Activation of Latent TGF-β1 by Thrombospondin-1 is a Major Component of Wound Repair

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Purpose: Thrombospondin 1 (TSP1) is a matrix glycoprotein that regulates cell adhesion, migration, and proliferation, and is a natural inhibitor of angiogenesis. Recent evidence suggests that TSP1 is a major physiologic activator of latent transforming growth factor-β1 (TGF-β1), and that TGF-β1 is important for wound healing. The purpose of this study was to examine whether excisional wound healing in TSP1-deficient mice is compromised as a result of deficient TGF-β1 activation.

Materials and Methods: Punch wounds were made on the dorsum of TSP1 deficient and wild-type mice and the area of granulation tissue, number of microvessels, and inflammatory cell infiltration was evaluated over a period of 28 days.

Results: TSP1 deficient mice showed impaired wound healing with persistent granulation tissue, decreased collagen content over time, and delayed arrival of macrophages compared to wild-type littermates. The number of microvessels in wounds of TSP1-deficient mice was approximately two-fold greater than in wild-type littermates 10 days after injury. Topical application of TSP1, or KRFK (a peptide derived from TSP1 that activates latent TGF-β1), to wounds of TSP1-deficient mice rescued wild-type patterns of wound repair and partially recovered local levels of TGF-β1 expression. Topical application of anti-TGF-β neutralizing antibody impaired the ability of KRFK to rescue normal patterns of wound neovascularization in TSP1-deficient mice.

Conclusions: These results demonstrate that TSP1 plays a key role in the orchestration of wound healing, and that TSP1-mediated activation of local TGF-β1 is an important step in this process.

Key words: angiogenesis, neovascularization, oral cavity, apoptosis

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INTRODUCTION

Thrombospondin-1 (TSP1) is a modular matricellular protein that has been shown to interact with other matrix molecules, proteoglycans, integrins and cationic proteins in serum and in the extracellular compartments (Lawler, 1986; DiPietro, 1997; Sheibani et al, 1997; Krutzsch et al, 1999). TSP1 mediates cell-cell interactions and also plays a role in cell-substrate interactions since many cells have been shown to attach, spread and migrate on insoluble TSP1 (Lawler et al, 1988; Good et al, 1990; Sage and Bornstein, 1991). TSP1 is a potent inhibitor of angiogenesis (Good et al, 1990; DiPietro et al, 1994; Bornstein, 1995; Polverini, 1995; Iruela-Arispe and Dvovak, 1997; Iruela-Arispe et al, 1999; Sheibani and Frazier, 1999) that functions through the endothelial cell membrane receptor CD36 to induce endothelial cell apoptosis *in vitro* and *in vivo* (Dawson et al, 1997; Guo et al, 1997; Freyberg et al, 2000; Jimenez et al, 2000; Nör et al, 2000). In addition, it modulates the processes of migration, differentiation, and proliferation of diverse cell types such as epithelial and mesenchymal cells that participate in the healing

process (Raugi et al, 1987; DiPietro et al, 1996; Simon et al, 1996; Nissen et al, 1998; Roth et al, 1998). These include among others keratinocytes, inflammatory cell populations such as platelets and macrophages, and components of granulation tissue such as fibroblasts, myofibroblasts, and endothelial cells. TSP1 has been shown to activate transforming growth factor-β1 (TGF-β1), that has a major role in embryogenesis and angiogenesis, and in the regulation of inflammatory processes and wound healing (Schultz-Cherry et al, 1994, 1995; Crawford et al, 1998; Lawrence, 1996; Massague, 1998; Murphy-Ullrich et al, 2000).

A tightly regulated angiogenic response is a key step in wound healing. It has been recently shown that an initial angiogenic stimulus is supplied in healing wounds by fibroblast growth factor-2 (FGF-2), followed by a subsequent and more prolonged induction of angiogenesis mediated by vascular endothelial growth factor (VEGF) (Nissen et al, 1998). However, seven to 14 days after injury thrombospondin expression is enhanced around the wound microvessels and this coincides with downregulation of wound neovascularization (Raugi et al, 1987). We have reported previously that the application of antisense TSP1 oligonucleotides to mouse skin wounds markedly retards wound healing (DiPietro et al, 1996). Antisense treated wounds exhibited a marked delay in repair that included a decrease in the rate of epithelialization, delayed dermal organization and maturation, and a reduction in the accumulation of mature macrophages (DiPietro et al, 1996), which have an important role in the orchestration of wound healing processes (Polverini, 1996, 1997). TSP1 also functions as an inhibitor of several proteases, including neutrophil elastase and cathepsin G and perhaps certain proteolytic enzymes of the fibrinolytic pathway, including plasmin and urokinase plasminogen activators (Hogg, 1994). Each of these enzymes is present at the sites of injury and thus TSP1 may influence the balance of proteolytic activity necessary for appropriate tissue repair and remodeling.

