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WOUND-HEALING PROPERTIES OF TRANSFORMING GROWTH FACTOR β (TGF- β) INDUCIBLE EARLY GENE 1 (TIEG1) KNOCKOUT MICE

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Abstract

Transforming growth factor beta (TGF- β) has a broad effect on wound healing, but many questions remain about the regulation of TGF- β during the healing process. TGF- β inducible early gene 1 (TIEG1) is a primary response gene for TGF- β that controls the activities of the TGF- β /Smad pathway, the primary TGF- β signaling pathway. The purpose of this study was to investigate the role of TIEG1 in cutaneous wound healing using TIEG1 knockout mice. The wound healing in TIEG1 knockout mice and wild-type controls was evaluated by wound breaking strength, Western blot, and histology at postoperative days 3, 7, and 14. Although re-epithelialization of both groups was similarly complete at day 7, the TIEG1 knockout mice had a significantly lower wound breaking strength than the controls at postoperative day 14. These results suggest that TIEG1 expression may be an important factor involved in the initiation and support of normal cutaneous wound healing.

Keywords

Wound healing; Breaking strength; TIEG; TGF- β ; Smad

INTRODUCTION

Wound healing is a complex and highly regulated process that relies heavily on a large number and variety of molecules. Recently, growth factors have been shown to play an important role in these processes. Of the many cytokines that have been implicated in wound healing, transforming growth factor beta (TGF- β) has the broadest effects on soft tissue healing, such as cell proliferation, cell migration, and extracellular matrix (ECM) deposition.¹¹⁻¹⁶ The effect of TGF- β is exerted through its type I (T β RI) and type II (T β RII) serine/threonine kinase receptors. Although several different signaling pathways have been suggested to play a significant role in the regulation of TGF- β , the TGF- β /Smad pathway appears to be the primary TGF- β signaling pathway.⁵ In the TGF- β /Smad pathway, the activated T β RI phosphorylates intracellular receptor-regulated Smad proteins (R-Smad). R-Smads (Smad2 and Smad3) then complex with the common mediator Smad4, and the complex translocates to the nucleus where it activates the transcription of target genes by a variety of mechanisms. The latter involves both a direct binding of Smad proteins to DNA as well as an indirect activation via binding to other transcription factors.⁴ Negative feedback to the Smad pathway occurs through the

induction of the inhibitory Smad7, which blocks TGF- β signaling by binding to T β RI; this prevents R-Smad phosphorylation and increases the turnover of T β RI.⁸

TGF- β inducible early gene 1 (TIEG1), a widely expressed three-zinc-finger, Krüppel-like transcription factor, was originally identified as the product of a TGF- β -inducible gene from an osteoblastic cell population.²¹ The induced expression of TIEG1 is one of the earliest known events in the TGF- β /Smad pathway. Overexpression of TIEG1 has been shown to mimic TGF- β activity in many cell types.^{3,6,17,23} All three TGF- β isoforms (TGF- β 1, TGF- β 2, TGF- β 3) induce TIEG1 expression in a similar pattern and intensity.⁷ TIEG1 overexpression enhances the TGF- β induction of Smad-binding element reporter activity by suppressing the inhibitory Smad7 gene activity and the activation of Smad2.⁸

Based on the above observations and the role of TGF- β in wound healing, we hypothesize that the loss of TIEG1 would have an effect on cutaneous wound healing. However, to our knowledge, no studies have been published that have investigated the effects of TIEG1 on wound healing. The purpose of this study is to investigate the role of TIEG1 in wound healing using TIEG1 knockout mice. Exploration of the wound-healing process in TIEG1 null mice provides a means for further understanding of TGF- β and the role of the TGF- β /Smad pathway on the regulation of wound healing.

MATERIALS AND METHODS

Specimen Preparation

Thirty C57Black/129 mice consisting of 15 TIEG1 knockout mice (TIEG^{-/-}) and 15 wild-type controls (TIEG^{+/+}) were used in this study. All National Institutes of Health (NIH) and Institutional Animal Care and Use Committee (IACUC) protocols for the care of animals were followed. Prior to incision, mice were anesthetized using 0.02–0.04 mL of 2.5 mL ketamine HCl + 0.5 mL xylazine in the hind limb and their backs were shaved, prepped with SURGI-PREP (Sparta Surgical Corp., Concord, CA, USA), and draped with a sterile drape. A 2-cm transverse, linear, full-thickness incision symmetrically down to the level of the panniculus carnosus was made below the inferior edge of the scapula. The incision was then closed using five 5-0 Prolene interrupted sutures (Ethicon, Inc., Somerville, NJ, USA).

Both TIEG^{-/-} mice and controls were assigned into three subgroups for the evaluation of the time periods of wound healing (postoperative days 3, 7, and 14; five mice for each subgroup). Mice were sacrificed with an overdose of CO₂. Their backs, including the wound, were harvested for further analysis by making a rectangular excision 2.0 cm in width and 3.0 cm in length. The sutures were then removed from the wound. The central portion of the wound (1 cm wide) was used to measure the wound breaking strength, and both lateral parts were used for analysis of histology and Western blot.

