Factor VII Deficiency Impairs Cutaneous Wound Healing in Mice

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Skin keratinocytes express tissue factor (TF) and are highly associated with skin wound healing. Although it has been demonstrated that perivascular TF expression in granulation tissue formed after dermal injury is downregulated during healing, studies of the mechanism of factor (F) VII, a TF ligand, in skin wound healing are lacking. We reported the use of a dermal punch model to demonstrate that low-expressing FVII mice (~1% of wild type (WT)) exhibited impaired skin wound healing compared with WT controls. These low-FVII mice showed defective reepithelialization and reduced inflammatory cell infiltration at wound sites. This attenuated reepithelialization was associated with diminished expression of the transcription factor early growth response 1 (Egr-1). *In vitro*, Egr-1 was shown to be essential for the FVIIa-induced regulation of keratinocyte migration and inflammation. Both Egr-1 upregulation and downstream inflammatory cytokine appearance in keratinocytes depended on FVIIa/TF/protease-activated receptor 2 (PAR-2)-induced signaling and did not require subsequent generation of FXa and thrombin. The participation of Egr-1 in FVIIa-mediated regulation of keratinocyte function was confirmed by use of Egr-1–deficient mice, wherein a significant delay in skin wound healing after injury was observed, relative to WT mice. The results from these studies demonstrate an *in vivo* mechanistic relationship between FVIIa, Egr-1 and the inflammatory response in keratinocyte function during the wound healing process.

Online address: http://www.molmed.org
doi: 10.2119/molmed.2009.00171

INTRODUCTION

Injury to the skin initiates a dynamic and complex process of wound healing that is characterized by an inflammatory phase, wherein factors are produced that induce migration of cells (for example, keratinocytes and fibroblasts); a proliferative phase, in which a series of biological steps allow for a provisional matrix to be deposited; and a final remodeling process (for example, apoptosis) that removes cells that are no longer required. These healing steps also involve an array of effectors, including coagulation cascade proteins (1) and vasoactive mediators (2). Many types of cells, including platelets (3), neutrophils (4), macrophages (5), mast cells (6), fibroblasts (7) and keratinocytes (8) are also involved in steps leading to healing of the wound. In addition, cellular processes, such as angiogenesis (9), fibroplasia and granulation tissue formation (10,11), collagen deposition (12) and epithelialization (13) contribute to the repair. Although the process of wound healing has been extensively studied, unresolved issues remain, including those surrounding the interplay between environmental and genetic factors, which allow the healing process to occur normally.

It has been demonstrated that the coagulation and fibrinolytic systems are critical to several steps of wound healing, and the outcome of this repair process is modulated by alterations of these components. With regard to the fibrinolytic system, it has been demonstrated that degradation of fibrin in the provisional wound matrix is important for timely wound healing. Thus, wound healing is delayed by factors that affect fibrin degradation and matrix resolution, for example, a plasminogen (Pg) deficiency (14) or a double deficiency of two critical activators of Pg, urokinase-type Pg activator and tissue-type Pg activator (15). Not unexpectedly, then, a deficiency in Pg activator inhibitor-1 accelerates the rate of wound healing in mice in a dermal punch model (16). A fibrinogen deficiency attenuates wound healing (17), and the mechanism of this response is multifaceted, involving a loss of fibrin in the wound field that is required for effective plasmin generation from Pg via tissue-type Pg activator, a loss of the protection of fibrin-bound plasmin from inactivation by its natural plasma inhibitor, α_2-antiplasmin, and the diminished availability of bioactive fibrin fragments in the provisional matrix. Linkages between these pathways and involvement

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Submitted November 19, 2009; Accepted for publication February 9, 2010; Epub (www.molmed.org) ahead of print February 11, 2010.

of matrix metalloproteinases also exist (17,18).

The demonstration of increased levels of thrombin and thrombomodulin in human scars (19) also links blood coagulation with wound healing. Studies on wound healing in hemophilia B mice ($FIX^{-/-}$) showed that macrophage infiltration and wound healing were delayed, potentially because of insufficient thrombin-mediated generation of fibrin degradation–based chemoattractant agents and insufficient fibrin scaffolding for reepithelialization of the wound area (20). Other studies have demonstrated that in $FXIII^{-/-}$ mice, a delay in reepithelialization of the wounded area occurs that is rescued with FXIII treatment (21). Similar observations have been made in patients with inherited FXIII deficiencies (22). Additional studies have demonstrated that perivascular tissue factor (TF) expression is downregulated in granulation tissue during the healing process, which potentially provides a method of preventing thrombosis of neovessels formed during angiogenesis (23).

Clearly, procoagulant proteins are fundamentally important to the process of wound healing, and procoagulant proteases may be major contributors to these events. Indeed, as the initiation point of the extrinsic coagulation cascade, FVII has been implicated in the process of wound healing. For example, with the use of the HaCaT keratinocyte cell line, FVIIa has been shown to induce expression of genes associated with early steps of wound healing, such as connective tissue growth factor, mitogen-activated protein kinase and epidermal growth factor (EGF) (24,25). In addition, PAR-2 has been implicated in FVIIa-induced signaling (26). However, despite many studies conducted using various cell lines, the *in vivo* significance of FVII in wound healing has yet to be demonstrated. This is probably because of the fact that mice with a complete deficiency of FVII ($FVII^{-/-}$) suffer fatal perinatal bleeding, thus precluding any studies with adult mice (27). To overcome this difficulty, we used a gene-targeting strategy to develop a novel line of mice that express very low levels of FVII ($FVII^{tTA/tTA}$) (28). In a C57Bl/6 background, $FVII^{tTA/tTA}$ mice produce less than 1% of WT FVII levels, but nevertheless survive to adulthood. This mouse line provides one of the better available tools to study the *in vivo* function of FVII in an animal model. We used these mice to study the effects of FVII deficiency and its functions, directly and indirectly, as a regulator of the inflammatory response in regulating skin wound healing after injury. The results of this study are summarized herein.