The current study was undertaken to define further the role of TSP1 during wound healing by examining this phenomenon in mice genetically deficient in TSP1, and by determining if TGF-β1 was involved in the orchestration of the wound response mediated by TSP1. The results reported here demonstrate that mice deficient in TSP1 exhibit aberrant wound healing characterized by delayed onset of granulation tissue, and delayed infiltration of macrophages to the wound bed. In later stages, TSP1-deficient mice presented a significant accumulation and persistence of granulation tissue, microvessels, and macrophages at the site of injury that is associated with delayed healing of the wound. In addition, we found that topical application of KRFK, a peptide from TSP1 domains known to activate TGF-β1, significantly attenuated wound neovascularization and the accumulation of granulation tissue and accelerated wound healing in TSP1-deficient mice. We also observed an increase in expression of local TGF-β1 in wounds of TSP1-deficient mice treated with TSP1 or KRFK. The application of KRFK together with an anti-TGF-β neutralizing antibody partially impaired the ability of KRFK to rescue wild-type patterns of wound healing in TSP1-deficient mice. These results demonstrate that TSP1 influences multiple components of the wound response and indicate that TGF-β1 is an important mediator of TSP1's function in wound healing.

MATERIALS AND METHODS

Animals and Skin Wounds

Age- matched male and female mice genetically deficient in TSP1 (Lawler et al, 1998) and their wild-type littermates weighing between 20 and 24g were anesthetized with ketamine and xylazine. The hair covering the mid-dorsal region of each mouse was shaved and six full-thickness punch wounds through the panniculus carnosus, each separated by 1cm, were made in each animal using a 3mm diameter sterile disposable Baker's dermal biopsy punch. Animals were sacrificed from one to 28 days after injury. At least 18 wounds from three independent TSP1-deficient mice and wild-type littermates were used for quantification of the wound area, microvascular density, and macrophage infiltration at each time period. Samples used for immunohistochemical analysis were fixed in 2.5% paraformaldehyde overnight at 4°C before embedding in paraffin. All procedures were conducted in accordance with the University of Michigan Animal Care and Use Committee guidelines.

Administration of TSP1, TSP1 Peptides into Wounds

Forty-two TSP1-deficient and wild-type mice were used for these studies. Either the full-length TSP1, the TGF-β1 activating peptide ($K^{412}RFK^{415}$) based on the sequence located between the first and second type I repeats of TSP1, or an inactive peptide with similar molecular weight (KQFK) was applied topically at the wounds of each animal. Peptides were synthesized by the UAB Comprehensive Cancer Center Peptide synthesis core facility, purified by HPLC, and determined to be >95% pure by mass spectroscopy. Wounds were treated with either a 50 or 100μM solution of the peptide mixed with 25% F127 surfactant gel (BASF Wyandotte Corporation, Wyandotte, MI). A single application of 50μl of the oligomer/gel solution was performed in each wound, and a transparent sterile dressing (Tegaderm, 3M, Minneapolis, MN) was used to keep the gel in place throughout the experiment. Only mice that retained the Tegaderm in place at the time of sacrifice were included in the study. The monoclonal anti-mouse TGF-β antibody (MAB1835, R&D Systems, Minneapolis, MN) was used for

neutralization of TGF-β bioactivity. The mice were monitored daily and sacrificed from one to 21 days after the wounding. Immediately after sacrifice, the wounded skin was surgically removed, fixed with 2.5% paraformaldehyde at 4°C overnight and embedded in paraffin. At least nine wounds from three independent mice were evaluated per condition and time point.

RNA Preparation and Analysis

Total RNA was prepared from tissue samples as previously described (DiPietro et al, 1996). Briefly, total cellular RNA (10µg/lane) was subjected to electrophoresis through 0.8% agarose, 2mM formaldehyde gels in 20mM MOPS buffer, pH 7.0, containing 5mM sodium acetate and 1mM EDTA. Gels were blotted onto Gene Screen Plus (DuPont-NEN, Wilmington, DE), hybridized, and washed according to manufacturer's directions. The murine TSP1 probe (a gift of Vishva Dixit) was labelled with ^{32}P by random priming to a specific activity of at least 10^8cpm/µg.