Wound Breaking Strength Measurements

The wound breaking strength was measured in all five mice per subgroup at each time period. The skin specimen was fixed using clamps with interdigitating grooves in a mechanical testing machine (MTS, Minneapolis, MN, USA) and distracted at a rate of 20 mm/min. Maximum forces required to break the wound at the sutured sites were recorded. All specimens were kept moist throughout testing with a spray of saline mist. The data obtained from the wound breaking strength were analyzed statistically by two-factor analysis of variance (ANOVA). When significance was detected, the Tukey–Kramer test was applied to determine the statistical difference between data pairs. All statistical tests were two-sided, and *p*-values less than 0.05 were considered significant.

Histological Analysis

The lateral part of the wound was fixed in 10% (v/v) buffered formalin for 2 hours before being processed and embedded in paraffin wax. Sections were then cut at a thickness of 5 μ m longitudinally. Hematoxylin and eosin (H&E) staining was performed. Histological analysis of wound healing was performed in a standard manner and was evaluated according to cell proliferation, cell re-epithelialization, inflammatory changes, and collagen deposition.

Western Blot

Skin specimens were resolved in 500 μ L of SDS sample buffer. The samples were boiled for 5 minutes to denature proteins and then stored at -80°C until use. Each sample was electrophoresed in a 15.0% (w/v) polyacrylamide gel containing SDS (SDS-PAGE) and transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA, USA). The membranes were first incubated for 2 hours in TBS-Tween containing 5% (w/v) bovine serum albumin (BSA). The blocked membranes were then incubated for 2 hours with primary antibodies against TGF- β 1 (1:400 dilution; Sc-146, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), TGF- β 2 (1:200 dilution; Sc-90, Santa Cruz Biotechnology), TGF- β 3 (1:200 dilution; Sc-82, Santa Cruz Biotechnology), or actin (1:1000 dilution; Sc-1616, Santa Cruz Biotechnology). Following incubation with primary antibodies, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000 dilution; Amer-sham Pharmacia Biotech, Buckinghamshire, UK) for 1 hour. The blots were detected using the enhanced chemiluminescence detection system (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. The band density was quantified with image analysis software (Scion Image, Scion Corp.) and normalized to that of actin.

RESULTS

Under gross examination, the TIEG $^{-/-}$ mice were phenotypically normal and the breeding characteristics appeared to be normal. The direct observation of wounds in both groups showed similar wound healing at all time points and revealed a tendency of less scar formation in TIEG $^{-/-}$ mice.

During mechanical testing, all skin specimens broke at the original incisional site. The wound breaking strength in TIEG $^{-/-}$ mice at postoperative days 3, 7, and 14 was $0.14 \text{ N} \pm 0.10 \text{ N}$, $1.19 \text{ N} \pm 0.38 \text{ N}$, and $2.96 \text{ N} \pm 0.22 \text{ N}$, respectively. The wound breaking strength in controls at days 3, 7, and 14 was $0.36 \text{ N} \pm 0.18 \text{ N}$, $0.98 \text{ N} \pm 0.25 \text{ N}$, and $4.48 \text{ N} \pm 0.40 \text{ N}$, respectively. Although there was no significant difference in wound breaking strength between the TIEG $^{-/-}$ mice and controls at postoperative days 3 and 7, the TIEG $^{-/-}$ mice had a significantly lower wound breaking strength than the controls at day 14 ($p < 0.05$) (Fig. 1).

In histological analysis, epidermal thickness and overall dermal thickness of the wounds were similar in both TIEG $^{-/-}$ mice and controls. Although some delay of re-epithelialization was shown in TIEG $^{-/-}$ mice at postoperative day 3, re-epithelialization of both groups was similarly complete at day 7. Rather weak inflammatory changes and granulation tissue formations were also observed in TIEG $^{-/-}$ mice (Fig. 2).

Western blot analysis was performed to investigate the effect of the loss of TIEG on the expression of TGF- β in cutaneous wound healing (Fig. 3). Although the expression level of TGF- β 1 decreased in TIEG $^{-/-}$ mice during wound healing, increased expression of TGF- β 2 and TGF- β 3 was seen in TIEG $^{-/-}$ mice at postoperative days 3 and 7 and at day 7, respectively (Table 1). The expression of all TGF- β isoforms decreased in TIEG $^{-/-}$ mice at postoperative day 14.

DISCUSSION

Because the TGF- β /Smad pathway appears to be the primary TGF- β signaling pathway,¹² the effects of exogenous and endogenous expression of Smad have recently been investigated to further understand the exact role of TGF- β during wound healing.^{1,2,18,22} Proteins that modulate the Smad pathway may provide a means of accelerating wound healing or potentially decrease scarring.