MATERIALS AND METHODS

Mice

Mice producing low levels (<1% of WT) of FVII ($FVII^{tTA/tTA}$) were as described (27). Egr-$1^{-/-}$ mice were purchased from Taconic (Hudson, NY, USA); PAR-$1^{-/-}$ and PAR-$2^{-/-}$ mice were obtained from S. Coughlin (University of California, San Francisco). Low-producing (~1% of WT) TF mice [$TF^{-/-}$hTF(tg)], which provide TF from a nontargeted single allelic transgenic (tg) insertion of the human TF cDNA, with the endogenous gene encoding mouse TF totally inactivated through breeding with $TF^{\pm}$ mice (29), were obtained from N Mackman (University of North Carolina). In all cases, we used C57Bl/6 male mice, 8–12 wks old. The local institutional animal care and use committee approved all animal protocols.

Wound-Healing Model

Mice were anesthetized with rodent cocktail (0.075 mg ketamine/0.015 mg xylazine/0.0025 mg aceprozamine per gram weight of mouse). The backs were shaved and disinfected. A full-thickness skin excision was made on the dorsal midline using an 8-mm dermal biopsy punch. Each wound was measured with a caliper every other day. Changes in the wound areas were expressed as the percentages of the initial wound areas. These measurements were made under conditions in which the investigators were blinded to the genotypes of the mice.

Histochemistry and Immunohistochemistry

Skin tissue samples were excised at d 5 after wounding, fixed in periodatelysine-paraformaldehyde fixative and embedded in paraffin. Sections (4 μm) were subjected to anti-CD45 and anti-cytokeratin immunostaining. The primary antibodies were rat antimouse CD45 (Pharmingen, San Diego, CA, USA) and mouse antihuman cytokeratin (DakoCytomation, Carpinteria, CA, USA), respectively. Secondary antibodies were biotinylated rabbit antirat IgG and biotinylated rabbit antimouse IgG, respectively. The slides were developed by using streptavidin-horseradish peroxidase, followed by diaminobenzidine, after which they were counterstained with hematoxylin QS.

Primary Keratinocyte Isolation and Stimulation Assays

Primary epidermal keratinocytes were isolated from the skins of newborn mice with the aid of a commercial kit (Cascade Biologics, Portland, OR, USA). Whole skins of the mice were dissected and treated with a splitting solution, which also contained dispase (Cascade Biologics), for 3–4 h at 4°C. The epidermis was peeled from the dermis with forceps and treated with trypsin-EDTA for 20 min at 37°C. The liberated keratinocytes were sequentially centrifuged, suspended in keratinocyte serum-free medium (Cascade Biologics), seeded onto 10-mm plates precoated with coating matrix (Cascade Biologics) and then cultured in EpiLife medium with Ca^{2+} plus keratinocyte growth supplement (HKGS-V2; Cascade Biologics). After confluence, keratinocytes comprised >95% of the total cell population. There were no variations in the protocol needed for isolating keratinocytes from the various gene-altered mice.

The isolated keratinocytes were employed in *in vitro* models by use of murine FVIIa (mFVIIa; a gift from LC Petersen, NovoNordisk, Maalov, Denmark) and/or lipopolysaccharide (LPS) (*Escherichia coli* serotype O111:B4; Sigma, St Louis, MO, USA) as stimulants. An endotoxin detection assay (QCL-1000

chromogenic limulus amoebocyte lysate endpoint assay; Lonza Group, Basel, Switzerland) was used to determine the LPS level in mFVIIa. Fondaparinux (Arixtra) was from GlaxoSmithKline (Brentford, UK) and Lepirudin (Refludan) was a product of Berlex (Montville, NJ, USA). PAR-1– and PAR-2–specific activation peptides were purchased from Bachem (Torrence, CA, USA). For these studies, keratinocytes were seeded at equal densities into 12-well plates (5×10^5 cells/well) and then starved without growth supplement for 24 h before challenge. After challenge, the cells were incubated at 37°C for 1 h for assay of Egr-1, and for 2 h for examination of macrophage inflammatory protein-2 (MIP-2) transcript levels by quantitative RT-PCR. Quantitative Q-RT-PCR was performed on the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using these specific primers and probes for Egr-1, MIP-2 and RPL19: Egr-1 (amplicon size 79 bp) forward primer 5′-CAGCGCCTTCAATCC TCAAG-3′, reverse primer 5′-CCATC GCCTTCTCATTATTCAGA-3′, probe 5′-AGCACCTGACCACAGAGTCCTTTTC TGACAT-3′; MIP-2 (amplicon size 101 bp) forward primer 5′-CAGACAGAAG TCATAGCCACTCTCA-3′, reverse primer, 5′-CCTTTGTTCAGTATCTTTTG GATGATTT-3′, probe 5′-CAGACAGAAG TCATAGCCACTCTCA-3′; RPL19 (amplicon size 233 bp) forward primer 5′-ATGTATCACAGCCTGTACCTG-3′, reverse primer 5′-TTCTTGGTCTCCTCC TCCTTG-3′, probe 5′-TTTCGTGCTT CCTTGGTCTTAGACCT-3′.

The probes were 5′ labeled with the reporter dye 6-carboxyfluorescein and 3′ labeled with the black hole fluorescence quencher-1. Reverse transcription and PCR were coupled in a single reaction using the relative quantification plate assay.

Keratinocyte Migration Assays

Measurements were carried out in Boyden chambers using 8-μm high-throughput screening membranes (BD Biosciences, San Jose, CA, USA). Primary keratinocytes were starved for 24 h before the migration assay. The bottom chamber contained EGF (20 ng/mL) in keratinocyte serum-free medium as a chemoattractant. The chambers were then incubated for 24 h. Afterward, the fluorescent dye cyquant (Invitrogen, Carlsbad, CA, USA) was used to stain the cells that migrated to the bottom chamber. The number of cells was quantified by measuring the fluorescence intensity at $\lambda_{Ex} = 485$ nm and $\lambda_{Em} = 520$ nm using a BMG FLUOstar Galaxy microplate reader (BMG Labtech, Offenburg, Germany). The emigrated cells were visualized and confirmed by fluorescence microscopy.

