The Pro-Healing Effect of Protamine-Hydrolysate Peptides on Skin Wounds Involves TGF-β/Smad Signaling

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Abstract: This study characterized the molecular mechanisms of the effects of protamine-hydrolysate peptides (p-h peptides) on skin wound healing in rats by analyzing the transforming growth factor (TGF)-β signaling pathway. TGF-β was expressed in experimentally wounded skin tissues in fibroblasts and in keratinocytes. In p-h peptides-treated animals, the skin wounds exhibited an increased expression of TGF-β and of TGF-β target genes compared with control saline-treated skin wounds. Treatment with p-h peptides accelerated wound epithelialization and induced protein expression of TGF-β, CTGF and VEGF. The expression of tumor necrosis factor (TNF)-α was decreased in fibroblasts of p-h peptides-treated skin wounds. In addition, treatment with p-h peptides significantly enhanced the phosphorylation of Smad3 and Smad4 in fibroblasts and also elevated the phosphorylation of Stat3 in skin wound tissues. In conclusion, treatment with p-h peptides activated the Smad-dependent TGF-β signaling pathway, enhanced the differentiation of myofibroblasts and accelerated skin wound closure.

Key words: Protamine-hydrolysate peptides, Skin wound, TGF-β, Smad

Introduction

Wound healing is an inherent process that restores the integrity of the skin as quickly as possible. Restoration of the skin is essential due to its importance in survival through the prevention of infection, fluid loss and other vital functions. It has been suggested that wound healing evolved for speed, to allow wounds to heal quickly and reduce the risk of infection¹.

The biological basis of pathological scar tissue formation is comprised of three closely associated processes: 1) the sustained vigorous proliferation of fibroblasts after the epithelialization of wounds relative to apoptosis inhibition, 2) the imbalance in the synthesis and degradation of the collagen-rich extracellular matrix, and 3) the abundant supply and prolonged existence of specific growth factors. Upon skin injury, a series of events takes place aiming at the reconstruction of the wounded tissue. Wound repair is one of the most complex biological processes and involves multiple pathways, which are immediately activated after injury, including inflammation, granulation tissue formation and tissue remodeling. This complex process is executed and regulated by an equally complex signaling network that involves numerous growth factors, cytokines and chemokines²,³. Among them, transforming growth factor (TGF)-β has emerged as the major modulator of wound healing since it regulates the proliferation of fibroblasts and stimulates extracellular matrix deposition and angiogenesis²,⁴. In the process of tissue fibrosis, TGF-β is likely to facilitate the expression of extracellular matrix-encoding genes to increase the synthesis and deposition of collagen, fibronectin and proteoglycan⁵,⁶. Simultaneously, TGF-β decreases the yield of cathepsin and enhances the synthesis of cathepsin inhibitors. In addition, TGF-β may strengthen intercellular adhesion by increasing integrin levels in the extracellular matrix (ECM)⁷. Upon binding of TGF-β to heterodimeric receptor complexes, the latter
are autophosphorylated and activate downstream signaling molecules belonging to the Smad family of transcription factors\(^2\)\(^\text{-}\)\(^5\). Activated TGF-β receptors induce the phosphorylation of Smad2 and Smad3, which form a hetero-oligomeric complex with the common mediator Smad4\(^6\)\(^\text{-}\)\(^10\). These complexes are translocated into the nucleus and regulate ligand-induced gene transcription. After full-thickness incisional wounding, Smad3-null mice exhibit an enhanced rate of epithelialization associated with a reduction in the number of fibroblasts, leading to an overall decrease in wound size\(^1\)\(^1\)\(^1\)\(^4\)\(^1\(^\text{-}\)\(^5\). The proinflammatory cytokine tumor necrosis factor (TNF)-α, which is expressed by macrophages during the wound healing response\(^10\), has long been known to possess an antifibrotic ability in that it suppresses the expression of ECM-encoding genes\(^17\) and the TGF-β induction of collagen and connective tissue growth factor (CTGF) (also called CCN2), a downstream mediator of TGF-β\(^14\)\(^\text{-}\)\(^16\). CTGF promotes fibroblast proliferation, matrix production and granulation tissue formation\(^19\). Mice lacking the TNF-α receptor p55 show, after skin wounding, increased angiogenesis, collagen content and re-epithelialization\(^17\)

