

Effects of thymosin β_4 on wound healing of rat palatal mucosa

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Abstract. The objective of the present study was to investigate the effect of thymosin β_4 ($T\beta_4$) on the wound healing of rat palatal (RP) mucosa and related cellular properties. Cell viability, adhesion and migration of primary cultured RP cells were observed in the presence of $T\beta_4$ at various concentrations ranging from 1 to 1,000 ng/ml. The mRNA and protein expression of matrix metalloproteinase 2 (MMP2) and vascular endothelial growth factor (VEGF) in $T\beta_4$ -treated RP cells was assessed by quantitative polymerase chain reaction (RT-qPCR) and western blot analysis, respectively. For the *in vivo* assay, $T\beta_4$ was applied to excisional wounds (3 mm in diameter) that were made in the center of the palate (n=6). Images of the wound areas were captured and assessed histologically one week after surgery. $T\beta_4$ did not affect cell viability and adhesion, but RP cell migration was stimulated by $T\beta_4$ at concentrations of 100 and 1,000 ng/ml. $T\beta_4$ also increased the mRNA and protein expression of MMP2 and VEGF in RP cells. In the animal model, palatal wound closure was significantly enhanced in rats treated with $T\beta_4$. The results of the present study indicated that $T\beta_4$ promotes the wound healing of RP mucosa. Enhancement of RP cell migration and angiogenesis is likely to be involved in the promotion of wound healing.

Introduction

Thymosin β_4 ($T\beta_4$), an oligopeptide consisting of 43 amino acids, is known to sequester G-actin monomers. Upregulation of $T\beta_4$ by cDNA-mediated transfection was found to cause actin depolymerization in fibroblast cells (1), which demonstrated

that the modulation of $T\beta_4$ affected the polymerization state of actin cytoskeleton, as well as other cell processes associated with the organization of actin cytoskeleton, such as cell migration. Ito *et al* (2) showed that overexpression of $T\beta_4$ enhanced the formation of actin-based pseudopodia and cell motility in prostate cancer cells, providing direct evidence that $T\beta_4$ plays a role in cell migration. Colon cancer cells overexpressing $T\beta_4$ also exhibited enhanced cell migration, due in part to the activation of the Rac1 signaling pathway (3). In addition, extracellular, administered $T\beta_4$ promoted the cell migration of various types of cells including cardiomyocytes, human umbilical vein endothelial cells and conjunctival epithelial cells (4-6). The mechanism by which exogenous $T\beta_4$ influences cell migration remains to be elucidated. However, results of a recent study showed that $T\beta_4$ was rapidly internalized by cells, suggesting the involvement of an intracellular receptor in the effects of the peptide (7). Besides the effect on cell migration, exogenous $T\beta_4$ was found to have multifunctional activities such as angiogenesis, anti-apoptosis, anti-oxidative stress and anti-inflammation (8-11), which emphasize its therapeutic potential in the repair of damaged tissues or wound healing.

$T\beta_4$ is distributed ubiquitously in most tissues and cells, and is also known to concentrate highly at blood platelets (12). These findings suggest that endogenous $T\beta_4$ likely promotes the healing of damaged tissues. Exogenous $T\beta_4$ was also reported to accelerate the tissue repair of damaged cardiac, corneal and dermal tissues (4,6,13), which demonstrates potential for clinical applications in wound healing.

Generally, wound healing in the oral cavity is known to occur more quickly and scar less than dermal tissue, which may be due to the elements in saliva and unique phenotype of oral fibroblasts (14-16). Despite the relatively rapid wound healing, however, tissues damaged during periodontal and implant surgery are continuously challenged by bacterial infection in the oral cavity, necessitating meticulous maintenance of oral hygiene and additional plaque control. Prevention of bacterial contamination is even more important in the case of gingival graft surgery because a significant amount of tissue is lost at a palatal donor site. Autogenous gingival grafts are often accompanied by discomfort, pain and retarded tissue repair depending on a patient's condition. To avoid these post-operative problems, topical application of an antimicrobial treatment is recommended (17). Furthermore, several dressing materials,

