Pigment epithelium-derived factor as a multifunctional regulator of wound healing

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Wound healing is an orchestrated physiological event consisting of several distinct yet overlapping phases composed of complex processes that return the affected tissue to homeostasis (28) (summary, Fig. 8A). During wound repair, the vascular network sprouts and regresses in a reproducible temporal pattern (62). Following a burst of hypoxia-driven angiogenesis during the proliferative phase of healing, neovessels that are immature and leaky (8) are systemically targeted for removal in the remodeling phase (62), whereas blood vessels essential for tissue homeostasis are preserved and reinforced by the recruitment of mural cells, e.g., vascular smooth muscle cells and pericytes (32, 40). While angiogenesis is ongoing, fibroblasts (FBs) mediate the synthesis and reconstruction of the extracellular matrix (ECM) in the wound bed, and the two processes have reciprocal regulation (27, 31, 44, 52). The resolution of healing is thus characterized by both capillary regression and ECM maturation via collagen remodeling. Whereas proliferative mechanisms that stimulate angiogenesis and synthesis of the provisional ECM are now well defined (2, 46), the mechanisms that promote blood vessel regression and ECM maturation are poorly understood (62).

Skin wounds represent a predictable model of resolution that returns affected tissue to homeostasis, an event that is absent in many pathologies. Cancer has been described as an overhealing wound that is stalled in a proangiogenic, proliferative phase with no transition to vessel regression nor ECM maturation as occur naturally in wounds (20, 41, 51). Aberrant angiogenesis has also been described in the pathogenesis of cardiovascular diseases (48). Elucidating the mechanisms regulating wound resolution can thus provide insights into the understanding and treatment of various complex human diseases.

Pigment epithelium-derived factor (PEDF), also known as early population doubling level cDNA-1 and encoded by the SERPINF1 gene, is a 50-kDa glycoprotein and member of the noninhibitory serpin family (11, 34). Although initially described as a neurotrophic and neurodifferentiation factor (57), PEDF is now best recognized as one of the most potent endogenous angiogenic agents (17). PEDF has been shown to inhibit malignancy in a wide range of tumors, either by directly causing cancer cell death or by targeting the tumors’ abnormal vasculature (5, 12, 21). PEDF has also been found to have a protective role in atherosclerosis and is being considered as a therapeutic target in cardiovascular disease (48). PEDF is expressed in many mammalian tissues (21, 58) and exists at high levels in unwounded human dermis (23, 38) and in the blood (42). While various tumors (58) and ischemic hearts (49) express relatively low levels of PEDF, a recent study showed that high systemic levels of PEDF may be etiologic for impaired healing in diabetics due to excessive angiogenesis (45). PEDF is produced by quiescent FBs (15, 44) in normoxic conditions (49) and by keratinocytes (KCs)

NEW & NOTEWORTHY

Our study identified and characterized pigment epithelium-derived factor as a key multifunctional player involved in blood vessel network stabilization and ECM maturation in resolving skin wounds. We show that pigment epithelium-derived factor promotes blood vessel regression and matures the vascular microenvironment, thereby promoting dermal homeostasis following injury.

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(14), and it is readily secreted into the ECM. Analyses of its amino acid sequence (34) and its crystal structure (54) have revealed distinct binding sites for ECM components collagen-1 (35, 39) and glycosaminoglycans (3), including heparin (59, 66) and hyaluronan (6). Binding of PEDF to collagen-1 promotes antiangiogenesis directly (30) or indirectly by disrupting ECM-cell adhesion interactions that are crucial for angiogenesis (66). These studies suggest that the tissue microenvironment, including oxygen levels and ECM composition, influences PEDF expression and function. Intriguingly, PEDF targets immature neovessels while sparing perfused vasculature (17). The aggregate evidence of PEDF’s functions supports its recent classification as matricellular tissue homeostatic agent (11).

We hypothesized that PEDF exists at the axis of blood vessel and ECM regulation in resolving skin wounds. In this study, we used a reproducible model of excisional dermal wound repair in the mouse to evaluate PEDF’s contribution to the physiological regulation of angiogenesis and ECM remodeling via descriptive, loss-of-function, and gain-of-function experiments. Our results demonstrate that PEDF is a key endogenous factor in healing wounds that is produced by resident FBs and accumulates in the dermal ECM and epidermis. Furthermore, we show that PEDF controls blood vessel regression, microvascular integrity, and ECM maturation. These multiple functions of PEDF contribute to the resolution of the wound healing process, the return to and maintenance of dermal tissue homeostasis.

MATERIALS AND METHODS

Animals and Wound Model

BALB/c-strain 6-to-8 wk-old female mice (Harlan, Indianapolis, IN) were anesthetized by intraperitoneal injection of 100 mg/kg ketamine and 5 mg/kg xylazine solution, and their dorsal skin was shaved and cleansed with 70% isopropyl alcohol. Six or four excisional full-thickness dermal wounds (depending on experiment) were shaved and cleansed with 70% isopropyl alcohol. Six or four excisional full-thickness dermal wounds (depending on experiment) were made on the dorsal surface of each mouse, symmetrically on both sides of the midline, using a sterile 3-mm punch-biopsy instrument (Acu Punch, Acuderm, Ft. Lauderdale, FL). Standard aseptic techniques were followed. The excised skin during wounding was used as normal, unwounded skin control.

At different time points postinjury (depending on the experiment), animals were euthanized and the wounds harvested. For real-time RT-PCR analyses, samples were placed in RNAlater (Sigma, St. Louis, MO) and stored at −20°C. For ELISA protein analyses, wound samples were snap frozen and stored at −80°C. For immunofluorescent histochemical analyses, wound samples were embedded in HistotPrep compound (Fisher Scientific, Waltham, MA), snap frozen, and stored at −80°C. To account for contraction of murine excisional wounds and standardize the amount of unwounded tissue surrounding each excised wound sample, 5-mm punch-biopsy instruments were used for the collection of samples until the day 5 time point, and 3-mm punch-biopsy instruments were used for wound harvesting during later time points. To identify the wound area during later time points (i.e., after day 20 postinjury), the presence of a scar, often characterized by the lack of hair or unusual pattern of hair regrowth, was observed; additionally, photographs taken of the animals throughout the course of the experiments aided in the tracking of wound locations.

