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Thymosin beta 4 improves dermal burn wound healing via downregulation of receptor of advanced glycation end products in db/db mice

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ABSTRACT

Background: The delay of dermal burn wound healing caused by vascular disorders is a critical problem for many diabetic patients. Thymosin β_4 ($T\beta_4$), identified by subtractive cloning of endothelial cells on plastic versus basement membrane substrates, has been found to promote angiogenesis and dermal wound repair in rats, aged mice, and db/db diabetic mice. However, previous studies involving the role of $T\beta_4$ in wound repair were limited to mechanical damage and dermal impairment. Thus, this study aimed to evaluate the improvement of healing of burn wounds by $T\beta_4$ in relation to advanced glycation end products (AGE), which are pathological factors in diabetes.
Methods: We adapted a dermal burn wound in vivo model in which the dorsal skin of db/db mice was exposed for 10 s to 100 °C heated water to produce a deep second-degree burn 10 mm in diameter. Five mg/kg of $T\beta_4$ was then injected intradermally near the burn wound twice a week for 2 weeks.
Results: After treatment, $T\beta_4$ improved wound healing markers such as wound closure, granulation, and vascularization. Interestingly, $T\beta_4$ reduced levels of receptor of AGE (RAGE) during the wound healing period.
Conclusions: $T\beta_4$ exerts effects to remedy burn wounds via downregulation of RAGE.
General significance: Our results suggest the potential importance of $T\beta_4$ as a new therapy for impaired burn wound healing that is associated with diabetes.

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1. Introduction

Diabetes mellitus resulting in vascular disorders may lead to critical risks from burns and impaired wound healing [1]. The combination of burns with risk factors such as diabetes can critically delay wound healing, and is a problem for people worldwide [2]. The main type of burn injury in diabetic patients is lower extremity burns resulting from intentional exposure to sources of heat without recognition of the risk of burning, in consequence of degeneration of peripheral nerves [3]. Burn injury is often associated with significant complications such as diabetes, and is common and responsible for the majority of limb amputation in diabetic patients, as a result of subsequent infection. Multifactorial causes of increased infection rates are in part due to impaired immune functioning of damaged skin, the primary immune defense organ, which may be further affected by inadequate glucose control among diabetic patients [4]. Few therapeutic agents have been developed and adapted to promote significant burn wound healing in diabetic patients [5].

Advanced glycation end products (AGE) are formed by reactions of both sugars and oxidized lipids with proteins. These nonenzymatic glycation reactions between reducing sugars and macromolecules are considered central to the vascular complications that occur in diabetic patients [6]. The formation and accumulation of AGE are a characteristic feature of tissues of elderly people, especially in patients with diabetes mellitus; these products have also been strongly implicated in the pathogenesis of age-related and diabetic complications [7]. Previous studies have reported that AGE are involved in impaired wound healing in diabetes [8] and thermal injury [9]. AGE induce deleterious effects on oxidative stress, inflammation, angiogenesis, fibroblast migration, and collagen deposition during wound repair [10]. As a result, AGE may delay burn wound healing in diabetic patients. Receptor of AGE (RAGE) is the best-characterized AGE receptor and is responsible for most of the deleterious effects of AGE. RAGE is highly expressed in the skin tissue of diabetic model animals when compared with control animals [11].

Thymosin β_4 ($T\beta_4$) is a small, naturally occurring 43-amino acid peptide that was initially isolated in 1981 from the thymus [12]. $T\beta_4$ binds to actin, blocks actin polymerization, and is the major actin-sequestering molecule in eukaryotic cells [13,14]. $T\beta_4$ has been identified as an angiogenic factor among early genes induced in endothelial

Abbreviations: $T\beta_4$, thymosin β_4; AGE, advanced glycation end products; RAGE, receptor of AGE; α-sma, alpha-smooth muscle actin

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cell differentiation in vitro [15], as well as being involved in enhancing vasculogenesis during development [16]. Moreover, previous studies have demonstrated the wound healing effect of $T\beta_4$ on mechanical dermal wounds in normal rats as well as diabetic mice [17,18] and a recovery effect in peripheral neuropathy in diabetic mice [19]. However, the effect of $T\beta_4$ on healing of burn wounds in diabetes has not yet been investigated.

The present study shows that $T\beta_4$ improves burn wound healing in a mouse model of type II diabetes (db/db mice). Additionally, we investigated the role of $T\beta_4$ in attenuating the effects of RAGE as well as those of vascular disorders in burn injuries. The results of the present study suggest that $T\beta_4$ may serve as a novel therapeutic agent for the treatment of burn injuries in diabetes.