P-h peptides sheets® (Rohto Pharmaceutical Co., Ltd, Osaka, Japan), developed for dry mouth, has a sheet shape and dissolve in saliva. P-h peptides sheets® consist of common salt, thickening agents, an emulsifier, a sugar substitute and so on. It is noteworthy that p-h peptides sheets® contain novel protamine-reduced peptides (PRPs), including arginine (Maruha Nichiro Holdings, Inc., Tokyo, Japan), as a preservative, and these PRPs are effective against Candida albicans.

Although there has been much research conducted on wound healing, understanding of its pathophysiology and potential therapeutic agents remains unclear. Some therapies have been shown to be adverse to wound healing, such as the over-inhibition of fibronectin synthesis. These effects have puzzled investigators in recent decades since they suggest the existence of an undetermined specific target protein possessing important biological effects on signaling pathways. The efficient and specific regulation of such a protein could play a significant role in the expression of its downstream signals, thus affecting wound healing. Taking into account the pivotal role of TGF-β in wound healing, the present study analyzed whether the salutary effects of treating skin wounds with p-h peptides involves the TGF-β signaling pathway. First, we examined TGF-β expression in skin tissue, in fibroblasts and in keratinocytes. Second, we characterized whether the treatment of skin wounds in rats with p-h peptides activates the TGF-β signaling pathway and affects the expression of TGF-β and of TGF-β target genes.

Materials and Methods

Animals and surgery

Ten 12-week-old male SD rats, each weighing 150 to 170 g, were obtained from Japan SLC (Shizuoka, Japan). The rats were allowed food and water ad libitum and were maintained on a 12-h light/dark cycle (lights on from 8:00 to 20:00) at 23±1 °C with 60±10 % humidity for 1 week before use. All animals were maintained and used in accordance with the guidelines of the Nihon University Intramural Animal Use (No. AP11MD0021). All animals were injected intravenously with 35 mg/kg sodium pentobarbital (Somnopentyl®, Kyoritsu Seiyaku, Tokyo, Japan). The back of each rat was shaved, aseptically prepared for surgery, and a full-thickness incisional wound (approximately 20 mm), was then created on the back of each animal. The open-skin wounds were treated with p-h peptides or with saline only as a control. The percentage of reduction in each incisional wound area was recorded. The treatment was repeated for 1 week post-surgery, after which the rats were sacrificed by CO₂ inhalation. To collect specimens, the wound sites were excised. The specimens were fixed in 4 % paraformaldehyde and prepared for histology analysis.

Tensile strength testing

The skin samples were loaded into the grips of a mechanical testing system (Test Resources, model: 100P/Q, MN, USA) such that the incision line was orthogonal to the direction of tension. The grips were set at a distance of 0.5 inch and the scar was centered between the grips. Samples were then loaded under tension at a rate of 5 mm/min to failure. The ultimate tensile strength at failure (breaking strength) was recorded.

Immunohistochemistry

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded experimental rat skin wound specimens. Primary antibodies used were an anti-TGF-β antibody (1:100; ab66043, Abcam, Tokyo, Japan), an anti-phospho Smad3 antibody (1:250; NBP1-77836, Novus Biologicals, Littleton, CO), an anti-Smad4 antibody (1:100; ab40759, Abcam, Tokyo, Japan), an anti-CTGF antibody (1:100; ab6992, Abcam, Tokyo, Japan), an anti-phospho Stat3 antibody (1:100; ab76315, Abcam, Tokyo, Japan), an anti-VEGF antibody (1:50; sc152, Santa Cruz Biotechnology, Inc., Dallas, TX) and anti-TNF-α antibody (IW PA1079, IHC WORLD, LLC, Woodstock, MD). A Catalyzed Signal Amplification (CSA) system (DAKO, Carpinteria, CA) was employed to detect primary antibody binding. Sections were initially immersed in Target Retrieval Solution (DAKO) at 97 °C for 20 min, and subsequent steps were performed according to the manufacturer’s instructions. The immunostaining of all specimens was performed simultaneously to ensure the same antibody reaction and DAB exposure conditions.

