Role of TGFβ-Mediated Inflammation in Cutaneous Wound Healing

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Among many molecules known to influence wound healing, transforming growth factor β1 (TGFβ1) has the broadest spectrum of actions, affecting all cell types that are involved in all stages of wound healing. Both positive and negative effects of TGFβ1 on wound healing have been reported. However, the underlying mechanisms are largely unknown. We observed that endogenous TGFβ1 was elevated in a narrow window of time after injury, and transgenic mice constitutively overexpressing wild-type TGFβ1 in keratinocytes (K5.TGFβ1^{wt}) exhibited a significant delay in full-thickness wound healing as compared to non-transgenic mice. Delayed wound healing was associated with profound inflammation throughout all stages of wound healing in K5.TGFβ1^{wt} mice. Our data suggest that excessive and prolonged TGFβ1 at the wound site does not benefit wound healing, which is partially owing to its pro-inflammatory effect. Future studies need to be conducted to assess whether tightly regulated TGFβ1 expression will benefit wound healing. To this end, we have developed a gene-switch TGFβ1 transgenic system that allows TGFβ1 induction in keratinocytes temporally with desired levels. These mice will provide a tool to study stage-specific effects of TGFβ1 on cutaneous wound healing.

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INTRODUCTION

As the first barrier to the body, skin remains the first line of defense against injury and disease. The cutaneous wound-healing process is essential for the maintenance of skin homeostasis. Impaired wound healing may lead to chronic skin disorders (e.g., diabetic ulcers) accounting for significant levels of morbidity as well as severe decrease in quality of life. Excessive scarring resulting from deregulated wound repair can also cause loss of joint motion or major body deformation (Harding *et al*., 2002). A better understanding of the cellular and molecular mechanisms during normal wound healing will be instrumental for the development of effective therapies for patients with aberrant wound healing. Cutaneous wound healing proceeds via three overlapping phases: inflammation, tissue formation, and tissue remodeling (Singer and Clark, 1999). When the skin is injured, blood constituents are released from disrupted blood vessels and a blood clot forms. The platelets of the blood clot release numerous chemotactic factors, particularly transforming growth factor beta1 (TGFβ1), which in turn rapidly recruit leukocytes to accumulate at the site of injury and initiate the inflammation phase. These leukocytes secrete chemokines and inflammatory cytokines to augment the inflammatory response that usually reach the peak within a few days after injury. As the inflammation phase subsides, the tissue formation phase takes place. The tissue formation phase involves re-epithelialization and granulation tissue formation. Upon injury, keratinocytes at the wound edge detach from the basement membrane to acquire motility and migrate to cover the wound. Initially, the re-epithelialization uses the blood clot as a provisional matrix, which will be gradually replaced with granulation tissue. A series of biological events take place to promote granulation tissue formation, including fibroblast activation and myofibroblast differentiation, extracellular matrix degradation and production, and neovascularization. The last stage of wound repair is the tissue-remodeling phase, which includes changes in both the epidermis and dermis. Particularly, the dermis underlying the re-epithelialized epidermis undergoes a transition from granulation tissue to fibrotic tissue, marked by the cessation of neovascularization and the change of collagen synthesis from collagen III to collagen I (Eckes *et al*., 2000). Finally, the collagen synthesis slows down in the fibrotic tissue and a scar form at the site of injury.

Normal wound healing is orchestrated by numerous molecules, including growth factors, chemokines, inflamma-

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Abbreviations: Co-Smad, common Smad; MMP, matrix metalloproteinase; PCNA, proliferating cell nuclear antigen; R-Smad, receptor-specific Smad; TGFβ, transforming growth factor beta