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which are supposed to aid in tissue repair, are commonly applied to palatal wounds during the healing process (18,19). Accelerated regeneration of palatal mucosa was reported to occur following treatment with a basic fibroblast growth factor impregnated in collagen-gelatin scaffold (20). The aforementioned studies showed that dressing materials or chemicals employed for the treatment of dermal wound healing can also be effective for the regeneration of oral mucosa. Therefore, $T\beta_4$, which is known to enhance the regeneration of different types of tissue, is also expected to accelerate mucosal wound healing. In a previous study, $T\beta_4$ was documented to be a natural component of saliva. The concentrations in human saliva ranged from 0.2 to 3.6 $\mu\text{g/ml}$, varying with age and state of disease (21). The $T\beta_4$ levels in gingival crevicular fluid from patients with periodontal disease were higher than those from healthy patients in the control group (22). Considering the various functions involved in wound healing, $T\beta_4$ in saliva or gingival crevicular fluid is thought to promote the repair of damaged oral tissues. The aim of this study was to evaluate the effect of $T\beta_4$ on palatal wound closure in a rat model. As $T\beta_4$ already existed in saliva, we applied relatively high concentrations of $T\beta_4$ with carboxymethyl cellulose (CMC) ointment. We also evaluated the effects of $T\beta_4$ on the growth, adhesion and migration of rat palatal (RP) cells. Furthermore, the mRNA and protein expression of matrix metalloproteinase 2 (MMP2) and vascular endothelial growth factor (VEGF) were analyzed in $T\beta_4$ -treated RP cells.

Materials and methods

Chemical reagents and cell culture. Cell culture medium and reagents were purchased from Gibco-BRL (Grand Island, NY, USA). Other experimental reagents were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA), unless otherwise specified.

RP cells were obtained from the palatal tissues of 5-week-old male Sprague-Dawley (SD) rats. Isolated palatal tissues were washed with phosphate-buffered saline (PBS), minced into sections, and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotic solution (100 U/ml of penicillin-G and 100 $\mu\text{g/ml}$ of streptomycin) at 37°C in a 5% CO_2 humidified incubator. After 20 days of culture with medium changes every 3 days, RP cells were collected and subcultured under the same conditions. Passages 5-8 were used for the present study.

Cell adhesion and proliferation assay. Adhesion and proliferation of RP cells on the polystyrene surface of culture plates were observed in the presence of $T\beta_4$. For the adhesion assay, 1×10^4 palatal cells were incubated in the wells of a 96-well plate with $T\beta_4$ at various concentrations of 1-1,000 ng/ml for 4 h. The wells were gently washed three times with PBS, and the number of attached cells was quantified using 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8; Dojindo Laboratories, Kumamoto, Japan). The cells were incubated in 100 μl of WST-8 solution for 1 h at 37°C in a humidified atmosphere (5% CO_2 /95% air). The absorbance was measured at a wavelength of 450 nm using a plate reader (Sunrise; Tecan Austria GmbH, Salzburg, Austria). To observe proliferation, palatal

cells were treated with $T\beta_4$ for 24 h, and the number of cells was measured by using WST-8.

In vitro wound closure assay. For the *in vitro* wound closure assay, a culture insert (ibidi GmbH, Martinsried, Germany) was employed to create a wound in cell culture. The culture insert was placed on a culture dish, and 70 μl of RP cell suspension (5×10^5 cells/ml) was added into the two wells of the insert. The RP cells were incubated at 37°C for 18 h, and were serum-starved for 24 h. Following serum starvation, the culture insert was carefully removed, and the cells were exposed to $T\beta_4$ at various concentrations of 0-1,000 ng/ml. The wound closure was observed and recorded at intervals under a phase contrast microscope (Olympus, Tokyo, Japan). This experiment was replicated three times.