Quantification of Granulation Tissue

The area of granulation tissue in hematoxylin and eosin-stained sections was quantified using a Zeiss Zidas digitizing tablet. Tissue sections were projected (at 200x) onto the surface of a digitizing tablet and outlined. At least five serial sections each separated by 20µm were used to determine the area of granulation tissue per wound, and nine wounds from three independent mice were evaluated per time point.

Quantification of Microvessels and Macrophages

Von Willebrand factor (factor VIII-related antigen) and F4/80 antigen were used for the identification of microvessels and macrophages respectively at the wound sites. Sections were deparaffinized and then treated with a solution of 0.1% trypsin and 0.1% calcium chloride for 45 minutes at 37°C for antigen retrieval. Monoclonal rabbit anti-human factor VIII-related antigen (Dako Corp., Carpinteria, CA) was used at 1/200 dilution for one hour at 37°C. A 1/10 dilution of F4/80 rat anti-mouse mature macrophage (Serotec Ltd., Raleigh, NC) was applied to sections for two hours at 370C. The ABC Vectastain Elite kit (Vector Laboratories Inc., Burlingame, CA) standard protocol was then carried out. Positive cells were stained with a solution of 0.014 g 3-amino-9-ethyl carbazole (AEC, Sigma, St. Louis, MO) in 2.5ml N,N- dimethylformamide (Sigma) for about seven minutes and counterstained with hematoxylin for one minute. Microvessels were quantified by a modification of the method of Weidner and collaborators that enumerates vessels in the most vascular portion of the injured tissue. Sections were examined under low power (40x to 100x) to identify the regions of highest vessel density. The number of microvessels and macrophages was determined in 10 microscopic fields (400x) from at least nine wounds of three independent mice.

Determination of Collagen Content of Wounds

As an indication of total wound collagen content, hydroxyproline concentration was determined as previously described (Woessner, 1961). Briefly, tissues were hydrolyzed in 2ml of 6 N HCl for three hours at 130°C. The solution was neutralized to pH 7 with 2.5 N NaOH and diluted 40-fold with H_2O. Two ml of diluted solution were mixed with 1ml of 0.05 M chloramine T solution and incubated for 20 minutes at room temperature. One ml of 3.15 M perchloric acid was added and the solution incubated an additional five minutes at room temperature. One ml of 20% p-dimethylamino-benzaldehyde was then added and the solution incubated for 20 minutes at 60°C. The absorbency of each sample at 557nm was determined, and the amount of hydroxyproline determined by comparison to a standard curve.

Determination of TGF-β1 Expression Levels in Wounds

Individual wounds were homogenized in 1ml of PBS containing 2mM phenyl-methyl-sulfonyl fluoride and 1µg/ml each of antipain, aprotinin, leupeptin, and pepstatin A, followed by sonication for two minutes on ice. Homogenates were briefly centrifuged to remove debris, and filtered through a 1.2µm pore membrane. Murine TGF-β1 concentration in wounds was assessed by a TGF-β1 specific ELISA (R&D Systems) according to the manufacturer's directions. Briefly, 100µl of sample or standard were mixed with 100µl of diluent and then added to individual wells of the assay plate. After incubation for three hours at room temperature, plates were washed four times, 200µl of TGF-β1 conjugate solution were added to each well, and the plates were incubated for an additional 1.5 hours at room temperature. Plates were then washed four4 times and 200µl of substrate solution was added to each well. After a 20-minute incubation, 50µl of stop solution was added and the absorbency determined at 450nm in a microplate reader.

RESULTS

TSP1 Deficient Mice Exhibit Aberrant Granulation Tissue and Persistent Macrophage Infiltration during Wound Healing

Microscopic examination of the skin of TSP1-deficient mice demonstrated no major structural defects in the organization of the dermis, epidermis, or underlying mesenchymal tissue. We observed a slight increase in the number of microvessels, but this difference was not statistically relevant when compared to wild-type litter-

mates (data not shown). In contrast, the process of wound healing (Fig 1) was significantly affected by the lack of expression of TSP1 in null mice (Fig 2). Wound healing was delayed in TSP1-deficient mice and granulation tissue persisted in the wound bed for much longer than in wild-type littermates (Fig 1).

