Attenuation of the Transforming Growth Factor β–Signaling Pathway in Chronic Venous Ulcers

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Transforming growth factor β (TGFβ) is important in inflammation, angiogenesis, reepithelialization and connective tissue regeneration during wound healing. We analyzed components of TGFβ signaling pathway in biopsies from 10 patients with nonhealing venous ulcers (VUs). Using comparative genomics of transcriptional profiles of VUs and TGFβ-treated keratinocytes, we found deregulation of TGFβ target genes in VUs. Using quantitative polymerase chain reaction (qPCR) and immunohistochemical analysis, we found suppression of TGFβRI, TGFβRII and TGFβRIII, and complete absence of phosphorylated Smad2 (pSmad2) in VU epidermis. In contrast, pSmad2 was induced in the cells of the migrating epithelial tongue of acute wounds. TGFβ-inducible transcription factors (*GADD45β*, *ATF3* and *ZFP36L1*) were suppressed in VUs. Likewise, genes suppressed by TGFβ (*FABP5*, *CSTA* and *S100A8*) were induced in nonhealing VUs. An inhibitor of Smad signaling, Smad7 was also downregulated in VUs. We conclude that TGFβ signaling is functionally blocked in VUs by downregulation of TGFβ receptors and attenuation of Smad signaling resulting in deregulation of TGFβ target genes and consequent hyperproliferation. These data suggest that application of exogenous TGFβ may not be a beneficial treatment for VUs.

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INTRODUCTION

Chronic wounds, including venous ulcers, represent a challenging clinical problem. It is estimated that, in the US, each year more than 8 million patients develop chronic nonhealing wounds, including pressure, venous and diabetic ulcers and burns (1). Although venous and arterial insufficiencies are well-known etiological factors involved in the pathogenesis of chronic wounds, little is known about the molecular events leading to chronic wounds. We previously reported that keratinocytes at the nonhealing edges of chronic wounds do not properly execute activation or differentiation pathways, resulting in a thick, hyperproliferative, hyper- and parakeratotic epidermis (2). In addition, resident fibroblasts are senescent and unresponsive to growth factors (3–6). Transforming growth factor β (TGFβ) is a pleiotropic cytokine that participates in maintenance of epidermal homeostasis. It is also known as growth-inhibitory cytokine, particularly in epithelial tissues (7). In addition, TGFβ coordinates the wound-healing response (8) and regulates reepithelialization, inflammation, granulation-tissue formation and wound contraction (6,9–11). TGFβ mediates its signaling by binding to TGFβ receptor II (TGFβRII) followed by heterodimerization and phosphorylation of TGFβ receptor I (TGFβRI) (12,13).

Activated TGFβRII binds and phosphorylates receptor-activated Smad2 or Smad3, which heterodimerize with Smad4 (14,15). This complex translocates into the nucleus, binds to Smad-binding promoter elements and regulates expression of target genes. Members of the third group, inhibitory Smad6 and Smad7, are also induced by TGFβ. They prevent phosphorylation and/or nuclear translocation of receptor-associated Smads, acting as a negative feedback loop (16,17).

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After acute injury, TGFβ1 is rapidly upregulated and secreted by keratinocytes, platelets, monocytes, fibroblasts and macrophages (18). Although TGFβ and its receptors are highly expressed in acute wounds, TGFβ1 and TGFβRII expression is reduced in chronic wounds (19–22). In addition, *in vitro* studies have revealed that VU-derived fibroblasts have reduced levels of the TGFβRII (4) and are therefore unresponsive to TGFβ1. Numerous studies have shown that exogenously applied TGFβ accelerates acute wound healing (23,24). Furthermore, application of recombinant TGFβ improves healing in animal models (25,26). However, despite these studies in animal models (23,27), treatment of human chronic ulcers with TGFβ has not met expectations, due to its limited effects (28,29).

To study the mechanism by which TGFβ signaling participates in the pathogenesis of VUs, we analyzed proteins of the TGFβ/Smad signaling cascade and target gene expression in biopsies derived from nonhealing edges of 10 patients with VUs. Using qPCR and immunohistochemistry, we found downregulation of TGFβRI, TGFβRII and TGFβRIII as well as signaling molecules pSmad2 and Smad7 in the epidermis of nonhealing VUs. Comparative genomics and qPCR revealed that expression of TGFβ target genes in VUs is the opposite from that found in TGFβ-treated keratinocytes. Thus, in nonhealing VUs, TGFβ signaling is attenuated by downregulation of all three TGFβ receptors, failed activation of Smad2 and downregulation of Smad7 resulting in deregulation of TGFβ target genes.