Thioglycollate Challenge Model

Mice were injected intraperitoneally with 1 mL of a 4% Brewer thioglycollate medium solution (Difco, Detroit, MI, USA). At various times after stimulation, the mice were euthanized by cervical dislocation. The peritoneal cavity was then exposed and the exudates collected by washing the cavity with 3 mL sterile PBS/10 mmol/L EDTA. The percentage of viable cells from the peritoneal lavages was determined in triplicate by using Guava ViaCount dye (Guava Technologies, Hayward, CA, USA). Diluted cells were incubated with Guava PCA-96 ViaCount Flex reagent (Guava Technologies) at room temperature for 10 min. Samples were measured on the Guava EasyCyte System using the Guava ViaCount application. For peritoneal cell differentials, a total of 100 peritoneal cells/sample were subjected to cytospin onto a glass slide, stained with DiffQuick and manually counted.

Statistical Analyses

All data are expressed as the mean ± SEM. The Student *t* test was used for data analysis and comparison of single pairs, with a value of $P < 0.05$ used to void the null hypothesis.

RESULTS

Low-Expressing FVII Mice Display Impaired Wound Healing Kinetics

Dermal wounds (8-mm diameter) were made in age-matched male $FVII^{tTA/tTA}$ and WT mice, and wound sizes were monitored every other d. The initial skin wounds were similar between WT and $FVII^{tTA/tTA}$ mice at d 1 post wound (Figure 1A, D). In WT mice, there was a gradual contraction of the wound (Figure 1B) and loss of the wound crust around d 12 (Figure 1C), which fully healed by d 15. $FVII^{tTA/tTA}$ mice showed significantly larger wound sizes, compared with WT mice, during the wound contraction phase (d 5 to d 11). In addition, the crust was attached to the injured skin in $FVII^{tTA/tTA}$ mice by d 12 (Figure 1E, F). However, the wound in $FVII^{tTA/tTA}$ mice eventually resolved, and the final outcome of the wound recovery (d 15) was similar between the two genotypes (Figure 1G).

Impaired Reepithelialization in Low-Expressing FVII Mice during Wound Healing

Impaired reepithelialization is a distinguishing feature of the chronic ischemic wound. The dysfunction of keratinocytes is directly associated with defective reepithelialization because of their inability to function as a mechanical support and to protect from injury (30). Keratinocytes are also actively involved in regulating inflammatory cytokines and growth factors that are critical for the healing process of the wound (31). Because reepithelialization is a crucial event during skin wound healing, keratinocyte migration in the wound areas was investigated by cytokeratin immunostaining of histological sections. Although the keratinocytes in WT mice showed significant migration at d 5 post wound (Figure 2A), the keratinocytes in $FVII^{tTA/tTA}$ mice remained adjacent to the edge of the nonwounded skin tissue at this time point (Figure 2B). Morphologically, the shape of the epidermal tongue was sharp edged in WT mice compared with blunt edged in $FVII^{tTA/tTA}$ mice. The leading edge of keratinocytes in $FVII^{tTA/tTA}$ mice was just above the zone of the proliferating epidermis, without the additional intrusion into the wound crust that was seen in WT mice. These findings suggest that FVII regu-