mRNA expression analysis of MMP2 and VEGF. To investigate the effect of $T\beta_4$ on the mRNA expression of genes related to cell migration and angiogenesis, mRNA expression of MMP2 and VEGF was analyzed by quantitative polymerase chain reaction (RT-qPCR) assay. Following serum starvation for 24 h, the palatal cells were treated with $T\beta_4$ for 6 and 24 h and the total RNA was isolated using RNA extraction reagent (WelPrep Total RNA isolation reagent; Welgene Inc., Daegu, Korea). From the total RNA, cDNA was prepared using a cDNA synthesis kit (Power cDNA Synthesis kit; Intron Biotechnology, Seongnam, Korea) and RT-qPCR was performed in an ABI PRISM 7500 Sequence Detection System Thermal Cycler (Applied Biosystems, Foster City, CA, USA) with 20 μl reaction volumes containing 10 μl SYBR Premix Ex Taq (Takara Bio, Otsu, Japan), 0.4 μl ROX reference dye II (Takara Bio), cDNA, and primers. The primers for gene amplification were: VEGF, sense: 5'-GAGTATATCTTCAAGCCGTCCTGT-3' and antisense: 5'-ATCTGCATAGTGACGTTGCTCTC-3'; MMP2, sense: 5'-CAGGGAATGAGTACTGGGTC TATT-3' and antisense: 5'-ACTCCAGTTAAAGGCAGCATCTAC-3'; GAPDH, sense: 5'-TGTGTCCGTCGTGGATCTGA-3' and antisense: 5'-CCTGCTTACCACCTTCTTGAT-3'. The PCR conditions were 95°C for 30 sec, followed by 40 cycles of denaturation at 95°C for 5 sec and annealing at 60°C (34 sec) for MMP2 and 63°C (34 sec) for VEGF. The reactions were run in triplicate. Gene expression was evaluated based on the threshold cycle (Ct value) and normalized to the amount of GAPDH transcript.

Western blot analysis. Western blot analysis was performed to examine the protein expression of MMP2 and VEGF in $T\beta_4$ -treated palatal cells. After treatment with $T\beta_4$ for 6 h, the cells were centrifuged and re-suspended in an extraction buffer containing 50 mM Tris base-HCl (pH 8.0), 150 mM NaCl, 0.5% Triton X-100, and 1 tablet of protease inhibitor cocktail (1 tablet/10 ml; Roche Applied Science, Mannheim, Germany) for 45 min on ice. Extracts containing equal amounts of protein were run on 10% sodium dodecyl sulfate polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The blots were incubated with rabbit polyclonal antibodies against VEGF, MMP2 or GAPDH in PBST (PBS containing 0.1% Tween-20) for 1.5 h, washed three times with PBST, and then probed with goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase. The anti-