Mice were housed in groups of five at 22 to 24°C on a 12-h:12-h light/dark cycle; food and water were provided ad libitum. Animal protocols used in these studies were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Illinois at Chicago. All animal procedures were conducted in accordance with the “Guide for the Care and Use of Laboratory Animals” (National Institutes of Health).

Culture of Human KCs and FBs

Normal human epidermal KCs (NHKCs) (ATCC, Manassas, VA) were cultured in dermal cell basal medium and keratinocyte growth kit (ATCC). Normal human FBs (NHFBs) (PromoCell, Heidelberg, Germany) were cultured in DMEM with 10% FCS. Cell cultures were grown in six-well plates to 70–80% confluency and harvested using TRIzol (Invitrogen, Carlsbad, CA) for total RNA extraction.

Purification of Human Recombinant PEDF

Purification of human recombinant PEDF (rPEDF) from the medium of stable baby hamster kidney cell transfectants overexpressing and secreting the protein was performed as previously described (56). Purity of protein extracts was verified using spectrophotometry and by SDS-PAGE followed by Coomassie blue staining and immunoblotting using anti-PEDF antibody (BioProducts MD, Middletown, MD) and commercial rPEDF as positive control (BioProducts MD). Purified rPEDF was further tested for biological activity in vitro and was found to induce apoptosis in cultured human microvascular endothelial cells (ECs) (data not shown). Recent work from our laboratory used the same batch of purified rPEDF to assess its effects on human KCs, further validating rPEDF’s biological activity (14).

Amino acid alignment analysis using basic local alignment search tool (BLAST) was performed to assess similarity between human and mouse PEDF orthologs; results are as follows: 87% identity, 94% similarity, 0% gaps in alignment, and 0.0 E value. The extremely high degree of similarity between PEDF orthologs ensures the reliability of using human PEDF in mouse studies.

Treatment of Wounds with Recombinant Proteins and Antibodies

Treatment of healing dermal wounds was performed while animals were under anesthesia via isoflurane inhalation using a SurgiVet isoflurane vaporizer and oxygen mixing apparatus (Smiths Medical, Dublin, OH). Mice were randomly selected into experimental and control groups before treatment. Photographs were taken of each mouse on a daily basis for tracking of wounds and measurements of wound closure.

Treatment of wounds with rPEDF. Purified rPEDF was applied to wounds daily following injury at a dose of 2 µg per wound, at a time course and concentration determined most effective in preliminary studies (data not shown). rPEDF was applied topically onto the open wound before scab formation, and after 3 days postinjury, rPEDF was directly administered into each wound via intradermal injection using a short 3/10-ml insulin syringe with a 30-gauge, 8-mm needle. For topical applications, rPEDF was dissolled in a controlled-release Pluronic gel made from Pluronic F-127 (Sigma) to a consistency of 25% w/vol, as previously described (63), to a concentration of 200 µg/ml (10 µl applied per wound). For intradermal injections, rPEDF was dissolved in sterile phosphate-buffered saline (PBS) to a concentration of 100 µg/ml (20 µl injected per wound); the control group was vehicle (Pluronic gel for topical; PBS for injection). Wound samples were harvested at day 10 postinjury.

Treatment of wounds with neutralizing antibody against PEDF. To inhibit endogenous PEDF, healing wounds were treated with a neutralizing antibody against human PEDF (PEDF-Ab) (BioProducts MD) (33). To keep immune reaction to a minimum, antibodies were applied 4 days apart and at a maximum dose of 0.5 µg per mouse. PEDF-Ab was administered directly into each wound via intraderal injection using a short 3/10-ml insulin syringe with a 30-gauge, 8-mm needle. PEDF-Ab was dissolved in sterile PBS to a concentration of 6.25 µg/ml (20 µl injected per wound). The control group received intradermal injections of rabbit IgG (Sigma) at the same concentra-
tion. Both antibodies did not contain sodium azide preservative. Time points for administration of antibodies were days 8, 12, 16, and 20 postwounding; wound samples were harvested at days 16, 20, and 24 postinjury. Five mice from each group were randomly selected for death and tissue harvest at days 16 and 20.

**Wound Size Measurements**

Throughout wound treatment experiments, photographs of all animals were taken from a set distance in standardized conditions and camera settings, with a ruler in the field of view as reference. Photographs were opened in Fiji image processing software (http://fiji.sc), the scale was calibrated to ruler, and sizes of wounds determined for each animal. Values were exported to Microsoft Excel; changes in wound area were expressed as percentage of original wound area and calculated as follows: (measured wound area)/(original wound area) × 100. All four wounds per animal were analyzed and the four values averaged to produce a unique value for each animal.

**Total RNA Extraction and Real-Time RT-PCR for PEDF**

Total RNA extracted using TRIzol (Invitrogen) was treated with DNase I and subjected to reverse transcription using a Retro-script kit (Invitrogen). Semiquantitative mRNA expression of PEDF was examined using a SYBR Green PCR mix and gene specific primers. The sequences of mouse PEDF (Serpinf1) and Gapdh primers were previously published (25, 65). The sequences of human PEDF (SERPINF1) and GAPDH primers were previously published (14). For in vitro comparison of NHFBs versus NHEKs, data were normalized to NHEKs. For in vivo time-course studies, data were normalized to normal, unwounded skin.