MATERIALS AND METHODS

Skin Specimens

Healthy skin specimens (n = 4) were obtained as discarded tissue from patients 46–72 years of age who were undergoing elective plastic surgery. Skin biopsies derived from nonhealing edges of VUs were collected from tissue normally discarded after surgical debridement procedures, per institutional review board–approved protocol. Patients (n = 10) were included after their consent for the use of their discarded skin samples was obtained and their diagnosis was confirmed (1). None of the patients had diabetes. Evaluation for ischemia was performed either by noninvasive flow exams (ankle-brachial index of <0.9) or arteriogram, and ischemia was ruled out in all patients. Patients were between 43 and 83 years of age. All patients underwent debridement in the operating room under monitored anesthesia care or general anesthesia, and local lidocaine injection was used for local anesthesia. The nonhealing wound edges used in this study were clinically identified by a surgeon as the most proximal skin edge to the ulcer bed. Skin biopsies were then processed as follows: samples were (a) embedded in OCT (optimal cutting temperature) compound (Fisher Scientific, Pittsburgh, PA, USA), (b) stored in formalin for paraffin embedding and (c) stored in RNAlater (Ambion/Applied Biosystems, Foster City, CA, USA) for subsequent RNA isolation. Samples were standardized as previously described (2). Tissue morphology was evaluated using hematoxylin and eosin staining. All specimens showed characteristic hyperproliferative, hyper- and parakeratotic epidermis and the nuclear presence of β-catenin (30).

Human Skin *ex vivo* Wound-Healing Model

Healthy skin samples were used to generate acute wounds as previously described (30–33). *Ex vivo* human experimental wound models have been extensively used to study wound healing in human skin. Moreover, comparative analyses between *ex vivo* wound models and acute human wounds confirmed similar expression patterns for multiple genes involved in epithelilization and wound healing at both protein and mRNA levels (34–37), suggesting that this model is reliable and useful in studying human epidermal healing.

Under sterile conditions, subcutaneous fat was trimmed from skin before generating wounds. A 3-mm punch (Acuderm, Fort Lauderdale, FL, USA) was used to make wounds in the epidermis through the reticular dermis, and 3-mm discs of epidermis were excised using sterile scissors. Skin discs (6 mm) with the 3-mm epidermal wound in the center were excised using a 6-mm biopsy punch (Acuderm). Specimens of wounded skin were immediately transferred to the air–liquid interface with DMEM (BioWhittaker, Walkersville, MD, USA) supplemented with antibiotics-antimycotics and fetal bovine serum (Gemimi Bio-Products, West Sacramento, CA, USA). The skin samples were incubated at 37°C in a humidified atmosphere of 5% CO_2. Tissues were fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA).

Immunohistochemistry

Frozen sections were used for staining with antibodies against TGFβRI and TGFβRII. (Abcam, Cambridge, UK). Sections were fixed with acetone, rinsed in Tris-buffered saline (TBS) plus 0.025% Triton X-100, blocked in 1% bovine serum albumin in TBS and incubated with rabbit polyclonal anti-TGFβRI (1:100) or rabbit anti-TGFβRII (1:250) antibody overnight at 4°C. Samples were rinsed in TBS plus 0.025% Triton X-100 and incubated with the secondary antibody (Alexa-Flour) for 1 h and mounted with propidum iodide mounting medium (Vector Labs, Burlingame, CA, USA). For detection of TGFβRIII, paraffin sections were dewaxed and rehydrated. Antigens were retrieved by microwaving sections in citrate buffer. Endogenous peroxidase was blocked by incubation in 1% serum supplied by the Vectastain kit (Vector Labs). The primary antibody, rabbit anti-TGFβRIII (1:100; LifeSpan Biosciences, Seattle, WA, USA) was applied at 4°C overnight. The secondary antibody was applied, and antigens were visualized by DAB (3, 3′-diaminobenzidine) according to the manufacturer's instructions (Vector Labs).

Paraffin sections were also used for staining with anti–phospho-Smad2 (Cell Signaling, Danvers, MA, USA) and anti-

Smad7 antibodies (LifeSpan Biosciences). Antigen retrieval was performed using Dako retrieval solution (Dako, Carpinteria, CA, USA), and endogenous peroxidase activity was quenched. Unspecific protein binding was blocked and antibodies were applied according to instructions in the Vectastain Universal Kit (Vector Labs). For visualization DAB (Sigma, St. Louis, MO, USA) tablets were used. Samples were counterstained with hematoxylin, dehydrated and mounted. Specimens were analyzed with a Nikon Eclipse E800 microscope. Digital images were collected using the SPOT Camera Advanced program.

Cell Culture

Normal human epidermal keratinocytes were initiated using 3T3 feeder layers for storage as described (38). The keratinocytes were grown, without feeder cells, in defined serum-free keratinocyte medium supplemented with epidermal growth factor and bovine pituitary extract (Keratinocyte-SFM; Gibco, Carlsbad, CA, USA) (31,32,39). Cells were expanded through two 1:4 passages and grown to 80% confluence after being washed with 1XPBS several times before incubation in basal keratinocyte medium (Gibco) that was custom made without phenol-red, hydrocortisone and thyroid hormone. Keratinocytes were incubated for 24 h in the presence or absence of 40 pmol/L recombinant human TGFβ1 (R&D Systems, Minneapolis, MN, USA).

Comparison of Gene-Array Data

We used the LOLA programs for comparing lists of genes (40,41). Lists of genes regulated in biopsies obtained from patients with chronic VUs (2) were compared with the list of genes regulated by TGFβ1 treatment in primary human keratinocytes (Blumenberg M and Zavadil J, unpublished data).

