determined as the distance between the tip of the renewing epithelium on one side and the tip of the renewing epithelium on the other side (these points are indicated by filled arrows in Fig. 1).

For immunohistochemistry, the sections were treated with 0.3% hydrogen peroxide. Antigen retrieval was performed with heat-activated citric acid, and the sections were blocked with 10% normal goat serum. The specimens were then incubated overnight at 4°C with primary antibodies: polyclonal rabbit anti-mouse HSP70 antibody (1:200; Cell Signaling Technology, Danvers, MA, USA), monoclonal rat anti-mouse Ki67 antibody (1:200; Dako Japan, Tokyo, Japan) or monoclonal rat anti-mouse TnC antibody (1:100; R&D Systems, Minneapolis, Mn, USA). The specimens were subsequently incubated for 1 hour at room temperature with biotin-conjugated secondary antibodies: goat anti-rabbit IgG (Nichirei Biosciences Inc., Tokyo, Japan) or goat anti-rat IgG H&L (Abcam, Tokyo, Japan). The biotin-conjugated secondary antibodies were detected using streptavidin-peroxidase (Vector Laboratories Inc., Burlingame, CA, USA) and visualized with 3,3'-diaminobenzidine (DAB; Nichirei Biosciences Inc.). The sections were counterstained with hematoxylin, dehydrated and mounted for observation. Omission of the primary antibody was used as a negative control. Additionally, as a positive control, the expression of TnC was confirmed in palatal tissue from mouse embryos.

Cell proliferation assay

The proliferative activity of cells within a

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rectangular region containing the front of the renewing epithelium or the wound margin area (Fig. 2) was calculated as the number of Ki67-positive cells divided by the number of hematoxylin-positive cells.

Extraction of RNA and real-time polymerase chain reaction (PCR)

Ulcer lesions (wound area), surrounding tissues (wound margin area) and cultured cells were collected at 4 hours, 12 hours, 24 hours and 48 hours after laser irradiation or thermal stimulation and then homogenized in TRIzol reagent (Thermo Fisher Scientific, Tokyo, Japan). Total RNA was extracted as per the manufacturer's recommendations, and cDNA was synthesized using PrimeScript™ II reverse transcriptase (Takara Bio Inc., Otsu, Japan). Amplification was performed in a CFX96™ Real-Time PCR Detection System using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA). The mRNA expressions of alpha-smooth muscle actin (α -SMA), HSP47, HSP70, platelet and endothelial cell adhesion molecule-1 (PECAM-1), TnC, vascular endothelial growth factor (VEGF), collagen-I α 1 (Col-I α 1), periostin (Pstn), TGF- β 1, TGF- β 2 and TGF- β 3 were examined by real-time PCR. Fold-differences in gene expression were calculated based on the $2^{-\Delta\Delta CT}$ method with normalization to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers are described in Table 1.

Temperature measurement

A micro-thermometer (AM3000; Anritsu Meter Co., Tokyo, Japan) was used to measure the temperature changes at the palatal surface caused by 1 W or 3 W CO₂ laser irradiation. The temperature was measured within the palatal epithelium at a depth of 0.4 mm. The temperature during laser irradiation was recorded every second for two minutes.

Cell culture

Fibroblast cells derived from human periodontal ligament (a kind gift from Prof. Tsuruga) were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12; Gibco, Thermo Fisher Scientific) (Tsuruga et al., 2002). HaCaT epithelial cells derived from human skin keratinocytes (Cosmo Bio Co., Tokyo, Japan) were cultured in DMEM (Gibco, Thermo Fisher Scientific) containing 10% fetal bovine serum (Gibco, Thermo Fisher Scientific) and 1% penicillin-streptomycin (Roche Diagnostics, Berlin, Germany) (Boukamp et al., 1988). Cells at passage 3-8 were used for all experiments. In experiments assessing the effects of thermal stimulation, the culture medium was replaced by medium heated to 45°C or 60°C, and the cells were then cultured for 4 hours or 24 hours in an incubator set to 37°C. In experiments assessing the effects of CO₂

laser irradiation, the cells were exposed to irradiation at a power of 1 W (power, 1.0 W; pulse duration, 1 ms) for 13 min or 3 W (power, 3.0 W; pulse duration, 2 ms) for 4.5 min and then cultured for 4 hours or 24 hours; the laser light was delivered from a distance of 10 mm above the cell dish. The number of living cells in each dish was counted after staining of the cells with trypan blue.

CO₂ laser therapy

For the *in vivo* experiment, a CO₂ laser (Opelaser Pro; Yoshida Dental Mfg Co. Ltd, Tokyo, Japan) was used to irradiate the ulcer and a surrounding region (about 1 mm beyond the ulcer margin) of palatal mucosa at 24 hours after creation of the ulcer (i.e. on day 1). Laser irradiation was delivered from a distance of 10 mm from the surface of the palatal mucosa. The ulcer was exposed to radiation at a power of either 1 W (power, 1.0 W; pulse duration, 1 ms) for 30 s or 3 W (power, 3.0 W; pulse duration, 2 ms) for 10 s, corresponding to an energy density of 7.0 J/cm². Mice with ulcers not irradiated with CO₂ laser were used as a control group. For the *in vitro* experiments, CO₂ irradiation of the cells was performed in a rapid circular motion and with blowing of air onto the culture medium to displace it from the surface of the cells (which enabled the CO₂ laser to be positioned closer to the cells during their irradiation). The method is illustrated in Fig. 6C. For the *in vitro* experiments, the settings used for 1 W irradiation (power, 1.0 W; pulse duration, 1 ms) and 3W irradiation (power, 3.0 W; pulse duration, 2 ms) were the same as for the *in vivo* experiments; however, since the area being irradiated was much larger than that in the *in vivo* experiments, CO₂ laser irradiation was applied at 1 W for 13 min or 3 W for 4.5 min.

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of TnC in the medium bathing cultured cells were measured using an ELISA kit (27767; IBL, Fujioka, Gunma, Japan). Samples of culture medium were added to buffer solution in 96-well plates and incubated for 1 hour at 37°C. Horseradish peroxidase-conjugated mouse monoclonal anti-TnC antibody (4F10TT) was added to each well and incubated for 30 min at 4°C. Substrate solution was then added to each well and incubated for 30 min at room temperature. Subsequently, stop solution was added to each well, and the level of TnC was determined with a microplate reader at 450 nm (ImmunoMini NJ-2300; NJ InterMed, Tokyo, Japan). The ELISA data were normalized to the number of living cells, which was counted for each plate. All data were normalized to those of the control.

Statistical analysis

Temperature measurements are expressed as the

mean \pm standard deviation (SD). All other data were analyzed using non-parametric methods and are presented graphically as box-plots showing the median, interquartile range and full range. Statistical comparisons were made using the Kruskal-Wallis test followed by the Mann-Whitney U test (as a post-hoc test). $P < 0.05$ was taken to indicate statistical significance.

Results

CO₂ laser therapy accelerated ulcer healing in mice

Acetic acid was applied to the palatal mucosa to create artificial ulcers in mice (day 0), and CO₂ laser irradiation was performed 24 hours after ulcer creation (day 1). Wound healing was subsequently observed on