**Protein Extraction and ELISA for PEDF**

Wound samples were homogenized in 500 μL radioimmunoprecipitation assay buffer (Sigma) with a protease inhibitor cocktail (1/100 dilution; Sigma). Samples were centrifuged at 13,000 rpm at 4°C for 15 min. The resulting supernatants were collected and total protein concentrations were determined using the Pierce BCA protein assay kit according to the manufacturer’s instructions (Thermo Fisher Scientific, Rockford, IL). PEDF protein content was evaluated using the ELISAtest Antibody PEDF Sandwich ELISA kit according to the manufacturer’s instructions (BioProducts MD). PEDF content was normalized to each sample’s total protein concentration; normal-

**PEDF localization studies.** Wound sections were air dried for 10 min and rehydrated in PBS for 10 min. Sections were fixed in precooled (−20°C) acetone for 2 min, followed by 80% methanol for 5 min, washed 3 × 3 min with PBS, and blocked using normal goat serum (10% in PBS; Sigma) for 30 min. Slides were double-stained, overnight, using 1) rabbit anti-human PEDF (BioProducts MD) and 2) either rat anti-mouse CD31 (BD Pharmingen, San Diego, CA) or chicken anti-mouse vimentin (Abcam, Cambridge, MA) primary antibodies; Rabbit IgG was used as an isotype control for PEDF (Vector, Burlingame, CA). Slides were washed 3 × 5 min with PBS and incubated for 45 min using 1) Alexa Fluor 594 goat anti-rabbit, and 2) either Alexa Fluor 488 goat anti-rat or FITC-conjugated goat anti-chicken fluorescent secondary antibodies (Invitrogen). Slides were washed 3 × 5 min with PBS and mounted using 50% glycerol containing 4',6-diamidino-2-phenylindole (DAPI) for staining of cell nuclei.

**Vessel density, proliferation, and maturity studies.** Wound sections were air dried for 10 min and rehydrated in PBS for 10 min. Sections were fixed in precooled (−20°C) acetone for 10 min, washed 3 × 3 min with PBS, and blocked for 30 min using normal goat serum (Sigma). Slides were double stained using 1) rat anti-mouse CD31 antibody (BD Pharmingen) and 2) either mouse anti-mouse FITC-conjugated α-smooth muscle actin (α-SMA) antibody (Sigma) or rabbit anti-mouse Ki67 antibody (Abcam) for 1 h, washed 3 × 5 min with PBS; followed by 45 min incubation using 1) Alexa Fluor 594 goat anti-rat and 2) for Ki67 staining. Alexa Fluor 488 goat anti-rabbit secondary antibodies (Invitrogen). Slides were mounted using with 50% glycerol containing DAPI.

**Visualization and quantification.** All slides were visualized under a Carl Zeiss microscope at ×10, ×20, ×40, or ×100 magnifications, and multiphoton images were taken using Axiovision software (Carl Zeiss Microscopy, Thornwood, NY). Image files were randomized and deidentified for analysis, and a coding system was used to later identify analyzed values. Photomicrographs were evaluated and fluorescence staining was quantified using Fiji.

**Quantification of PEDF and CD31 colocalization.** Images of sections taken using different filters at magnification of ×20 were opened separately in Fiji and converted to 16 bit, and threshold was applied via the Otsu algorithm at standardized intensity level. Images from the PEDF and CD31 filters were combined to generate tricolor images showing separate colors for PEDF, CD31, and their colocalization. Pixel area of each color was quantified. The amount of wound microvasculature bound by PEDF was evaluated by measuring the percentage of CD31 colocalized with PEDF and was calculated as follows: (PEDF + CD31 colocalized area)/(CD31 area) × 100. At least three histological slides per animal and two images per slide were analyzed and averaged to produce a unique value for each animal.

**Quantification of CD31 area.** For rPEDF- or PBS-treated wounds harvested at day 10 postinjury, images of sections were taken at magnification of ×20 centered on wound bed. For PEDF antibody- or IgG-treated wounds harvested at days 16, 20, and 24 postinjury, images of sections were taken at magnification of ×10 to better identify wound margins and ensure that analysis of CD31 area be restricted to the wound bed. For both analyses, images showing CD31 fluorescence were opened side by side with DAPI, the two windows synced, and DAPI-stained image was used to select the area of wound bed simultaneously in the CD31-stained image. Threshold was applied to selected area via the Otsu algorithm at standardized intensity level. Pixel areas for total wound bed and for CD31 staining were quantified. Microvessel density was evaluated by measuring the percent CD31 area in wound bed and was calculated as follows: (CD31 area in wound bed)/(total area of wound bed) × 100. At least four histological slides per animal and two images per slide were analyzed and averaged to produce a unique value for each animal.

**Quantification of CD31 and α-SMA colocalization.** Images of sections taken using different filters at magnification of ×20 were...
opened in Fiji. Analysis was identical to that described above for the colocalization of PEDF and CD31. Histological maturity of wound microvascular networks was evaluated by measuring the percentage of CD31 colocalized with α-SMA and was calculated as follows: 
\[
(CD31 + \alpha\text{-SMA colocalized area})/[(colocalized area) + (CD31 area)] \times 100
\]
At least three histological slides per animal and two images per slide were analyzed and averaged to produce a unique value for each animal.

Quantification of EC proliferation. Images of sections taken using different filters at magnification of ×20 were opened in Fiji. Analysis was identical to that described above for the colocalization of PEDF and CD31, except individual areas of CD31 and Ki67 colocalization were counted using Fiji’s analyze particle function. The extent of EC proliferation was evaluated by counting the numbers of Ki67-positive microvessels in the wound bed. At least two histological slides per animal were analyzed and averaged to produce a unique value for each animal.