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tory cytokines, and angiogenic factors produced by a variety of cell types, including kerationcytes, fibroblasts, leukocytes, and endothelial cells. Out of these molecules known to influence wound healing, TGFβ1 has the broadest spectrum of actions, affecting all cell types that are involved in all stages of wound healing (O'Kane and Ferguson, 1997). TGFβ is a family of pluripotent cytokines consisting of three isoforms: TGFβ1, 2, and 3. All TGFβ isoforms are secreted as latent forms, in which the N-terminal latency-associated peptide remains non-covalently bound to the C-terminal mature TGFβ (Lawrence, 1991). The latent forms have to be activated via proteolysis to release the mature TGFβ before functioning. TGFβ acts via two types of transmembrane serine/threonine kinase receptors. The major intracellular mediators of TGFβ family members are Smad proteins (for reviews, see Derynck and Zhang, 2003; Shi and Massague, 2003; Feng and Derynck, 2005), which are divided into three groups: receptor-specific Smads, a common Smad, and inhibitory Smads. To date, eight Smad proteins (Smad1 to Smad8) have been identified in mammals. Among them, Smad2 and Smad3 function as receptor-specific Smads specifically responsible for TGFβ-mediated signaling (Liu *et al.*, 1997; Souchelnytskyi *et al.*, 1997; Goto *et al.*, 1998; Miyazawa *et al.*, 2002; Derynck and Zhang, 2003). When TGFβ binds to a type I–type II receptor complex, the type I receptor kinase binds and phosphorylates receptor-specific Smads. The phosphorylated receptor-specific Smads form oligomeric complexes with Smad4, the common Smad, which translocate into the nucleus and regulate gene expression transcriptionally. The inhibitory Smads, Smad6 and Smad7, inhibit the TGFβ-signaling cascade by either blocking phosphorylation and subsequent nuclear translocation of signaling Smads (see for a recent review, Massague *et al.*, 2000; ten Dijke and Hill, 2004) or degradation of the receptors via specific ubiquitin–proteasome pathways (Kavsak *et al.*, 2000; Izzi and Attisano, 2004). Although Smads are critical for TGFβ signal transduction, compelling evidence has suggested that Smad-independent pathways may also mediate TGFβ signaling. For instance, TGFβ has been found to activate mitogen-activated protein kinases, phosphoinositide 3-kinase, and Rho family guanosine triphosphatases to regulate cell growth and apoptosis (Izzi and Attisano, 2004). TGFβ has been implicated in cutaneous wound healing in regulating both Smad-dependent and-independent pathways (Saika, 2004; Saika *et al.*, 2004).

PARADOXICAL ROLE OF TGFβ1 IN CUTANEOUS WOUND HEALING

After injury, TGFβ1 is rapidly upregulated and secreted by keratinocytes, platelets, and macrophages (Singer and Clark, 1999). TGFβ1 is essential for initiating inflammation and granulation tissue formation (McCartney-Francis and Wahl, 1994). Additionally, TGFβ1 may be required for cell migration during wound repair. Some of the proteases that are involved in cell migration, such as matrix metalloproteinase (MMP)1, MMP2, MMP3, and MMP9, are transcriptionally upregulated by TGFβ1 (Madlener *et al.*, 1998; Santibanez *et al.*, 2000; Ellenrieder *et al.*, 2001; Verrecchia *et al.*, 2001). Cell migration-associated integrins, such as β1, α5, αv, β5, and β6, are also regulated by TGFβ1 (Gailit *et al.*, 1994; Zambruno *et al.*, 1995). These integrins affect keratinocyte and fibroblast migration (Gailit *et al.*, 1994; Zambruno *et al.*, 1995). TGFβ1 has also been shown to stimulate wound contraction (Montesano and Orci, 1988) through its direct induction of α-smooth muscle actin expression in fibroblasts (Desmouliere *et al.*, 1993). Furthermore, TGFβ1 stimulates the production of extracellular matrix molecules, including collagens and fibronectin, which strengthen the repaired wound. Reduced TGFβl expression has been observed in humans with impaired wound healing (Schmid *et al.*, 1993; Cowin *et al.*, 2001; Jude *et al.*, 2002), particularly in diabetic foot ulcers and chronic venous leg ulcers (Cowin *et al.*, 2001; Jude *et al.*, 2002). In addition, many animal models of impaired wound healing exhibit reduced TGFβ1 expression and the rate of healing is improved by the application of exogenous TGFβ1 (Pierce *et al.*, 1989; Salomon *et al.*, 1990; Beck *et al.*, 1991, 1993). Based on the above studies, it is not surprising that TGFβ1 has long been considered as a promising potential therapeutic agent for impaired wound healing. Despite that early studies showing that injection of TGFβ1 to wounds accelerate healing in experimental animals (Mustoe *et al.*, 1987; Sporn and Roberts, 1993), clinical trials that exogenously administered TGFβ1 to human chronic ulcers have achieved very limited efficacy (Bennett *et al.*, 2003; Mulder, 2004).