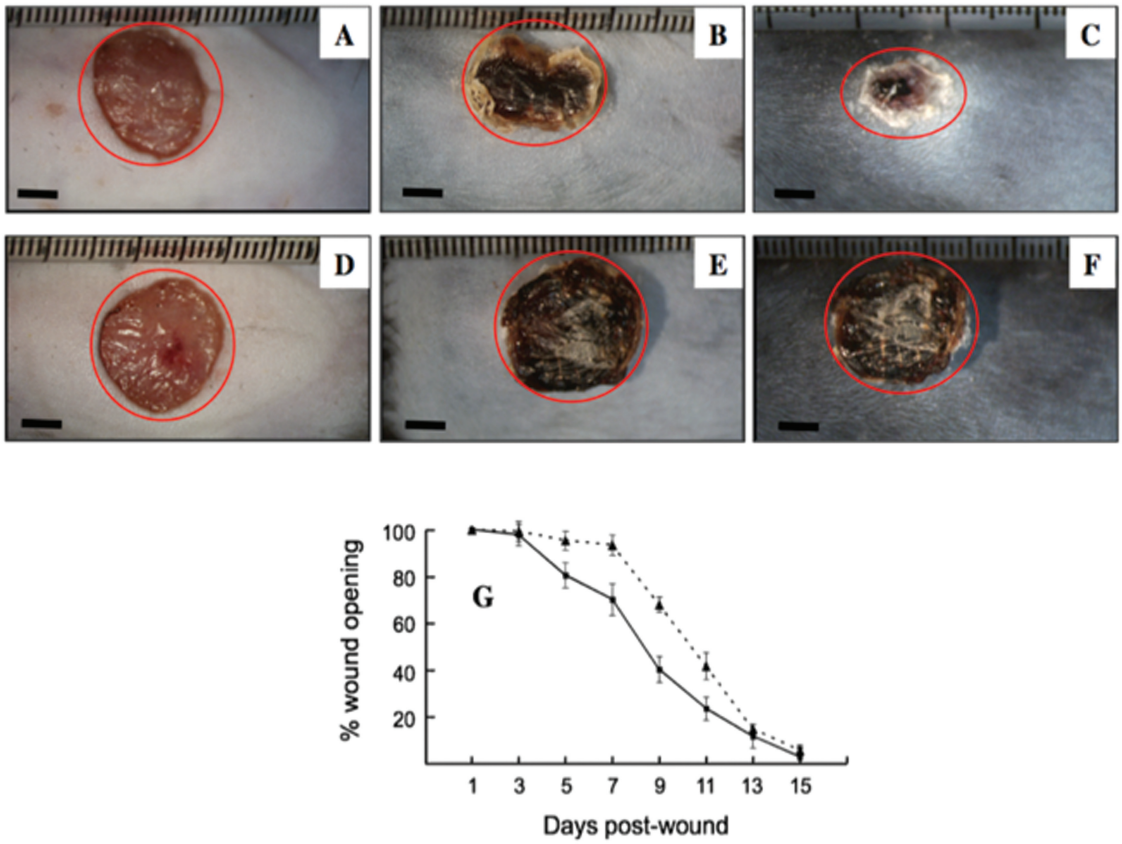

Figure 1. Time course of dermal wound healing in WT and FVII$^{tTA/tTA}$ mice. Representative post wound photographs at d 1 (A), d 9 (B) and d 12 (C) in WT mice and at d 1 (D), d 9 (E) and d 12 (F) in FVII$^{tTA/tTA}$ mice. The wound area is marked by red circle. The scale bar represents 1 cm in length. (G) The kinetics of skin wound closure in WT (solid line) and FVII$^{tTA/tTA}$ (dashed line) mice. The values represent mean ± SEM, N = 7 for WT and N = 8 for FVII$^{tTA/tTA}$ mice. *$P < 0.05$ at d 5, 7, 9 and 11 after injury.

lates keratinocyte migration and reepithelialization during wound healing.

FVII Regulates Leukocyte Migration during Wound Healing

It has been well established that inflammation is a critical phase involved in wound healing, in which bacteria and debris are removed mainly by phagocytosis via macrophages. In addition, various cytokines/chemokines are released to prepare for the cell migration and proliferation stages necessary for proper wound healing. Previous studies from our laboratory have established that FVII regulates the inflammatory response in endotoxemia (32). To determine the inflammatory state of skin wound healing between WT and FVII$^{tTA/tTA}$ mice, anti-CD45 immunostaining was performed. At d 5 in WT mouse skin, a large number of leukocytes infiltrated the entire hypodermis and upper dermal compartment of the wound (Figure 2C). On the other hand, leukocytes showed much less intense infiltration in the wounded area in the skins of FVII$^{tTA/tTA}$ mice (Figure 2D).

FVIIa Signaling Is Mediated through Egr-1 in Keratinocytes

The FVIIa/TF complex initiates the extrinsic coagulation cascade, ultimately resulting in fibrin formation as well as protease signaling. Undoubtedly, FVIIa-induced fibrin clot formation is needed to control active bleeding immediately after wounding occurs. However, the extended delay of wound healing up to d 11 post wound, as well as the aberrant keratinocyte migration and the diminished inflammation in FVII$^{tTA/tTA}$ mice, suggested that the role of FVII in wound healing could reach beyond the coagulation cascade. Therefore, the involvement of FVIIa-mediated signaling in this model of wound healing was investigated.

We examined the potential role of Egr-1 in mediating FVIIa signaling in wound healing. Such a relationship has been previously reported by our laboratory in a setting of lethal endotoxemia (32). Furthermore, Egr-1 is a critical transcriptional factor that controls a wide spectrum of gene expression, including growth factors, adhesion molecules, cytokines and proteases (33). This regulator has been shown to be an important mediator in FGF-dependent cell growth and migration (34) and has been implicated as a potential drug target in wound healing (35). Therefore, we studied Egr-1

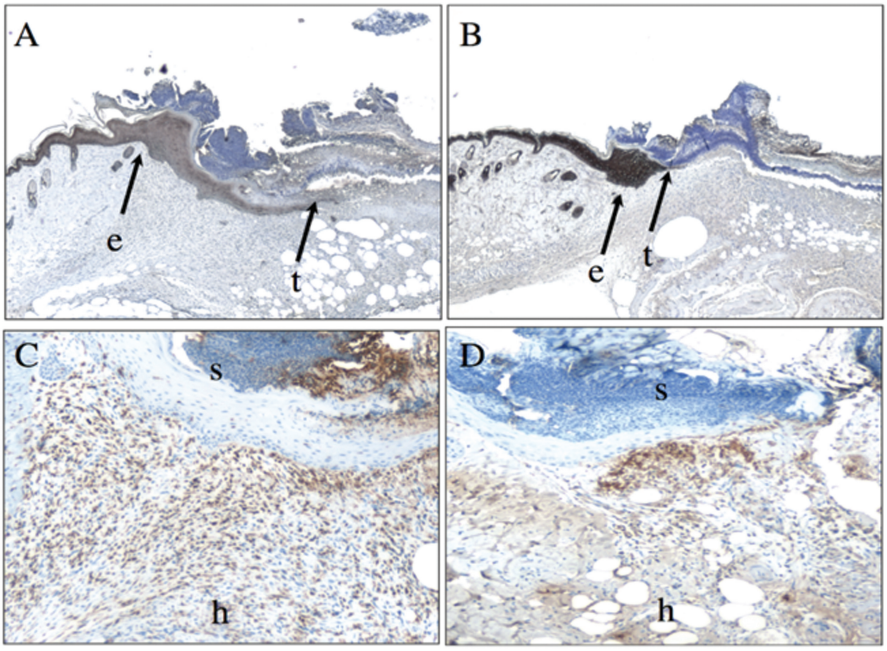

Figure 2. Low FVII impairs reepithelialization and leukocyte migration during wound healing. (A–D) Immunohistochemical stains of d 5 wounds. Representative cytokeratin stains of wounded skin at d 5 postinjury in WT (A) and FVII$^{tTA/tTA}$ (B) mice. Original magnification 40×. The arrows indicate the distance from the edge of the wound (e) to the tip (t) of the keratinocytes. Representative CD45 stains (brown) at d 5 after wound induction in WT (C) and FVII$^{tTA/tTA}$ (D) mice. The orientation is marked with the location of the scar tissue (s) and hypodermis (h). Original magnification 200×.

as a mediator of FVII downstream inflammatory signaling in the setting of wound healing, using primary keratinocytes at an early passage number (P1–P2). Indeed, primary keratinocytes challenged with mFVIIa induced a dose-dependent increase in Egr-1 mRNA levels (Figure 3A). This finding suggested that Egr-1 expression might be a downstream mediator of FVIIa-induced signaling in wound healing.