Functional Analysis of Vascular Maturity

In a separate experiment, following daily treatment of wounds with rPEDF and PBS as described above, FITC-conjugated high-molecular weight dextran (Sigma) was reconstituted in sterile PBS to a concentration of 15 mg/ml, and 200 μl were injected into the retro-orbital venous plexus of all mice 30 min before the animals were euthanized at day 10 postinjury. Harvested wound samples were snap frozen in HistoPrep, sectioned, and stained for CD31 and DAPI as described above.

Images of sections taken at magnification of ×20 were opened separately in Fiji and converted to 16 bit, and threshold was applied via the Otsu algorithm at standardized intensity level. Images from the CD31 and FITC-dextran filters were combined to generate tricolor images showing separate colors for CD31, dextran, and their colocalization. Pixel area of each color was quantified. Microvessel leakiness was evaluated by measuring extravascular dextran (not colocalized with CD31) area in the wound bed and was calculated as follows: (dextran area in wound bed)/(total area of wound bed) × 100. Functional maturity of the wound microvascular networks was further evaluated by measuring the ratio of intravascular dextran (percentage of dextran colocalized with CD31) compared with total dextran. This measurement is independent of how many vessels were actually perfused by the procedure, which is quite variable because of vasocstriction caused by smooth muscle cells surrounding mature microvessels, and it was calculated as follows: 
\[
(CD31 + dextran colocalized area)/[(colocalized area) + (dextran area)] \times 100
\]
At least two histological slides per animal were analyzed and averaged to produce a unique value for each animal.

Picrosirius Red Analysis for Collagen Maturity

Wound samples were fixed in formalin, embedded in paraffin, sectioned, and stained using Picrosirius red via standard methods to visualize collagen content and maturity. Slides were evaluated under a polarized microscope (Carl Zeiss Microscopy); photomicrographs were taken at ×10 and ×20 magnification, randomized, and analyzed in Fiji using standardized color thresholds to identify and quantify areas of mature (red-orange) and immature (green-yellow) collagen. Total collagen content in the wound bed was calculated as follows: 
\[
[(red-orange area) + (green-yellow area)]/(total area of wound bed) \times 100
\]
Percentage of mature collagen in relation to total collagen was calculated as follows: 
\[
(red-orange area)/(red-orange area) + (green-yellow area) \times 100
\]
At least three histological slides per animal and two images per slide were analyzed and averaged to produce a unique value for each animal.

Statistical Analysis

Average values for each animal were exported to GraphPad Prism 5.0 (GraphPad Software, San Diego, CA) for statistical analyses and graph creation. Data were analyzed to yield means ± SE values for each group. For analysis of experimental group versus control, a two-tailed Student’s t-test was used; for analysis of more than two groups (i.e., for multiple time points postinjury), one-way ANOVA followed by Bonferroni’s posttests was used. Statistical significance of values between groups was considered when P < 0.05.

RESULTS

Localization and Production of PEDF in Unwounded Mouse Skin

We first sought to investigate the distribution of PEDF in mouse skin. With the use of indirect immunofluorescence, positive staining for PEDF was seen in epidermis, areas of the dermis, and in hair follicles (Fig. 1A). Previous studies have shown that PEDF is produced by FBs in various mammalian tissues (44), including in the uninjured human dermis and associated appendages (23, 38). In mouse skin, PEDF colocalized with vimentin, a marker for dermal FBs (19), in spindle-shaped structures that resemble the morphology of dermal FBs (Fig. 1B, white triangles). PEDF present in the dermis that was not colocalized with vimentin appeared as patches of positive staining adjacent to dermal FBs, suggesting a considerable extracellular component of PEDF (Fig. 1B, white dotted outlines).

To determine the primary cell type responsible for PEDF production in mammalian skin, the relative PEDF transcript levels were assessed in NHEKs and NHFBs. PEDF expression in NHFBs was over 300-fold greater than in NHEKs (Fig. 2A). This finding was substantiated by mined results from publicly available microarrays that showed consistently greater expression of PEDF by human dermal FBs versus matched KCs (Fig. 2, B and C), as well as by FB, smooth muscle, and stromal cell lines versus endothelial and epithelial cell lines (Fig. 2D).

PEDF is known to interact with ECs via membrane-associated proteins in other tissues (5). In the dermis, PEDF colocalized with CD31, a marker for ECs, in elongated tubelike structures resembling capillaries (Fig. 1C, white triangles). Extracellular patches of PEDF that were not colocalized with CD31 or DAPI were again observed (Fig. 1C, white dotted outlines).

The aggregate results demonstrate that PEDF is ubiquitous in unwounded mouse skin, its spatial distribution is related to that of dermal FBs and ECs, and much of PEDF is localized in the dermal ECM in addition to the epidermis.

Distinct Pattern of PEDF Production During Excisional Skin Wound Healing

To investigate whether PEDF may play a role during skin healing, we measured levels of PEDF transcript and protein throughout the time course of excisional wound repair in mice. Analysis of our previously published transcriptome of 1-mm excisional wounds (13) demonstrated a distinct expression profile for PEDF (encoded by the Serpinf1 gene) during healing (Fig. 3A). To validate this data, we used real-time RT-PCR to examine PEDF mRNA levels in full-thickness, 3-mm wounds at 1, 3, 5, 7, 10, 14, 21 and 28 days postwounding, time points that correspond to important milestones in the healing in this model (Fig. 3A).

