

# TGF- $\beta$ 1 Latency Associated Peptide Promotes Remodeling of Healing Cutaneous Wounds in the Rat

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## Abstract

**Background:** The process of wound healing involves integrated events including inflammation, granulation tissue formation, matrix deposition and remodeling. Growth factors play a key role in the process. Among them transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) is known to accelerate tissue repair by promoting the synthesis and deposition of extracellular matrix proteins. However, persistence or overactivity of TGF- $\beta$ 1 during the remodeling phase can potentially lead to fibrosis. The primary objective of this study was, therefore, to determine the effects of TGF- $\beta$ 1 inactivation, by its latency associated peptide (LAP), on the cutaneous healing wounds.

**Methods:** Excisional wounds were generated on the back of male adult rats. Wounds received TGF- $\beta$ 1 or LAP during the post-inflammatory phase. Expression of type I collagen and  $\alpha$ -smooth muscle actin was evaluated by Western blotting. Wound maturation was further assessed by histology and immunohistochemical methods using specific antibody for proliferating cell nuclear antigen (PCNA).

**Results:** Wounds treated with TGF- $\beta$ 1 showed a marked increase in the level of type I collagen, whereas no significant changes were observed in the wounds treated with LAP as compared to that in control. Expression of  $\alpha$ -smooth muscle actin was markedly reduced in the wounds treated with LAP but was slightly increased in the wounds treated with TGF- $\beta$ 1. Both neodermis and newly-formed epidermis exhibited a higher degree of maturation in the LAP-treated wounds as compared to TGF- $\beta$ 1 treated wounds.

**Conclusion:** Local administration of LAP seems to be beneficial to tissue remodeling. It promotes wound maturation and, may prevent fibrosis and hypertrophic scarring.

**Iran J Med Sci 2006; 31(2): 65-69.**

**Keywords** • TGF- $\beta$ 1 • latency associated peptide • wound healing • fibrosis

## Introduction

The complex process of adult wound healing involves inflammation, granulation tissue formation, matrix deposition and remodeling.<sup>1,2</sup> Transforming growth factor beta (TGF- $\beta$ 1) is known to play an important role in the

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process. TGF- $\beta$ 1 modulates immune responses, stimulates proliferation and differentiation of fibroblasts and modulates growth and survival of epithelial cells.<sup>3-5</sup> It also induces the expression of extracellular matrix (ECM) proteins, and inhibits synthesis and activity of ECM degrading enzymes.<sup>6,7</sup> Moreover, TGF- $\beta$ 1 controls angiogenesis by modulating endothelial cell growth and apoptosis.<sup>8</sup> Collectively, these activities lead to granulation tissue formation and deposition of the nascent matrix.

Wound contraction is essential for wound closure, and myofibroblasts are known to be involved. In response to injury, migrating fibroblasts and perivascular satellite cells differentiate to myofibroblasts and express alpha-smooth muscle actin.<sup>9-11</sup> The filamentous actin in myofibroblasts are expressed in the form of stress-fibers which are arranged parallel to the axis of the cell, generating the force of contraction.<sup>11</sup> Contraction helps wound closure by narrowing its margins. During the remodeling phase, gradually the granulation tissue matures and is replaced by a permanent matrix while progressing to neodermis.<sup>10</sup> Wound maturation requires re-orientation of collagen fibers, elimination of myofibroblasts and reduction in cellularity through apoptosis of endothelial cells and fibroblasts. These alterations are fundamental to normal wound healing.<sup>12</sup>

Persistence or over activity of TGF- $\beta$ 1 during the remodeling phase can potentially delay maturation of the granulation tissue, leading to hypertrophic scarring and fibrosis.<sup>13</sup> Although the positive effects of TGF- $\beta$ 1 during the early phase of wound healing is well established, its role in the remodeling phase is controversial.<sup>14</sup> The primary objective of this study, therefore, was to inactivate TGF- $\beta$ 1 by its latency associated peptide (LAP) during the post-inflammatory phase, and evaluate its effects on the remodeling phase of healing in cutaneous wounds.

## Material and Methods

**Materials:** Recombinant TGF- $\beta$ 1 and latency associated peptide (LAP) were purchased from R & D System (Minneapolis, USA). Anti  $\alpha$ -smooth muscle actin, anti proliferating cell nuclear antigen (PCNA) and anti type I collagen monoclonal antibodies and biotin conjugated secondary antibody were purchased from Sigma (St. Louis, USA). Diaminobenzidine (DAB) substrate kit was from Zymed (San Francisco, USA).