Accumulating reports from recent studies begin to challenge whether TGFβ1 can be used as a therapeutic agent for impaired wound healing. The first surprise was from wound healing using Smad3 knockout mice, in which TGFβ signaling is partially abolished. Instead of a predicted delay in healing, Smad3 null mice exhibited accelerated wound healing, featured by increased keratinocyte proliferation and migration, and reduced monocyte infiltration (Ashcroft *et al.*, 1999). Consistently, deletion of the secretory leukocyte protease inhibitor, a molecule that is required for normal wound healing, results in delayed wound healing that is characterized with increased TGFβ1 activation and can be attenuated by TGFβ1 antibody (Ashcroft *et al.*, 2000). More direct evidence comes from recent studies, in which transgenic mice expressing the TGFβ1 transgene in keratinocytes exhibited delayed healing after burn injury (Yang *et al.*, 2001; Tredget *et al.*, 2005). Conversely, TGFβ1 knockout mice showed accelerated re-epithelialization during incisional wound repair, in comparison with wild-type mice (O'Kane and Ferguson, 1997; Koch *et al.*, 2000). Thus, ideally, a better wound-healing outcome may be achieved by selectively blocking the negative effects of TGFβ1. In supporting this notion, transgenic mice overexpressing a dominant-negative TGFβ receptor exhibit accelerated re-epithelialization in skin wounds. This is associated with an increased proliferation and reduced apoptosis in keratinocytes at the wound edge owing to the resistance of keratinocytes to TGFβ-mediated growth arrest and apoptosis (Amendt *et al.*, 2002). Similarly, administration of exogenous Smad7, the antagonist of TGFβ signaling, to mouse eyes accelerates corneal wound healing via promoting epithelial

cell migration and inhibiting monocyte/macrophage invasion to the wounds (Saika, 2004; Saika *et al.*, 2005). Taken together, selective suppression of TGFβ signaling in certain cell types in cutaneous wounds may benefit wound healing despite the dogma that TGFβ plays a key role in wound healing.

CONSTITUTIVE OVEREXPRESSION OF TGFβ1 IN KERATINOCYTES DELAYED CUTANEOUS WOUND HEALING OWING TO AT LEAST, IN PART, ELICITING SKIN INFLAMMATION

Previously reported studies have prompted us to speculate that endogenous TGFβ1 may have a negative impact on wound healing at least partially owing to its role in the induction of inflammation. TGFβ1 has long been known for its dual effects on inflammatory response and immune modulation. The pro-inflammatory effect of TGFβ1 has been overlooked ever since studies have shown that TGFβ1 knockout mice exhibit inflammation in multiple organs and autoimmune conditions, which highlight an anti-inflammatory role of TGFβ1 (Shull *et al.*, 1992; Kulkarni *et al.*, 1993). However, the *in vivo* role of TGFβ1 in regulating inflammatory/immune response may vary in different organs. For instance, TGFβ1 knockout mice do not develop any inflammatory phenotypes in the skin (Shull *et al.*, 1992; Kulkarni *et al.*, 1993) and are devoid of Langerhans cells in the epidermis (Borkowski *et al.*, 1996, 1997). Considering that TGFβ1 is a potent chemotactic cytokine for virtually all leukocytes as well as endothelial cells and fibroblasts (Wahl *et al.*, 1987, 1993; Wahl, 1992, 1994), all of which are involved in the development of inflammation, the pro-inflammatory effect of TGFβ1 is likely to predominate in the skin. In supporting this notion, overexpression of TGFβ1 in basal keratinocytes and hair follicles initiates chronic skin inflammation, marked by epidermal hyperplasia, leukocyte infiltration, and angiogenesis (Liu *et al.*, 2001; Li *et al.*, 2004). In addition, we observed that during cutaneous wound healing, endogenous TGFβ1 increased rapidly upon injury and reached a peak level 3 days post a 6-mm full-thickness wounding (Figure 1), which coincide with the peak of the inflammation phase during early stages of wound healing.