2. Materials and methods

2.1. Chemicals

$T\beta_4$ was purchased from Tocris Bioscience (Bristol, UK). Saline and other basic reagents were purchased from Sigma (St. Louis, MO, USA). Primary antibodies to RAGE and β-actin were purchased from Cell Signaling Technology (Beverly, MA, USA). Immunohistochemical primary antibody to Ki-67 was purchased from Millipore (Temecula, CA, USA). Cy3-conjugated alpha-smooth muscle actin (α-sma) primary antibody was purchased from Sigma (St. Louis, MO, USA). Secondary antibody (i.e., anti-rabbit IgG antibody conjugated with horseradish peroxidase) was obtained from Millipore (Temecula, CA, USA). The immunohistochemical staining kit with 3,3′-Diaminobenzidine (DAB) was purchased from Sigma. All other chemicals and reagents were of analytic grade.

2.2. Animals and experimental burn procedure

Forty-five male C57BL/KsJ db/db (db/db) and twenty-five male m/m mice, free of murine viruses, bacteria, and parasites, and approximately 8 weeks of age on arrival, were purchased from Japan SLC Inc. (Hamamatsu, Japan). C57BL/KsJ m/m (normal) mice were used as normal controls. Mice were maintained in micro-isolator cages under pathogen-free conditions on a 12 h light/dark cycle, fed a standard laboratory pellet diet and water ad libitum, and housed at controlled temperature (23 ± 3 °C) and humidity (about 60%). All animals were cared for in accordance with institutional ethical guidelines for the care and use of experimental animals at Chonbuk National University. When the mice were aged 10 weeks, all mice were anesthetized by intraperitoneal injection of zoletile (30 mg/kg), their dorsal hair was shaved, and they were depilated with a hair removal cream (Shiseido Co., Tokyo, Japan). The dorsal skin was exposed for 10 s to water heated to 100 °C to produce a deep second-degree burn 10 mm in diameter. Each mouse of the normal control group (each n = 5, total 25; D-0, D-3, D-7, D-14 and D-20) and db/db control group (each n = 5, total 25; D-0, D-3, D-7, D-14 and D-20) was intradermally injected with 100 μl saline cross-regionally near the site of the burn wound twice a week for 2 weeks, while each mouse of the db/db $T\beta_4$ group (each n = 5, total 20; D-3, D-7, D-14 and D-20) was intradermally injected with 5 mg/kg of $T\beta_4$ in 100 μl saline in the same locations (Fig. 1a). To decide this optimal dosage of treatment, we did many repeated experiment using various concentration of $T\beta_4$. Through the entire experiment period, the mice were individually caged and their wounds were not dressed. The investigators observing samples were blinded to group and treatment. Each wound was photographed daily and then excised with a margin of healthy skin for histological, real-time RT-PCR, and immunoblotting analysis.

2.3. Analysis of wound healing and angiogenesis

Wound area was evaluated by macrophotography. Digital photographs of the dorsal wounds were taken on the specified days (D-0, D-3, D-7, D-14, and D-20) until the end of the experiment for all experimental mice. Photographs of the wounded areas were analyzed by tracing the wound margins and calculating pixel areas using the Image Pro analysis program. The remaining wound area was calculated as a percentage area of the original wound area. After photography of the wound area, wound area tissue was removed and the reverse side of the removed skin was photographed.

2.4. Histological and immunohistochemical analysis

Wound samples from all experimental groups were evaluated for histological and immunohistochemical studies. Paraffin-embedded 6-μm-thick tissue blocks were sectioned and stained with hematoxylin & eosin (H&E). The sections were also stained with anti-Ki-67 and Cy3-conjugated anti-α-sma antibodies. The H&E stained sections were examined for granulation tissue formation. The thickness of granulation tissue formation was determined vertically at the center of each section, from the surface of the granulated tissue to the margin of the dermis and the subcutis. Ki-67 immunostained sections were stained with DAB solution following the manufacturer's protocol and Cy3-conjugated α-sma immunostaining of sections was likewise performed as per the manufacturer's protocol. All prepared sections were observed and photographed under an optical/fluorescence microscope using an Observer A1 microscope at 100× magnification (Carl Zeiss, Germany).

