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Pigment epithelium-derived factor attenuates angiogenesis and collagen deposition in hypertrophic scars

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Abstract

Scar forming wounds are often characterized by higher levels of vascularity than non-scarring wounds and normal skin, and inhibition of angiogenesis has been shown to inhibit scar formation in some model systems. The rabbit ear hypertrophic scar (HS) model has been widely used to study the mechanisms that underlie the development of HS as well as the effectiveness of various treatments. Although the rabbit ear HS model is well characterized in terms of scar formation, the rate and level of angiogenesis has not been investigated in this model, and the cause-effect relationship between angiogenesis and rabbit HSs has not been examined. In the current study, full-thickness excisional wounds were created on the ventral side of New Zealand White rabbit ears to induce HS formation, and the dynamic pattern of angiogenesis and the expression of angiogenic regulatory factors were examined over time. Blood vessel density was found to peak at 2.7% on day 14 post-wounding, decreasing to 1.7% by day 28. mRNA levels of the proangiogenic factor VEGF-A peaked at day 14, while the expression of the antiangiogenic factors pigment epithelium-derived factor (PEDF) and thrombospondin 1 (TSP1) peaked at day 28 post-wounding. To examine whether inhibition of angiogenesis influences HS formation in this model, wounds were treated with exogenous soluble antiangiogenic agents including recombinant PEDF (rPEDF) and a functional PEDF peptide (PEDF-335). rPEDF and PEDF-335 were administered intradermally from day 4 post-wounding every 3 days until day 19. Intradermal injection of rPEDF

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AUTHOR CONTRIBUTIONS

Da Ma, Lin Chen, Jack Henkin, and Luisa A. DiPietro designed the study; Da Ma, Lin Chen, Yan Zhao, Junhe Shi, Shruti Vasani, Kevin Chen, and Bruna Romana-Souza performed the experiments; Da Ma, Lin Chen, and Luisa A. DiPietro wrote and revised the manuscript; all authors reviewed and approved the manuscript.

CONFLICT OF INTEREST

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

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

or PEDF-335 both led to decreased angiogenesis and decreased collagen deposition at the wound site. The results support the utility of antiangiogenic therapies, including rPEDF/PEDF-335, as a potential new strategy for the prevention or treatment of HSs.

1 | INTRODUCTION

Hypertrophic scars (HSs) are a fibroproliferative disorder of the skin characterized by an excessive accumulation of extracellular matrix deposition after skin injury.^{1,2} Hypertrophic scars may impair functionality as well as appearance, and often create physiologic and psychological difficulties. Increased microvascular content and collagen accumulation in the reticular layer are characteristic of HSs.^{1–5} HSs can arise following injury or irritation, including burn, surgery, trauma, insect bites, vaccination, skin piercing, acne, folliculitis, chickenpox, and herpes zoster infection.² Genetic predisposition, inflammation, and tension are believed to be the major factors contributing to the development of HSs.^{1,2} Several methods are used clinically to limit or reduce HSs, including scar revision surgery, silicone tape, corticosteroids, cryotherapy, radiotherapy, laser therapy, and pressure and massage. However, treatment efficacy is highly variable, and in many cases the outcomes are unsatisfactory.^{1,2}

The formation of scars has been linked to a high level of angiogenesis or capillary density in several models as well as in human scars.^{4–7} The utility of antiangiogenic therapy to limit scar formation has been explored by us and others.^{7–9} Our previous studies show that inhibition of wound angiogenesis either by blocking the major pro-angiogenic factor in wounds (VEGF-A) or via the application of an antiangiogenic factor (recombinant pigment epithelium-derived factor [rPEDF]), significantly impedes scar formation in mouse skin wounds.^{7,8} Therefore, antiangiogenic treatment could be a potential novel strategy to prevent or treat the formation of the excessive scar.