Granulation tissue, comprised principally of proliferating fibroblasts, myofibroblasts and new capillary blood vessels, is a transient tissue essential for an efficient wound repair. We assessed this component of wound response over time in both wild type and TSP1-deficient mice by measuring the area occupied by granulation tissue in multiple contiguous sections. In wild-type mice, granulation tissue was observed within three days of injury occupying an area of approximately 1mm^2 (Fig 3a). Granulation continued to accumulate through day five, attaining maximal growth and occupying an area of 1.6mm^2 (Fig 3a). By day seven, wound resolution and replacement by scar tissue became evident and the area occupied by granulation tissue steadily declined through day 21 (Fig 3a). By day 21, the wound had completely healed and granulation tissue was now replaced by scar tissue (Fig 3b). The collagen content of the wound at day 21 was slightly higher than pre-injury levels (Fig 3b), which is an expected consequence of the process of scaring. This orderly ingrowth of granulation tissue and its replacement by scar tissue seen in wild-type mice was in marked contrast to events occurring in TSP1-deficient mice. Detectable granulation tissue was clearly evident only five days after injury. Once present, the granulation tissue was aberrant showing a significant increase in area that persisted for a longer time compared to wild-type littermates (Fig 3a). The area of granulation continued to increase through day seven and persisted through day 14. A relative decrease in collagen content in the wounds was evident in TSP1-deficient mice over time compared to wild-type littermates. At 14 and 21 days after injury, we observed a significant decrease ($p<0.05$) in the collagen content of wounds compared to wild-type mice (Fig 3b).

TSP1-deficient mice showed a generalized increase in the number of inflammatory cells at the site of injury compared to wild type, especially during later stages of wound healing (Fig 3c). When counts of mononuclear and polymorphonuclear leukocytes at the wound sites were evaluated, we found that macrophages presented the most significant increase among inflammatory cell populations (data not shown). Macrophages first became evident at the wound site by day one in wild-type mice and steadily increased in number through day five (Fig 3c). At later time periods, the number of macrophages was substantially reduced such that by day 14 or 21 they have essentially disappeared from the wound site. In contrast, TSP1-defi-

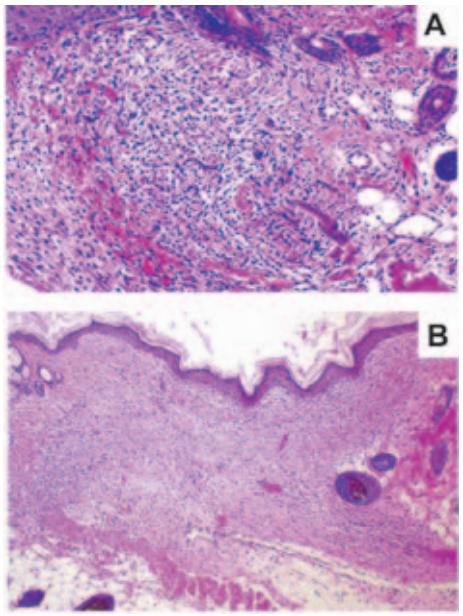

Fig 1 TSP1-deficient mice presented delayed wound healing and persistence of granulation tissue in the wound bed. Representative histological sections of excisional wounds (3mm in diameter) made in TSP1-deficient **(a)** and wild-type littermates **(b)**. Seven days after injury, the wound bed of TSP1-deficient mice **(a)** still presented a large area of granulation tissue and intense neovascularization. In contrast, the wound bed of wild-type mice **(b)** was less cellular and the wound was completely covered by newly formed epidermis. **(a, b)** Hematoxylin-eosin stain. Magnification: 100x.

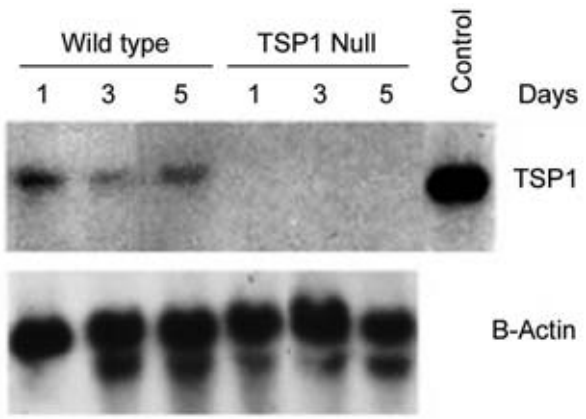

Fig 2 Northern blot confirmed the lack of expression of TSP1 in the wounds of TSP1-deficient mice. Total RNA was extracted from wounds of wild type and TSP1-deficient mice one, three and five days after injury. A murine TSP1 probe was used to determine TSP1 expression in wounds, and a murine β-actin probe served as a control for equal loading.