RNA Isolation and qPCR Analysis

Tissue was homogenized and RNA isolation and purification was performed using an miRVana RNA isolation Kit (Ambion/Applied Biosystems).

For real-time qPCR, 0.5 μg of total RNA from healthy skin and chronic wounds was reverse transcribed using an Omniscript Reverse Transcription kit (Qiagen). Real-time PCR was performed in triplicates using the Opticon2 thermal cycler and detection system and an iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). Relative expression was normalized for levels of *HPRT1*. The primer sequences used were:

HPRT1, forward (5′-AAAGGACCCC ACGAAGTGTT-3′) and reverse (5′-TCAAG GGCATATCCTACAACAA-3′); *Smad7*, forward (5′-ACTCCAGATACCCGATG-GATTT-3′) and reverse (5′-CCTCC CAGTATGCCACCAC-3′); *TGFβRI* forward (5′-ACGGCGTTACAGTGTTTCTG-3′) and reverse (5′- GCACATACAAACGGC CTATCT-3′); *TGFβRII*, forward (5′-CCAAG GGCAACCTACAGGAG-3′) and reverse (5′-GTGGAGGTGAGCAATCCCA-3′); *TGFβRIII* forward (5′-ACCTGTCAGT GCCTCCCAT-3′) and reverse (5′-GAGCA GGAACACAACAGACTT-3′); *Smad2* forward (5′-GCCATCACCACTCAA AACTGT-3′) and reverse (5′-GCCTG TTGTATCCCACTGATCTA-3′); *Smad3* forward (5′-GAACGTCAACACCAA CTGCAT-3′) and reverse (5′-ACGCA GACCTCGTCCTTCT-3′); *Smad4* forward (5′-ATGTGATCTATGCCCGTCTCT-3′) and reverse (5′-AGGTGATACAACTCG TTCGTAGT-3′); *GADD45β* forward (5′-ACAGTGGGGGTGTACGAGTC) and reverse (5′-ATGAGCGTGAAGTGG ATTTGC-3′); *ATF3* forward (5′-TCGGG GTGTCCATCACAAAAG-3′) and reverse (5′-GGCCGATGAAGGTTGAGCA-3′); *ZFP36L1* forward (5′-ACTCCAGCCG CTACAAGAC-3′) and reverse (5′-CGTAG GGGCAAAAGCCGAT-3′); *S100A8* forward (5′-TGATAAAGGGGAATTTCCAT GCC-3′) and reverse (5′-ACACTCGGTC TCTAGCAATTTCT-3′); *CSTA* forward (5′-AACCCGCCACTCCAGAAATC-3′) and reverse (5′-CACCTGCTCGTACCT TAATGTAG-3′) and *FABP5* forward (5′-ATGAAGGAGCTAGGAGTGGGA-3′) and reverse (5′-TGCACCATCTGTAAA GTTGCAG-3′). Statistical comparisons of expression levels from chronic wound versus healthy skin were performed using the Student *t* test. Statistically significant differences between VUs and healthy skin controls were defined as $P < 0.05$.

RESULTS

TGFβ Signaling Is Reduced in VUs

To analyze TGFβ signaling in the epidermis of the wound edge of VUs, we compared transcriptional profiles of biopsies obtained from VU patients (2) with profiles of primary human keratinocytes treated with TGFβ (Blumenberg M and Zavadil J, unpublished data) using the LOLA program (32,40,42). RNA isolated from VU biopsies predominantly originated from hyperproliferative epidermal keratinocytes, and the amount of dermal RNA was minimal according to expression of the mesenchymal tissue marker vimentin1 (data not shown). The genomic comparison identified an inverse correlation between the genes deregulated in VU and TGFβ-regulated genes. Specifically, 73 genes that are suppressed by TGFβ treatment were induced in VU ($P = 1.43e^{-44}$), whereas 67 genes induced by TGFβ treatment were downregulated in VUs ($P = 2.34e^{-30}$). These data suggest deregulation of TGFβ signaling in the nonhealing edge of VUs.

Expression of TGFβ Receptors Is Reduced in VUs

TGFβ plays a crucial role in overall maintenance of epidermal tissue homeostasis by delivering cytostatic signals (7), in contrast to the epidermis of the nonhealing edge of VUs, which is hyperproliferative (2,30). Thus, to assess all the components of the TGFβ pathway in nonhealing VUs, we used patient biopsies from the nonhealing edge of VUs and normal skin tissue for comparison. We analyzed the mRNA levels of *TGFβRI*, *TGFβRII* and *TGFβRIII* using qPCR. *TGFβRI* and *TGFβRIII* mRNA levels were reduced in nonhealing edges of VUs compared with steady-state levels of healthy skin. Expression levels of *TGFβRII* were not significantly altered in VUs (Figure 1A). Interestingly, immuno-