The regulation of wound angiogenesis involves both pro- and anti-angiogenic signals,¹⁰ with VEGF known to be the most prominent proangiogenic factor¹¹. Several factors, including Sprouty2 and PEDF, are responsible for the antiangiogenic signals in wounds.^{8,12} Our previous studies show that the addition of exogenous PEDF can reduce blood vessel density and decrease vessel leakiness in mouse skin wounds. This PEDF induced refinement of the angiogenic response results in increased collagen maturity and reduced scar formation in mouse skin wounds, without affecting wound closure.^{8,13} Although these results imply that a refinement of the angiogenic response could reduce scar formation after injury, the mouse model is highly dissimilar to humans due to differences in skin architecture and the lack of a true hypertrophic scar in mouse. In the present study, we sought to test the effect of PEDF treatment on angiogenesis and scar formation in a well-established rabbit HS model.^{14,15} This model, first described by Joseph and Dyson in 1966¹⁶ and later improved by Morris,¹⁵ has been used in many studies to study HS formation and the effect of various treatments.^{14,15,17–20} However, the role of angiogenesis in rabbit HS model has not been investigated. The current study first characterizes the angiogenic response in the rabbit HS model, and then investigates the utility of anti-angiogenic therapy with rPEDF and a PEDF

peptide on scar formation. The results demonstrate that both rPEDF and a PEDF peptide (PEDF-335) inhibit angiogenesis and impair collagen deposition in rabbit HS.

2 | MATERIALS AND METHODS

2.1 | Rabbit HS model

All procedures were approved by University of Illinois at Chicago Institutional Animal Care and Use Committee before the start of the animal experiments. Nine rabbits (~3.5 kg female New Zealand White rabbits (Charles River Laboratories, Wilmington, MA) were anesthetized by intramuscular administration of 45 mg/kg of ketamine and 5 mg/kg of xylazine. The area on the ear to be wounded was injected with 1% lidocaine to assist with pain control. The animals were monitored every 10 minutes while anesthetized for heart rate, respiratory rate, temperature, and oxygen saturation. Before wounding, the hair on the ventral side of the ear was removed using Nair depilatory cream (Church & Dwight, Ewing, NJ), and the skin was then thoroughly cleaned with gauze moistened with sterile deionized water, and was disinfected with Betadine and 70% alcohol. Full-thickness wounds were produced on the ears as described by Kloeters et al.¹⁴ Briefly, four 8 mm diameter wounds were created on the ventral side of each ear down to bare cartilage using a sterile biopsy punch (Acu Punch, Acuderm, Ft. Lauderdale, FL). The epidermis, dermis, and perichondrium were thoroughly and carefully removed. A thin layer of Mastisol adhesive (Ferndale Laboratories, INC. Ferndale, MI) was applied to the area surrounding the wounds, and then Tegaderm (3 M, St. Paul, MN) was placed. Wounds were photographed at multiple time points after injury using a digital camera. For tissue analysis, wounds were harvested either stored in RNALater (Sigma-Aldrich, St. Louis, MO), embedded in Tissue-Plus OCT Compound (Fisher Scientific, Hampton, NH), or fixed in formalin (Sigma-Aldrich) for real-time PCR, IHC/apoptosis quantification, and measurement of scar elevation index (SEI), respectively.

2.2 | Treatment with antiangiogenic factors

To examine the effect of antiangiogenic treatment on wound closure, angiogenesis, HS formation, and collagen deposition, rabbit wounds were treated with recombinant PEDF (rPEDF) purified from the medium of stable baby hamster kidney cell transfectants⁸ or PEDF peptide 335 (PEDF-335). rPEDF was prepared as described by us in a previous study.⁸ PEDF-335, a 7 amino acid peptide, was synthesized and modified in the house (patent: US10081668B2, SEQ ID NO 10). rPEDF (30 µg/wound) and the PEDF-335 (120 µg/wound) were administered intradermally in 60 µL normal saline using a 1 mL insulin syringe six times from day 4 post-wounding (every 3 days) until day 19. PEDF-335 was also applied topically in 30 µL normal saline six times from day 4 post-wounding (every 3 days) until day 19. For all applications, normal saline was used as control. Each treatment group included eight wounds.