Additional studies were performed to determine whether Egr-1 was a necessary transcription factor in regulating FVIIa-induced inflammatory signaling, using Egr-1$^{-/-}$ keratinocyte, and MIP-2 expression as an end point. The physiological plasma concentration of FVII in humans is approximately 10 nmol/L. However, a supraphysiological level of FVIIa, 50 nmol/L, was used for the subsequent experiments to stimulate keratinocytes. This is mainly because of two considerations: a therapeutic dosage of FVIIa for bleeding control, as in patients with postoperative refractory bleeding or hemophilia A or B, is typically higher than physiological concentration, and the FVII expression level has been demonstrated to be upregulated during challenges, such as inflammation and malignancy (32,36). In particular, TF expression in cutaneous tissue is upregulated immediately after wounding (37). In addition, although 10 nmol/L of mFVIIa induced significant Egr-1 upregulation in the primary keratinocytes, 50 nmol/L of mFVIIa treatment elicited a more robust response (Figure 3A).

WT and Egr-1$^{-/-}$ mouse keratinocytes were treated with mFVIIa ± LPS. Treatments with mFVIIa resulted in an increase of MIP-2 mRNA levels in WT keratinocytes, but only marginally so in Egr-1$^{-/-}$ keratinocytes (Figure 3B). This finding suggests that Egr-1 is a key transcriptional mediator in FVIIa-mediated signaling in keratinocytes. LPS was used as a positive control of inflammatory responses in keratinocytes. Under conditions of LPS stimulation, increased inflammation was observed, and a similar trend of reduction in MIP-2 mRNA in Egr-1$^{-/-}$ keratinocytes was also found (Figure 3B), suggesting that Egr-1 is a general mediator involved in inflammatory responses in keratinocytes. In addition, mFVIIa, or LPS or a combination of mFVIIa and LPS treatment did not induce a statistically significant upregulation of MIP-2 in Egr-1$^{-/-}$ keratinocytes, which underscores the critical role of Egr-1 in inflammatory responses during wound healing.

FVIIa Signaling Depends on TF and Protease-Activated Receptor 2 in Keratinocytes

We also sought to determine aspects of the molecular mechanism of FVIIa-induced signaling in wound healing. Protease-activated receptor (PAR)-mediated signaling is a critical pathway induced by several coagulation proteases, which could exert a variety of biological activities in many physiological and pathological conditions. To establish the relevance of PAR-mediated signaling in keratinocytes in this study, WT keratinocytes were subjected to challenge by either PAR-1 or PAR-2 activation peptide (PAR-1/AP and PAR-2/AP). Both Egr-1 and MIP-2 expression levels were significantly increased by PAR-1/AP and PAR-2/AP, suggesting that these inflammatory mediators are among the downstream targets of PAR-1– and PAR-2–based signaling (Figure 3C, D). PAR-1/AP appears to be a more potent inducer for Egr-1 expression compared with PAR-2/AP in keratinocytes.

Next, we sought to dissect the components of the FVIIa signaling pathway in keratinocytes. To determine whether TF is necessary for FVIIa-mediated signaling events leading to Egr-1 and MIP-2 production, mFVIIa was added to keratinocytes isolated from low