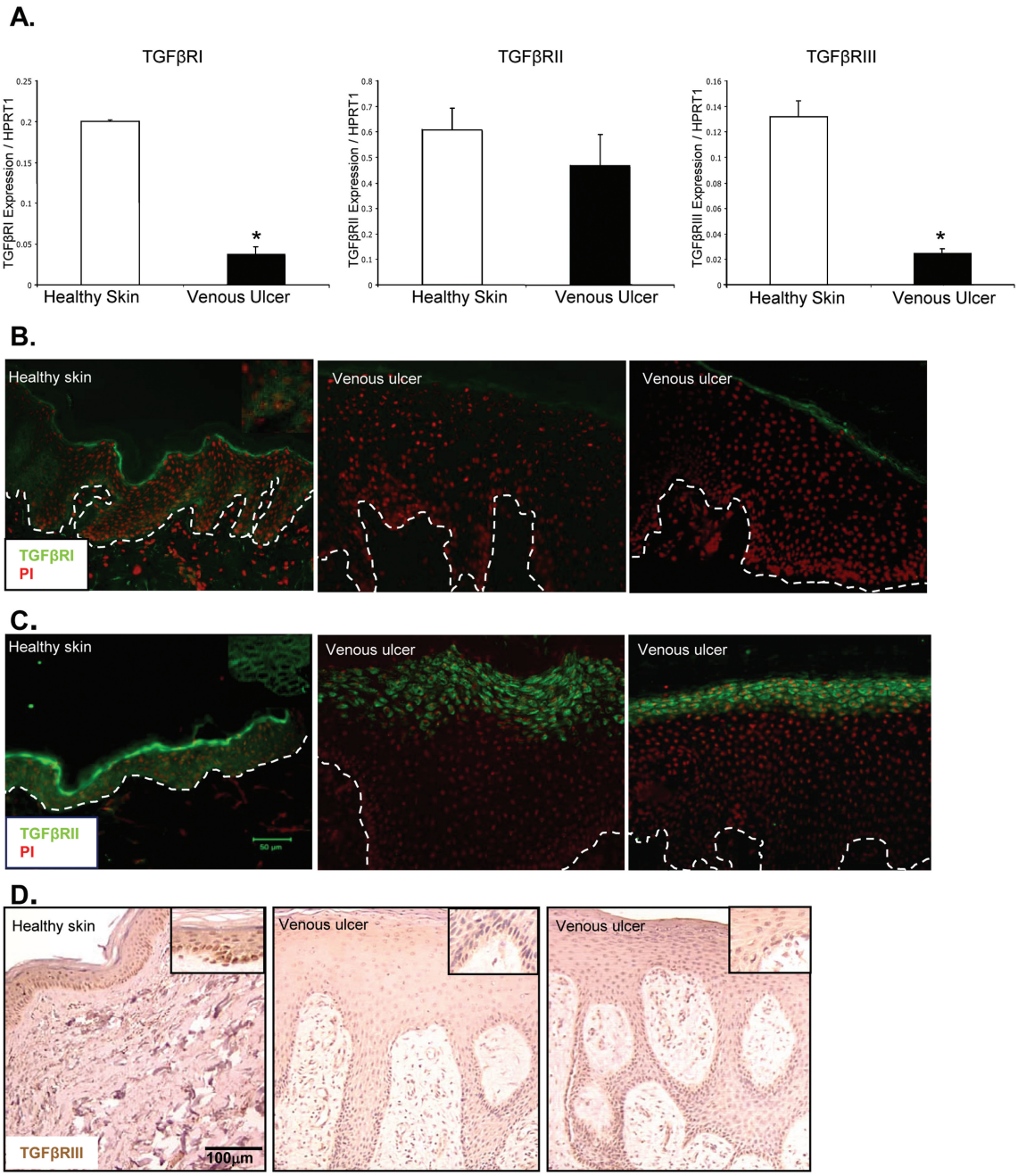

Figure 1. Deregulation of TGFβRI, TGFβRII and TGFβRIII in VUs. (A) Expression levels of *TGFβRI*, *TGFβRII* and *TGFβRIII* by qPCR. Mean values are represented after normalization to the expression level of *HPRT1*. Error bars indicate mean ± SD. *Statistically significant differences between VUs and healthy skin were defined as $P < 0.05$ (A). Immunofluorescence microscopy of healthy skin shows TGFβRI staining in the basal layer of epidermis. TGFβRI is absent in VUs (B). TGFβRII is expressed throughout the epidermis of healthy skin, but not in VUs (C). TGFβRIII is downregulated in VUs (D).

histochemistry revealed dramatic downregulation of both TGFβRI and TGFβRII in VUs compared with healthy skin (Figure 1B, C), suggesting that TGFβRII suppression occurs on a posttranscriptional level. TGFβRI was predominantly expressed in the basal layer of healthy skin, but was not detectable in any of the VUs. Similarly, TGFβRII was expressed throughout the epidermis of healthy skin, whereas it was absent in basal and suprabasal layers of hyperproliferative VU epidermis. For TGFβRIII, we detected downregulation on both the mRNA and protein level (Figure 1A, D).