2.5. RNA preparation and real-time RT-PCR

Total RNA prepared from skin tissue was precipitated with Ribo EX (Geneall, Daejeon, Korea) and dissolved in DEPC-treated distilled water on the specified day, D-7. The mRNA was reverse transcribed to cDNA using a Maxime RT PreMix (Intron, Seongnam, Korea) as per the manufacturer's instructions. For real-time RT-PCR, cDNA was amplified using a Mastercycler Gradient 5331 Thermal Cycler (Eppendorf, Germany). Real-time PCR was monitored by measuring the fluorescence signals after each cycle with an ABI Step One Plus Sequence Detection System (Applied Biosystems, Singapore). Specific primers for each gene were designed using Primer Express software (Applied Biosystems). The following real-time RT-PCR sense primer and antisense primers were used: forward 5′-GTACCTCCACCATGCCAAGT-3′ and reverse 5′-ACACAGGACGGCTTGAAGAT-3′ for vascular endothelial growth factor (VEGF); forward 5′-CCCTCACACTCAGATCATCTTCT-3′ and reverse 5′-GCTACGACGTGGGCTACAG-3′ for tumor necrosis factor-α (TNF-α); forward 5′-GCAACTGTTCCTGAACTCAACT-3′ and reverse 5′-ATCTTTTGGGGTCCGTCAACT-3′ for interleukin-1β (IL-1β); forward 5′-GGCTGTATTCCCCTCCATCG-3′ and reverse 5′-CCAGTTGGTAACAATG CCATGT-3′ for β-actin, housekeeping gene. All experiments were performed three or more times.

2.6. Immunoblotting analysis

Proteins in skin tissue were subjected to SDS-polyacrylamide gel electrophoresis in a 10% gel and electrophoretically transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 5% skim milk in PBS, and then incubated individually with each primary antibody diluted 1:1,000 in 1% skim milk in PBS overnight at 4 °C. The blots were further incubated with each secondary antibody diluted 1:10,000 at room temperature for 1 h. Immunoreactions were visualized using SuperSignal West Dura Extended Duration Substrate (Thermo, CA, USA) and analyzed using a Chemi Imager (Alpha Innotech, San Leandro, CA, USA).

2.7. Statistical analyses

The results are presented as mean ± standard error. The data were analyzed using Student's *t*-test (for two groups), one-way ANOVA, and Tukey's test (for more than two groups). A p value < 0.05 was considered statistically significant. All analyses were performed using the