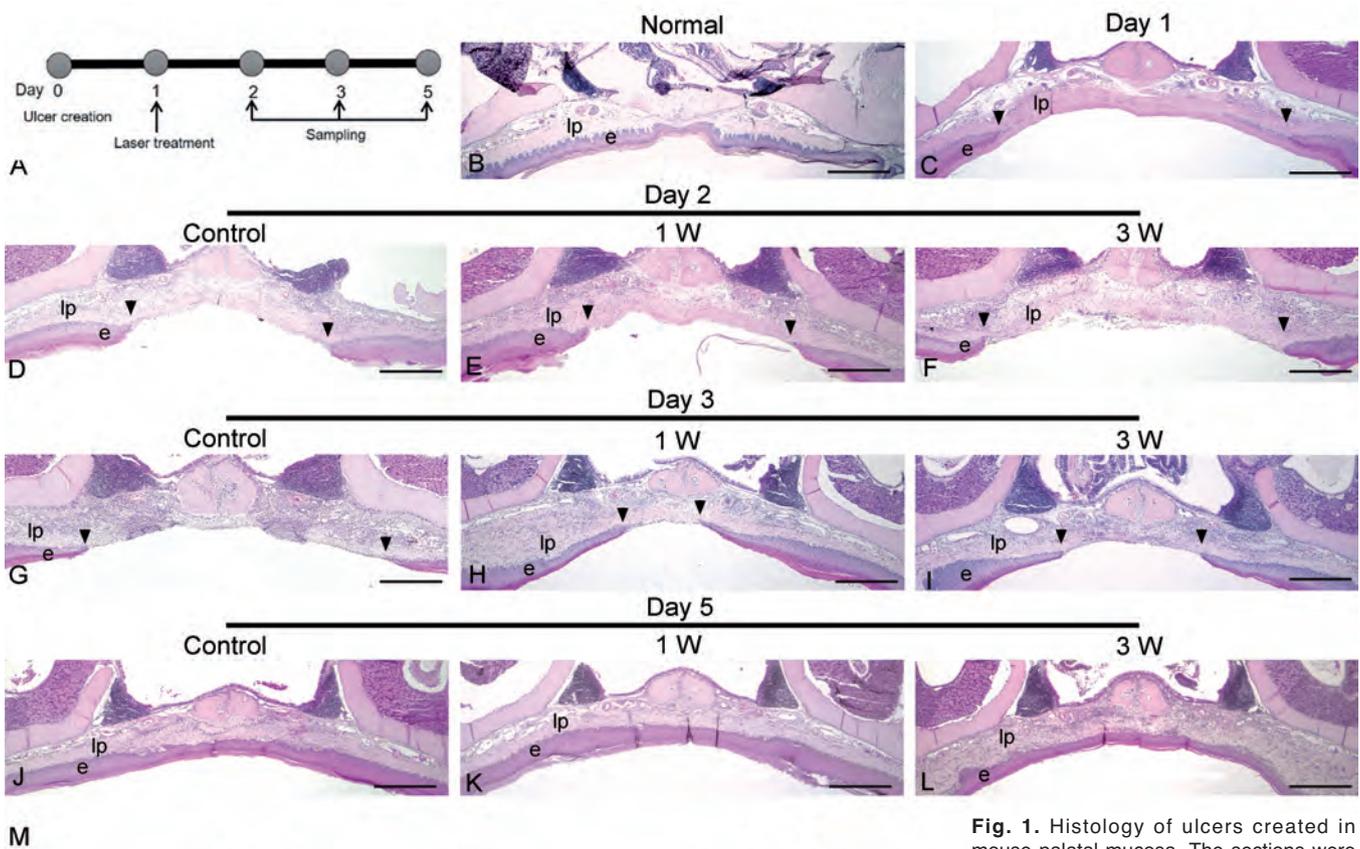


Fig. 1. Histology of ulcers created in mouse palatal mucosa. The sections were stained with hematoxylin and eosin. **A.** Schematic description of the experimental procedure showing the time points at which the ulcer was created, laser irradiation was performed and sampling was carried out. **B-L.** Representative tissue sections from each experimental group to illustrate ulcer histology at various time points. **B.** Normal palatal mucosa before ulcer creation. **C.** Palatal mucosa 24 hours after ulcer creation. **D.** Control group (no irradiation with laser) on day 2. **E.** 1 W CO₂ laser group on day 2. **F.** 3 W CO₂ laser group on day 2. **G.** Control group on day 3. **H.** 1 W CO₂ laser group on day 3. **I.** 3 W CO₂ laser group on day 3. **J.** Control group on day 5. **K.** 1 W CO₂ laser group on day 5. **L.** 3 W CO₂ laser group on day 5. e: epithelium; lp: lamina propria. **J.** Wound size in the control group (Control), 1 W CO₂ laser group (1 W) and 3 W CO₂ laser group (3 W) on days 2 and 3. Data are presented as box-plots showing the median, interquartile range and full range (n=4 per group). Kruskal-Wallis test: $P=0.241$ (day 2), $P=0.028$ (day 3). * $P < 0.05$ vs. the control group (Mann-Whitney U test). Scale bars: 300 μ m.

J. Wound size in the control group (Control), 1 W CO₂ laser group (1 W) and 3 W CO₂ laser group (3 W) on days 2 and 3. Data are presented as box-plots showing the median, interquartile range and full range (n=4 per group). Kruskal-Wallis test: $P=0.241$ (day 2), $P=0.028$ (day 3). * $P < 0.05$ vs. the control group (Mann-Whitney U test). Scale bars: 300 μ m.

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days 2, 3 and 5 in histological sections stained with H&E. To examine the effect of CO₂ laser irradiation on ulcer healing, wound size (the distance between the epithelial margins of each wound; arrowheads, Fig. 1C-I) was measured on days 2 and 3 and compared between the control and laser-treated groups. The normal hard palate (in the absence of an ulcer) was lined by a continuous, thick, stratified squamous epithelium (Fig. 1B). This normal epithelium was lost 1 day after creation

of an ulcer (Fig. 1C). On day 2, the distance between the epithelial margins of the wound did not differ significantly between the 1 W laser, 3 W laser and control groups (Fig. 1D-F,M). However, on day 3, wound size was significantly smaller in the 1 W laser and 3 W laser groups than in the control group ($P < 0.05$; Fig. 1G-I,M). On day 5, the wound area was completely covered by renewing epithelium in all groups (Fig. 1J-L).