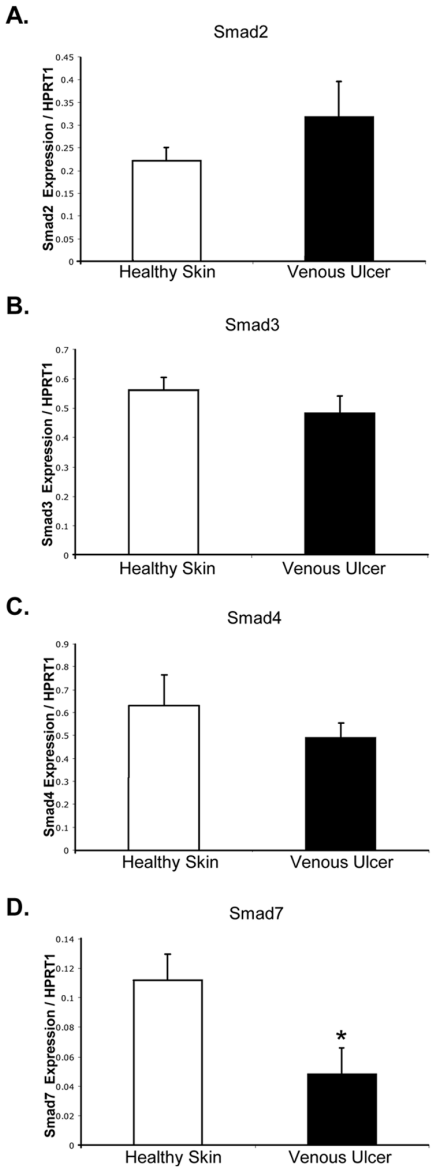

Figure 2. Expression of *Smad2*, *Smad3*, *Smad4* and *Smad7* in VUs and healthy skin. Real-time qPCR results for the expression of *Smad2* (A), *Smad3* (B), *Smad4* (C) and *Smad7* (D). Error bars indicate mean ± SD. *Statistically significant differences between VUs and healthy skin were defined as $P < 0.05$.

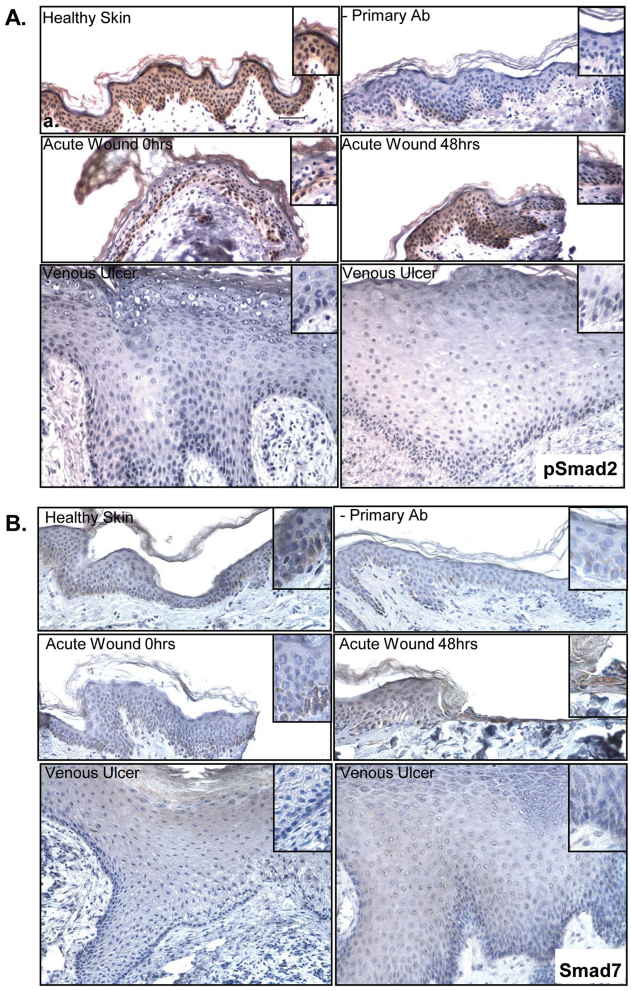

Figure 3. Phosphorylated Smad2 is absent and Smad7 does not contribute to the attenuation of TGFβ signaling in VUs. (A) Immunohistochemistry shows nuclear pSmad2 in basal keratinocytes in healthy skin, in acute wounds immediately upon wounding and in the migrating epithelial tongue 48 h after wounding. In contrast, no nuclear pSmad2 was observed in keratinocytes of VUs. (B) Smad7 is predominantly expressed in the basal layer in healthy skin and at the acute wound edge immediately after wounding. It is upregulated in migrating epithelial tongue 48 h after wounding and downregulated in VUs.

TGFβ Signaling via Smads Is Attenuated in Venous Ulcers

To further analyze expression of components of the TGFβ-signaling cascade, we determined mRNA levels for *Smad2*, *3*, *4* and inhibitory *Smad7* by qPCR in nonhealing edges of VUs (Figure 2). No significant changes in expression of *Smad2*, *3* and *4* genes were found. However, phosphorylated Smad2 (pSmad2) was absent in VUs as shown by immunohistochemistry, whereas high immunoreactivity for pSmad2 was observed in healthy skin. The levels of pSmad2 were also elevated in an *ex vivo* acute wound model. Furthermore, pSmad2 was present in the nuclei of cells comprising migrating epithelial tongue (Figure 3A). These data indicate that although mRNA levels of total Smad2 are not reduced in the edges of VUs compared with healthy skin, TGFβ signal transduction via Smad2 is impaired.