### *Surgery Procedure*

The protocol for excisional wound model is approved by the Institutional Animal Care and Use Committee. Adult male Sprague-Dawley

rats (200-230 g) were used in this study. For acclimatization the animals were housed in plastic cages in an air-conditioned room ( $22\pm 2^\circ\text{C}$ ), 12-h light dark cycle and fed Purina rat chaw and water *ad libitum* for one week.

In brief, on the day of surgery, rats were anesthetized by an intraperitoneal injection of pentobarbital sodium (Nembutal 30 mg/kg), and received an intramuscular injection of the pain killer butorphanol (Sedatol, 0.2 mg/kg). The dorsum was shaved with electric clippers and clean washed with betadine solution followed by sterile saline. Four circular full thickness punch biopsies of 6 mm in diameter were created in paravertebral region of the dorsum. Using sterile procedure, the skin and *panniculus carnosus* were removed but fat and fascia were left intact. The wounds were equally spaced with respect to right-left and head-tail alignment. In case of hemorrhage, direct pressure was gently applied to stop bleeding. Animals were then placed on a pre-warmed electric blanket for 1-2 h and then transferred to individual metal cages. The pain killer was given every 24 h for 5 days. Animals were kept in an isolated clean room until the last day of experiment.

### *Treatments and Wound Harvesting*

The wound size was measured manually, on day five or day 10 post-wounding, to assure similar rate of healing. Animals were then anesthetized and wounds were moisten using a pre-soaked sterile gauze in normal saline. Five  $\mu\text{l}$  of transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1; 0.5  $\mu\text{g}$ ), latency associated peptide (LAP; 3  $\mu\text{g}$ ) or bovine serum albumin (BSA; 25  $\mu\text{g}$ ), the carrier of TGF- $\beta$ 1 and LAP, were injected to wounds using a 28 G needle (Becton Dickinson, New Jersey, USA). The wounds treated on day 5 were harvested on day 14 and those treated on day 10 were harvested on day 21 post-wounding, using a 8 mm Biopsy Punch (Fray, Amherst, USA). For preparation of histology sections, the tissue was immediately fixed in phosphate-buffered saline with 4% paraformaldehyde for 24 h at  $4^\circ\text{C}$ . The tissue was then processed, embedded in paraffin blocks, and 4  $\mu\text{m}$  sections were prepared using standard techniques. Tissue sections were used for histological analysis and immunohistochemistry.

### *Immunohistochemistry*

Wound tissue sections were de-paraffinized in xylene, rehydrated in graded ethanol and washed in PBS. For proliferating cell nuclear antigen (PCNA) staining, the sections were processed as described previously.<sup>16</sup>

### *Western blotting analysis*

Wound tissue extracts (25  $\mu$ g/lane) were resolved by sodium dodecyl sulfat polyacrylamide gel electrophoresis (10%) and electrotransferred to polyvinylidene fluoride membranes. The nonspecific bindings were blocked by incubation in Tris-buffered saline (TBS) containing 10% non-fat milk and 0.5% Tween. The blots were then washed and incubated with the primary antibody for 1 h at room temperature followed by extensive wash in TBS solution. Subsequently, blots were incubated with conjugated secondary antibody. The ECL detection system (Amersham, Piscataway, USA) was used according to the manufacturer instructions.

### Results

Fig 1 shows Western blot analysis of type I collagen expression in the wound tissues. The expression of type I collagen was markedly increased in the wounds treated with TGF- $\beta$ 1 on day 5 post-wounding whereas, the expression of type I collagen in the wounds treated with LAP remained unchanged when compared with that in the control wounds. As shown in Fig 2, injection of TGF- $\beta$ 1 or LAP into the wounds on day 5 post-wounding moderately inhibited the expression of  $\alpha$ -smooth muscle actin. However, injection of LAP on day 10 post-wounding markedly reduced the expression of  $\alpha$ -smooth muscle actin whereas injection of TGF- $\beta$ 1 was ineffective.