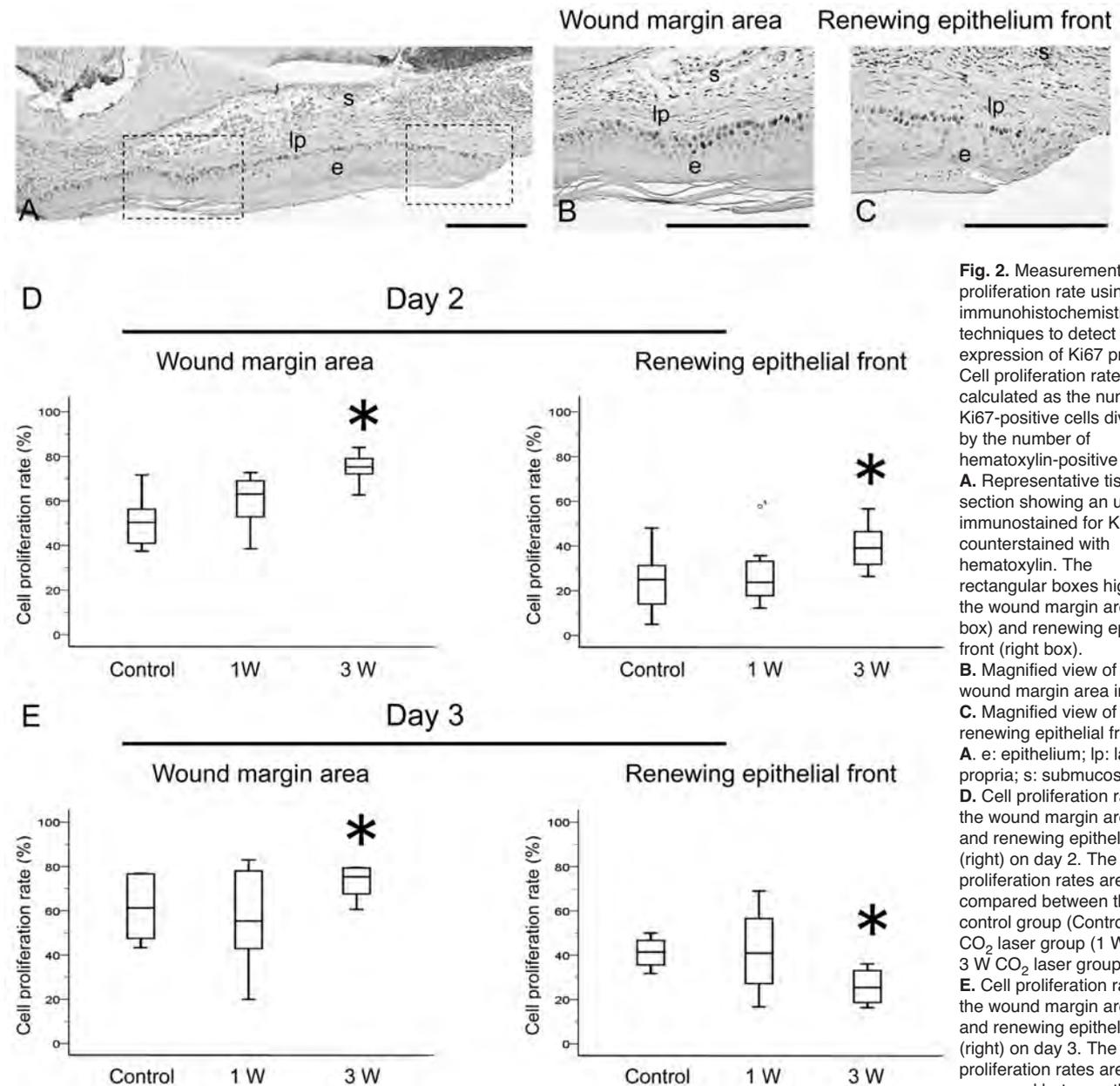


Fig. 2. Measurement of cell proliferation rate using immunohistochemistry techniques to detect the expression of Ki67 protein. Cell proliferation rate was calculated as the number of Ki67-positive cells divided by the number of hematoxylin-positive cells. **A.** Representative tissue section showing an ulcer immunostained for Ki67 and counterstained with hematoxylin. The rectangular boxes highlight the wound margin area (left box) and renewing epithelial front (right box). **B.** Magnified view of the wound margin area in **A.** **C.** Magnified view of the renewing epithelial front in **A.** e: epithelium; lp: lamina propria; s: submucosa. **D.** Cell proliferation rates in the wound margin area (left) and renewing epithelial front (right) on day 2. The cell proliferation rates are compared between the control group (Control), 1 W CO₂ laser group (1 W) and 3 W CO₂ laser group (3 W). **E.** Cell proliferation rates in the wound margin area (left) and renewing epithelial front (right) on day 3. The cell proliferation rates are compared between the

control group (Control), 1 W CO₂ laser group (1 W) and 3 W CO₂ laser group (3 W). Data are presented as box-plots showing the median, interquartile range and full range (n=4 per group). Kruskal-Wallis test: $P = 0.048$ (day 2, wound margin area), $P = 0.0495$ (day 2, renewing epithelial front), $P = 0.047$ (day 3, wound margin area), $P = 0.094$ (day 3, renewing epithelial front). * $P < 0.05$ vs. the control group (Mann-Whitney U test). Scale bars: 200 μm .

Irradiation with 3 W CO₂ laser induced epithelial cell proliferation

Since recovery of the epithelium was accelerated by CO₂ laser irradiation, we conducted a cell proliferation assay based on immunohistochemical staining of Ki67. We selected two regions for evaluation of cell proliferation rate: the wound margin, which lay outside the boundary between normal and renewing epithelium, and the renewing epithelial front within the wound region (Fig. 2A-C). The epithelial cell proliferation rate in the wound margin area after 3 W laser irradiation was significantly higher than that in the control group on days 2 and 3 ($P < 0.05$; left panels, Fig. 2D,E). In the renewing epithelial front, cell proliferation rate in the 3 W laser group was also higher than that in the control group on day 2 but was lower than that in the control group on day 3 ($P < 0.05$; right panels, Fig. 2D,E). 1 W CO₂ laser irradiation did not significantly affect epithelial cell proliferation rate in the wound margin or the renewing epithelial front, as compared with controls (Fig. 2D,E).

Irradiation with CO₂ laser increased the mRNA expressions of HSP70 and TnC

We next explored whether the histological changes described above might be associated with changes in the mRNA expressions of HSP47, HSP70, α -SMA, PECAM-1, VEGF, TnC, Col-I α 1, Pstn, TGF- β 1, TGF- β 2 or TGF- β 3. These experiments were carried out using normal palatal mucosa irradiated with 1 W or 3 W CO₂ laser. At 4 hours after 1 W CO₂ laser irradiation there was a dramatic increase in HSP70 mRNA expression but no significant changes in the expressions of the other genes (Fig. 3A). HSP70 mRNA expression was also markedly enhanced at 4 hours after 3 W CO₂ laser irradiation, but this was accompanied by smaller increases in the expressions of PECAM-1, VEGF and TnC (Fig. 3A). There were no significant changes in the expressions of any of the genes at 12 hours after 1 W CO₂ laser irradiation (Fig. 3B). However, at 12 hours after 3 W CO₂ laser irradiation, the mRNA expressions of HSP70 and TnC remained significantly elevated, and there were also significant increases in the mRNA expressions of HSP47, VEGF, TGF- β 1 and TGF- β 3 (Fig. 3B). Interestingly, at 4 hours after laser irradiation there were no changes in the mRNA expressions of any of the TGF- β cytokines, which are known to be associated with wound healing. By contrast, at 12 hours after 3 W laser irradiation, TGF- β 1 and TGF- β 3 were significantly increased compared with normal tissue.

Irradiation with CO₂ laser increased the protein expression of HSP70

In subsequent experiments, we focused on the pattern of HSP70 expression during the wound healing process. The protein expression of HSP70 (detected by

immunohistochemistry) in the wound area and wound margin area at day 3 (48 hours after laser irradiation) are shown in Fig. 4A-D. In normal palatal mucosa, HSP70 expression was observed in the epithelium (brown staining in Fig. 4A). HSP70 expression was diminished in the renewing epithelial front in the control, 1 W CO₂ laser and 3 W CO₂ laser groups (arrows, Fig. 4B-D). Interestingly, after treatment with 3 W CO₂ laser, HSP70 expression was also observed in the lamina propria near the wound margin (arrowhead, Fig. 4D). Compared with the control group, the mRNA expression of HSP70 in the wound margin area was significantly upregulated in the 3 W laser and 1 W laser groups at 4 hours after laser irradiation but not at 48 hours (Fig. 4E). We also performed Western blot experiments to examine the HSP70 protein expression at 48 hours after irradiation:

Table 1. Primers used for real-time PCR experiments.

Primer sets	Orientation	Sequence (5' to 3')
Mouse α -SMA	Forward	GGCTCTGGGCTCTGTAAGG
	Reverse	CTCTTGCTCTGGGCTTCATC
Mouse HSP47	Forward	AGGGCAGCCTAGCTCTTTTT
	Reverse	ATCATTTTAAGGATGGGCAGTTCT
Mouse HSP70	Forward	GGTGGTGCAGTCCGACATG
	Reverse	TTGGGCTGTGCGCCGT
Mouse Col-I α 1	Forward	GGGTCCCTCGACTCCTACA
	Reverse	TGTGTGCGATGACGTGCAAT
Mouse PECAM-1	Forward	CTGCCAGTCCGAAAATGGAAC
	Reverse	CTTCATCCACCGGGGCTATC
Mouse VEGF	Forward	AGTCCCATGAAGTGATCAAGTTCA
	Reverse	ATCCGCATGATCTGCATGG
Mouse Pstn	Forward	CAGTTGGAATGATCAGCTCTTGG
	Reverse	CAATTTGGATCTTCGTCTTGCAG
Mouse TGF β 1	Forward	GATGTCAGCTCTGGCAAAGATTAG
	Reverse	CAGGCTGAGCTTCATGCCTTTAC
Mouse TGF β 2	Forward	AATGGTTGCACCACAAGCAAGA
	Reverse	TTCCCAGGGCTGAGATGATAAGAG
Mouse TGF β 3	Forward	TGATGCTGAAACCATGCTGGA
	Reverse	GCTACATGACCAACTCAGGCAGAA
Mouse TnC	Forward	GGAGCAAGCTGATCCAAACCA
	Reverse	CCAGTGCTTGAGTCTTGTCACCA
Mouse GAPDH	Forward	TGTGTCCGTCGTGGATCTG
	Reverse	TTGCTGTTGAAGTCGCAGGAG
Human HSP70	Forward	CGTCTATAAGGATGGCCGGG
	Reverse	TGAGCTTGTGGATCACTGGAG
Human TGF β 1	Forward	GCAGCACGTGGAGCTGTA
	Reverse	CAGCCGGTTGCTGAGGTA
Human TnC	Forward	AGTTTCTGGATGGCATCGG
	Reverse	CAGTCTCTCTGGGTTCTCG
Human GAPDH	Forward	GCACCGTCAAGGCTGAGAAC
	Reverse	TGGTGAAGACGCCAGTGGA

α -SMA, alpha-smooth muscle actin; Col-I α 1, collagen type I alpha 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HSP47/70, heat shock protein-47/70; PECAM-1, platelet endothelial cell adhesion molecule-1; Pstn, periostin; TGF β 1/2/3, transforming growth factor beta-1/2/3; TnC, tenascin C; VEGF, vascular endothelial growth factor.