The inhibitory Smad protein Smad7 can bind to Smad complexes and inhibit Smad2 phosphorylation and nuclear translocation. Furthermore, Smad7 can cause degradation of TGFβ receptors. Therefore, we analyzed the presence of Smad7 in VUs. *Smad7* mRNA and Smad7 protein were

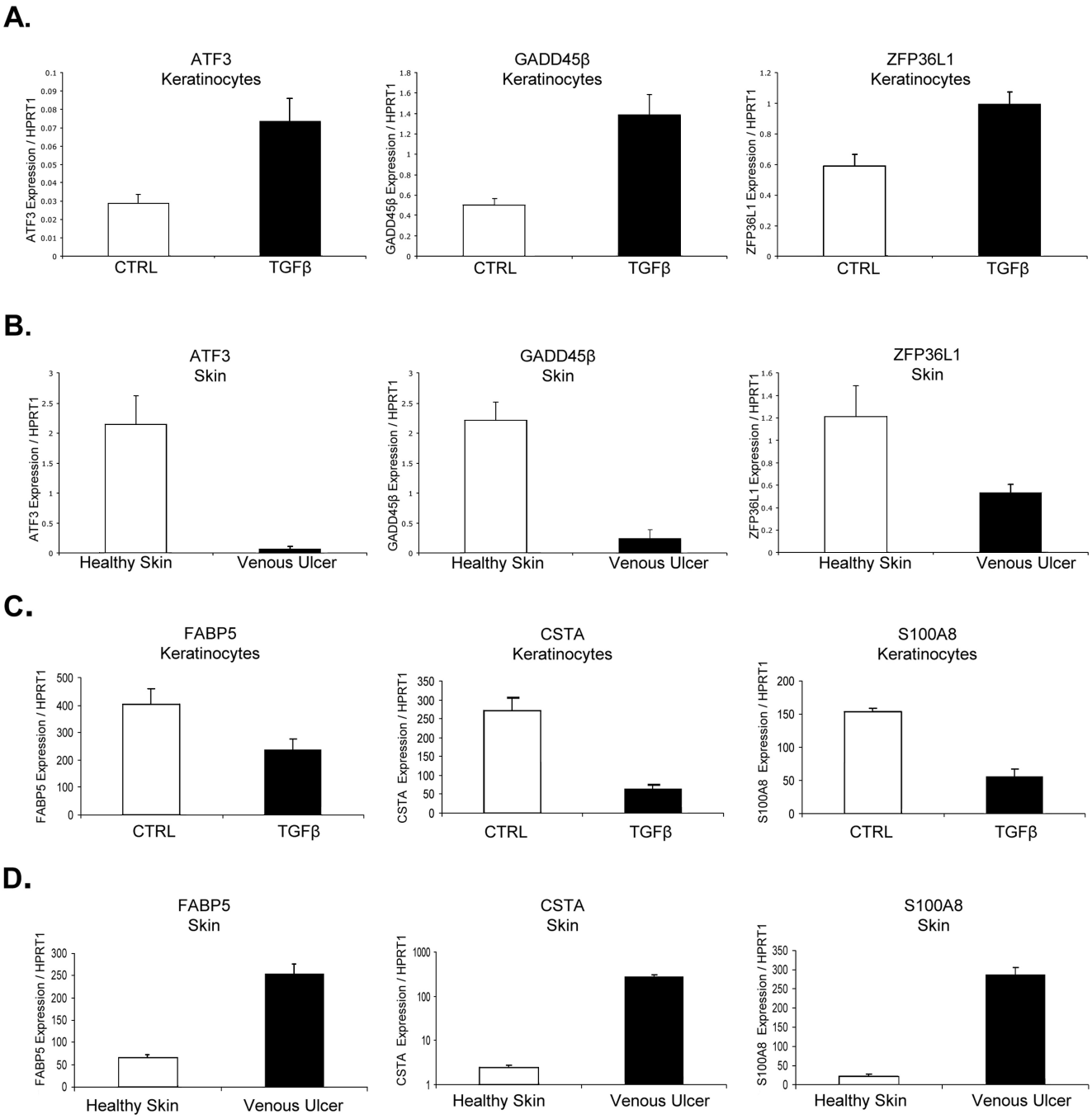

Figure 4. *ATF3, GADD45β* and *ZFP36L1* are suppressed while *FABP5, S100A8* and *CSTA* are induced in VUs. mRNA levels of *ATF3, GADD45β, ZFP36L1* (A, B) and mRNA levels of *FABP5, S100A8* and *CSTA* (C, D) in TGFβ-treated keratinocytes and VUs measured by qPCR. Error bars indicate mean ± SD.

strongly downregulated in the epidermis of all VU tested compared with healthy skin (Figure 2D, 3B). The absence of Smad7 was most prominent in the basal layer of the VU epidermis (Figure 3B). These data suggest that in VUs TGFβ signaling via Smad2 is abrogated at the receptor level, and Smad7 does not contribute to decreased TGFβ signal transduction.