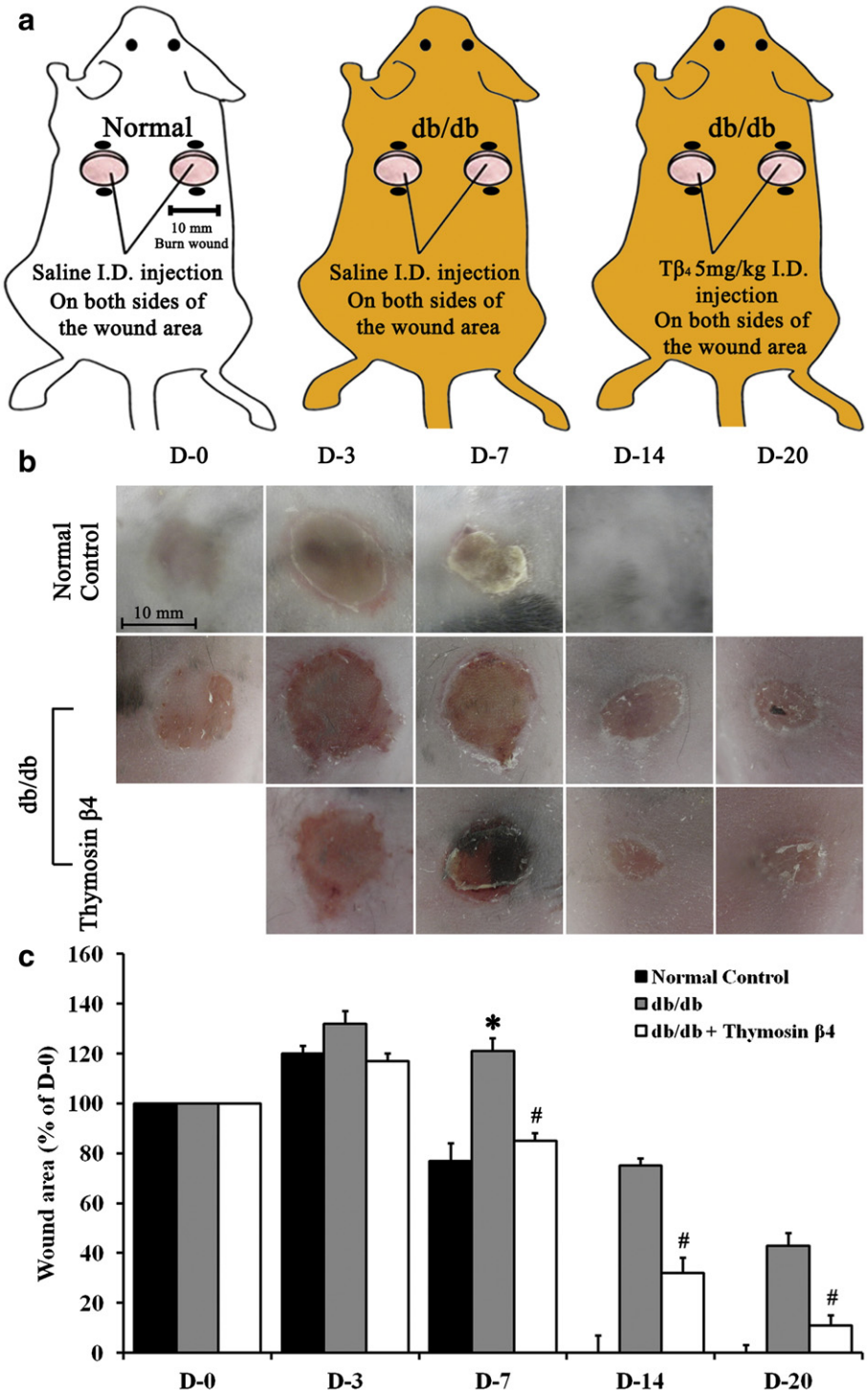

Fig. 1. Wound closure analysis of the effect of Tβ4 on burn-injured db/db mice. (a) Schematic overview of the study design for the burn wound model. (b) Photography of the burn wound area on consecutive days. The wound area treated with Tβ4 after circular burn injury decreased significantly starting at D-7. (c) Graph of quantification of wound closure. Values are mean ± SEM for five animals in each group. $^{*}p < 0.05$, compared with normal control. $^{\#}p < 0.05$, compared with db/db.

Statistical Package for Social Sciences version 13.0 for Windows (SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. Tβ4 accelerates the healing of burn wounds in db/db mice

To examine the effects of Tβ4 treatment on healing of burn wounds, we observed gross changes in the wounded skin. Fig. 1b shows the gross appearance of the burn wounds during the 20 days of treatment. Analysis of the images is presented in Fig. 1c. On D-0, the db/db control group exhibited more redness and destroyed epidermis than did the normal control group. Levels of redness and destroyed epidermis in all the db/db mice reflected the greater severity of damage induced by burns than occurred in the normal control group. On D-3, burn injuries of both the normal control group and the db/db control group had progressed significantly compared to D-0. The connective tissues of the burn wound had regenerated with some concurrent local wrinkling

by D-7. Interestingly, the db/db Tβ4 group exhibited a significant acceleration of wound closure compared to the db/db control group. Wound closure progressed more rapidly in the db/db Tβ4 group than in the db/db control group through D-20. In the normal control group, burn wounds were completely abolished at D-14, and the db/db Tβ4 group exhibited scarring at D-20. These results demonstrated that Tβ4 treatment is effective in accelerating burn wound closure and subsequent healing in db/db mice.

3.2. Tβ4 accelerates dermal regeneration of burn wounds in db/db mice

To examine the dermal regeneration effects of Tβ4 treatment, we analyzed granulation change and cell proliferation during the experimental period. Fig. 2a shows the thickness of the granulation tissue in burn wounds treated with Tβ4 in db/db mice. Granulation tissue formation in the db/db Tβ4 group began to show significant ($p < 0.01$) enhancement at D-7 compared to the db/db control group. There were no significant differences in granulation tissue formation in the db/db control group until D-7. Granulation tissue formation in the db/db control group was delayed relative to the other experimental groups. Fig. 2c shows a representative immunolocalization of Ki-67 in burn wounds treated with Tβ4. Ki-67 positive cells (colored brown) were mainly observed at the basal layer of the epidermis. There was no significant difference in the expression of Ki-67 between the normal control group and the db/db Tβ4 group during the experimental period. However, positive staining for Ki-67 was more prevalent in the db/db Tβ4 group than in the db/db control group. The number of cells positive for Ki-67 in the db/db Tβ4 group was significantly higher than that in the db/db control group at D-7, a result that is consistent with enhanced granulation tissue formation. Thus, these results indicate that Tβ4 significantly accelerated dermal regeneration at the wound site.

3.3. Tβ4 induces angiogenesis in burn wounds in db/db mice

To examine the effects of Tβ4 on skin neovascularization, blood vessels were examined on the reverse side of skin tissue harvested from the burn wound area on specified days. Images at D-0 did not reveal significant differences in blood vessel formation among the experimental groups (data not shown). As shown in Fig. 3a, Tβ4 significantly promoted angiogenesis during the treatment period after burn injury, which was characterized by dense neovascularization. By D-3, burn injuries had induced a progressive absence of blood vessels in the normal control and db/db control groups, while Tβ4 restored blood vessels significantly more quickly than occurred in the other experimental groups. Moreover, the db/db Tβ4 group revealed significant neovascularization compared with other experimental groups at D-7. Thick and long blood vessels in the db/db Tβ4 group indicate the potent healing properties of Tβ4 in burn wounds. Below panel expressed quantification of blood vessels following count number of blood vessels. Accordingly, immunohistochemistry for α-sma (to identify smooth muscle cells) was performed in order to determine the structure of blood vessels in histological sections. As shown in Fig. 3b, the db/db Tβ4 group revealed a strongly fluorescent red color compared with the db/db control group throughout the experimental period. This result corresponds with that of Fig. 3a.