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the level of HSP70 protein in the wound area was not significantly different from that of the control (data not shown).

Irradiation with CO₂ laser increased the protein expression of TnC

The protein expression of TnC in the wound area

and wound margin area at day 3 (48 hours after laser irradiation) are shown in Fig. 5A-D. There was little or no expression of TnC in normal palatal mucosa (Fig. 5A). However, in the control group, TnC was widely expressed in the lamina propria of the wound area during healing (Fig. 5B). The expression of TnC in the lamina propria of the wound area was increased in mice irradiated with 1 W CO₂ laser (arrowhead, Fig. 5C).

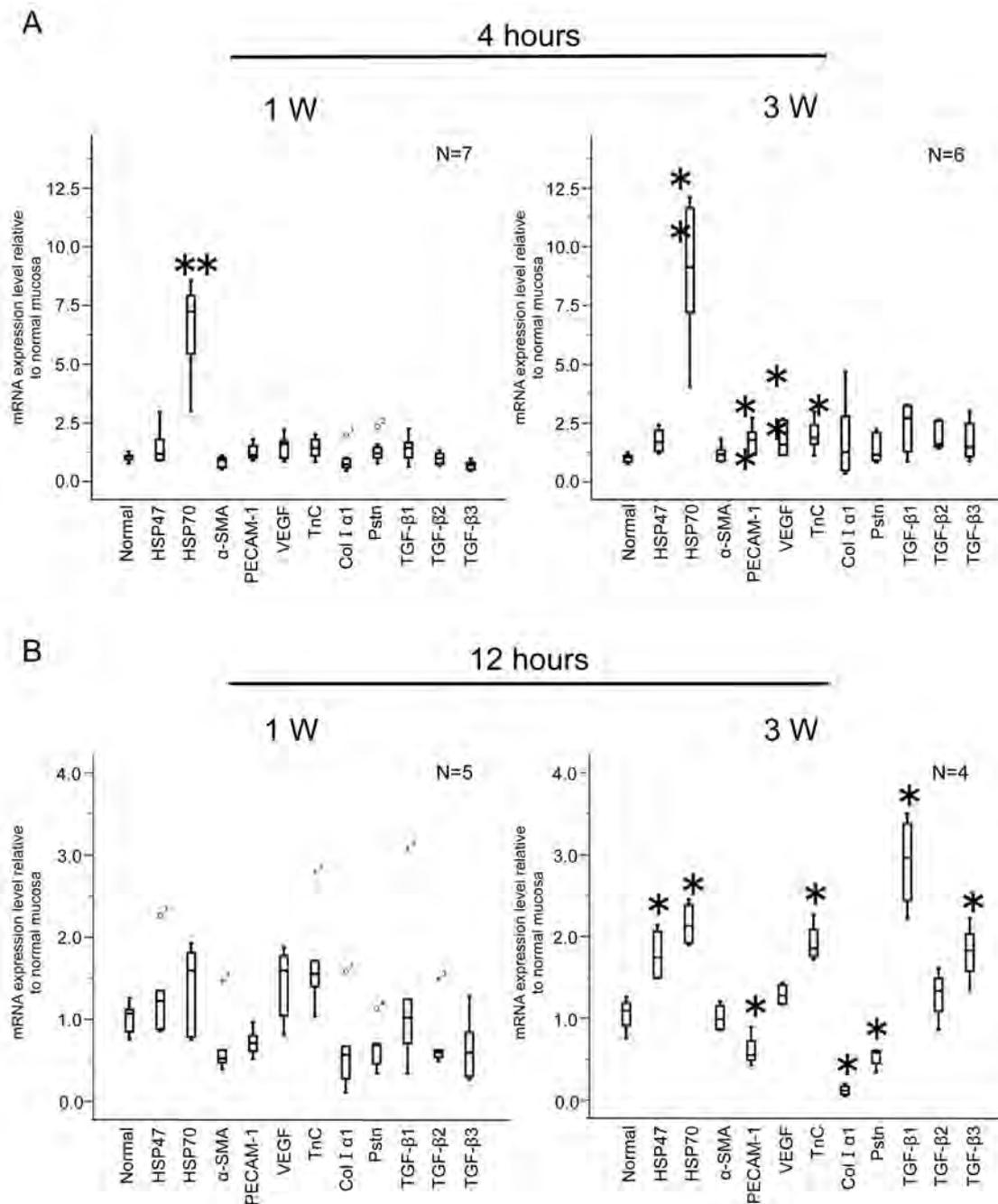


Fig. 3. Real-time PCR analysis of the effects of CO₂ laser irradiation on the expressions of various genes in the palatal mucosa. The mRNA expression of each gene was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and then expressed relative to that measured in normal palatal mucosa. **A.** Gene expression levels at 4 hours after irradiation with 1 W or 3 W CO₂ laser. **B.** Gene expression levels at 12 hours after irradiation with 1 W or 3 W CO₂ laser. Data are presented as boxplots showing the median, interquartile range and full range (the number of samples is indicated in the figure). * P<0.05, ** P<0.01 vs. normal palatal mucosa (Mann-Whitney U test). α-SMA: alpha-smooth muscle actin; Col-I α1: collagen-I α1; HSP47: heat-shock protein-47; HSP70: heat-shock protein-70; PECAM-1: platelet and endothelial cell adhesion molecule-1; Pstn: periostin; TnC: tenascin C; TGF-β1/2/3: transforming growth factor beta-1/2/3; VEGF: vascular endothelial growth factor.

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However, after irradiation with 3 W CO₂ laser, TnC expression was increased only in the lamina propria of the wound margin area and just beneath the newly formed epithelium (arrowheads, Fig. 5D). Interestingly, TnC expression in the wound margin area was significantly upregulated in the 3 W and 1 W laser groups at 4 hours after irradiation (as compared with the control group) but not at 48 hours (Fig. 5E). We also carried out a Western blot analysis, which revealed that TnC protein expression at 48 hours after irradiation was

not significantly different from that of the control (data not shown). In additional experiments, it was found that the expression of TGF- β 1 was not altered following CO₂ laser irradiation (Fig. 5F).

Comparison of the effects of CO₂ laser irradiation and thermal stimulation on the mRNA expressions of HSP70, TnC and TGF- β 1 in cultured cells