We further assessed the wound-healing process in K5.TGFβ1^{wt} transgenic mice, which spontaneously develop an inflammatory skin disorder (Li *et al.*, 2004). Notably, almost all K5.TGFβ1^{wt} mice developed spontaneous skin ulcers in the friction-prone areas by 4–5 months of age (Figure 2). Histology on skin ulcers revealed an absence of epidermis, massive inflammatory cells in the dermis, and prominent angiogenesis, resembling granulation tissues during wound healing (Figure 2). We then performed wound-healing studies using 6-mm punch biopsies on the dorsal skin of 8-week-old transgenic mice and non-transgenic littermates. At this age, the hair follicles are synchronized at the telogen (resting) phase (Muller-Rover *et al.*, 2001), and the inflammation and epidermal hyperplasia are not yet severe in transgenic skin. Five mice in each group were evaluated at each post-wounding time point. Both male and female mice were used in the study, and there was no noticeable

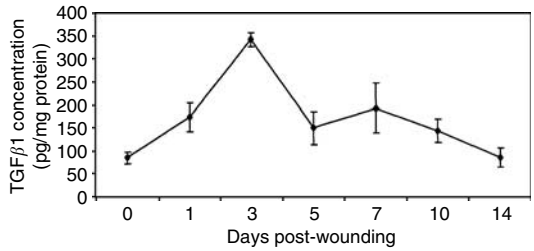

Figure 1. Expression levels of endogenous TGFβ1 determined by ELISA on wound biopsies. Total protein was extracted from individual wound biopsies from normal ICR mice at different time points. The value at each time point represents the average level of TGFβ1 detected in samples from three mice.