Expression of TGFβ Target Genes Is Deregulated in VUs

To determine whether reduction of TGFβ signaling via the Smad signaling cascade affects expression levels of

TGFβ-dependent genes, we analyzed nonhealing edges of VUs for the expression levels of three TGFβ-inducible transcription factors and three genes that are suppressed by TGFβ (Figure 4), all found to be differentially regulated by comparative genomics. Specifically, we focused on TGFβ-inducible transcription factors, including growth arrest and DNA-damage-inducible β (GADD45β), activating transcription factor 3 (ATF3) and zinc finger protein 36L1 (ZFP36L1). GADD45β is involved in the regulation of growth and apoptosis (43). ATF3 is a member of the mammalian activation transcription factor/cAMP responsive element-binding (CREB) protein family of transcription factors and a common target of TGFβ and stress signals (44). Primary human keratinocytes treated with TGFβ for 24 h served as controls. In conformity with published data using human keratinocyte cell line HaCaT (44,45), mRNA levels of *ATF3*, *GADD45β* and *ZFP36L1* were upregulated in primary human keratinocytes after stimulation with TGFβ (Figure 4A). In contrast, the mRNA levels of *ATF3*, *GADD45β* and *ZFP36L1* were downregulated in biopsies from VUs compared with healthy skin (Figure 4B).

We next analyzed expression levels of TGFβ target genes that are found to be suppressed by TGFβ in keratinocytes: fatty acid binding protein 5 (*FABP5*), cystatin A (*CSTA*) and S100 calcium binding protein A8 (*S100A8*). FABP5 was first identified as being upregulated in psoriatic tissue (46) and has recently been described as a novel marker of human epidermal transit amplifying cells (47). CSTA (Stefin A) is one of the precursor proteins of the cornified cell envelope and plays a role in epidermal development and maintenance. Stefins have also been proposed as prognostic and diagnostic tools for cancer (48). S100A8 is upregulated in hyperproliferative and psoriatic epidermis and is a marker of inflammation. TGFβ suppresses *S100A8* expression in murine fibroblasts (49). Expression levels of all three genes were reduced after stimulation of keratinocytes with TGFβ, and mRNA levels were significantly increased in wound edges of VUs compared with healthy skin (Figure 4C, D). Specifically, *CSTA* was 110-fold higher in VUs than in healthy skin ($P < 0.01$), and *S100A8* levels were 13-fold higher in VUs than in healthy skin ($P < 0.01$). Taken together, our data demonstrate that deregulation of TGFβ signaling in nonhealing VUs occurs at multiple levels, all of which contribute to the overall pathogenesis of VUs. Deregulation is not only due to reduced TGFβ receptor expression, but also to abrogation of signal transduction via the Smad2 signaling cascade, leading to deregulation of TGFβ target genes.

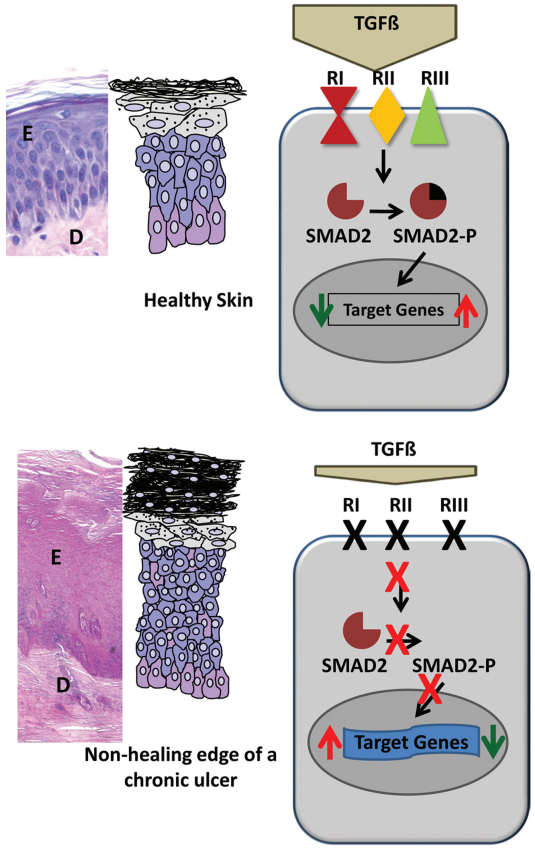

Figure 5. Attenuation of TGFβ signaling in VU. Top, histology of healthy skin. Cartoon summarizes a simplified signaling cascade in healthy epidermis. Bottom, histology of VU. In addition to decreased levels of TGFβ, downregulation of receptors followed by subsequent loss of pSmad2 leads to deregulation of TGFβ target genes and hyperproliferative epidermis of nonhealing VU.

DISCUSSION

In this study, we investigated the mechanism of deregulation of TGFβ signaling in chronic VUs. The TGFβ signaling cascade is attenuated by downregulated expression of all three major receptors, loss of Smad2 activation, and deregulation of TGFβ target genes (Figure 5).