The results of the *in vivo* experiments suggested that

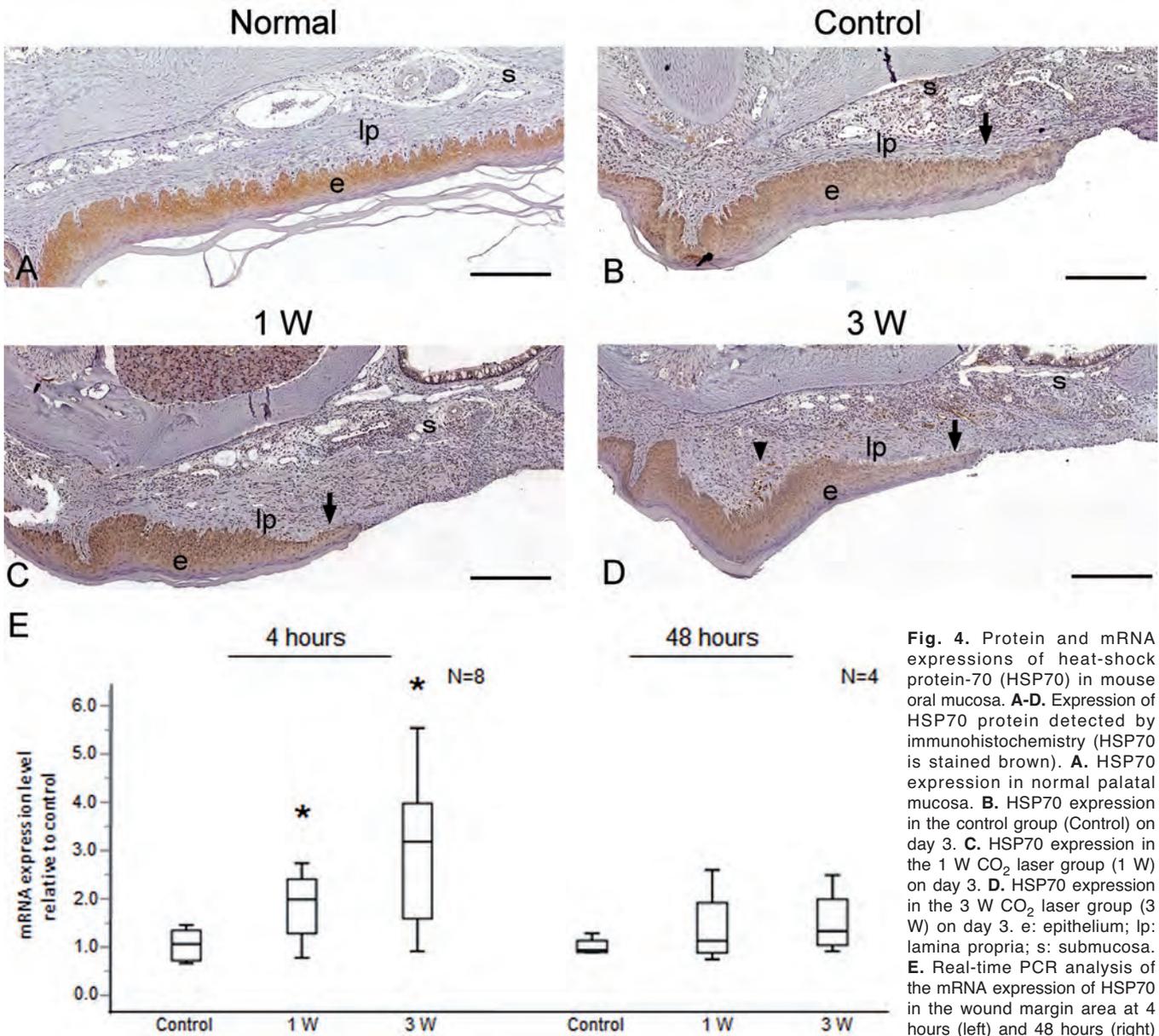


Fig. 4. Protein and mRNA expressions of heat-shock protein-70 (HSP70) in mouse oral mucosa. **A-D.** Expression of HSP70 protein detected by immunohistochemistry (HSP70 is stained brown). **A.** HSP70 expression in normal palatal mucosa. **B.** HSP70 expression in the control group (Control) on day 3. **C.** HSP70 expression in the 1 W CO₂ laser group (1 W) on day 3. **D.** HSP70 expression in the 3 W CO₂ laser group (3 W) on day 3. e: epithelium; lp: lamina propria; s: submucosa. **E.** Real-time PCR analysis of the mRNA expression of HSP70 in the wound margin area at 4 hours (left) and 48 hours (right)

after laser irradiation. The mRNA expression level of each gene was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and then expressed relative to the level in the control group. Data are presented as box-plots showing the median, interquartile range and full range (the number of samples is indicated in the figure). Kruskal-Wallis test: $P=0.014$ (4 hours), $P=0.437$ (48 hours). * $P<0.05$ vs. the control group (Mann-Whitney U test). Scale bars: 200 μ m.

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increases in the expressions of HSP70 and TnC might be key factors contributing to the acceleration of wound healing after CO₂ laser irradiation. To explore whether the changes in gene expressions induced by CO₂ laser irradiation were due solely to thermal effects in the

tissue, *in vitro* experiments were performed using cultured fibroblasts and epithelial cells. To mimic the temperature changes in the tissue caused by irradiation with 1 W and 3 W CO₂ laser, the cells were cultured in medium that had been heated.

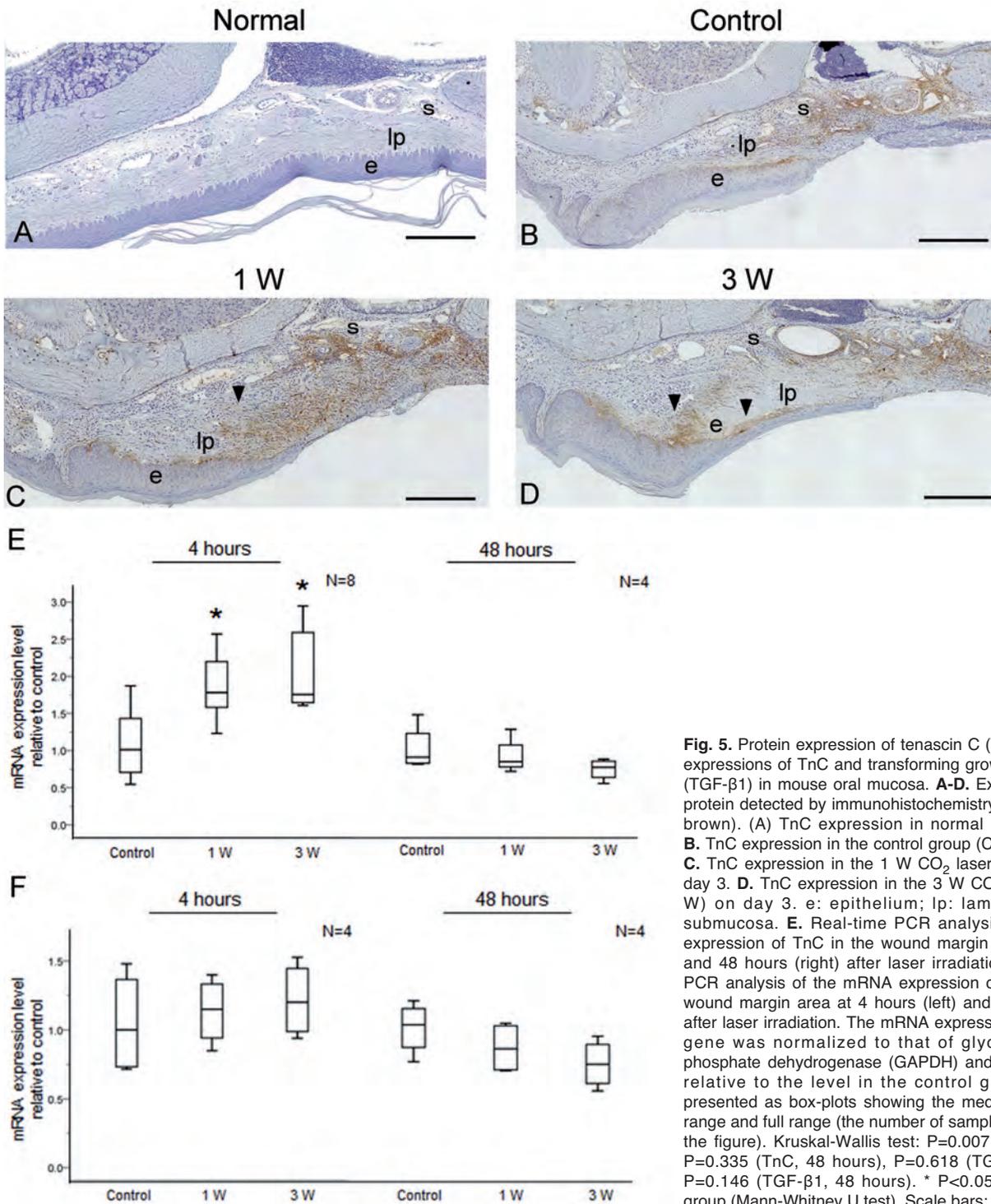


Fig. 5. Protein expression of tenascin C (TnC) and mRNA expressions of TnC and transforming growth factor beta-1 (TGF-β1) in mouse oral mucosa. **A-D.** Expression of TnC protein detected by immunohistochemistry (TnC is stained brown). (A) TnC expression in normal palatal mucosa. **B.** TnC expression in the control group (Control) on day 3. **C.** TnC expression in the 1 W CO₂ laser group (1 W) on day 3. **D.** TnC expression in the 3 W CO₂ laser group (3 W) on day 3. e: epithelium; lp: lamina propria; s: submucosa. **E.** Real-time PCR analysis of the mRNA expression of TnC in the wound margin at 4 hours (left) and 48 hours (right) after laser irradiation. **F.** Real-time PCR analysis of the mRNA expression of TGF-β1 in the wound margin area at 4 hours (left) and 48 hours (right) after laser irradiation. The mRNA expression level of each gene was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and then expressed relative to the level in the control group. Data are presented as box-plots showing the median, interquartile range and full range (the number of samples is indicated in the figure). Kruskal-Wallis test: P=0.007 (TnC, 4 hours), P=0.335 (TnC, 48 hours), P=0.618 (TGF-β1, 4 hours), P=0.146 (TGF-β1, 48 hours). * P<0.05 vs. the control group (Mann-Whitney U test). Scale bars: 200 μm.