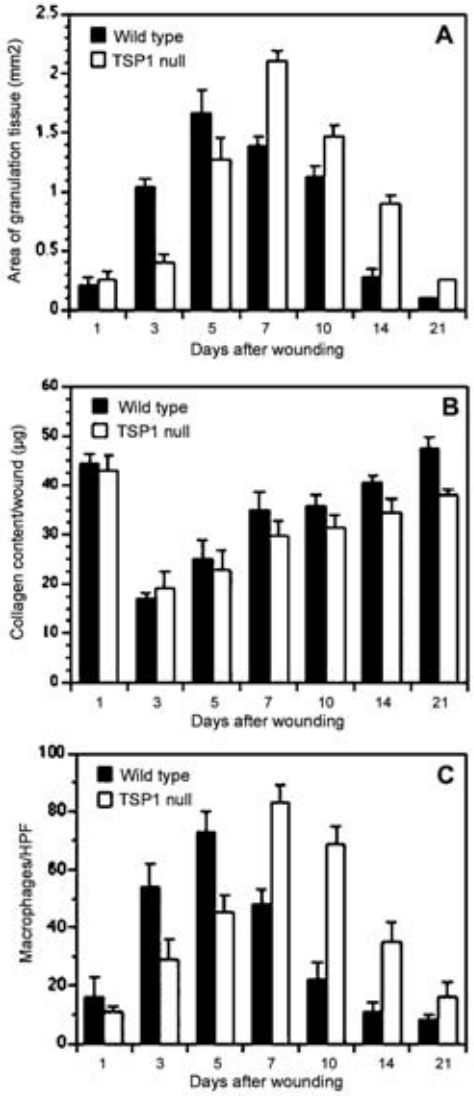

Fig 3 Wounds in TSP1-deficient mice presented persistent granulation tissue, decreased collagen content, and delayed arrival of macrophages to the wound bed. Excisional wounds were performed in TSP1-deficient and wild-type mice that were sacrificed one to 21 days after injury. The area of granulation tissue **(a)** was maximal five days after injury in wild-type mice and decreased steadily thereafter. In contrast, TSP1-deficient mice showed an initial delay in the organization of granulation tissue **(a)** that persisted in the wound bed for longer than in wild-type littermates. The collagen content **(b)** in the wounds of TSP1-deficient mice was lower than in wild-type mice five days after injury and thereafter. Macrophages had a delayed arrival to wound beds of TSP1 deficient mice and persisted for longer than in wild-type littermates **(c)**. The number of macrophages was determined in 10 microscopic fields (400x) from at least nine wounds of three independent mice.