The degree of re-epithelialization or wound closure is shown in Fig 3. All wounds were closed on day 14 post-wounding, indicating that the rate of wound closure was not affected by the treatments. However, the architecture of newly-formed epidermis was profoundly affected by both LAP and TGF- $\beta$ 1. When compared with the control wounds, the wounds treated with TGF- $\beta$ 1 exhibited a thinner epidermis with a disrupted basal layer showing many pyknotic and apoptotic cells (Fig 3). Besides, cells stained positive for immunoreactive PCNA were significantly reduced in the

epidermis of the TGF- $\beta$ 1-treated wounds when compared to that of the controls or LAP-treated wounds. In contrast, the LAP-treated wounds displayed a much thicker epidermis with a perfect basal layer showing more cells stained positive for immunoreactive PCNA (Fig 3). In addition, the cellularity, mainly fibroblasts, of neodermis was markedly affected by TGF- $\beta$ 1 but not by LAP. Although LAP did not have marked effects on the fibroblast population of neodermis, wounds treated with TGF- $\beta$ 1 exhibited a neodermis with a larger fibroblast population when compared to that of control or LAP-treated wounds (Fig 3).

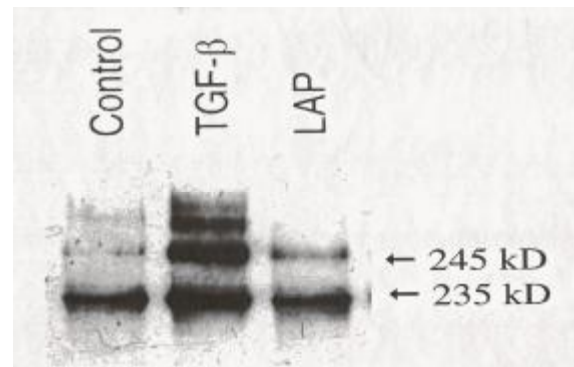


Fig. 1: Representative Western blot of type I collagen protein (245 and 235 kD) from wound tissues treated on day 5 post-wounding.

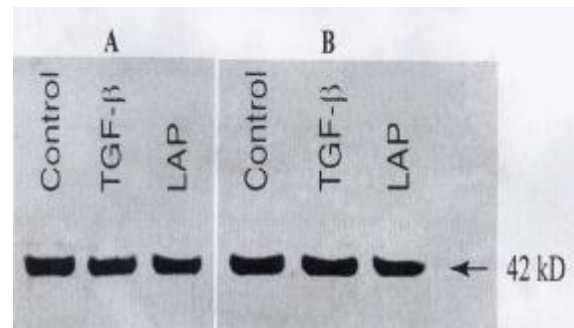


Fig. 2: Representative Western blot of  $\alpha$ -smooth muscle actin protein (42 kD) from wound tissues. A: Wounds were treated on day 5 post-wounding. B: Wounds were treated on day 10 post-wounding.

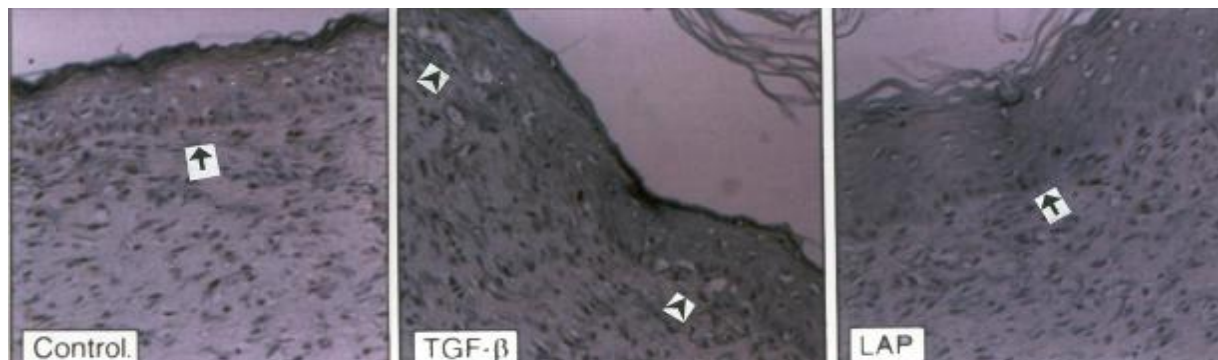


Fig. 3: Representative photomicrograph of immunohistochemistry staining for PCNA in wound tissues. Wound treated with TGF- $\beta$ 1 exhibits a thinner epidermis with disrupted basal layer (arrow heads). LAP-treated wound shows a thicker epidermis with intact basal layer, having more cells stained positive for immunoreactive PCNA similar to that in control (arrows).