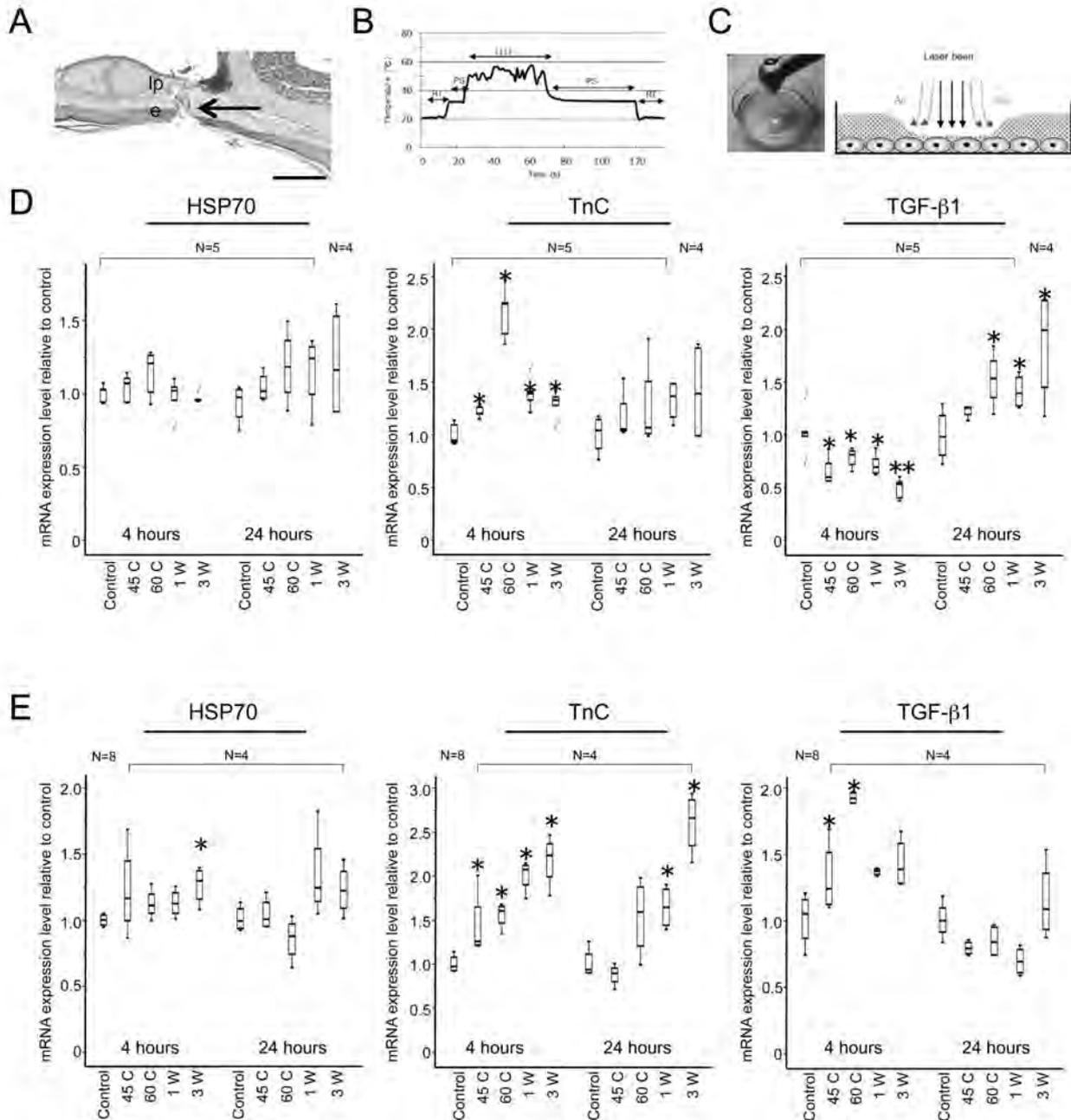
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Fig. 6. Comparison of the effects of CO₂ laser irradiation and thermal stimulation on gene expression in cultured cells. **A.** A section of tissue that was stained with hematoxylin and eosin after the temperature changes within the tissue had been measured using a micro-thermometer (the arrow indicates where the sensor had penetrated through the palatal surface). e: epithelium; lp: lamina propria. **B.** The temperature changes on the surface of the palatal epithelium before, during and after irradiation with CO₂ laser. LLLI: 1 W laser irradiation; PS: palatal surface; RT: room temperature. **C.** Photograph (left) and illustration (right) to show the technique used for laser irradiation of cells *in vitro*. Air was blow onto the surface of the culture medium to displace it from the cells being exposed to laser light. **D.** Real-time PCR analysis of the mRNA expressions of heat-shock protein-70 (HSP70), tenascin C (TnC) and transforming growth factor beta-1 (TGF-β1) in cultured epithelial cells at 4 hours and 24 hours after stimulation with heated culture medium (45°C and 60°C) and after irradiation with 1 W and 3 W CO₂ laser. **E.** Real-time PCR analysis of the mRNA expressions of HSP70, TnC and TGF-β1 in cultured fibroblasts at 4 hours and 24 hours after stimulation with heated culture medium (45°C and 60°C) and after irradiation with 1 W and 3 W CO₂ laser. The mRNA expression level of each gene was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and then expressed relative to the level in cells not exposed to heated medium or laser light. 45 C: exposed to culture medium heated to 45°C; 60 C: exposed to culture medium heated to 60°C; 1 W: irradiation with 1 W CO₂ laser; 3 W: irradiation with 3 W CO₂ laser. Data are presented as box-plots showing the median, interquartile range and full range (the number of samples is indicated in the figure). Kruskal-Wallis test: P=0.582 (HSP70 in epithelial cells at 4 hours), P=0.459 (HSP70 in epithelial cells at 24 hours), P=0.001 (TnC in epithelial cells at 4 hours), P=0.427 (TnC in epithelial cells at 24 hours), P=0.002 (TGF-β1 in epithelial cells at 4 hours), P=0.042 (TGF-β1 in epithelial cells at 24 hours), P=0.086 (HSP70 in fibroblasts at 4 hours), P=0.023 (HSP70 in fibroblasts at 24 hours), P<0.001 (TnC in fibroblasts at 4 hours), P=0.003 (TnC in fibroblasts at 24 hours), P=0.001 (TGF-β1 in fibroblasts at 4 hours), P=0.014 (TGF-β1 in fibroblasts at 24 hours). * P<0.05, ** P<0.01 vs. non-stimulated (control) cells (Mann-Whitney U test). Scale bar: 200 μm.