2.3 | mRNA quantification by real-time PCR

Total RNA was extracted from the wounds using TRIzol (Invitrogen, San Diego, CA) according to the manufacturer's instructions. cDNA was synthesized from 1 µg DNase I (Invitrogen) treated total RNA using a High-Capacity cDNA Reverse Transcription Kit

(ThermoFisher Scientific, Waltham, MA). Semi-quantitative PCR was carried out using a StepOne Plus real-time PCR system (Applied Biosystems, Carlsbad, CA) with the SYBR Green mix (Roche, Basel, Switzerland) and specific primers for collagen IA, collagen IIIA, VEGF-A, Sprouty-2 (SPRY 2), PEDF, thrombospondin-1 (TSP-1), TGF- β 1, and CTGF (Table 1). GAPDH was used for normalization. All primer sequences are listed in Table 1. Relative expression was calculated using the 2^{-CT} method. A normal skin sample was used as the baseline to calculate the relative expression of all other samples.

2.4 | Wound area and thickness measurements

External measurements were used to examine the rate of wound closure. Using photographs, the wound size was determined in square mm using AxioVision (ZEISS, Oberkochen, Germany) software. In some experimental groups, the thickness of the ear including the ventral side scar, cartilage, and dorsal skin was physically measured at the thickest part using a slide caliper at days 18, 22, 24, 26, and 28 post-wounding.

2.5 | Calculation of scar elevation index

Tissues fixed in formalin were embedded in paraffin, sectioned at 5 μ m thickness, and subjected to a standard hematoxylin and eosin (H&E) stain. The SEI was calculated as the ratio of the tissue height in the total wound area to the area of normal tissue below the hypertrophic dermis as shown in Figure 1B. An SEI of one indicates that the healed wound is essentially flat, with no scar hypertrophy, and an SEI greater than one represents a raised, hypertrophic scar.¹⁴

2.5.1 | Immunofluorescent staining for quantification of vascularity, fibroblasts, and myofibroblasts—To determine capillary density in wounds and skin tissues, indirect immunofluorescent staining of the endothelial cell marker CD31 was performed as described in our previous publication.¹³ Briefly, 8 μ m frozen sections were prepared from wound tissues embedded in OCT. Sections were air-dried, rehydrated in PBS, and fixed in cold acetone for 10 minutes. After being blocked with 10% goat serum for 30 minutes, the sections were incubated with mouse anti-human CD31 which cross-reacts with rabbit CD31 (1:200 dilution, Clone C31.3&JC/70A, ScyTek, Logan, UT) followed by Alexa fluor 594 goat anti-mouse IgG (1:1000 dilution, Invitrogen, Carlsbad, CA). To confirm that CD31 positively stained structures were indeed blood vessels, some sections were double-stained with mouse anti-CD31 (ScyTek) and sheep anti-human Von Willebrand Factor (vWF, 1:50 dilution, Abcam, Boston, MA) which cross-reacts with rabbit vWF followed by Alexa fluor 594 goat anti-mouse IgG (1:1000 dilution, Invitrogen) and donkey anti-sheep Alexa fluor 488 (1:1000, Abcam). To identify fibroblasts and myofibroblasts, sections were incubated with chicken anti-mouse/human vimentin (1:400 dilution, Abcam) or goat anti-human α -SMA (1:800 dilution, Novus, Littleton, CO), which cross-react with rabbit vimentin and α -SMA respectively, followed by donkey anti-chicken Alexa Fluor 594 (1:1000 dilution, Sigma-Aldrich) or donkey anti-goat IgG Alexa Fluor 488 (1:800 dilution, Abcam). All procedures were performed at room temperature. Sections were analyzed using a fluorescence microscope (Axioskop 40, ZEISS, Oberkochen, Germany). The wound bed was identified, and ImageJ analysis was used to determine the percent area occupied by CD31 stained blood vessels and vimentin+ fibroblasts in total defined wound area. The

number of α -SMA+ myofibroblasts was counted in the wound bed of each sample within 3 to 10 $\times$ 20 fields. Counts were averaged to produce a single data point for each wound.