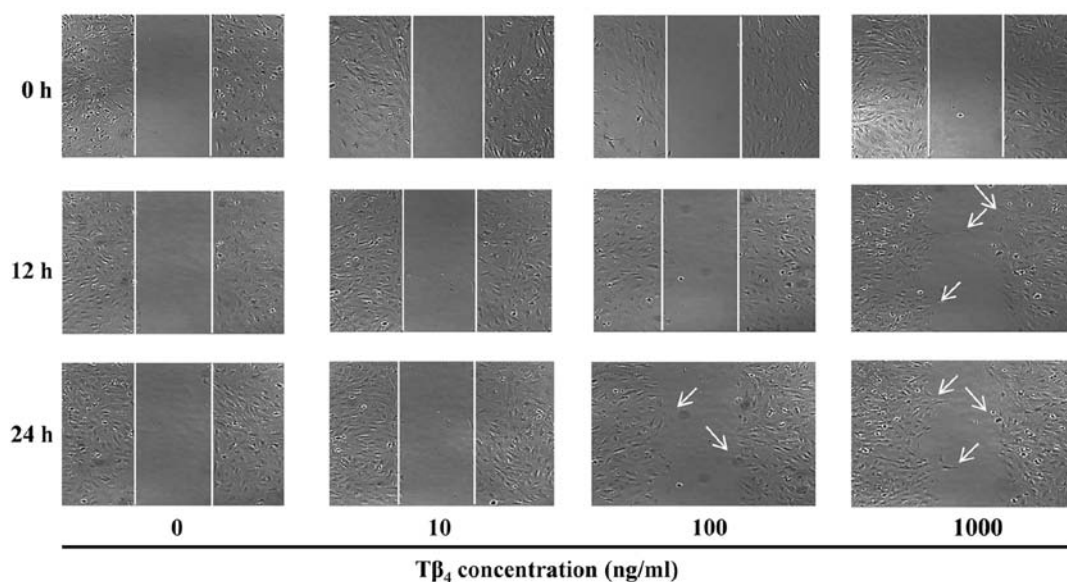


Figure 1. Effect of thymosin β_4 ($T\beta_4$) on rat palatal (RP) cell migration. Cells were incubated with $T\beta_4$ at different concentrations of 0-1,000 ng/ml, and cell movement was observed at 0, 12 and 24 h (magnification, x40). The arrows show cell motility during incubation.

bodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The blots were developed using a chemiluminescence kit (West-Zol plus western blot analysis detection system; Intron Biotechnology). Chemiluminescence was detected using the LAS 1000 Plus Luminescent Image Analyzer (Fuji Photo Film Co., Ltd., Tokyo, Japan).

RP wound healing assay. The effect of $T\beta_4$ on the wound healing of palatal tissue was investigated in a RP wound model. Thirteen-week-old SD male rats, weighing 300-350 g, were used in this study. Animal experiments were performed under the control of the animal welfare committee of Seoul National University Institutional Animal Care and Use Committee. Under general anesthesia, punch wounds were made on a central area of hard palate with a disposable 3-mm diameter biopsy punch (Kai Industries Co., Ltd., Gifu, Japan), exposing a circular area of bare bone. The wound area was covered with 30% CMC ointment containing 0 or 1 mg/ml $T\beta_4$. Six rats were used for each group. After the surgery, the animals were fed a standard diet of pellets and water with enrofloxacin. The agents were re-applied on day 2 and 4 to reduce stress by anesthesia, and the rats were sacrificed on day 7. The maxillae were separated, and wound area was observed by stereoscopic microscope (Nikon, Tokyo, Japan) and by histological analysis. The wound areas in the microscopic images were calculated using CellSense Dimension 1.6 software (Olympus).

The palatal specimens were fixed in 10% formalin for at least 24 h, decalcified in Calci-Clear Rapid solution (National Diagnostics, Atlanta, GA, USA) for 35 h, and processed for histological analysis. Serial sections, 5 μ m apart, were cut across the wound, perpendicular to the palatal midline at the widest diameter of the wound, and stained with hematoxylin and eosin (H&E). The sections were examined under a light microscope (Olympus), and the distance of wound margins in each section was measured with a calibrated ocular micrometer (Olympus).

Statistical analysis. Each experiment was performed in triplicate unless otherwise specified. Data were presented as the mean \pm SD. Statistical analyses were performed by the Student's t-test. $P < 0.05$ was considered statistically significant.

Results

Effects of $T\beta_4$ on the adhesion, proliferation and migration of palatal cells. To investigate the effects of $T\beta_4$ on the adhesion and proliferation of palatal cells, the cells were incubated for 4 and 24 h in the presence of $T\beta_4$. At concentrations of 1-1,000 ng/ml, $T\beta_4$ did not exert any significant effects on the adhesion and proliferation of palatal cells (data not shown), whereas cell migration was affected (Fig. 1). Untreated control cells did not exhibit any movement during 24 h, possibly due to the serum-starved test conditions. However, cell movement was observed when $T\beta_4$ was present at concentrations >100 ng/ml. The migration effect of $T\beta_4$ was more rapid at the higher concentrations and cell motility was observed at 12 h when incubated with 1,000 ng/ml $T\beta_4$.