3.4. Cytokine gene expression is regulated by Tβ4 in db/db mice

As shown in Fig. 4, we examined the effect of Tβ4 on cytokine gene expression in burn wound tissue on D-7, based on previous results. To assess the differences in cytokine mRNA expression between each group, β-actin was used as a reference housekeeping gene and cytokine mRNA levels were normalized to β-actin. The expression of VEGF in the db/db control group was significantly lower than in the normal control group, while the expression levels of IL-1β and TNF-α were significantly higher than in the normal control group. The expression patterns for the db/db Tβ4 group significantly ameliorated or even reversed the changes seen in the db/db control group relative to the normal controls. Indeed, previous study demonstrated that Tβ4 reduced TNF-α followed by inflammatory [20,21]. These results mean that the diabetic condition of db/db mice may induce an inflammatory response at the burn wound area and exhibit a decreased wound repair response via VEGF reduction. Moreover, Tβ4 improved healing of burn wounds via suppression of inflammatory genes such as IL-1β and TNF-α and induction of angiogenesis factors such as VEGF.

3.5. Tβ4 attenuates RAGE expression in burn wounds in db/db mice

RAGE plays a major role in the pathogenesis of diabetes associated with AGE. Therefore, we investigated levels of RAGE protein via immunoblotting of tissue lysates prepared from burn wounds. As shown in Fig. 5, upon burn injury, RAGE expression in the normal and db/db control groups rapidly increased by D-3. Moreover, RAGE expression in the db/db control group was significantly higher than in the normal control group. However, Tβ4 significantly reduced RAGE expression in db/db mice. The RAGE protein expression in tissues taken from animals in the db/db Tβ4 group was significantly different from that in the db/db control group on every day from D-3 to D-20. These results indicate that Tβ4 treatment significantly attenuated the potent induction of RAGE at the burn wound sites of db/db mice.

4. Discussion

In the last decade, a great deal of evidence has suggested that Tβ4 may exert a therapeutic effect in wound healing [22] as well as in improving diabetic complications such as peripheral neuropathy [19], peripheral vascular diseases [19], and retinopathy [23]. Indeed, it has been demonstrated that Tβ4 is involved in protection and recovery from diabetic injury and exerts its properties via anti-apoptotic, anti-inflammatory, and angiogenic functions both in vitro and in vivo [17,20,21]. However, the role played by Tβ4 in burn injuries, which are highly prevalent among, and can cause critical damage to, diabetic patients has not yet been investigated.

Diabetes is characterized by increased cellular stress. Hyperglycemic conditions induce various manifestations of cellular stress, including increased apoptosis, decreased vascular recovery, an aberrant inflammatory response, and delayed cellular turnover in diabetic wounds, resulting in impaired wound healing [1,10,24,25]. Although previous studies have indicated various causes for impaired wound healing in diabetes, AGE have been recognized as major factors in the pathogenesis of impaired wound healing in diabetes. The accumulation of AGE has been recognized as an important pathophysiological mechanism in the development of impaired wound healing. AGE exert their deleterious effects upon being recognized by RAGE, and the expression of both AGE and RAGE is increased in type 2 diabetic skin tissue relative to normal skin tissue [11]. AGE binding to RAGE on various cell types leads to impairment of growth factors [25]. The hyperglycemia-induced advanced glycation pathway has an important role in the pathogenesis of microvascular and macrovascular disorders in diabetic skin injury, and the oxidative stress-related RAGE response may play a key role [26]. Thus, we investigated the therapeutic effect of Tβ4 on burn injury-induced impaired wound healing in diabetes, and identified an association of the major target, RAGE, in the present study.

Wound closure is the main strategic goal in the development of therapies for impaired wound healing in diabetes. We initially examined the evaluation of wound closure induced by burn injury in db/db mice and have confidence about the therapeutic effect of Tβ4 on burn injuries based on our positive results. As shown in Fig. 1B and C, Tβ4 dramatically improved wound closure compared with the db/db control group. Notably, the redness of burn wounds caused by hemorrhage was also attenuated by Tβ4. Furthermore, we confirmed dermal regeneration via granulation tissue size and Ki-67 staining. Ki-67 is a nuclear protein