2.5.2 | Assessment of apoptotic cells—An In Situ Cell Death Kit, AP (Roche, Basel, Switzerland) that identifies TUNEL activity was used to detect apoptotic cells in the wounds in frozen wound sections per the manufacturer's instructions. Fluorescence images were taken, and the number of apoptotic cells in the wound bed of each sample on 3 to 5 $\times$ 20 fields was counted. Counts were averaged to produce a single data point for each wound.

2.5.3 | Assessment of collagen density—To evaluate the collagen deposition in day 28 wounds, tissues were fixed in formalin and then subjected to Masson's trichrome staining as described in our previous publications.^{13,21} The density of collagen was then determined using ImageJ.²²

2.5.4 | Statistical analyses—Results are expressed as means $\pm$ SEM. Two-way analysis of variance (ANOVA) followed by Bonferroni's posttest was performed using GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA) or Wilcoxon test was performed using SAS Version 9.4 (SAS, Cary, NC). *P* values equal to or less than .05 were considered statistically significant.

3 | RESULTS

3.1 | Validation of the rabbit hypertrophic scar model

To validate the model, we created four 8 mm wounds on each ear of each rabbit as described by Kloeters et al.¹⁴ External observation demonstrated that HSs were reproducibly formed (Figure 1A). To provide quantitative data on HS formation, the scar elevation index (SEI) was measured in histologic sections (Figure 1B). The results demonstrate that hypertrophic scars formed in a reproducible manner, as the mean SEI on days 14, 18, 22, and 28 post-wounding was 3.60, 4.95, 5.46, and 3.09, respectively (Figure 1C) and was significantly greater than normal skin ($P < .05$) at all-time points. As a further confirmation, the mRNA expression of two pro-fibrogenic factors, CTGF and TGF- β 1, was quantified. CTGF showed a nonsignificant trend to an elevation over the period of wound closure. TGF- β 1 levels rose significantly, mirroring the generation of the HS (Figure 1D,E). The expression of collagen I and collagen III also gradually increased, and was significantly increased in the wounds from days 22 to 28 (Figure 1F,G). Similar to other species, levels of apoptosis increased during the time period of vascular regression in the rabbit HS model. In the rabbit HS model, the number of apoptotic cells was significantly increased over that of normal skin at all-time points examined (Figure 2A,B). The expression of caspase 3, a central mediator of apoptosis, also went up during the period of vascular regression in the rabbit HS (Figure 2C). Together, the results validate the rabbit HS model.^{14,20,23,24}

3.2 | Significant angiogenesis is present in the hypertrophic scars

Although the rabbit HS model has been widely used, the pattern of angiogenesis has not been investigated in this model. Angiogenesis was measured in wound samples at days 14, 18, 22, and 28 post-wounding as well as normal skin by image analysis quantification

of CD31 immunofluorescent staining (Figure 3A). To confirm that CD31 positive stained structures are indeed blood vessels, multiple sections from day 14 wounds were double-stained with anti-CD31 and anti-vWF antibodies. The results showed that 100% of CD31 positive structures were also vWF positive (Figure 3B). As compared with normal skin, vessel density was significantly increased in HS from day 14 to day 28 ($P < .01$, Figure 3C), with the highest density observed at day 14 ($2.68 \pm 0.18\%$). Following day 14, capillary density gradually declined to $1.67 \pm 0.13\%$ at day 28 post-wounding (Figure 3C). These results suggest that the rabbit HS model includes a vigorous neovascularization response. To assess the pattern of production of pro- and antiangiogenic factors, the mRNA levels of a panel of pro- and antiangiogenic factors that are known to play a role in wound healing were examined. Prior studies by us and others have shown that pro and antiangiogenic factors are produced in wounds in a reproducible and temporal manner.^{8,12} In tandem with the observed increase in vessels, the mRNA expression of a key pro-angiogenic factor, VEGF-A, was significantly increased at day 14 post-wounding (Figure 4). As expected, the expression of known wound anti-angiogenic factors exhibited a different pattern than pro-angiogenic factors. PEDF, Sprouty 2, and TSP1 began to increase at day 14 with a peak at day 28 (Figure 4).