Effects of $T\beta_4$ on mRNA expression of MMP2 and VEGF genes. The mRNA expression of MMP2 and VEGF was quantified to investigate the effects of $T\beta_4$ on cell migration and angiogenesis at molecular levels. As shown in Fig. 2, $T\beta_4$ enhanced the gene expression of MMP2 and VEGF after 6 h. The expression of MMP2 in the $T\beta_4$ -treated group was significantly higher than that in the untreated cells. Although the expression did not increase in a dose-dependent manner, the highest concentration of $T\beta_4$ (1,000 ng/ml) led to the strongest induction of MMP2. $T\beta_4$ also enhanced mRNA expression of the VEGF gene in RP cells, inducing an ~ 1.4 -fold increase at 1,000 ng/ml at 6 h. A further increase of VEGF mRNA was obtained when exposed for 24 h. However, the expression level of the MMP2 gene was not maintained over the 24-h period, with expression levels decreasing below those of the untreated control (Fig. 3).

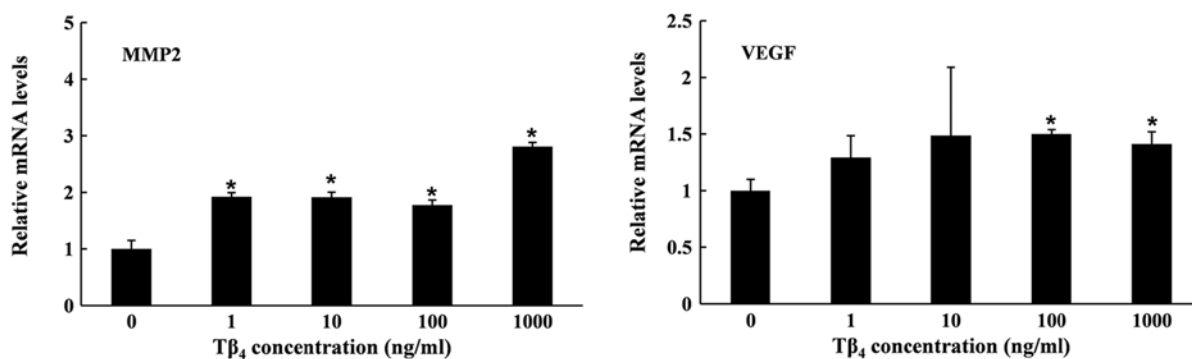


Figure 2. Effect of thymosin β_4 ($T\beta_4$) on mRNA expression of matrix metalloproteinase 2 (MMP2) and vascular endothelial growth factor (VEGF) in rat palatal (RP) cells. The cells were incubated with $T\beta_4$ for 6 h. The mRNA expression levels of each gene were normalized to GAPDH mRNA. Each value is the mean \pm SD of three independent experiments. *Significant difference of mRNA levels between the treated and untreated control cells ($p < 0.05$).

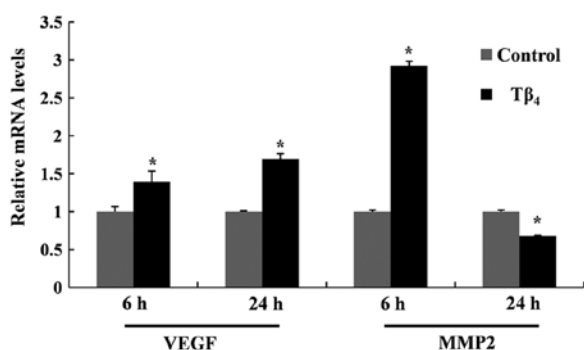


Figure 3. The mRNA expression of matrix metalloproteinase 2 (MMP2) and vascular endothelial growth factor (VEGF) genes in thymosin β_4 ($T\beta_4$)-treated rat palatal (RP) cells at different time points. RP cells were treated with $T\beta_4$ (1 $\mu\text{g/ml}$) for 6 and 24 h. Each value is the mean \pm SD of three independent experiments. *Significant difference of mRNA expression levels compared to those of the untreated control cells ($p < 0.05$).