Unwounded skin contained significantly more PEDF mRNA than all time points postinjury (P < 0.05, Fig. 3B).
Fig. 1. Localization of pigment epithelium-derived factor (PEDF) in unwounded mouse skin in relation to dermal fibroblasts (FBs) and microvascular endothelial cells (ECs). Immunofluorescent histochemistry for PEDF in unwounded mouse skin. A: positive staining using commercial anti-PEDF antibody. Major anatomical structures in murine skin are labeled epidermis, dermis, hair follicles. Arrowheads indicate defined areas of positive PEDF staining in the dermal layer. Dashed white line indicates boundary of epidermis and dermis. Isotype control demonstrates specificity of primary antibody. Arrow indicates hair follicle. B and C: immunohistochemical staining for PEDF and FB marker vimentin (B) or EC marker CD31 (C); 4’,6-diamidino-2-phenylindole (DAPI) was used to stain for nuclei. Representative photomicrographs are shown at ×40 and ×100 magnification. Arrowheads indicate examples of PEDF + vimentin (B) or PEDF + CD31 (C) colocalization. Dotted white outlines indicate patches of PEDF localized to the dermal extracellular matrix. Isotype controls demonstrate specificity of primary antibodies. Scale bar = 50 μm.
At days 3–7 time points corresponding to the inflammatory and early proliferative phases of healing (Fig. 8A), PEDF mRNA levels were about 75% less than in unwounded skin \((P < 0.01)\) and about 50% less than in day 21 wounds \((P < 0.05)\) (Fig. 3B). Similarly, PEDF protein levels in unwounded skin were significantly higher than all time points postinjury \((P < 0.01)\), except day 28 (Fig. 3C), and showed a pattern nearly identical to the PEDF transcript (Fig. 3B). These results demonstrate a considerable decrease in PEDF expression during the early phases of healing, followed by a gradual increase to near baseline levels of PEDF during wound resolution (Fig. 8B).

**Localization of PEDF in Relation to FBs and ECs During Excisional Skin Wound Healing**

Colocalization studies were performed to investigate patterns of PEDF spatial distribution during healing. At day 7, when provisional ECM dominates the wound bed (Fig. 8A), PEDF and vimentin staining were relatively weak and scattered, and the spatial pattern of PEDF was similar to that of vimentin (Fig. 4A, top row). At day 14, the extracellular staining for PEDF was more intense in the proliferating wound dermis (Fig. 4A, middle row). By day 28, the remodeling phase of healing (Fig. 8A), robust staining showed significant colo-
nascent vasculature (Fig. 4B, top row). PEDF staining was also diffuse, with minimal areas of colocalization with CD31 (Fig. 4B, top row). At day 14, a time point at the transition from angiogenesis to vessel regression (Figs. 8A and 4C), PEDF was distributed throughout the wound ECM, and colocalization between PEDF and CD31 remained sparse (Fig. 4B, middle row). At day 28, a time point well into the vessel regression phase of healing (Figs. 8A and 4C), CD31 and PEDF were more defined and showed robust colocalization (Fig. 4B, bottom row), a situation that is nearly identical to unwounded skin (Fig. 1B).

PEDF and CD31 colocalization was quantified over the time course of healing. Quantification revealed that nearly 70% of CD31 is colocalized with PEDF in unwounded skin and at day 28 (Fig. 4, D and E). Colocalization of CD31 and PEDF dropped to around 25% on days 7–14 (P < 0.01 vs. unwounded skin and day 28), rose to 50% at day 21, and returned to baseline level by day 28 (Fig. 4, D and E). The interaction of PEDF with wound microvasculature follows a pattern that is inversely proportional to microvascular density (Fig. 4, C vs. E).

Inhibition of Endogenous PEDF Delays Blood Vessel Regression and Collagen Maturation During Wound Resolution

To explore the functional role of PEDF in resolving skin wounds, mouse skin wounds were treated with exogenous neutralizing antibody against PEDF (PEDF-Ab) or an isotype control antibody (IgG) every 4 days starting at day 8 postinjury. The period of treatment with inhibitory PEDF-Ab corresponds to time points of healing when levels of endogenous PEDF are high (Figs. 3 and 8B) and when PEDF begins to interact strongly with the wound microvasculature (Fig. 4E). Whereas no differences in macroscopic external healing rates were seen between PEDF-Ab- and IgG-treated wounds (Fig. 5A), significant changes were observed in the wound microvasculature at 16, 20, and 24-days postinjury, time points corresponding to vessel regression in this wound model (Figs. 8A and 4C). At day 16 postinjury, PEDF-Ab-treated wounds had about 50% more (P < 0.01) microvessel content than control (Fig. 5, B and C, left). At days 20 and 24 postwounding, PEDF-Ab-treated wounds had higher levels of microvascular content than control, although the differences were not statistically significant (Fig. 5, B and C, middle and right). The effect of PEDF inhibition on the wound ECM maturation process was examined by using Picrosirius red analysis to assess collagen maturity. At day 16, PEDF-Ab-treated wounds had about 15% less (P < 0.05) mature collagen than IgG-treated wounds (Fig. 5, D and E). These results demonstrate that inhibition of endogenous PEDF delays microvessel regression and may negatively influence ECM maturation in wounds.

Exogenous PEDF Inhibits Angiogenesis and Increases Pericyte Coverage During the Proliferative Phase of Healing

Since the inhibition of PEDF delayed wound resolution, we next asked whether addition of PEDF might improve wound maturation. In a gain-of-function study, we treated mouse skin wounds with exogenous, purified rPEDF or sterile PBS. rPEDF was administered via intradermal injection until day 10, a time

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**Fig. 3.** Pattern of PEDF mRNA expression and protein production during excisional wound repair. A: PEDF mRNA expression profile mined from transcriptome microarray of 1-mm wounds (Chen et al. (13), GSE23006). Data were normalized to unwounded, normal skin (NS); n = 3 for all time points; P < 0.05 by 1-way ANOVA. B: PEDF mRNA expression levels were determined by real-time RT-PCR relative to GAPDH housekeeping gene on 3-mm wound samples harvested on days 1, 3, 5, 7, 10, 14, and 21 postinjury. Data were normalized to unwounded, NS and are expressed as means ± SE; n = 3 for all time points; *P < 0.05, **P < 0.01 vs. NS; #P < 0.05, ##P < 0.01 vs. day 21 by 1-way ANOVA and Bonferroni’s posttests. C: PEDF protein content was determined by ELISA on 3-mm wound samples harvested on days 1, 3, 5, 7, 10, 14, 21, and 28 postinjury. Data were normalized to total protein content and are expressed as means ± SE; n = 10 for all time points, except n = 5 for days 1 and 28, n = 7 for NS, and n = 9 for day 5; **P < 0.01 vs. NS; #P < 0.05 vs. day 28 by 1-way ANOVA and Bonferroni’s posttests.