3.3 | PEDF treatment inhibits angiogenesis and reduces collagen deposition in wounds

Our previous studies in mouse wound models demonstrated that partial inhibition of the angiogenic burst leads to refined capillary growth, better perfusion, and improved collagen ultrastructure. To test if the antiangiogenic treatment might modify healing outcomes in the rabbit model, wounds were injected with either rPEDF or PEDF peptide PEDF-335, a peptide previously shown to be highly antiangiogenic.²⁵ Wounds were injected once every 3 days from day 4 to day 19. Similar to prior studies in mouse wounds, the partial inhibition of wound angiogenesis had no effect on wound closure (Figure 5 A&B). In contrast, antiangiogenic treatments reduced vessel density from $2.98 \pm 0.15\%$ in the control group to $2.31 \pm 0.27\%$ in the PEDF-335 treated group ($P = .09$) and $2.36 \pm 0.23\%$ in the rPEDF treated group ($P < .05$) at day 28 (Figure 5C,D).

We then evaluated if rPEDF or PEDF-335 treatment could affect collagen I and III mRNA expression in the scars. Real-time PCR analysis results showed that rPEDF, but not PEDF-335, down-regulated collagen I mRNA expression (Figure 6A). However, both PEDF-335 and rPEDF significantly inhibited collagen III mRNA expression ($P < .05$ and $P < .01$, respectively, Figure 6B). We further investigated the collagen deposition in the scars using Masson's trichrome staining. Collagen density in day 28 scars of PEDF-335 and rPEDF treated groups were $24.92 \pm 2.77\%$ and $33.87 \pm 2.62\%$, respectively, both being significantly lower than the control group ($49.63 \pm 6.71\%$) ($P < .05$ – 0.01 , Figure 6C,D). Interestingly, while rPEDF/PEDF-335 treatment decreased collagen density, the treatment did not affect the SEI at day 28, or wound thickness as measured with an external caliper (Figure 6E,F). To examine whether topical application might also be effective, wounds were treated with topical PEDF-335 ($120 \mu\text{g}/\text{wound}$ in $30 \mu\text{L}$ saline) at doses comparable to injection. Topical treatment of wounds with PEDF-335 did not have a significant effect on either angiogenesis, collagen deposition, wound closure or the SEI/scar thickness (Figure 7A–E). Overall, the results demonstrate that the injection of rPEDF/PEDF-335 reduces

wound angiogenesis and leads to decreased collagen expression and accumulation in rabbit HSs.

To examine possible cellular targets of PEDF treatment, the amounts of vimentin+ fibroblasts and alpha α -SMA+ myofibroblasts were quantified in treated and control day 28 wounds (Figure S1). These results showed that, when compared with normal tissue, both fibroblast and myofibroblast levels were increased in both treated and untreated wounds. In addition, a modest yet non-significant decrease in α -SMA+ myofibroblasts was observed in the rPEDF treated wounds. This result suggests the possibility that rPEDF treatment may inhibit the fibroblast to myofibroblast conversion in wounds.

4 | DISCUSSION

A central dogma of wound healing has been that optimal tissue repair requires a robust and vigorous angiogenic response.^{26,27} Intuitively, this would seem to be a correct assumption, as wounds need oxygen and nutrients. However, at the time of maximal angiogenesis in wounds, many of newly formed capillaries are immature and dysfunctional, and most are not perfused.²⁸ Within wounds, the vessels are tortuous, disorganized, and exhibit high permeability, supporting increased infiltration of inflammatory cells and soluble factors in perivascular areas.²⁹ In addition, excessive capillary growth increases the apoptotic load that occurs during vascular regression in wounds. This increased apoptotic burden may induce compensatory proliferation of neighboring cells and contribute to fibrosis.^{30,31}