Based on the therapeutic effect of topical application in animal models (50), TGFβ has been a promising therapeutic option for impaired wound healing. In rats and humans with impaired wound healing, overall TGFβ levels in wound fluid were shown to be diminished, and the normal elevation of TGFβ1 found during acute wound healing was absent (21,51). Furthermore, differential expression of the TGFβ receptors in acute and

chronic wounds has been described (3,19,20). However, TGFβ1 failed to get into clinical trials (29). Our data demonstrate that TGFβ signaling is further impaired by reduced expression of the TGFβ receptors in the wound edge of VU in addition to decreased TGFβ levels. This impaired signaling leads to an abrogation of Smad2 activation and deregulation of TGFβ target genes. Functional loss of the TGFβ/Smad signaling cascade in VU offers an explanation for the limited ability of exogenous applications of TGFβ to accelerate wound healing in chronic wounds.

An important study analyzing TGFβ receptors using qPCR on biopsies of human nonhealing VUs has suggested that the absence of TGFβRII contributes to the chronicity of these ulcers (20). The study also looked at TGFβRI and found high expression throughout the thickened epidermal margin of the ulcers, including the suprabasal layers, and within fibroblasts in the dermal ulcer margin (20). In contrast, we found a complete absence of TGFβRI and TGFβRII and downregulation of TGFβRIII. These differences in findings might be attributed to the wound location from which biopsies were taken. To assure that biopsies obtained indeed originated from nonhealing tissue, we used a previously established marker (nuclearization of β-catenin) that demarcates nonhealing tissue within chronic wounds (30).

Inhibition of keratinocyte mitosis is an important step in the early stages of acute wound healing, when keratinocytes are being recruited to the wound edge to migrate and properly epithelialize the wound site (52). In addition to its role during cutaneous wound healing, TGFβ signaling plays a profound role in maintaining epithelial tissue homeostasis by suppressing proliferation (7). Because of this important role we focused on the TGFβ pathway in VUs compared with healthy skin and found suppression of signaling components. Our data suggest that attenuation of TGFβ signaling in VUs could contribute to the hyperproliferative phenotype of nonhealing epidermis (2,30). TGFβ responses include repression of growth-promoting transcription factors, most notably c-myc (53,54). This finding is in agreement with our previous data that showed elevated c-myc expression and the presence of β-catenin in the nucleus at the nonhealing wound edge of VUs (30). Thus, we conclude that the lack of TGFβ signaling might contribute to c-myc overexpression and loss of cytostatic control in nonhealing VU epidermis (Figure 5). The transcription factors we found suppressed in VUs, GADD45β and ATF3 are also known to participate in TGFβ-mediated growth control in epithelial cells (44,55). ATF3 is also a stress response gene that can be activated by cellular stresses and mechanical injury (56,57). Its suppression in VUs suggests that epidermis in chronic wounds may be unresponsive to both stress and growth factor therapy.

Loss of responsiveness to TGFβ is a hallmark of many types of cancers (54,58). Although carcinomas are documented to arise at the site of chronic wounds, they are a relatively infrequent complication (59). Similar to chronic wounds, conditional knockout of TGFβRII in stratified epithelia led to infrequent and localized tumor development only in older animals (60). Strong induction of CSTA in VUs may be an additional factor in providing protection against malignant transformation in VUs, because CSTA is reduced in skin cancers (61).

The role of Smad7, an inhibitor of the Smad signaling cascade, has not yet been studied in chronic wounds. Our study demonstrated profound downregulation of Smad7 in the basal layer of the epidermis of VUs, whereas Smad7 was upregulated in keratinocytes of acute wounds *ex vivo*. The human skin organ culture model has been extensively and reliably used to study epidermal wound healing (33–37,62); however, it was never used to analyze TGFβ signaling. Showing induction of Smad2 phosphorylation and upregulation Smad7, we confirmed activation of TGFβ signaling in an acute *ex vivo* wound model. In a mouse model of acute wound healing, increased levels of Smad7 coincided with delayed reepithelialization of wounds, whereas downregulation of Smad7 accelerated reepithelialization (63). In contrast, we observed reduced expression of Smad7 in the basal epidermis of nonhealing wounds in parallel to reduced levels of pSmad2 and deregulated target gene expression. Thus, downregulation of Smad7 did not rescue TGFβ-signaling activity *via* the Smad cascade. This finding indicates that Smad7 is not responsible for the inhibition of TGFβ signaling in VUs, but instead the strong decrease in TGFβ receptor levels causes a functional knockdown of TGFβ signaling.

In summary, this study demonstrates that in the nonhealing wound edge of VUs, TGFβ signaling is attenuated by decreased expression of receptors, resulting in diminished activation of the Smad signaling cascade and subsequent deregulation of TGFβ target genes leading to loss of tissue homeostasis and hyperproliferation. Our results provide strong evidence that disruption of the TGFβ signaling cascade may be the underlying factor for failure of exogenous TGFβ to accelerate healing in chronic wounds.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that

might be perceived to influence the results and discussion reported in this paper.

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