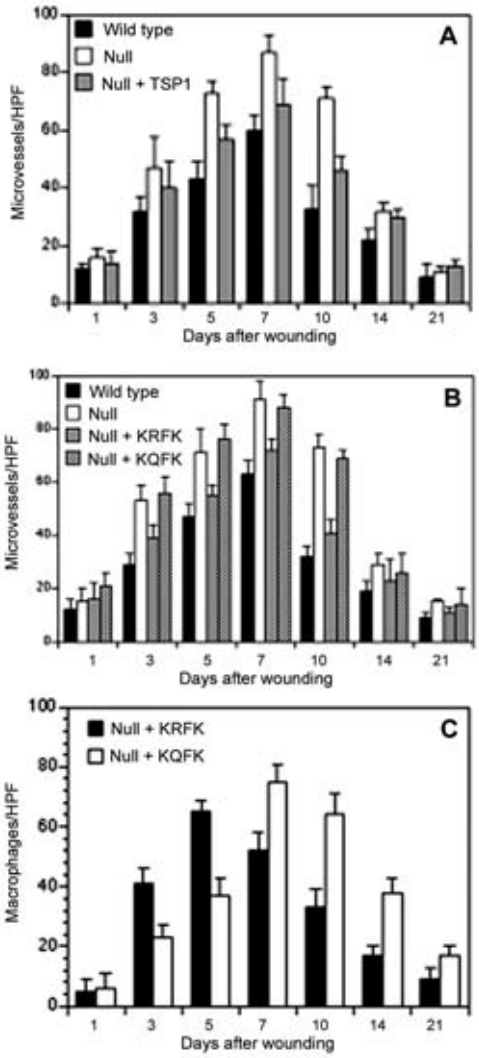

Fig 4 TSP1 and KRFK partially rescued neovascularization and macrophage content in wounds of TSP1 deficient mice. TSP1, KRFK, or the inactive control peptide KQFK, were applied topically to the wounds of TSP1-deficient mice that were sacrificed one to 21 days thereafter. Wound neovascularization in TSP1 deficient mice was reduced to levels more similar to wild-type mice after application of TSP1 **(a)** or KRFK **(a)**, and was not affected by the application of the inactive peptide KQFK **(b)**. The application of KRFK to wounds of TSP1 deficient mice **(c)** allowed for faster increase in macrophage numbers and reduced their persistency in the wound beds compared to the application of the inactive peptide KQFK.

cient mice showed both a delay in the onset of macrophage infiltration and a persistent accumulation at the wound site for up to 14 days after injury (Fig 3c). This is in marked contrast with wild-type mice, in which we observed only few macrophages at this phase of wound healing.

TSP1-Deficient Mice Exhibit Persistent Neovascularization during Wound Healing

The new microvessels that form during the repair process are a major component of the granulation tissue response, and serve to meeting the increased metabolic demands of the healing tissue. Once these metabolic

demands have been met and the angiogenic stimuli have been removed, they undergo rapid regression and the tissue microvasculature returns to a quiescent state. It is well established that TSP1 blocks angiogenesis and attenuates ongoing angiogenic responses *in vivo* (Nör et al, 2000). The transient nature of wound neovascularization was manifested in wild-type mice (Fig 4a). In wild-type mice, detectable vessel growth was initially observed at day three after injury, peaked at day seven, and showed a decline by day 10. By day 21 a neovascular response was no longer seen at the site of injury. When compared to wild-type mice, TSP1-deficient mice presented more microvessels at the wound sites five to 10 days after injury ($p<0.05$). At 10 days TSP1-deficient mice have a two-fold increase in wound microvessel density compared to wild-type mice, suggesting that lack of TSP1 prevents timely downregulation of neovascularization at late stages of wound healing. They also presented a luxuriant bed of granulation tissue that resembles the vigorous neovascularization seen in inflammatory pyogenic granulomas.

TSP1 or KRFK-Containing Peptides Partially Rescue Wound Neovascularization in TSP1-Deficient Mice

To assess more directly the role of TSP1 in the local activation of latent TGF-b1 in wound healing, we applied topically full-length TSP1 protein, a peptide comprising the TGF-b1 activating sequence (KRFK) from TSP1's type 1 repeats, or an inactive control peptide to the wounds of TSP1-deficient mice. The introduction of TSP1 or KRFK changed the pattern of wound healing in TSP1-deficient mice, making it more similar to wound responses seen in wild-type littermates. The area occupied by granulation tissue was markedly reduced at all time periods examined and complete wound resolution was observed to occur by day 14 (Fig 4b). A similar reduction in macrophage content of wounds was also observed and the total number of mononuclear leukocytes at days 10 and 14 was reduced by approximately one half in wounds treated with KRFK as compared to wounds treated with the inactive peptide KQFK (Fig 4c). The most significant changes were those involving wound microvessel density, where the application of KRFK rescued both the number of vessel and the duration of neovascularization to levels more comparable to wounds in wild-type mice. The application of KRFK to the wound was as effective, if not more, than the full-length TSP1 molecule (Fig 4a, b), while the application of the inactive control did not affect any of the wound healing responses observed in TSP-1 deficient mice (Fig 4b, c).

Effect of TGF-β1 Activation on Wound Maturation and Neovascularization

TSP1 has been shown to activate TGF-,1, a mediator that participates in the regulation of neovascularization *in vivo* (Crawford et al, 1998; Murphy-Ullrich and Poczatek, 2000). To determine the role that TGF-β1 may have in the wound response we measured the level of TGF-β1 in wild-type mice and in TSP1-deficient mice before and after the introduction of the TGF-β1 activation sequence of TSP1 (KRFK). Biochemical analyses revealed that TSP1-deficient mice exhibit reduced levels of TGF-β1 in wound sites over time, especially three to seven days after injury (Fig 5a). Topical application of KRFK increased the expression of TGF-β1 in wounds of TSP1-deficient mice (Fig 5a). This response was associated with a reduction of the number of new microvessels and shortening the duration of wound neovascularization to levels more comparable to wild-type mice (Fig 4a, 5b). The topical application of an anti-TGF-β neutralizing antibody to wounds of TSP1-deficient mice did not change significantly the ability of KRFK to reduce the number of microvessels in wounds in the initial phases of wound healing. However, this antibody impaired the ability of KRFK to mediate quicker removal of microvessels from the wound bed at later stages of healing, especially around 10 days after injury. Taken together these results demonstrate that TSP1 orchestrates wound neovascularization, at least in part, by modulating the content of TGF-β1 at the wound site.