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The pattern of PEDF localization in the wound ECM changed during healing, colocalization of PEDF with vimentin remained robust at all time points (Fig. 4A). This result suggests continuous production of PEDF by dermal FBs during excisional healing. Furthermore, PEDF accumulated in the epidermis during repair (Fig. 4A).
period when physiological levels of endogenous PEDF are low (Figs. 3 and 8B). The macroscopic external healing rates were the same for PEDF- and PBS-treated wounds (Fig. 6A). However, quantification of traced blood vessels revealed that rPEDF-treated wounds contained about two-thirds less (P < 0.05) macrovessels per area than PBS-treated wounds (Fig. 6, B and C). Histologic quantification of microvessel density revealed that rPEDF-treated wounds had about two-thirds less (P < 0.01) microvessel content than PBS-treated wounds (Fig. 6, D and E). Concurrently, rPEDF-treated wounds exhibited about twofold more (P < 0.01) microvessels colocalized with pericytes [identified using α-SMA (47)] than control wounds

Fig. 4. Localization of PEDF during excisional wound repair in relation to dermal FBs and microvascular ECs. A and B: wounds harvested at days 7, 14, and 28 postinjury were double stained for PEDF and FB marker vimentin (A) or EC marker CD31 (B); DAPI was used to stain for nuclei. Representative fluorescence photomicrographs are shown at ×40 magnification. Dashed white lines indicate boundary of epidermis and dermis. Arrowheads indicate examples of PEDF + vimentin (A) or PEDF + CD31 (B) colocalization. Dotted grey outlines (B, left) indicate boundaries of CD31 staining transferred to the PEDF panels to delineate differences in spatial distribution between PEDF and CD31. C: wound vascularity over the time course of healing was quantified by measuring the percent area of wound bed occupied by CD31 staining. Data are expressed as means ± SE; n = 4 for all time points, except n = 6 for unwounded NS, n = 5 for day 10, and n = 3 for day 3; *P < 0.05 vs. NS by 1-way ANOVA and Bonferroni’s posttests. D: representative composite photomicrographs showing colocalization of PEDF and CD31 in unwounded skin and days 10 and day 28 postinjury at ×40 magnification; white color represents colocalized pixels. Scale bar = 50 μm. E: percent CD31 colocalized with PEDF over the time course of healing was quantified by measuring the area of colocalized pixels compared with total CD31 area. Data are expressed as means ± SE; n = 4 for all time points, except n = 6 for NS, n = 5 for day 10, and n = 3 for day 3; *P < 0.05, **P < 0.01 vs. NS; #P < 0.05, ##P < 0.01 vs. day 28 by 1-way ANOVA and Bonferroni’s posttests.
Exogenous PEDF Decreases Wound EC Proliferation, Decreases Blood Vessel Permeability, and Increases Collagen Maturity During the Proliferative Phase of Healing

To explore the effect of exogenous PEDF on the proliferation of ECs in healing wounds, immunohistochemical analysis via Ki67 staining was performed. rPEDF-treated wounds had about half (P < 0.05) the number of proliferating ECs than PBS-treated wounds (Fig. 7, A and B). The data suggest that PEDF decreases nascent angiogenesis in healing wounds via inhibition of EC proliferation.

The effect of exogenous PEDF treatment on the functionality of wound microvascular networks was examined by perfusing mice with FITC-conjugated, high-molecular weight dextran and by determining the levels of intra- and extravascular FITC-dextran in wounds. rPEDF-treated wounds had about 75% less (P < 0.01) extravascular dextran content than PBS-treated wounds (Fig. 7, C and D). Concurrently, rPEDF-treated wounds had about 50% more (P < 0.05) intravascular dextran in net amount than control wounds (Fig. 7, C and E). These results demonstrate that rPEDF-treated wounds exhibit more physiologically mature microvascular networks with minimal vessel leakage.

When the effect of exogenous PEDF on wound ECM was evaluated, rPEDF-treated wounds had about 30% more (P = 0.05) collagen content than PBS-treated wounds (Fig. 7G). Quantification of relative collagen maturity revealed that rPEDF-treated wounds had about twofold higher levels (P < 0.01) of mature collagen than PBS-treated wounds (Fig. 7, F and H). These results demonstrate that exogenous PEDF causes a more mature vascular microenvironment, with enhanced functionality of blood vessel networks embedded in a more remodeled ECM.