Effect of $T\beta_4$ on VEGF and MMP2 protein level. The protein expression of MMP2 and VEGF was determined by western blot analysis. RP cells were treated with $T\beta_4$ for 6 h at various concentrations. The results showed that $T\beta_4$ upregulated the expression of VEGF and MMP2 proteins in a dose-dependent manner (Fig. 4). The quantitative measurement of VEGF and MMP2 protein showed that treatment with $T\beta_4$ (1,000 ng/ml) significantly enhanced the level of VEGF protein by 2.1-fold, and enhanced the level of pro-MMP2 and active MMP2 by 3.8- and 1.3-fold, respectively, when compared to the control (Fig. 4).

Effects of $T\beta_4$ on the palatal wound healing of rats. The wound healing effect of $T\beta_4$ was observed seven days after surgery, whereas the palatal wound gap was completely closed < 2 weeks in the untreated rats (Fig. 5A). As shown in Fig. 5B, the wound area was not completely epithelized in either the control or the test groups. However, microscopically smaller wound areas were observed in the $T\beta_4$ -treated test group, indicating that more advanced epithelization at the wound margin had occurred in the $T\beta_4$ -treated rats (Fig. 5B). The mean area of unepithelized surface in the $T\beta_4$ -treated rats was significantly smaller than that in the control group (Fig. 5C). The histological examination also demonstrated an accelerated epithelization by $T\beta_4$ (Fig. 5D). The mean distance

between the epithelized margin at the center of a palatal wound was $\sim 1,280 \mu\text{m}$ in $T\beta_4$ -treated rats, while $1,830 \mu\text{m}$ of the wound remained unepithelized, on average, in the control group (Fig. 5E). Therefore, we have clearly demonstrated that $T\beta_4$ significantly accelerated the epithelization of RP wounds.

Discussion

Wound healing is a series of events including inflammation, angiogenesis and re-epithelization (23). Previous studies (8-11) reported that $T\beta_4$ played a multi-functional role in wound repair processes, demonstrating a potential for the clinical use of $T\beta_4$ in wound care. In this study, we aimed to investigate the feasibility of using $T\beta_4$ for oral wound repair. As shown in Fig. 1, $T\beta_4$ promoted palatal cell migration. Previously, MMPs were reported to be upregulated during dermal wound repair in $T\beta_4$ -treated rats (24), and cell migration, promoted by $T\beta_4$, was blocked by a MMP inhibitor (25). The aforementioned studies demonstrated a critical role of MMPs in $T\beta_4$ -aided cell migration and wound healing. In this study, we investigated the expression of MMP2 as mucosal fibroblasts are known to mainly express MMP2 in the MMP family, which is associated with wound healing (26,27). As shown in Fig. 2, the mRNA and protein expression of MMP2 was enhanced by $T\beta_4$, suggesting that the promotion of palatal cell migration by $T\beta_4$ was mediated by MMP2. The precise mechanism underlying the induction of MMP expression by $T\beta_4$ is not yet fully understood. However, previous studies suggesting $T\beta_4$ as a hypoxia-responsible regulator (28,29) lead us to speculate that hypoxia-inducible factor (HIF)-1 α may be involved in the $T\beta_4$ -derived modulation of MMPs, because MMPs are known to be regulated by hypoxia or HIF-1 α in other cell types (30,31). Together with MMP2, VEGF, another main factor related to wound healing, is also a well-known target of HIF-1 α . In this study, we have shown that VEGF was upregulated by $T\beta_4$ (Fig. 4). Considering the ability of $T\beta_4$ to induce HIF-1 α stabilization, it is assumed that HIF-1 α was also involved in the enhanced expression of VEGF in the palatal cells.