DISCUSSION

TSP1 is a modular extracellular matrix molecule that regulates angiogenesis and influences a number of critical steps in tissue regeneration (Lawler, 1986; DiPietro, 1997; Sheibani et al, 1997). TSP1-deficient mice are viable but have subtle abnormalities during development (Lawler, 1998). The skin of these mice presents a minor elevation in the number of microvessels, which may be due to the absence of the angio-inhibitory effect of TSP1. In contrast, we found that the wound healing process of skin injuries was dramatically impaired in TSP1-deficient mice. Wounds in these mice present enlarged areas of granulation tissue that persist over time, low collagen content, and enhanced numbers of macrophages and microvessels compared to their wild-type littermates. A recent report demonstrates that transgenic mice that overexpress TSP1 present normal angiogenesis during development, while the wound healing-associated angiogenesis was impaired (Streit et al, 2000). These findings suggest that TSP1 plays a more active role in the regulation of wound healing than in the maintenance of skin microvascular homeostasis. We also found that TGF-β1 levels in the wounds of TSP1-deficient mice are low, and that topical application of a

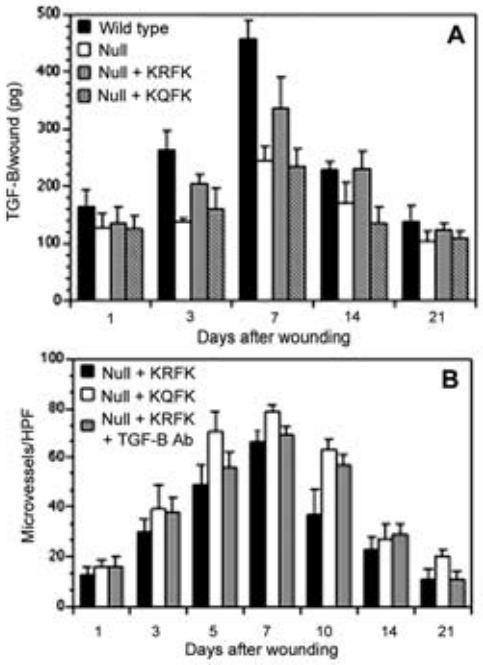

Fig 5 Topical application of KRFK partially rescues TGF-β1 expression in the wounds of TSP1 deficient mice. The expression of active TGF-β1 in the wounds was measured by ELISA **(a)**, and the number of microvessels **(b)** was counted one to 21 days after injury. KRFK, KQFK, or KRFK combined with a TGF-β1 neutralizing antibody were applied topically to the wounds of TSP1 deficient mice. The expression of active TGF-β1 in the wounds of TSP1 deficient mice is decreased compared to wild-type littermates, and topical application of KRFK to the wounds of these mice partially rescues expression of TGF-β1 **(a)**. Topical application of anti-TGF-β1 neutralizing antibody impaired the ability of KRFK to mediate down-regulation of neovascularization in the wound bed 10 days after injury, during the resolution phase of wound healing **(b)**.

TSP1-derived peptide (KRFK) enhances local expression of TGF-β1 in these mice and rescues the wound healing process. This study demonstrates that TSP1 has an important role in the orchestration of wound healing in skin and that TSP1's effects are mediated by the activation of latent TGF-β1.

The healing of skin wounds in TSP1-deficient mice was impaired and delayed compared to wild-type littermates. Full thickness skin wounds in wild-type mice are generally healed within 14 days. In contrast, the wounds in TSP1-deficient mice were not healed 14 days after injury and still presented extended areas of granulation tissue that were not replaced by a fibrous scar. Among the predominant cell populations in wounds are the macrophages, which are an important source of growth factors and cytokines, and are believed to play an important role in the orchestration of the wound healing process (Polverini, 1996, 1997). We observed a delay in the arrival of macrophages to the wounds of TSP1-deficient mice. This might be explained by the fact that TSP1 facilitates the migration of activated macrophages (Silverstein and Nachman, 1987). Macrophages have surface receptors for TSP1, and thus lay down TSP1 upon the existing extracellular matrix as a scaffold upon which macrophages migrate (Silverstein and Nachman, 1987). The delayed arrival of macrophages to the site of injury in TSP1-deficient mice may affect timely organization of the granulation tissue, and interfere with its substitution by scar tissue in the resolution phase of wound healing.