DISCUSSION

While much research has focused on evaluating the therapeutic potential of PEDF against a myriad of tumors (5), cardiovascular diseases (48, 49), and other pathologies (10, 11), little is known about the physiological function of PEDF. We present the first evidence of the important role for PEDF in the regulation of angiogenesis and ECM remodeling during dermal wound repair. Our data demonstrate that PEDF is a key natural multifunctional factor in healing wounds that controls blood vessel regression and promotes maturation of the vascu-
Fig. 6. Treatment of wounds with recombinant PEDF (rPEDF) decreases blood vessel density and increases pericyte coverage during the proliferative phase of healing. Murine excisional wounds were treated daily via topical application or intradermal injection of purified exogenous rPEDF or PBS vehicle until day 10 postinjury, when wound samples were harvested for analysis. A, C, E, and F: data are expressed as means ± SE; n = 6 for both treatment groups; *P < 0.05, **P < 0.01 between groups by 2-tailed t-tests. A: relative macroscopic wound sizes in PBS- and rPEDF-treated mice. Data were normalized to original wound size. B: the underside of day 10 wound tissues was photographed, images of wounds were cropped, and each visible macrovessel was traced. Representative original and traced photographs show extent of macrovessels in experimentally treated wound tissue. C: wound macrovascularity was quantified by measuring the percent area of wound tissue occupied by traced vessels. D: day 10 wounds were double stained for EC marker CD31 and pericyte marker α-smooth muscle actin (α-SMA). Representative fluorescence photomicrographs taken at ×20 magnification show areas of CD31 and α-SMA staining. D, right: colocalized pixels in white, indicating pericyte-supported vasculature in the wound bed. Scale bar = 100 μm. E: wound microvascularity was quantified by measuring the percent area of wound bed occupied by CD31 staining. F: percent CD31 colocalized with α-SMA was quantified by measuring the area of colocalized pixels compared with total CD31 area.
Fig. 7. Treatment of wounds with rPEDF inhibits EC proliferation, decreases blood vessel leakiness, and increases collagen maturity during the proliferative phase of healing. Murine excisional wounds were treated daily via topical application or intradermal injection of purified exogenous rPEDF or PBS vehicle until day 10 postinjury. A: day 10 wounds were double stained for EC marker CD31 and cell proliferation marker Ki67. Representative fluorescence photomicrographs taken at ×20 magnification show areas of CD31 (red) and Ki67 (green) staining; arrowheads show examples of Ki67-positive microvessels. B: wound EC proliferation was analyzed by number of Ki67-positive microvessels. Data are expressed as means ± SE; n = 5 for both treatment groups; *P < 0.05 between groups by 2-tailed t-test. C: wounds from mice were perfused with FITC-dextran 30 min before wound harvest. Representative fluorescence photomicrographs taken at ×20 magnification show areas of CD31 (red) and dextran (green) staining in PBS- and rPEDF-treated wounds. D and E: blood vessel leakiness was quantified by measuring the percent area of wound bed occupied by extravascular dextran (dextran not colocalized with CD31) (D) and by measuring the amount of intravascular dextran (percentage of total dextran colocalized with CD31) (E). Data are expressed as means ± SE; n = 5 for both treatment groups; *P < 0.05, **P < 0.01 between groups by 2-tailed t-tests. F: Picrosirius red staining was used to analyze wound collagen content and maturity; representative photomicrographs at ×10 (for orientation) and ×20 (for detail) magnifications are shown, where green-yellow indicates immature collagen and orange-red indicates mature collagen. G and H: wound collagen composition was quantified by measuring area of total collagen in the wound bed (G) and relative collagen maturity was quantified by measuring orange-red staining as a percentage of total collagen in the wound bed (H). Data are expressed as means ± SE; n = 3 for PBS, n = 6 for rPEDF; **P < 0.01 between groups by 2-tailed t-test. Scale bars = 100 μm.
lar microenvironment, thereby contributing to the reestablishment of dermal homeostasis.

We show that PEDF is abundant in unwounded mouse skin (Fig. 1A), localizes with dermal FBs (Figs. 1B and 4A) and binds to microvasculature (Figs. 1C and 4B), accumulates in the dermal ECM and epidermis (Fig. 1), and is produced primarily by confluent dermal FBs (Fig. 2A). The spatial distribution described here corresponds to PEDF's classification as a FB-secreted matricellular protein that binds collagen-1 (11), a ubiquitous structural component of quiescent mammalian skin (52). We recently found that KCs are capable of producing PEDF (14), a finding that is supported by mining publicly available databases. Direct comparison of recently published microarray data of matched human dermal FBs and KCs from keloids and control donor sites (29) (GSE44270) shows that PEDF expression is higher in FBs than in matched KCs (Fig. 2B). Analysis of microarray data of whole human skin, cultured skin substitute, as well as matched human dermal FBs and KCs (55) (GDS1505, GSE3204) confirms that skin FBs have much greater PEDF expression than KCs (Fig. 2C). With regard to the positive staining in murine hair follicles (Fig. 1A), a recent study found abundant PEDF content in human hair follicles, especially in inner and outer root sheath cells (38).

Review of a compendium of microarrays from various pure normal human cell cultures (GDS1402, GSE3239) shows that PEDF expression is higher in FB, smooth muscle, and stromal cell lines than in endothelial or epithelial cell lines (Fig. 2D). The colocalization of PEDF and vasculature in unwounded and healing mouse skin (Figs. 1B and 4E) thus probably represents the binding of extracellular, FB-secreted PEDF to ECs. These data, when considered in aggregate, point to a role for local PEDF in maintaining skin homeostasis, as PEDF secreted from resident FBs and binding to the ECM and neighboring ECs may function to prevent unnecessary sprouting of neovessels in the quiescent dermis.

We discovered a spatiotemporal pattern for PEDF production during skin wound healing that is reproduced at the transcript (Fig. 3, A and B), protein (Fig. 3C), and histological (Fig. 4) levels. There are several explanations for the observed low levels of PEDF in early phase wounds. First, dermal FBs do not appear in appreciable numbers until inflammation is resolved in skin wounds (28). Activated, proliferating dermal FBs, similar to those that populate the early wound, express significantly less PEDF than quiescent FBs (43, 44). Hypoxia has also been suggested to downregulate PEDF (49). Thus decreased FB numbers coupled with activated state of FBs due to hypoxia and inflammation likely account for the low levels of PEDF present in the early phases of excisional skin wound healing. The increase in PEDF production during later phases of healing probably represents a return to quiescent FB phenotypes as oxygen levels stabilize and cell-cell contact inhibition occurs after wound closure (Fig. 8C).

The spatial relationship of PEDF to endothelial structures changes considerably during healing. The percentage of PEDF-binding microvessels is inversely proportional to total microvessel content in the wound bed during the time course of healing (Fig. 4, C vs. E). These spatiotemporal differences in PEDF-EC localization point to PEDF’s hypothesized role as a matricellular protein that binds to ECs and activates antiangiogenic signaling pathways to initiate vessel regression in wounds (Fig. 8).