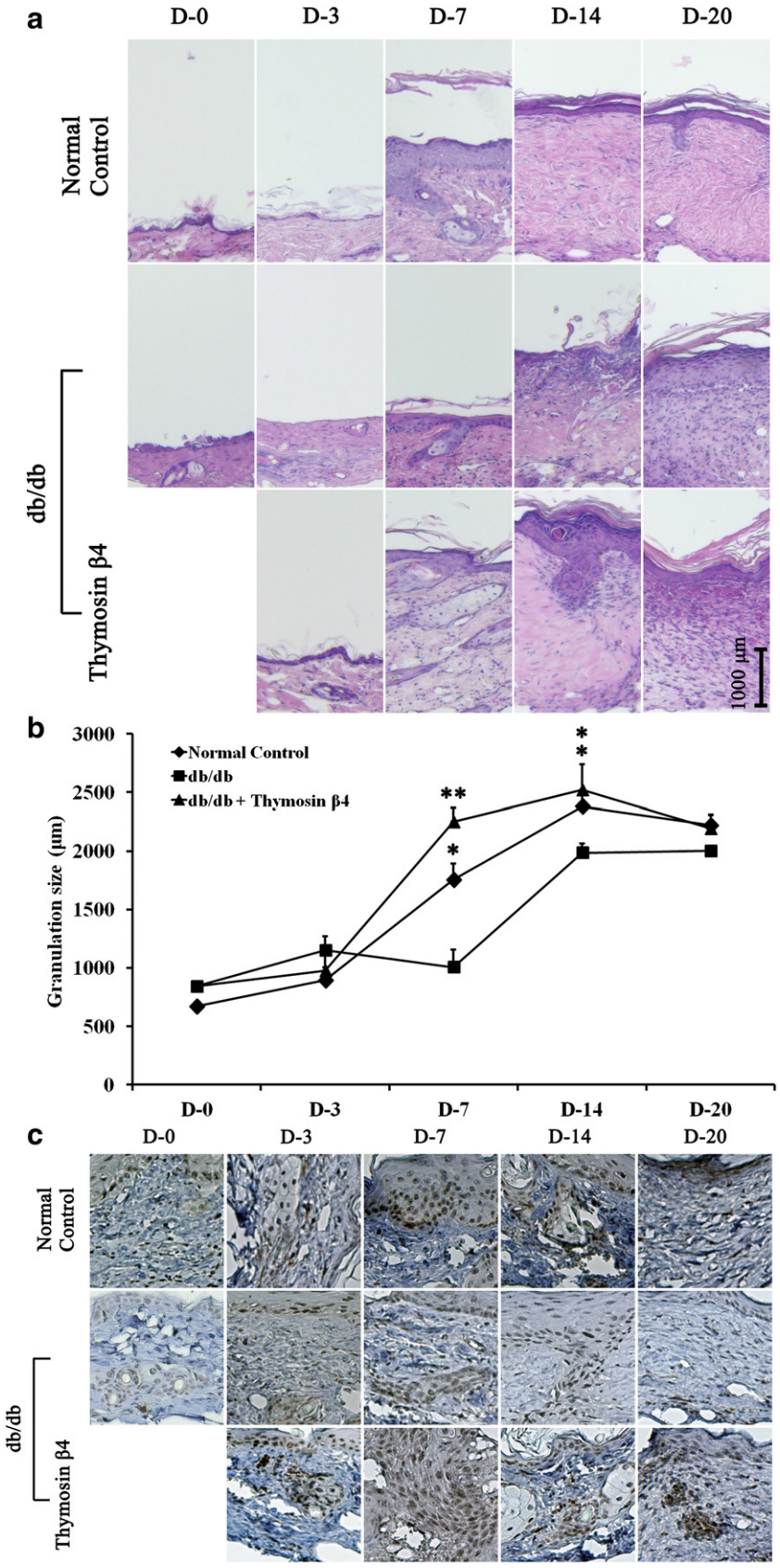

Fig. 2. Dermal regeneration analysis of the effect of $T\beta_4$ on burn-injured db/db mice. (a) Granulation tissue formation observed by H&E staining of wounded skin. Histological observation (100×) of newly formed granulation tissue in wounds on consecutive days. (b) Graph of quantification of granulation tissue. Values are mean ± SEM for five animals in each group. $^{*}p < 0.05$ and $^{**}p < 0.01$, compared with db/db. (c) Immunohistochemistry of Ki-67 in wounded skin. Ki-67 positive cells were mainly observed in the basal layer of the epidermis. The strongest positive staining for Ki-67 was observed in wounds treated with $T\beta_4$ at D-7.