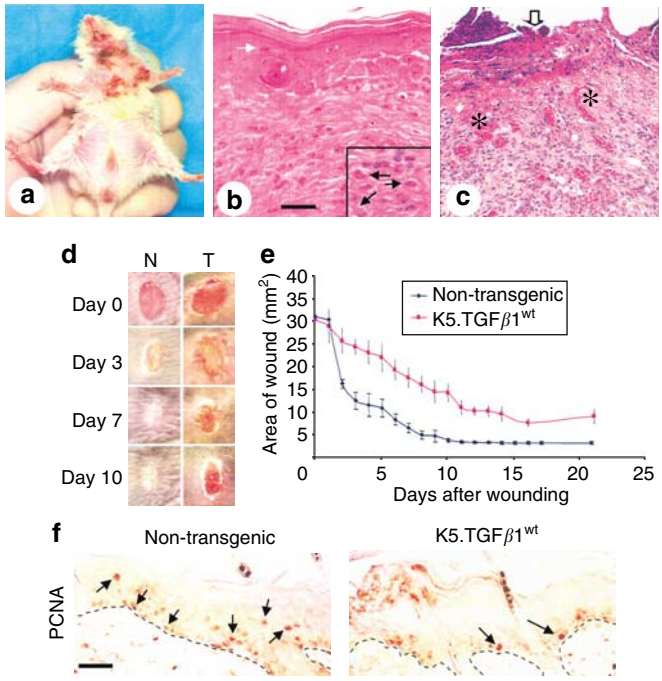

Figure 2. K5.TGFβ1^{wt} mice developed spontaneous skin ulcers and showed delayed re-epithelialization upon wounding. (**a**) A K5.TGFβ1^{wt} mouse displayed spontaneous skin ulcers. (**b**) Histological analysis of non-ulcerated transgenic skin showed epidermal hyperplasia, hyperkeratosis, acanthosis, and a diminished basement membrane. Numerous neutrophils were observed in both the epidermis (white arrow) and dermis (black arrows in the inset). (**c**) Histological analysis of ulcerated skin of a transgenic mouse revealed epidermal necrosis (open arrow), numerous inflammatory cells, and increased angiogenesis (asterisks indicate clustered red blood cells). The bar in panel (b) represents 40 μm for both (**b**) and (**c**). To further study the effects of TGFβ1 overexpression on cutaneous wound healing, both non-transgenic (N) and K5.TGFβ1^{wt} (T) mice were subjected to 6-mm full-thickness wounding and monitored for wound closure daily. (**d**) Macroscopic changes in skin wounds in a non-transgenic littermate and a K5.TGFβ1^{wt} mouse. (**e**) Changes in wound area (mm^2) at each time point. The data of each time point represents the average from five mice. (**f**) Proliferating cell nuclear antigen staining of day 3 wounds revealed a decreased number of positive nuclei (brown cells, arrows point out examples) of the wound edge in transgenic epidermis as compared to that of non-transgenic epidermis. The dotted line delineates the boundary between the epidermis and the dermis. Proliferating cell nuclear antigen staining at other time points provided similar results (data not shown). The bar represents 25 μm for both immunohistochemical sections.

difference in the wound healing kinetics between the sexes. Wound closure in non-transgenic mice occurred visibly during the first week, whereas the wound areas were barely changed in K5.TGF$\beta1^{wt}$ mice (Figure 2). The scabs on non-transgenic wounds detached at about day 10 post-wounding, but the scabs remained on transgenic wounds, even at day 21 post-wounding. Consistent with delayed wound closure, immunohistochemistry with an antiproliferating cell nuclear antigen–antibody revealed an approximately 3-fold decrease in keratinocyte proliferation in the migrating tongue of K5.TGF$\beta1^{wt}$ wounds compared to that in non-transgenic wounds (Figure 2). This observation is in contrary to that observed in unwounded skin, in which TGF$\beta1^{wt}$ induces expression of growth factors from fibroblasts and leukocytes, resulting in epidermal hyperplasia (Li *et al.*, 2004). Therefore, it appears that these growth factors are not sufficient to overcome TGFβ1-induced growth inhibition at the wound edge where keratinocytes demand a higher proliferation rate (Figure 2). Microscopically, K5.TGF$\beta1^{wt}$ wounds displayed delayed re-epithelialization and prolonged granulation tissue accompanied by a persistent inflammatory cell infiltration throughout in comparison with non-transgenic wounds (Figure 3).

Our data suggest that the delayed cutaneous wound healing in K5.TGF$\beta1^{wt}$ mice can be partially attributed to excessive inflammation throughout all stages of wound healing (Figure 3). For instance, inflammatory cells, especially macrophages at sites of injury, produce a large amount of MMPs, which may overdigest the basement membrane between the newly formed epidermis and the underlying granulation tissue, thereby suppressing re-epithelialization to cover the wound. This is consistent with an upregulation of genes encoding MMP2, MMP3, and MMP9, and a rapid degradation of the basement membrane in K5.TGF$\beta1^{wt}$ transgenic skin (Li *et al.*, 2004). Specifically, MMP9 knockout mice showed an accelerated epithelial cell migration in corneal wounds in association with reduced Smad2-mediated signaling, suggesting a suppressive role of MMP9 in re-epithelialization during wound healing (Mohan *et al.*, 2002). More importantly, elevated levels of MMPs, including MMP2 and MMP9, have been reported in wounds of human chronic ulcers, and MMP inhibitors have been considered for a new therapy (Mandal *et al.*, 2003). In addition, excessive TGFβ1 and other inflammatory cytokines expressed by inflammatory cells may directly inhibit expression of genes that promote keratinocyte migration.