Treatment of wounds with exogenous PEDF significantly inhibits angiogenesis during the proliferative phase of healing (Fig. 5), whereas inhibition of endogenous PEDF delays vessel regression (Fig. 6, C and E). In a previous study, we demonstrated a highly robust antiangiogenic phenotype in resolving skin wounds (18). Indeed, EC-mitigating mechanisms do activate during wound resolution, including those mediated by intracellular negative feedback against growth factor stimulation [e.g., Sprouty (63)], ECM remodeling processes that generate matrix-derived antiangiogenic peptides and secreted extracellular factors that promote EC apoptosis [reviewed in (62)]. Antiangiogenic factors include interferon-γ-induced protein 10 (CXCL10) (9) and matricellular proteins such as thrombospondins (36) (Fig. 8B). PEDF can now be added to the active compensatory mechanisms in the resolving wound and provides at least part of the signal for vessel regression.

ECs comprising the nascent microvasculature in wounds are highly plastic due to activation by factors such as hypoxia and VEGF (22, 53) (Fig. 8B), which sensitize the ECs to competing proangiogenic (53) and antiangiogenic and proapoptotic signals (61). Indeed, preactivation by VEGF is necessary for the potent antiangiogenic effects of PEDF on dermal microvascular ECs in vitro (4, 7, 17, 61). PEDF targets nascent, activated vessels while sparing mature, pericyte-stabilized blood vessels in the cornea (17), a mechanism that also seems active in wounds. PEDF-treated wounds exhibit less total microvessel density (Fig. 6E) via less endothelial proliferation (Fig. 7A), a greater percentage of pericyte-supported microvasculature (Fig. 6F), and less microvascular leakage (Fig. 7C). Our study and others establish PEDF’s role as an important homeostatic factor in mammalian skin (summarized in Fig. 8C) (5, 11, 66).

Our findings are consistent with other published studies of pathological skin conditions treated with PEDF. In one experiment, psoriatic skin lesions treated via intradermal injection with rPEDF or PEDF-derived peptides exhibited less microvascular content than controls (1). In another experiment, PEDF-overexpressing melanoma xenografts in mice showed less total vessel density but more mature vasculature than control tumors (24). In those studies, as well as in others (49), PEDF was therapeutic against pathological angiogenesis and improved outcomes (5). In our study, partial inhibition of physiological angiogenesis by intradermal injection with rPEDF did not affect wound closure kinetics (Fig. 6A) but did improve the quality of the healing tissue, as evidenced by more histologically mature (Fig. 6D) and functional (Fig. 7C) microvasculature as well as greater ECM maturation (Fig. 7F).

These results are in line with the emerging concept that the generation of excess immature vasculature in mammalian wounds may in fact be detrimental to the quality of the remodelled tissue, leading to scar formation (18, 64). From this perspective, a partial inhibition of wound angiogenesis, via blocking proangiogenic mechanisms and/or promoting antiangiogenic mechanisms, might improve the regenerative potential of the wound healing process in skin (18, 37, 60).

A recent study found that circulating PEDF was higher in diabetic patients with foot ulcers (45). Neutralizing systemic PEDF via anti-PEDF antibody perfusion or in PEDF knockout mice improved healing rates coincident with higher wound angiogenesis, whereas systemic overexpression of PEDF
slowed healing coincident with lower wound angiogenesis in diabetic mice. Since PEDF is a multifunctional protein (10, 11, 58), off-target effects of systemically altered PEDF levels were likely responsible for differences in healing rates in that study. In contrast, the present study investigated the role of locally produced and locally acting PEDF on sprouting angiogenesis, and we modulated local PEDF levels directly. In contrast to the other study, our work provides novel data about PEDF’s role in physiological vessel regression during wound resolution.

The data presented here have implications for antiangiogenic therapies in cancer (5), as the extensive highly permeable microvascular network in wounds during the proliferative phase (8) has been compared with that of solid tumors (20, 51). Similar to PEDF’s ability to improve microvascular integrity in wounds, PEDF may promote the maturation of the tumor blood vessel microenvironment, increasing the response to therapy via more efficient delivery of chemotherapeutic agents to cancer cells located deep inside tumors (26). Such mechanisms have already been suggested for other antiangiogenic cancer therapeutics.

Quiescent FBs in wounds mediate the maturation of the wound microenvironment, partly through the production of PEDF (44) as well as ECM maturation via ECM remodeling (31). PEDF binds to the mature ECM and initiates the apoptotic pathway in activated ECs (17, 61), leading to vessel regression; PEDF has also been found to inhibit EC migration (5) and proliferation. PEDF promotes ECM remodeling and EC quiescence leading to maturation of the wound microvasculature, which has positive feedback on reestablishing wound normoxia.
positive feedback, recruitment of nearby activated FBs in wounds and then cause quiescence of these FBs. This would accelerate FB-mediated ECM maturation, which would in turn positively affect remodeling of the microvascular network (16, 31, 62). PEDF’s maturation of wound vasculature may also positively impact FB quiescence via decreased wound hypoxia. In this way, PEDF may be at the axis of the cross-talk responsible for the dynamic reciprocity between blood vessel and ECM dynamics during wound resolution (summarized in Fig. 8C).

In conclusion, our study identified and characterized PEDF as a key multifunctional player involved in blood vessel network stabilization and ECM maturation in resolving dermal wounds. With the understanding of the details of how PEDF performs, the myriad physiological functions that we identified may lead to novel strategies to improve PEDF’s efficacy as a therapeutic agent. More generally, we hope that our work will inspire more interest in using wound healing as a model of a tissue’s proper return to homeostasis following insult.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES
PEDF REGULATES DERMAL WOUND HEALING