Statistical analysis

Statistical analysis was done by one-way ANOVA. A P value
Results

After incisional wounding, p-h peptides-treated animals showed accelerated wound healing, reduced granulation tissue formation, increased epithelization and decreased inflammation (Fig. 1A). A higher skin tension was determined in the p-h peptides-treated group [438.0±78.8g (p<0.01)] compared to the control group [281.4±44.8 g] (Fig. 1B). At day 7 after injury, macrophages were abundant in the control tissues. Endothelial cells had migrated into the clots and proliferated and formed new blood vessels. Fibroblasts had migrated into the wound tissue, where they proliferated and deposited ECM. The wounds were filled with granulation tissue in the p-h peptides-treated tissues at day 7. Fibroblasts has transformed into myofibroblasts, leading to wound contraction and collagen deposition. The wounds were
nearly covered with neoeipidermis (Fig. 1C).

In order to evaluate if p-h peptides could act locally, we analyzed the expression of TGF-β signaling in skin samples using immunohistochemistry. Strong immunoreactivity for TGF-β was found in epithelial and fibroblast cells of p-h peptides-treated skin wounds (Fig. 2). To further analyze the expression of components of the TGFβ-signaling cascade, we determined protein levels for phosphorylated Smad3 (pSmad3) and Smad4 using immunohistochemistry in skin wound tissues (Fig. 2). No significant changes in the expression of Smad3 and Smad4 genes were found in saline-treated skin wounds. High immunoreactivity for pSmad3 and Smad4 were observed in p-h peptides-treated wounds. CTGF, a downstream target of Smad signaling, was also highly expressed in p-h peptides-treated tissues (Fig. 3). Thus, treatment with p-h peptides increased the expression of several Smad family members known to mediate TGF-β signaling. Consistent with these data, the levels of pStat3 and VEGF were also elevated in p-h peptides-treated wound tissues compared with wound tissues of saline-treated animals (Fig. 4).

Interestingly, immunohistochemistry revealed a dramatically decreased expression of TNF-α in p-h peptides-treated skin wounds compared with saline-treated wounds (Fig. 4), suggesting that p-h peptides can suppress inflammation during the wound healing process. TNF-α was predominantly expressed in fibroblasts of saline-treated skin wounds compared to p-h peptides-treated skin wounds.

Discussion

Skin wound healing is initiated by the migration of epithelial cells adjacent to the injured surface into the wound to cover the denuded area. This epithelial restitution occurs within a period of minutes to hours. The effect of p-h peptides on skin wound healing was assessed in vivo by studying the migration of skin fibroblasts and epithelial cells. The present study confirms the pro-healing effect of p-h peptides on skin repair. We extend current knowledge by providing evidence that p-h peptides improve skin wound healing by activation of the TGF-β signaling pathway. In line with reports on the expression of TGF-β and its receptors in skin wounds, we detected increased TGF-β expression after wounding. Although the regulatory roles of TGF-β family members...
in wounded skin have been described in various experimental in vivo models, the exact mechanism of the action of TGF-β is not completely understood. In our study, p-h peptides markedly enhanced the phosphorylation of Smad3 in wound tissues. These data support the assumption that the action of p-h peptides involves the TGF-β signaling pathway.

Treatment with p-h peptides significantly enhances skin epithelial cell restitution in vivo (Fig. 1), and increases the expression of TGF-β at day 7 after surgery (Fig. 2). This result suggests that the mechanism by which p-h peptides increases the migration rate involves an increase in the production of TGF-β, a natural stimulus of the wound healing process. To confirm this hypothesis, signal transduction analysis was carried out to detect whether the transcription of this growth factor is affected. In the same experiment, some rats were treated with saline as a negative control.

The canonical TGF-β signaling pathway activates Smad transcription factors, and some effects of TGF-β on wound repair require Smad3. Thus, Smad3 and Smad4 may act as mediators, linking the expression of factors important in early wound healing (e.g., TGF-β) with factors important in the later remodeling phase. We found that the phosphorylation of Smad3 and Smad4 is regulated in skin wound healing and that p-h peptides-treated skin wounds demonstrate elevated phosphorylated Smad3 and Smad4 expression levels. These data suggest a critical role for the phosphorylation of Smad3 and Smad4 in skin wound healing. Given its role as a potential mediator regulating wound size, the phosphorylation of Smad3 and Smad4 is an enticing target for the treatment of disorders of wound healing.