In the animal model, $T\beta_4$ significantly accelerated the closure or re-epithelization of palatal wounds (Fig. 5). The multi-functional effects of $T\beta_4$ on cell migration, angiogenesis and inflammation are all thought to contribute to wound

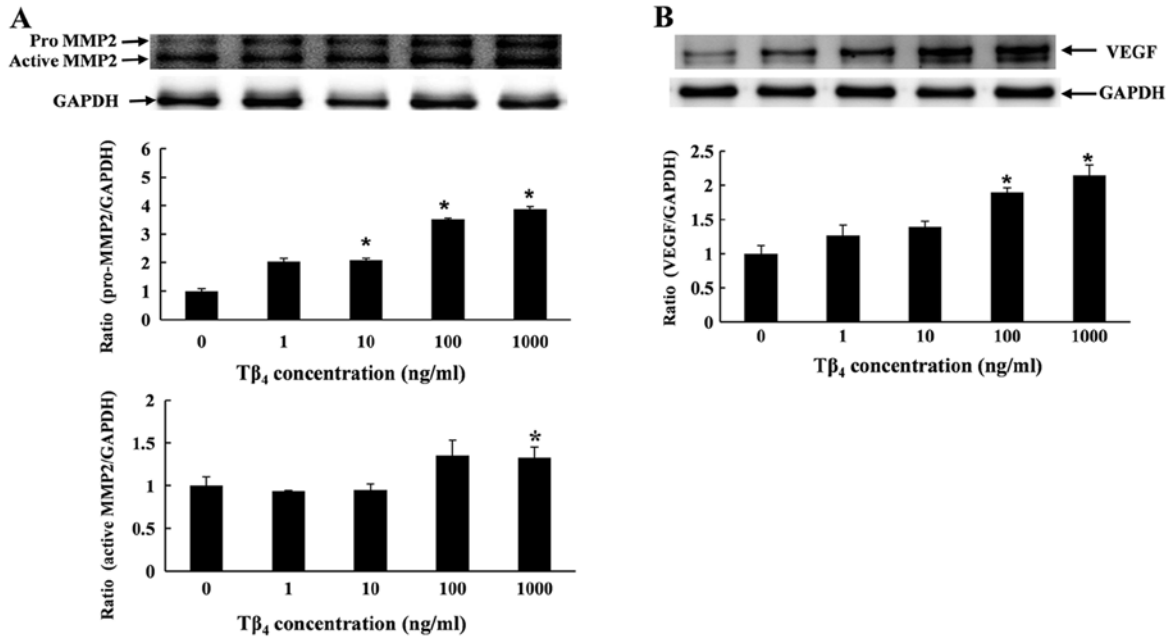


Figure 4. (A) Effect of thymosin β_4 ($T\beta_4$) on the protein expression of matrix metalloproteinase 2 (MMP2) and (B) vascular endothelial growth factor (VEGF). Rat palatal (RP) cells were treated with $T\beta_4$ ($1 \mu\text{g/ml}$) for 6 h. For quantification of the protein expression, the mean \pm SD of three independent experiments was obtained. Western blot analysis images are representative of each experiment. *Significant difference of the protein expression levels compared to the untreated control cells ($p < 0.05$).