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First, to confirm temperature changes in the tissue during laser treatment, we measured the temperature fluctuations on the surface and 400 μ m below the

surface of the palatal epithelium during irradiation with the CO₂ laser. Fig. 6A shows a section of tissue stained with H&E after measurement of the temperature

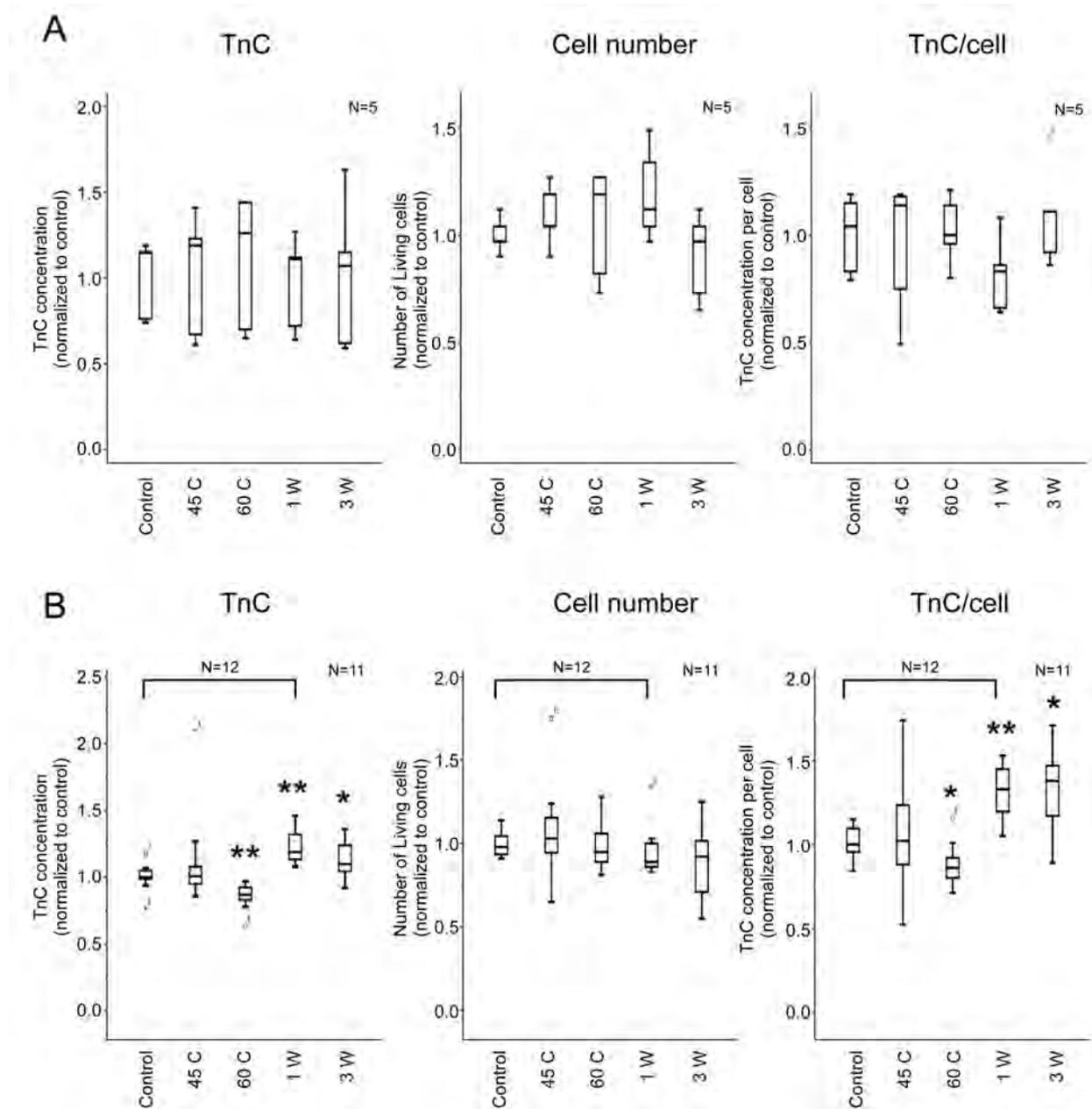


Fig. 7. Concentration of tenascin C (TnC) protein in the medium bathing cultured cells. **A.** Data for cultured epithelial cells obtained 24 hours after stimulation with heated culture medium (45°C or 60°C) or irradiation with 1 W or 3 W CO₂ laser. The graphs show the TnC concentration determined by ELISA (left), the number of living cells (middle) and the TnC concentration normalized to the number of cells (right). All data were normalized to those of the control. **B.** Data for cultured fibroblasts obtained 24 hours after stimulation with heated culture medium (45°C or 60°C) or irradiation with 1 W or 3 W CO₂ laser. The graphs show the TnC concentration determined by ELISA (left), the number of living cells (middle) and the TnC concentration normalized to the number of cells (right). All data were normalized to those of the control. 45°C: exposed to culture medium heated to 45°C; 60°C: exposed to culture medium heated to 60°C; 1 W: irradiation with 1 W CO₂ laser; 3 W: irradiation with 3 W CO₂ laser. Data are presented as box-plots showing the median, interquartile range and full range (the number of samples is indicated in the figure). Kruskal-Wallis test: P=0.86 (TnC concentration in medium bathing epithelial cells), P=0.31 (number of epithelial cells), P=0.43 (TnC expression per epithelial cell), P<0.001 (TnC concentration in medium bathing fibroblasts), P=0.136 (number of fibroblasts), P<0.001 (TnC expression per fibroblast). * P<0.05, **P<0.01 vs. non-stimulated (control) cells (Mann-Whitney U test).

changes. The arrow indicates where the sensor had penetrated through the palatal surface. The micro-thermometer did not cause any notable damage to the tissue. An example of the temperature changes measured in the region of the palatal surface during irradiation with 1 W CO₂ laser is presented in Fig. 6B. The technique used for laser irradiation of cultured cells is illustrated in Fig. 6C. The average temperature during 1 W CO₂ laser irradiation was 46.1±1.1°C at the palatal surface and 44.6±1.5°C at 400 μm below the epithelial surface. During 3 W laser irradiation, the average temperature increased to 71.5±5.1°C at the palatal surface and 60.3±5.6°C beneath the epithelium. Based on this, temperatures of 45°C and 60°C were applied to observe the effects of thermal stimulation *in vitro*.

In epithelial cells, both thermal stimulation and CO₂ laser irradiation increased TnC expression but slightly decreased TGF-β1 expression at 4 hours (all P<0.05; Fig. 6D). At 24 hours after stimulation, TGF-β1 expression was significantly increased by thermal stimulation at 60°C and by 1 W or 3 W CO₂ irradiation (Fig. 6D). Thermal stimulation and CO₂ laser irradiation also up-regulated TnC expression in cultured fibroblasts at 4 hours (Fig. 6E). Interestingly, TnC expression remained elevated at 24 hours in cells irradiated with 1 W or 3 W CO₂ laser. TGF-β1 expression in fibroblasts at 4 hours was significantly enhanced by thermal stimulation but not by CO₂ laser irradiation. At 24 hours after thermal or laser stimulation, the expression of TGF-β1 was not significantly different from that of the control (Fig. 6E).

CO₂ laser irradiation induces TnC in cultured fibroblasts but not epithelial cells

The results of the *in vitro* experiments shown in Fig. 6 indicated that CO₂ laser irradiation strongly induced the expression of TnC in fibroblasts. To examine this further, we used ELISA to measure the secretion of TnC protein into the culture medium following thermal or CO₂ laser stimulation. Since there was a possibility that thermal or laser stimulation would damage the cells, the number of living cells was counted in each group, and TnC protein expression was normalized to the number of surviving cells (Fig. 7). TnC secretion by epithelial cells was not affected by thermal stimulation or CO₂ laser irradiation (Fig. 7A). However, TnC secretion normalized to the number of surviving fibroblasts was significantly increased by 1 W and 3 W CO₂ laser irradiation (Fig. 7B).

Discussion

Aphthous stomatitis is a common ulceration of the oral mucosa caused by trauma, viral infection, thermal or chemical injury or defects of the immune system (Ship, 1996). In severe cases, aphthous stomatitis is accompanied by pain, and the relief of pain can greatly facilitate eating, swallowing and speaking. The clinical

efficacy of CO₂ laser irradiation at relieving the pain of aphthous stomatitis has been documented (Zand et al., 2009; Prasad and Pai, 2013). However, the mechanism by which CO₂ laser irradiation promotes wound healing has not been investigated previously.