In adult skin, CTGF normally is not expressed unless induced, for example, during the normal wound repair process. TGF-β induces the expression of CTGF by dermal fibroblasts, but not by keratinocytes, via consensus Smad elements in the CTGF promoter. CTGF promotes cell adhesion and migration in a wide variety of cell types as well as collagen matrix contraction in fibroblasts. TNF-α has also been proposed to suppress TGF-β signaling via the NF-κB induced expression of Smad7. Intriguingly, although TNF-α suppresses the TGF-β induced expression of CTGF, the over-expression of CTGF in scleroderma lesions, which occurs by a Smad-independent mechanism, is not suppressed by TNF-α.

Cells involved in wound healing release cytokines and growth factors that may act as paracrine factors for further VEGF expression. The important role of VEGF in the healing process is supported by several studies showing that the reduced expression of VEGF and/or its accelerated degradation is associated with wound healing defects. TGF-β induces VEGF transcription and secretion in keratinocytes. In support for a role of VEGF in wound repair, expression of the VEGF gene is strongly induced in p-h peptides-treated skin wounds, with keratinocytes and macrophages being the major producers, and this expression pattern suggests that VEGF-A stimulates wound angiogenesis.

Our results indicate that treatment with p-h peptides significantly stimulates the migration of skin epithelial cells through a TGF-β-dependent process, which could promote the healing of skin injuries. This mechanism may contribute to the demonstrated skin injury or inflammation in mice. Although these results were obtained using an in vivo system, the observed increase in the speed of restoration of the epithelial cells and fibroblasts suggests that p-h peptides could stimulate epithelial cell migration in damaged tissues of patients with skin or oral wounds. Therefore, p-h peptides may possess great potential as a new therapeutic agent for the treatment of injuries associated with skin inflammation.

The high levels of activated Stat3 suggest a specific function for Stat3 in the regulation of cell proliferation. Stat3 is a transcription factor oncogene that can support and regulate cell cycle progression and prevent apoptosis. The tissues from p-h peptides-treated animals were subjected to immunohistochemical analysis for Stat3, revealing that p-h peptides increase the phosphorylation of Stat3, which justifies the conclusion that the specific effect on inflammation and wound healing is due to treatment with p-h peptides.

Myofibroblasts have been shown to have two main functions during wound healing. Firstly, they exert contractile strength and align collagen via integrins to contract wound edges and, secondly, they deposit ECM collagen fibers to strengthen the wound. Thus, the activation and differentiation of myofibroblasts is a crucial step in wound contraction. As shown in the present study, myofibroblasts are induced in the p-h peptides-treated animals compared with the controls during skin wound healing. TGF-β has been shown to be a critical factor in myofibroblast activation via its stimulatory action on fibroblast proliferation, myofibroblast differentiation and matrix deposition. Therefore, the induction of TGF-β signaling may be responsible for the increased myofibroblast differentiation and the rapid dermal wound contraction observed in p-h peptides-treated animals. This significant effect deserves further study to more fully elucidate the mechanism of action of p-h peptides, since bioactive molecules that can act as anti-inflammatory agents inhibiting macrophage activation and at the same time favor the wound healing process, are promising therapeutics for epithelial wound healing.

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References
15. Flanders KC. Smad3 as a mediator of the fibrotic response. Int J Exp Pathol 85: 47-64, 2004
19. Gold LI, Sung JJ, Siebert JW and Longaker MT. Type I (RI) and type II (RII) receptors for transforming growth factor-beta isoforms are expressed subsequent to transforming growth factor-beta ligands during excisional wound repair. Am J Pathol 150: 209-222, 1997
31. Crean JK, Finlay D, Murphy M, Moss C, Godson C, Martin...


38. Swift ME, Kleinman HK and Dipietro LA. Impaired wound repair and delayed angiogenesis in aged mice. Lab Invest 79: 1479-1487, 1999