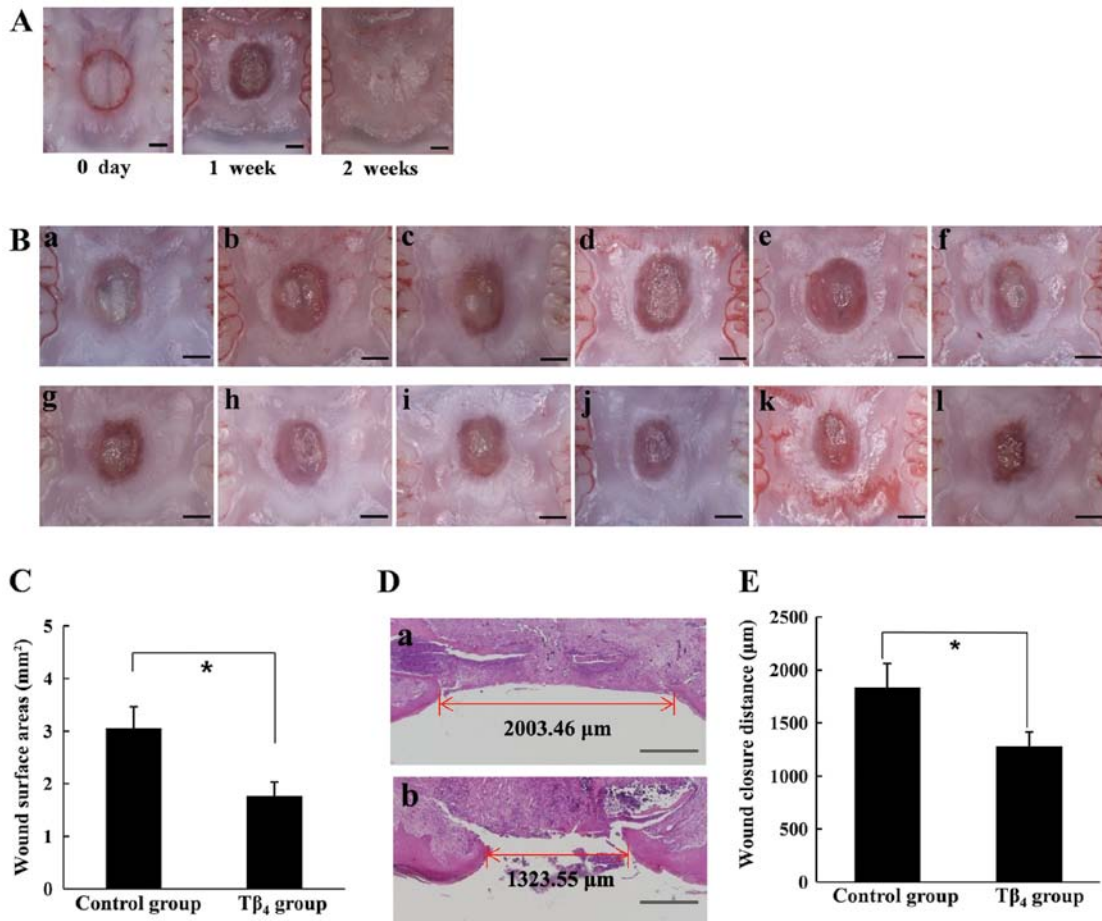


Figure 5. Effects of thymosin β_4 ($T\beta_4$) on the healing of palatal wounds. (A) Palatal wound of the untreated control. (B) Stereoscopic microscope images of the palatal wounds one week after surgery. (a-f) $T\beta_4$ was topically applied to test groups (g-l), and 30% CMC (vehicle) was applied to the control groups (scale bar, 1 mm). (C) Wound area calculated from the image of B. (D) Representative images of histological sections of palatal wounds. (a) The sections of control and (b) $T\beta_4$ -treated wounds were stained with hematoxylin and eosin (H&E) (scale bar, 500 μm). (E) Mean values of maximum palatal wound width calculated from the H&E-stained sections. *Significant difference of palatal wound width of $T\beta_4$ -treated groups compared to the untreated control groups ($p < 0.05$).

repair. Although $T\beta_4$ is an endogenous component of saliva, our results showed that exogenous administration of $T\beta_4$ was effective for the rapid healing of palatal wounds. In contrast to the applications of dermal wound healing, the administered $T\beta_4$ can be easily washed away by saliva in the oral environment. Therefore, we expect that a more appropriate delivery system, that supplies $T\beta_4$ continuously, would accelerate healing processes. This study employed normal healthy rats to investigate the effects of $T\beta_4$. Further experiments elucidating the efficacy of $T\beta_4$ in regeneration-impaired animals due to diabetes or advanced age would be highly beneficial for determining the clinical potential of this treatment.

In conclusion, the cell migration of RP cells was stimulated by $T\beta_4$. The protein and mRNA expression of MMP2 and VEGF were also enhanced by $T\beta_4$. The topical application of $T\beta_4$ greatly enhanced palatal wound healing in rats. Our results suggest that $T\beta_4$ can be used for the promotion of oral wound healing.

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