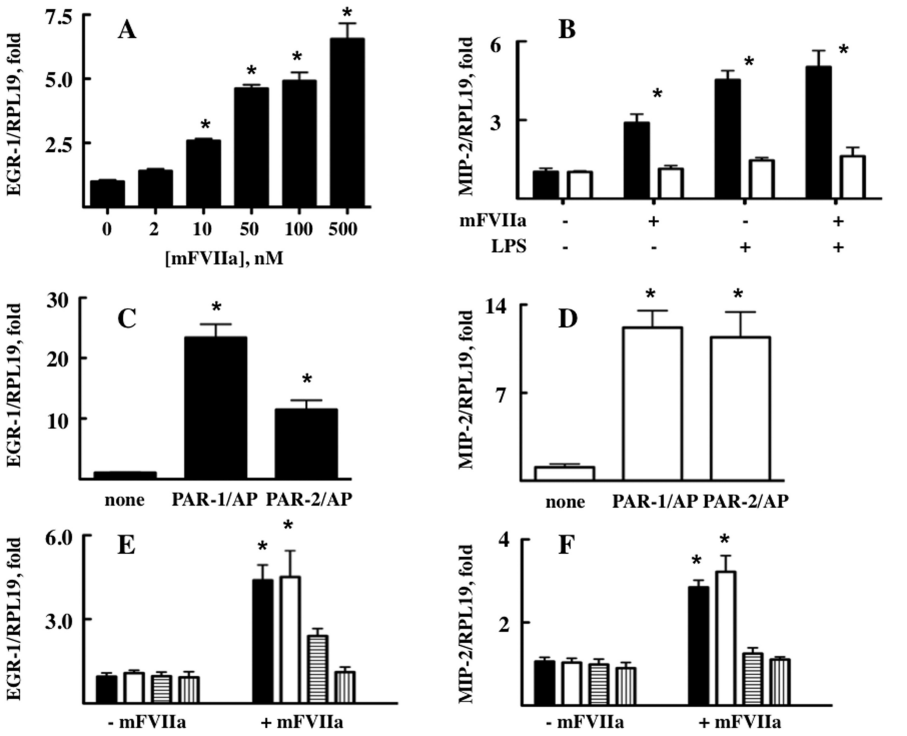

Figure 3. FVIIa signaling is mediated through Egr-1 and depends on TF and PAR-2 in keratinocytes. (A) Egr-1 mRNA levels in keratinocyte in response to addition of murine FVIIa. (B) MIP-2 mRNA levels from keratinocytes obtained from WT (filled bars) and Egr-1$^{-/-}$ (unfilled bars) mice after various combinations of mFVIIa (50 nmol/L) and LPS (10 μg/mL) were added to the culture. The Student *t* test was performed to compare WT and Egr-1$^{-/-}$ keratinocytes within each treatment group. (C–D) Egr-1 and MIP-2 are downstream targets of PARs signaling in keratinocytes. Expression levels of Egr-1 and MIP-2 are measured in WT keratinocytes before and after stimulation with either PAR-1/AP, 100 μmol/L, or PAR-2/AP, 100 μmol/L. (E, F) FVIIa-induced signaling in keratinocyte is mediated by TF and PAR-2. Expression levels of Egr-1 (E) and MIP-2 (F) in keratinocyte derived from WT (filled bars), PAR-1$^{-/-}$ (open bars), PAR-2$^{-/-}$ (horizontal striped bars) and TF$^{-/-}$hTF (tg) (vertical striped bars) mice, before (– mFVIIa) and after (+ mFVIIa) addition of 50 nmol/L mFVIIa for 2 h. *$P < 0.05$, N ≥ 3 for each genotype.

FVIIa-Induced Signaling Is Specific and Does Not Require Subsequent Generation of FXa and Thrombin in Keratinocytes

The FVIIa/TF pathway initiates the coagulation cascade, resulting in the generation of a series of activated coagulation factors, including FXa and thrombin. Both of these serine proteases may activate cell-signaling pathways through PARs. Therefore, we assessed the indirect contributions of FXa and thrombin in FVIIa-induced signaling in keratinocytes. In this regard, the roles of FXa and thrombin in FVIIa-induced signaling were dissected using fondaparinux and lepirudin, specific inhibitors of FXa and thrombin, respectively. The data (Figure 4A, B) demonstrate that neither fondaparinux nor lepirudin treatments altered the FVIIa-induced upregulation of Egr-1 or MIP-2 in keratinocytes. These data suggest that FVIIa-induced signaling is direct and specific in keratinocytes and does not require subsequent generation of FXa and thrombin.

Role of Egr-1 in Skin Wound Healing

Our data suggest that Egr-1 is a critical downstream mediator of FVIIa/TF/PAR-2 signaling during wound healing. To further examine the functional role of Egr-1 *in vivo*, skin wound–healing studies were performed in WT and Egr-1$^{-/-}$ mice. Similar to FVII$^{tTA/tTA}$ mice, Egr-1$^{-/-}$ TF-producing mice [(TF$^{-/-}$hTF(tg)]. Our data show that neither Egr-1 nor MIP-2 is upregulated in these cells (Figure 3E, F), indicating the TF dependence of FVIIa-induced signaling in keratinocytes. Similarly, when mFVIIa was added to PAR-2$^{-/-}$ keratinocytes, Egr-1 and MIP-2 mRNA levels were significantly reduced compared with WT keratinocytes (Figure 3E, F), whereas no such reduction in inflammation signals were found when PAR-1$^{-/-}$ keratinocytes were used. These results show that FVIIa inflammatory signaling in keratinocytes proceeds through the FVIIa/TF/PAR-2 axis.

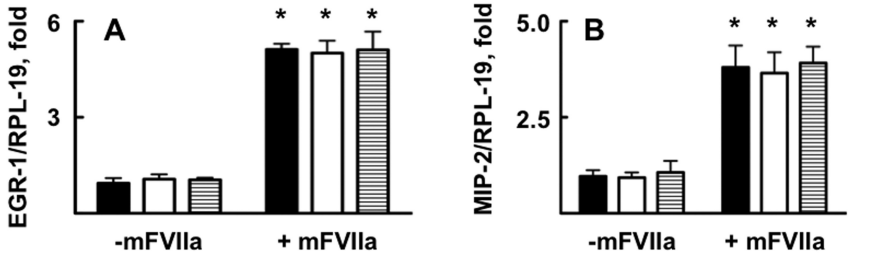

Figure 4. FVIIa signaling is specific and does not require subsequent generation of FXa and thrombin in keratinocyte. Expression levels of Egr-1 (A) and MIP-2 (B) in WT keratinocytes either without pretreatment (filled bars), or preincubated with fondaparinux (5 μg/mL, open bars), or lepirudin (4 μg/mL, horizontal striped bars) for 1 h, before (– mFVIIa) and after (+ mFVIIa) addition of 50 nmol/L mFVIIa for 2 h. *$P < 0.05$, N ≥ 3 for each genotype.

mice displayed a pronounced delay in wound healing from d 5 through d 15 post wound, compared with WT mice (Figure 5A), confirming the critical role of Egr-1 in wound healing. Furthermore, Egr-1$^{-/-}$ and WT keratinocyte migration results indicated that Egr-1$^{-/-}$ keratinocytes migrated toward this chemoattractant to a significantly lesser extent compared with WT keratinocytes (Figure 5B). In addition, because FVIIa influences Egr-1 mRNA levels, we examined whether FVIIa plays a role in regulating inflammatory cell migration via effects on Egr-1 by using a peritoneal thioglycollate challenge model in WT and Egr-1$^{-/-}$ mice. The results (Figure 5C) indicate that Egr-1$^{-/-}$ mice had fewer total leukocytes in the peritoneal lavage fluid compared with WT mice at 6, 24, 48, 72 and 96 h after thioglycollate challenge. Differential cell counts demonstrated that neutrophil migration was the prominent cell type, which diminished early during thioglycollate challenge (Figure 5D), whereas macrophage migration was greatly diminished late during challenge, in Egr-1$^{-/-}$ mice (Figure 5E). Taken together, these data suggest that as one of the downstream mediators of FVIIa/TF/PAR-2 signaling, Egr-1 plays a crucial role in the regulation of wound healing.