In the present study, we created a mouse model of oral mucosal ulceration to explore the possible mechanism by which CO₂ laser irradiation promotes the wound healing process. We found that CO₂ laser irradiation accelerated closure of the wound by the renewing epithelium. We then applied immunohistochemistry techniques to detect cells expressing Ki67 protein, which is present during the G1, S and G2 phases of the cell cycle. Immunohistochemical analysis of Ki67 expression has been widely used to measure cell proliferation (Oka et al., 2012). Interestingly, re-epithelialization of the wound occurred more rapidly after 1 W CO₂ laser irradiation than after 3 W irradiation, despite the cell proliferation rate being slower for wounds treated with 1 W laser irradiation than for those exposed to 3 W laser (Figs. 1, 2). A possible reason for this apparent inconsistency is that 3 W CO₂ laser irradiation caused more tissue damage (due to thermal or photobiogenic effects) than 1 W irradiation. Indeed, there was a trend for the wound size at day 2 to be largest for the group exposed to 3 W laser irradiation. Another notable, and perhaps surprising, observation was that the epithelial cell proliferation rate in the 1 W laser irradiation group was not significantly higher than that of the control group, despite re-epithelialization occurring much more quickly. Taken together, these observations suggest that the acceleration of wound healing by CO₂ laser treatment depends not only on epithelial cell proliferation but also on other factors such as epithelial cell migration or re-construction of the connective tissue of the wound.

HSPs are responsible for protein integrity during transcription in cells that are exposed to stressors such as thermal stimulation (Bellaye et al., 2014). HSPs are molecular chaperones that facilitate protein synthesis and degradation. HSP70 is the most studied HSP with regard to immunity, cancer and fibrosis (Glim et al., 2013). Kovalchin et al. (2006) reported that *in vivo* delivery of HSP70 accelerated wound healing by up-regulating macrophage-mediated phagocytosis. In our study, HSP70 was expressed mainly in the prickle cell layer and basal layer of the epithelium in normal palatal mucosa. During wound healing, HSP70 was also expressed in the palatal epithelium, including the renewing epithelium. Furthermore, HSP70 gene expression in the wound margin area was increased at 4 hours after laser irradiation but not at 48 hours (Fig. 4E). These results suggest that the acceleration of epithelial proliferation by CO₂ laser irradiation might be mediated through an induction of HSP70 expression during the early phase of wound healing. Interestingly, 3 W laser irradiation was associated with greater up-regulation of HSP70 expression than 1 W irradiation. Immunohistochemistry experiments detected the expression of HSP70

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in the lamina propria after irradiation with 3 W CO₂ laser (Fig. 4D). This suggests that the higher levels of HSP70 observed after 3 W laser irradiation may have been due to the induction of HSP70 expression in the lamina propria. It has been reported that irradiation with a diode laser can induce HSP70 expression in the epidermis, dermis and hypodermis of the dorsal skin of the rat (at 24 hours), especially near blood vessels, hair follicles and sebaceous glands (Souil et al., 2001). In addition to the up-regulation of HSP70, laser irradiation was also observed to increase the expression of PECAM1 and VEGF, which are genes associated with angiogenesis. Thus, it is possible that angiogenesis may contribute to the faster wound healing that occurs after laser irradiation.

The findings of the present study indicate that epithelial factors alone, such as epithelial cell proliferation and migration over the wound, cannot explain the acceleration of wound healing by CO₂ laser irradiation. We therefore assessed whether the deposition of ECM might play an important role in the migration of epithelial cells. TnC is a large hexameric ECM protein that is widely distributed in embryonic and regenerating tissues but whose expression is restricted in mature adult tissues. TnC has been shown to enhance cell migration in a trans-well chamber (Islam et al., 2014). In our experiment, TnC expression was observed in the lamina propria after ulcer creation, particularly beneath the epithelium (Fig. 4F). Notably, laser irradiation further increased the expression of both TnC mRNA (real-time PCR) and TnC protein (immunohistochemistry) *in vivo*.

Recently, it has been reported that CO₂ laser irradiation induces the proliferation and migration of cultured human dermal fibroblasts, possibly through the activation of Akt, ERK and JNK signaling pathways (Shingyochi et al., 2017). This illustrates that cultured cells may be useful model systems for exploring the mechanisms of CO₂ laser irradiation. We therefore carried out *in vitro* experiments to further investigate whether TnC expression was influenced by CO₂ laser irradiation. TnC expression was found to be much higher in cultured fibroblasts than in cultured epithelial cells, consistent with the expression of TnC mainly in the mesenchyme. TnC mRNA expression in fibroblasts was increased at 4 hours after thermal stimulation or laser irradiation. However, at 24 hours, TnC remained elevated (relative to the control) only in fibroblasts exposed to laser irradiation and not in those that received thermal stimulation. Thus, although hyperthermia can induce TnC expression during wound healing, CO₂ laser irradiation can induce a more prolonged up-regulation of TnC expression that is maintained for at least 24 hours after irradiation (Fig. 6). Measurements of TnC concentration in the culture medium also supported the induction of TnC expression by laser stimulation. Indeed, cell survival rate may be affected by stimulation with high temperature or laser irradiation. The factors determining whether laser irradiation stimulates cells or impairs their survival are not clear and merit further

investigation. Taken together with our other data, these findings imply that faster wound closure after CO₂ laser irradiation may involve an acceleration of epithelial cell migration as well as proliferation. Furthermore, TnC may be an important photobiogenic factor induced by CO₂ laser irradiation.

TGF- β signaling is the most intensively studied growth factor pathway involved in wound healing (Frank et al., 1996; Yang et al., 2001). Shortly after injury, TGF- β is secreted by platelets to stimulate the production of ECM components (such as collagen by fibroblasts) and inhibit ECM degradation, thereby leading to restoration of the tissue. However, overproduction of the TGF- β family of growth factors leads to fibrosis. Hypertrophic scar tissue and fibroblasts produce more TGF- β 1 mRNA and protein, hence TGF- β 1 may be important in hypertrophic scar formation (Wang et al., 2000). Schrementi et al. (2008) reported that oral wounds contained lower levels of TGF- β 1 than dermal wounds, which resulted in healing with less scarring. Embryonic wounds that heal without a scar have low levels of TGF- β 1 and TGF- β 2, and neutralizing TGF- β 1 and TGF- β 2 can mimic the scar-free embryonic profile (Ferguson and O'Kane, 2004). In our experiment, both 1 W and 3 W CO₂ laser irradiation suppressed the mRNA expression of TGF- β 1 in epithelial cells and fibroblasts at 4 hours after stimulation, whereas thermal stimulation increased TGF- β 1 expression in fibroblasts. At 24 hours, TGF- β 1 mRNA expression in epithelial cells was increased by both thermal stimulation and laser irradiation, but this effect was not observed in fibroblasts. This suggests that laser irradiation might suppress the over-production of cytokines that inhibit wound healing. Lensvelt et al. (2010) observed that when CO₂ was used for establishment of a pneumoperitoneum in patients, heating the carbon dioxide to body temperature resulted in a lower expression of active TGF- β 1 in peritoneal biopsy specimens as compared with unheated (room temperature) CO₂. It also has been reported that CO₂ laser irradiation of fibroblasts induced the secretion of bFGF but not TGF- β 1 (Nowak, 2000). Overall, our study has yielded the novel finding that CO₂ laser irradiation can accelerate wound healing while potentially avoiding excessive inflammation due to elevated expression of TGF- β .

In this study, we did not apply the CO₂ laser in the defocusing mode, which is commonly used in the clinical setting to minimize heat production during the treatment of ulcers in the oral mucosa of the lip or tongue regions. Instead, we used palatal mucosa to evaluate the effects of CO₂ laser irradiation on wound healing, which allowed us to observe clear anatomical and biological differences with minimal technical errors. Although it is possible that CO₂ laser irradiation in the focusing mode might damage the oral mucosa, we found that it did not cause any damage to the palatal mucosa. At the highest power used in this study (3 W), laser irradiation showed positive effects on the palatal

mucosa, and this was supported by the results of the proliferation assay based on Ki67 (Fig. 2). Furthermore, the expressions of HSP47, HSP70 and TnC, which facilitate wound healing, showed significant increases in a laser-dependent manner.

In conclusion, our experiments have provided new data consistent with the proposal that CO₂ laser irradiation accelerates wound healing by elevating the expression of HSP70 and TnC. Furthermore, our study provides evidence that CO₂ laser irradiation induces TnC expression in fibroblastic cells in the lamina propria while suppressing the expression of TGF- β 1. This latter finding implies that the influence of CO₂ laser irradiation on wound healing cannot be explained by a thermal effect alone.

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Competing financial interests. All authors have no conflicts of interest.

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