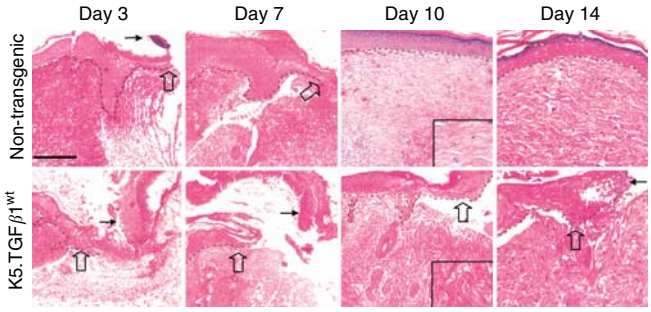

Figure 3. Histology of K5.TGF$\beta1^{wt}$ and non-transgenic wounds at different time points. In each panel, the dotted line delineates the boundary between the epidermis and the dermis. In these micrographs, the re-epithelialization was taking place from left to right. The open arrow points to the edge of the epidermal migrating tongue, and the solid arrow points to leukocytes, which have infiltrated into the scab. By day 3, the non-transgenic wound displayed an obvious epidermal migrating tongue and minimal leukocyte infiltration, whereas the transgenic wound had developed only a very small epidermal migrating tongue and massive leukocyte infiltration. On day 7, the non-transgenic wound exhibited a large migrating tongue, whereas the K5.TGF$\beta1^{wt}$ wound showed little sign of re-epithelialization with numerous infiltrating leukocytes. On day 10, re-epithelialization was complete in the non-transgenic wound, whereas the transgenic wound was still undergoing the re-epithelialization process. Also on day 10, the transgenic dermis displayed denser collagen deposition as compared to non-transgenic skin (insets of day 10 panels). On day 14, the non-transgenic wound displayed signs of tissue remodeling, whereas the K5.TGF$\beta1^{wt}$ wound still exhibited obvious inflammation and incomplete re-epithelialization. The bar in the first panel represents 100 μm for day 3–day 10 panels, and 40 μm for day 14 panels.

FUTURE PERSPECTIVES

The controversial data on the effects of TGFβ1 on cutaneous wound healing may reflect the complex nature of biological functions of TGFβ1, which may be cell type and context specific. As keratinocytes are one of the major sources of TGFβ1 after cutaneous injury (O'Kane and Ferguson, 1997), transgenic mouse models that overexpress TGFβ1 in keratinocytes will provide experimental tools to explore the molecular mechanisms of the effects of TGFβ signaling on wound healing. In previous studies, TGFβ1 expression levels were not temporally controlled, which makes it difficult to determine the direct effect of TGFβ1 at each specific stage. For instance, constitutive overexpression of TGFβ1 may result in excessive inflammation, which will over-ride other positive effects of TGFβ1 on wound healing. Furthermore, expression levels of TGFβ1 may also affect its functions. To study the role of TGFβ1 in wound healing in a stage-specific manner in the future, we developed a ''gene-switch'' transgenic system, which allows inducible expression of a transgene in keratinocytes temporally and for transgene expression levels to be controlled (Wang *et al.*, 1999; Lu *et al.*, 2004). Through inducing transgene expression at different levels and at different time points after skin punches on gene-switch transgenic mice, we will be able to analyze the effects of TGFβ1 on different wound-healing stages, including inflammation, tissue formation, and tissue remodeling. These studies will provide unique insights into a better understanding of stage- and dose-specific effects of TGFβ1 on skin wound healing, which will facilitate the development of new therapies for impaired wound healing through modulating TGFβ-elicited signaling.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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