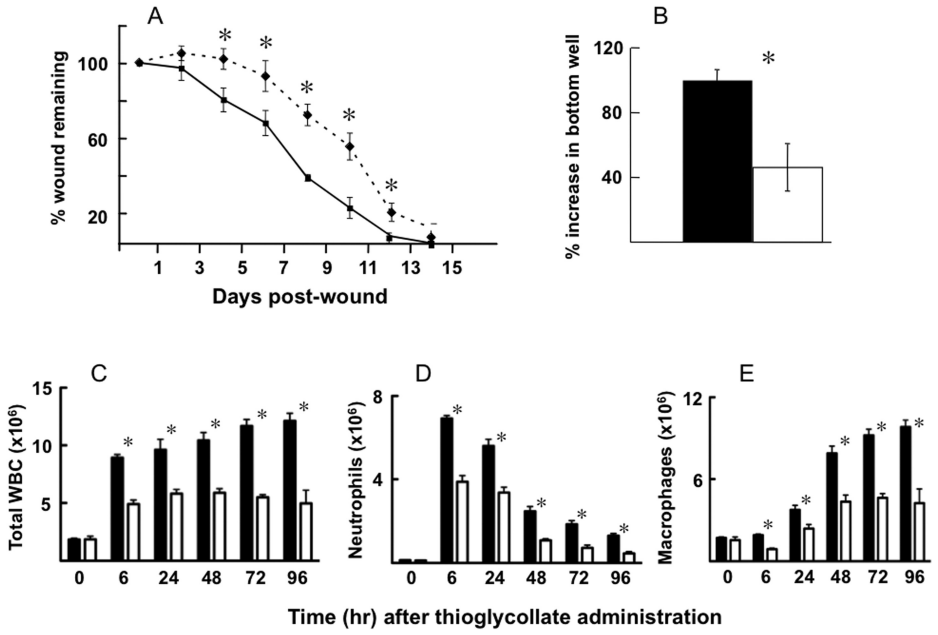

Figure 5. Egr-1 is a critical mediator in wound healing and cell migration. (A) Kinetics of skin wound healing in WT (solid line) and Egr-1$^{-/-}$ (dashed line) mice. The values represent the mean ± SEM (N = 4 for WT and N = 6 for Egr-1$^{-/-}$ mice). *P < 0.05 at d 5, 7, 9, 11, 13 and 15. (B) Primary keratinocytes (top chamber) from Egr-1$^{-/-}$ mice (unfilled bars) showed significantly reduced migration toward EGF (20 ng/mL in the bottom chamber) compared with WT keratinocytes (filled bars). (C, D) Inflammatory cell recruitment in WT (filled bars) and Egr-1$^{-/-}$ mice (unfilled bars) after thioglycollate challenge. (C) Total peritoneal leukocytes after thioglycollate challenge from WT and Egr-1$^{-/-}$ mice. Differential counts of peritoneal neutrophils (D) and macrophages (E). *P < 0.05, N = 3–8.

DISCUSSION

Healing of cutaneous wounds is an ordered process that requires a network of repair mechanisms. The initial immune responses to injured skin result in a proinflammatory state that is critical for inhibiting local infection and clearing the debris at the wound site. Coagulation factors are also critical during early stages of wound healing to stem the bleeding and to prevent additional blood loss after injury to the vessels. Thus, in response to inflammation, induction of TF, followed by formation of a provisional fibrin matrix and growth of new smooth muscle actin-positive vessels, are essential to the initial phases of repair. In addition, many of the serine proteases, for example, thrombin, downstream of FVIIa in the coagulation cascade elicit intracellular signaling, which regulates gene transcripts that are involved in wound healing (38). For example, administration of a 23-residue peptide derived from thrombin has been shown to accelerate wound repair (39), and enhancement of TF expression via gene transfer in wounds in diabetic mice restored the delayed wound closure times found in these mice to those of nondiabetic mice (37).

FVIIa initiates inflammatory cellular events that are important mechanisms for wound repair. The *in vivo* significance of FVIIa/TF signaling in inflammation has been demonstrated in disease models, for example, endotoxemia (32,40). Thus, the availability of low-FVII expressing mice provided us an excellent tool to investigate the role of FVII during wound healing, and it was found that delayed wound healing kinetics were significantly different between days 5–12 post wound in WT and FVII$^{tTA/tTA}$ mice. The histological findings at day 5 demonstrated that inflammatory cell infiltration was reduced in FVII$^{tTA/tTA}$ mice compared with WT mice. The attenuated inflammatory state of FVII$^{tTA/tTA}$ mice could result in insufficient clearing of the cell debris, as well as downregulation of target gene expression, which leads to insufficient fibroblast/keratinocyte migration and delayed closure of the wound. Taken together, these data suggest that FVII plays an important role in regulating inflammation via leukocyte migration and infiltration to the site of injury during wound healing.

It must be considered that FVII, as the initiator of the extrinsic coagulation cascade, may execute its regulatory function in wound healing through the activation of downstream coagulation factors, mainly FXa and thrombin. Indeed, FVIIa-initiated fibrin clot forma-

tion is critical to control active bleeding immediately after wounding occurs and FXa, thrombin and fibrin have been shown to affect wound healing through a number of mechanisms, for example, as chemotactic and mitogenic factors for leukocytes (41–43). However, several considerations encouraged an exploration of other mechanisms to identify roles of FVIIa, independent of thrombin generation, in regulation of wound healing: (a) restoring thrombin generation in hemophilia B did not normalize wound healing to the level of WT mice (44); (b) the response of FVIIa treatment in keratinocytes is abrogated by TF-neutralizing antibody, but is not affected by hirudin (26); (c) FVIIa signaling, independent of coagulation, affects smooth muscle cell migration and proliferation (45), as well as transcriptional responses in keratinocytes (26); and (d) FVIIa-mediated inflammatory signaling, independent of thrombin and FXa, has been shown to occur in the response of mice to lethal endotoxemia (32). In addition, the initial observations in this study, for example, the extended delay of wound healing up to day 11 post wound, the reduced keratinocyte migration, and the diminished inflammation in $FVII^{tTA/tTA}$ mice, suggest that the role of FVII in wound healing extends beyond the coagulation cascade. Therefore, we centered our focus on the investigation of direct FVIIa signaling in wound healing.