## Discussion

Taken together, these data show that neutralization of endogenous TGF- $\beta$ 1 by its LAP accelerates wound maturation and improves quality of healing. TGF- $\beta$ 1 is known as a potent fibrogenic factor, and, therefore, the healing tissues benefit from its actions during the early phase of healing process. However, persistence or overactivity of TGF- $\beta$ 1 during the post-inflammatory phase, day three on post-wounding, may delay wound maturation that may potentially lead to fibrosis and hypertrophic scarring. The ultimate goal of researchers is to simulate fetal wound healing in adults to prevent scar formation. Scarless fetal wound healing has been attributed, at least in part, to the underactivity of TGF- $\beta$ 1 in fetus.<sup>17,18</sup> In contrast, overactivity of TGF- $\beta$ 1 is known to be responsible for the scar-forming wound healing in adults.<sup>19,20</sup> Therefore, blocking the TGF- $\beta$ 1 activity during the post-inflammatory phase may prevent fibrosis and hypertrophic scarring.

Some investigators have suggested that LAP may be a potential new therapy in TGF- $\beta$ 1-driven fibrosing disorders, including scleroderma, liver and lung fibrosis and multiple sclerosis.<sup>21,22</sup> Our results, however, are in concert with these findings. TGF- $\beta$ 1 is secreted from almost all cells in a form of biologically inactive complex, latent TGF- $\beta$ 1, that consists of the mature TGF- $\beta$ 1 associated non-covalently with a protein designated as the latency associated peptide, LAP.<sup>23</sup> The latent TGF- $\beta$ 1 is not able to bind to its cellular receptor, and is biologically inert before being activated.<sup>23</sup> Therefore, activation of the latent TGF- $\beta$ 1 complex is the key event in regulation of TGF- $\beta$ 1 function.<sup>23</sup> The latent TGF- $\beta$ 1 can be activated by different mechanisms including extreme pH, heat and action of certain proteolytic enzymes, which lead to dissociation or altered-binding of LAP.<sup>24,25</sup> Because the dissociated mature TGF- $\beta$ 1 can be re-associated with the LAP, in situ high concentrations of LAP may inactivate mature TGF- $\beta$ 1 and prevent overactivity of this potent fibrogenic factor.<sup>21,22</sup>

Fibroblasts are the major cell population involved in matrix deposition and wound contraction. Previous studies have shown that myofibroblasts exclusion and reduction in collagen deposition during the remodeling phase are pivotal to wound maturation.<sup>12</sup> The up-regulating effects of TGF- $\beta$ 1 on the expression of type I collagen and  $\alpha$ -smooth muscle actin, a specific marker of myofibroblasts, indicates that TGF- $\beta$ 1 can delay tissue remodeling and wound maturation. In contrast, a significant reduction of the  $\alpha$ -smooth muscle actin expression in the wounds treated with LAP suggests

that LAP accelerates myofibroblast elimination, thereby, promoting wound maturation. Moreover, LAP blocked the TGF- $\beta$ 1-induced up-regulation of type I collagen expression and fibroblasts proliferation, major characteristics of fibrotic tissues.

The adverse effects of TGF- $\beta$ 1 on the newly formed epidermis are indicating that overactivity of TGF- $\beta$ 1 not only delays maturation of dermis, but hinders maturation of epidermis. In contrast, LAP not only reversed the inhibitory actions of TGF- $\beta$ 1 but also improved tissue maturation. Similar results have been shown in transgenic mice with elevated hepatic levels of bioactive TGF- $\beta$ 1.<sup>22</sup> In these animals administration of LAP has completely reversed suppression of the TGF- $\beta$ 1-induced proliferation after liver injury.<sup>22</sup> These data are in concert with our assumption that neutralization of endogenous TGF- $\beta$ 1 with its naturally occurring LAP improves tissue maturation and may prevent TGF- $\beta$ 1-induced fibrosis.

## Conclusion

The results of this study are indicating that latency associated peptide may be a new naturally occurring factor for prevention and/or treatment of fibroproliferative disorders. Whether the effects of latency associated peptide are fully induced by inactivation of the endogenous bioactive TGF- $\beta$ 1 or by itself remains to be elucidated.

## Acknowledgments

*We thank Dr. David N. Herndon Chief of Staff, Shriners Burns Hospital for Children, Galveston, Texas, USA. We gratefully acknowledge the excellent photographic technical assistance given by Miss. Z. Saffari from the Department of Education Development Center, Shiraz University of Medical Sciences, Shiraz, Iran. This work was supported by a Shrine Research Fellowship grant (M. Varedi), University of Texas, Medical Branch, Galveston, Texas, USA.*

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