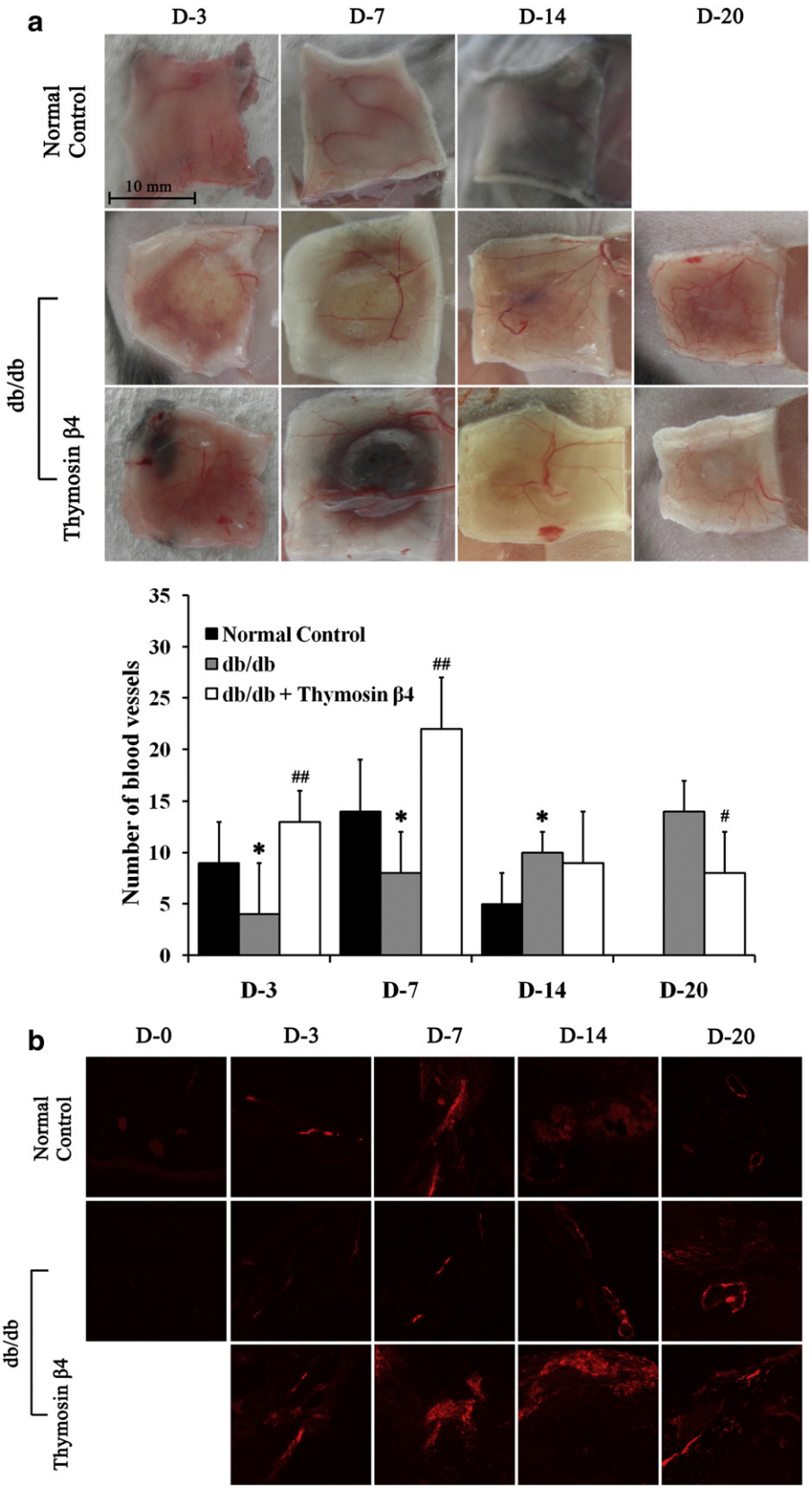

Fig. 3. Analysis of the effect of $T\beta_4$ on angiogenesis in burn-injured db/db mice. (A) Photography of blood vessel formation in the reverse side of wounded skin taken at indicated days after burn injury. Below panel expressed quantification of blood vessels following count number of blood vessels. Values are mean ± SEM for five animals in each group. $^{*}p < 0.05$, compared with normal control. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$, compared with db/db. (B) Immunohistochemistry of α-sma in wounded skin. The strongest positive staining for α-sma was observed in wounds treated with $T\beta_4$ at D-14.