Of note, TF is expressed in keratinocytes under physiological conditions (46), suggesting an environment in which FVIIa/TF signaling might be functioning. Indeed, a previous study showed that stimulating a human HaCa-T keratinocyte cell line with FVIIa induced gene transcripts with a pattern very similar to those found in a wound-type response (26). To investigate the mechanism of FVIIa signaling in a context resembling *in vivo* conditions, very low passage primary keratinocytes were used in this study to examine FVIIa-based signaling and its consequences in regulating inflammation and migration.

Egr-1 is an important transcriptional factor that controls expression of a variety of genes relevant to wound healing, such as growth factors, adhesion molecules, cytokines and proteases (33). Egr-1 has been implicated as a potential drug target in wound healing (35), and delivery of Egr-1 expression was shown to accelerate wound healing (47). Furthermore, previous studies from our laboratory have established links between FVIIa and Egr-1 in the setting of lethal endotoxemia in mice (32). Thus, we sought to study Egr-1 as a mediator of FVII downstream inflammatory signaling in wound healing. Primary keratinocytes challenged with mFVIIa induced an increase of Egr-1 transcripts, suggesting that FVIIa-induced signaling involves the regulation of Egr-1. The possibility that the induction of Egr-1 by FVIIa is due to an LPS contamination in FVIIa was excluded by assays that demonstrated undetectable levels of LPS in the proteins that were employed. Because Egr-1 has been shown to be a central transcription factor leading to inflammation, we tested the hypothesis that FVIIa-induced inflammation depends on Egr-1. When Egr-1–deficient keratinocytes were challenged with FVIIa, decreased MIP-2 levels were detected compared with WT keratinocytes. These results indicate that Egr-1 is a key downstream mediator involved in FVIIa-induced signaling.

It has been shown that FVIIa/TF induced enhancement of a series of genes related to wound response *in vitro* in a human keratinocyte cell line (26). Evidence for this relationship has been strengthened *in vivo* in the mouse wound-healing model reported here. In addition, FVIIa-mediated inflammatory signaling via PAR-2 has been proposed to occur in a breast carcinoma cell line (48,49). In the current study, the cellular mechanism of FVIIa-mediated inflammatory signaling in keratinocytes was investigated using keratinocytes isolated from mice with gene deficiencies that have been implicated in FVIIa-induced cellular signaling. This approach led to the discovery that keratinocytes employ the FVIIa/TF/PAR-2 system to enhance production of inflammatory cytokines, such as MIP-2, that are dependent on upregulation of the transcription factor, Egr-1.

Notably, our data suggest that PAR-2 is an important, but not an exclusive, mediator for FVIIa/TF signaling, particularly in the case of Egr-1 expression. Indeed, it has been suggested that the TF cytoplasmic domain may be phosphorylated at Ser253 and Ser258 (50). Consequently, this may regulate cell signaling and many cellular events, such as NF-κB activation, proinflammatory cytokine production, leukocyte recruitment and reactive oxygen species production in monocytes (51,52). Furthermore, our data demonstrate that FVIIa-induced signaling in keratinocytes is direct and specific, and independent of subsequent generation of FXa and thrombin. More surprisingly, it has been demonstrated that perivascular TF near the wound was absent 1 day after cutaneous wounding, and returned only by day 8. Neither endothelial cells nor granulation tissue to repair wounds showed TF expression (23,53). The mechanism associated with this observation is not well understood, but it has been speculated that it may serve to prevent thrombosis near newly wounded vasculature, thereby providing an adequate blood supply to granulation tissue for wound repair. Nevertheless, it highlights the complexity of wound healing, and suggests that the function of FVIIa/TF during wound healing occurs in a tissue- and cell type-specific manner.

Lastly, we sought to determine the functional consequences of Egr-1–mediated FVIIa/TF/PAR-2 signaling in wound healing. Because Egr-1–deficient keratinocytes display a diminished inflammatory response compared with WT keratinocytes, the migratory properties of these cells were investigated as the likely consequence of inflammation. The results indicated that Egr-1–deficient keratinocytes migrated toward EGF at a reduced rate compared with WT keratinocytes. Furthermore, Egr-1 plays an important role in regulating inflammatory cell migra-

tion, because Egr-1$^{-/-}$ mice had fewer total leukocytes in the peritoneal lavage fluid compared with WT mice after thioglycollate challenge. To further investigate the *in vivo* relevance of this finding, we demonstrated that Egr-1–deficient mice showed impaired wound healing kinetics in a dermal punch model, which is consistent with a recent observation by Wu *et al.* (54). Furthermore, our data suggested that Egr-1 is a critical regulator of keratinocyte and inflammatory cell migration, whereas Wu *et al.* demonstrated similar findings in fibroblast. Taken together, these results indicate that Egr-1 is an essential transcription factor in regulating multiple cell types and events of wound healing. Interestingly, Egr-1$^{-/-}$ mice show an even more pronounced delay of wound healing compared with FVII$^{tTA/tTA}$ mice. This finding suggests that Egr-1 is a general transcriptional factor involved in the regulation by signaling molecules in addition to FVIIa, which also contribute to wound healing. In addition, it underscores the critical role of Egr-1 in wound healing.

In conclusion, the current study demonstrated that a deficiency in FVII impairs skin wound healing. The mechanism involved is associated with delayed keratinocyte migration and less pronounced inflammation, similar to that observed in FIX$^{-/-}$ hemophiliac mice. The diminished, but not complete, abrogation of wound healing in coagulation-deficient mice implicates other pathways in regulating this process. Furthermore, we demonstrate that Egr-1 is a key transcriptional factor regulating FVIIa/TF/PAR-2–induced inflammatory signaling and contributes to the healing process of cutaneous wounds.

ACKNOWLEDGMENTS

We acknowledge the work of the animal husbandry core of the Freimann Life Sciences Center in the breeding and care of the needed mice, J Fu for genotyping, D Donahue for assistance with the mouse surgeries and A Carmony for cell culturing. This work was supported by NIH grants HL019982 and HL073750.

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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