TIEG1 was originally cloned from human osteoblasts as a primary response gene to TGF- β treatment.²¹ TIEG1 overexpression in MG63 human osteosarcoma cells mimics TGF- β effects by increasing alkaline phosphatase and decreasing osteocalcin secretion.⁶ In addition, TIEG1 overexpression in pancreatic carcinoma, hepato-carcinoma, and mink lung epithelial cells inhibits cell growth and induces apoptosis similar to that of TGF- β treatment.^{3,17,23} The TGF- β -like actions of TIEG1 are due to its regulation of the TGF- β /Smad pathway by a dual mechanism, in which it limits negative feedback through the inhibitory Smad7 via transcriptional repression⁹ while simultaneously activating the expression of Smad2.¹⁰ Recently, *in vitro* studies have revealed that loss of TIEG1 abrogated the activity of the Smad pathway in the presence of autocrine or exogenous TGF- β stimulation.⁸ To investigate whether loss of TIEG1 has effects on *in vivo* wound healing, we examined TIEG1 knockout mice developed by targeted disruption of the TIEG1 gene.²⁰

Wound strength is one of the most important aspects in the healing of surgical incision. The wound breaking strength of acute incisional wounds may be affected by the rate of ECM deposition in the early healing phase.^{13,15,19} TGF- β is an important regulator of cell growth, ECM deposition, fibroplasia and collagen deposition, ECM degradation, and upregulation of the synthesis of protease inhibitors.¹⁴ Exogenously applied TGF- β 1 and TGF- β 2 can increase the wound breaking strength and matrix deposition.^{13,24}

The results of this study demonstrate that the wound breaking strength in TIEG1 knockout mice is significantly decreased at postoperative day 14, but not at days 3 and 7, compared with controls. The re-epithelialization of wound in both TIEG^{-/-} mice and controls was complete at post-operative day 7. These findings indicate that loss of TIEG1 affects the function of TGF- β and the TGF- β /Smad pathway *in vivo*, possibly by decreased stimulation of fibroblasts and induction of the ECM deposition, resulting in decreased wound breaking strength. The decreased expression of all TGF- β isoforms in TIEG^{-/-} mice at postoperative day 14 may also affect wound healing, since it correlates with the lower wound breaking strength.

Modulation of TIEG1 expression may provide a means of modulating TGF- β expression. Increased expression of TGF- β 2 and TGF- β 3 in TIEG^{-/-} mice may be the result of compensatory mechanisms in the altered TGF- β /Smad pathway in the TIEG^{-/-} mice. The high ratio of TGF- β 3 to TGF- β 1 in TIEG^{-/-} mice may be a factor in the rather weak inflammatory response and granulation tissue formation which we saw at postoperative day 7.

In this study, we demonstrated that the absence of TIEG1 had significant effects on full-thickness incisional wounds, resulting in decreased wound breaking strength at postoperative day 14. The results suggest that TIEG1 expression is an important factor needed to initiate and sustain normal cutaneous wound healing. Further research into the TIEG1 pathway may provide mechanisms for manipulation of the cutaneous healing pathway and modulation of scar formation.

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Fig. 1.

The results of wound breaking strength. Results are mean $\pm$ standard deviation. $n = 5$ for each time point and group. TIEG^{-/-} mice had a significantly lower wound breaking strength than the controls on postoperative day 14 (* $P < 0.05$).

Fig. 2.

Above: Histology of **(a)** TIEG^{-/-} mice and **(b)** controls at postoperative day 3. Some delay of re-epithelialization was observed in TIEG^{-/-} mice at postoperative day 3 compared with controls. Below: Histology of **(c)** TIEG^{-/-} mice and **(d)** controls at postoperative day 7. Re-epithelialization was complete at day 7 in both TIEG^{-/-} mice and controls. ECM deposition was decreased in TIEG^{-/-} mice. (H&E staining; original magnification, $\times 100$).

Fig. 3. Western blot analysis for TGF- β 1, TGF- β 2, and TGF- β 3. Actin was used as an internal standard to quantify the loaded protein of each lane.

Table 1

Relative Expression Level of TGF-β1, TGF-β2, and TGF-β3 Against Actin.

	+/+ Day 3	-/- Day 3	+/+ Day 7	-/- Day 7	+/+ Day 14	-/- Day 14
TGF-β1	0.91	0.69	0.34	0.26	0.30	0.13
TGF-β2	0.43	0.87	0.84	1.03	0.28	0.12
TGF-β3	0.05	0.03	0.13	0.86	0.28	0.05

Note: The level of TGF-β1 decreased in TIEG^{-/-} mice during wound healing. Increased expression of TGF-β2 and TGF-β3 was seen in TIEG^{-/-} mice at postoperative day 3 and day 7, respectively. The level of all TGF-β isoforms decreased in TIEG^{-/-} mice at postoperative day 14.