These observations are in contrast with the recently reported evaluation of wound healing in thrombospondin 2 (TSP2) knockout mice (Kyriakides et al, 1999). Wounds performed in TSP2-deficient mice healed at an accelerated rate and with less scarring than wild-type mice, even though collagen organization in the wounds was somehow abnormal. Therefore, despite the fact that both TSP1 and TSP2 have been characterized as anti-angiogenic proteins, they seem to have different roles in wound healing. Our findings suggest that TSP1 plays a more crucial role in the resolution phase of wound healing since its absence delays significantly this step of the process.

We observed that an intense neovascular response persisted in the wounds of TSP1-deficient mice for a longer period of time than in wild-type mice. This correlates with the well-documented role of TSP1 as an inhibitor of angiogenesis *in vitro* and *in vivo* (DiPietro et al, 1994; Dawson et al, 1997; Guo et al, 1997; Iruela-Arispe et al, 1999; Freyberg et al, 2000; Jimenez et al, 2000; Nör et al, 2000). Wounds in null mice that were treated with either TSP1, or the active peptide KRFK, presented a more physiological process of healing than control wounds. We then reasoned that the effect of TSP1 in wound healing may be mediated via TGF-β1 since there is evidence that demonstrates that TSP1 can activate TGF-β1 *in vitro* and *in vivo*, and TGF-β1 has been implicated in scarring and tissue fibrosis (Schultz-Cherry et al, 1994; Schultz-Cherry et al, 1995; Crawford et al, 1998; Murphy-Ullrich et al, 2000).

Furthermore, immunodeficient TGF-β1 knockout mice present a delay of approximately one week in each phase of wound healing (Crowe et al, 2000). We have reported that the activation sequence of TSP1 (KRFK) interacts with the latency-associated peptide (LAP) as a biologically active complex that regulates the activation of latent TGF-β1 (Ribeiro et al, 1999; Yehualaeshet et al, 1999). Therefore, binding of TSP1 to LAP may modify the interaction of LAP with the mature domain of TGF-β1, inducing a conformational change that renders the TGF-β1 active (Ribeiro et al, 1999). To determine if the expression of TGF-β1 in wounds was affected by the lack of TSP1, we measured the level of active TGF-β1 in wounds and found that it is lower in TSP1-deficient mice compared to wild-type littermates. The delayed arrival of macrophages to the wound site observed in TSP1-deficient mice may also contribute to downregulation of TGF-β1 since macrophages can secrete latent TGF-β1 that is activated locally by extracellular matrix molecules such as plasmin (Yehualaeshet et al, 1999), or tissue transglutaminases (Gosiewska et al, 1999).

To further our understanding of the role of activated TGF-β1 in wound healing, we applied topically KRFK and found that it was able to partially rescue TGF-β1 expression in the wounds to levels more similar to what is found in wounds of wild-type mice. Enhanced activation of TGF-β1 was associated with the rescue of a more physiological wound healing process in TSP1 deficient mice, as reported above. We also used a neutralizing antibody for TGF-β in conjunction with KRFK and determined that this antibody impaired KRFK's ability to rescue wild-type pattern of neovascularization in wounds made in TSP1-deficient mice. The most noticeable effect of the neutralizing antibody was observed around day 10 after injury in TSP1-deficient mice. This suggests that activated TGF-β may be particularly necessary for coordinated removal of microvessels from the wounded area during the resolution phase of wound healing.

It has been previously shown that TGF-β1 and TSP1 knockout mice have similar histological abnormalities in several organ systems and that some of the defects observed in TSP1 knockouts could be reversed by treatment with KRFK peptide (Crawford et al, 1998). In this study we showed that TSP1-deficient mice have aberrant healing of wounds, and that the application of KRFK peptides is sufficient to mediate an increase in local expression of TGF-β1 and to rescue a more physiological pattern of wound healing. Taken together, the results reported here underline the role that TSP1 exerts in the orchestration of physiological wound healing, and demonstrate that TGF-β1 is an important mediator of TSP1's function *in vivo*.

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