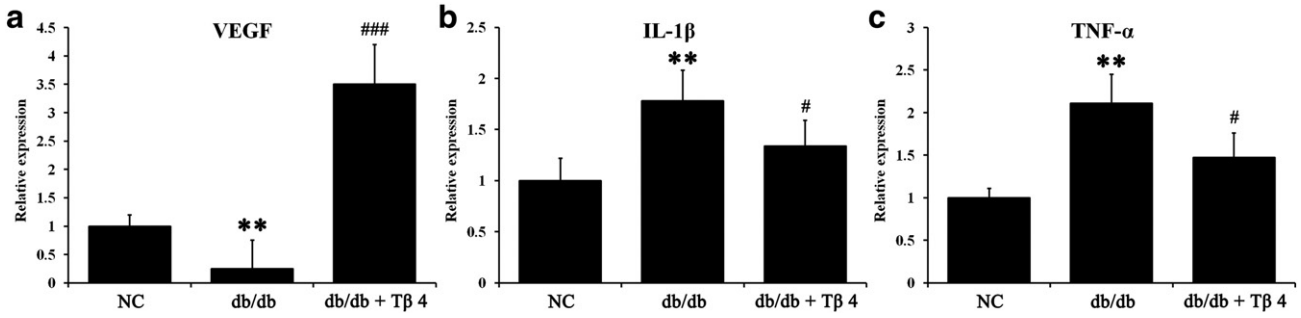

Fig. 4. Analysis of the effect of $T\beta_4$ on mRNA expression in burn-injured db/db mice. VEGF (a), IL-1β (b), and TNF-α (c) gene expression in burn-injured skin treated with $T\beta_4$ as determined by real-time RT-PCR. Values are mean ± SEM for five animals in each group. $^{**}p < 0.01$, compared with normal control. $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$, compared with db/db.

and is widely used as a proliferation marker [27]. The expression of Ki-67 protein is thought to be an indicator of growing cells within the overall cell population. As shown in Fig. 2, $T\beta_4$ induced more granulation tissue formation and cell proliferation in db/db mice. We then evaluated angiogenesis in burn wounds treated with $T\beta_4$ in db/db mice. Angiogenesis has a key role in wound healing, and factors which promote angiogenesis have been proposed to promote rearrangement of skin tissue [22]. Moreover, we evaluated α-sma expression, which is a transient marker of smooth muscle differentiation in neovascularization used to determine blood vessel formation. During the wound healing process, α-sma is found throughout granulation tissue, and its amount and distribution over time correlate with the rate of wound closure [28]. Our results revealed thick and long blood vessels induced by $T\beta_4$ on D-7, and significant neovascularization compared to the other experimental groups. mRNA expression of VEGF was increased by $T\beta_4$, and expression of inflammatory genes such as IL-1β and TNF-α was decreased. Taken together, these results indicate that $T\beta_4$ improves recovery from burn injuries.

Based on these results, we measured RAGE protein expression in skin tissue of burn wounds. A previous study indicated that blockage of RAGE significantly suppresses inflammatory factors, including IL-1β and TNF-α, while enhancing wound closure in db/db mice [29]. This study suggests that interaction of AGE with RAGE can trigger inflammatory signals and compromise angiogenesis in diabetes, and leads to impaired diabetic wound healing. As shown in Fig. 5, $T\beta_4$ attenuated RAGE expression significantly in db/db mice throughout the wound healing period. This result indicates a previously undiscovered function of $T\beta_4$ in diabetic diseases associated with AGE. Although the interaction of $T\beta_4$ with AGE has been incompletely elucidated, our further research will identify the nature of this interaction.

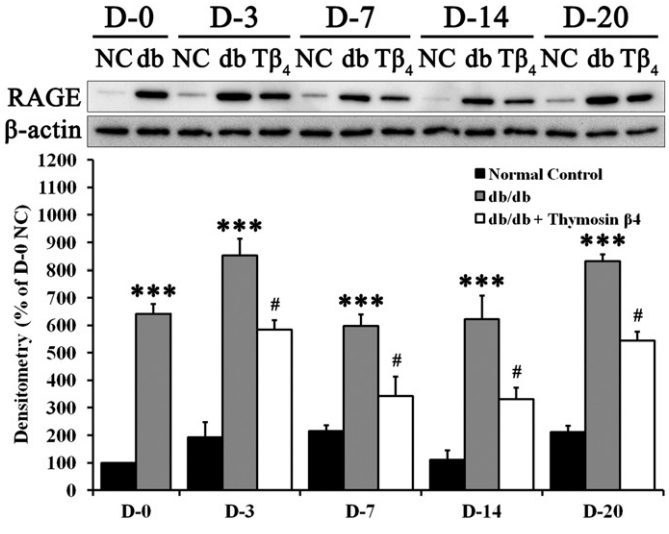

Fig. 5. Analysis of the effect of $T\beta_4$ on RAGE protein expression in burn-injured db/db mice. RAGE protein expression was determined by immunoblotting; β-actin was used to normalize protein loading. Images are representative of four independent experiments. The graph represents densitometry of blots. NC; normal control, db; db/db mice, and Tβ4; db/db + Thymosin β4. Values are mean ± SEM for five animals in each group. $^{***}p < 0.001$, compared with normal control. $^{\#}p < 0.05$, compared with db/db.

5. Conclusions

In conclusion, the results of the present study show for the first time that $T\beta_4$ improves impaired wound healing induced by burn injury in diabetic mice, and plays a pivotal role as a key anti-diabetic factor that attenuates RAGE in diabetic skin during wound healing. Based on our findings, we suggest a novel therapeutic strategy for the treatment of burn injuries in chronic diabetic patients, consisting of exogenous $T\beta_4$ administrated locally.

Acknowledgments

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