Several studies, including some from our lab, show that full-thickness wounds can heal well when angiogenesis is reduced.^{7-9,32-35} Our prior studies demonstrate that either antibody neutralization of VEGF or treatment with the antiangiogenic mediator PEDF can lead to an ~50% reduction in peak vascularity in adult skin wounds. When angiogenesis is reduced by such treatments, the normal cellular inflammatory response is unaffected, and wounds close normally. Most interesting is that wounds in which the angiogenic response is blunted show a significant reduction in wound scar width and an improved collagen architecture that is much more like native skin.^{7,8} Recent studies of human scars reinforce the concept that hypertrophic scar formation is linked to increased microvascular content,^{4,5} and the use of antiangiogenic therapy to reduce scar formation has been suggested by us⁷ and others.^{6,7,36} In this study, we sought to investigate the profile of angiogenesis in the rabbit HS model and tried to elucidate the cause-effect relationship of angiogenesis and HSs.

Our findings confirm that full-thickness excisional skin wounds on the ventral side of rabbit ears heal with significantly raised scars similar to human HSs histologically. The expression of the fibrogenic factor TGF- β 1, as well as collagen I and collagen III, was observed to gradually increase as the wounds closed. Within the scars, microvascular density in the wound bed was significantly increased compared with normal skin, a finding that is consistent with human HSs.^{1,3-5} The increased neovascularization was accompanied by elevation of the key pro-angiogenic factors, VEGF-A, which occurred at the relatively early stage of healing, whereas antiangiogenic factors, PEDF, SPRY2, and TSP1, appeared at the later stage of the healing or just before vessel regression. Similar to other species, levels of apoptosis also increased during the time period of vascular regression in the rabbit HS

model. During the vascular regression phase, overall cellular content in wounds is reduced, including the number of fibroblasts and immune cells, so many cells contribute to the overall measurement of apoptosis. Although our studies did not allow a determination of the type of cells that contribute to the apoptotic load in HS model, the findings demonstrate that the pattern of wound angiogenesis, angiogenic regulator production, and apoptosis in the rabbit ear HS model is similar to that seen in mouse models of skin repair.^{8,12}

PEDF is a 50KD glycoprotein and a member of the serpin family that is also known as serpin F1 (SERPINF1). PEDF was initially identified as a neurotrophic factor³⁷ but was later found to be a multifunctional secreted glycoprotein with antiangiogenic, anti-tumorigenic, anti-oxidant, anti-thrombotic, and anti-inflammatory properties.³⁸ PEDF is a potent endogenous antiangiogenic factor that is multifunctional.³⁹ PEDF significantly decreased VEGF-A expression in both retinal capillary endothelial cells (RCEC) and Muller cells by competitively binding to VEGF receptor,⁴⁰ and inhibited VEGF-induced sprouting in a spheroid sprouting assay.⁴¹ Moreover, PEDF can induce apoptosis of endothelial cells by activating the FAS-FAS ligand death pathway to remodel vascular formations.⁴² The natural antiangiogenic activity of PEDF has been proposed as a therapeutic inhibitor of pathological neovascularization, such as retinal neovascularization,⁴³ cardiovascular disease,⁴⁴ and malignant tumors.^{45–47} Our prior studies show that in skin wounds, PEDF is primarily produced by fibroblasts, with lesser production by keratinocytes.^{8,48} There is a distinct spatiotemporal production of PEDF in the wounds during the process of skin wound healing: down-regulation of PEDF occurs at the inflammatory and early proliferative stages, and then significant up-regulation of PEDF is seen at the remodeling stage when vessel regression occurs.⁸ In wounds, the application of exogenous PEDF inhibits the proliferation of endothelial cells, reduces blood vessel density, and decreases vessel leakiness.⁸ More importantly, PEDF treatment of wounds leads to enhanced collagen maturity in wounds, suggesting that a reduction in the formation of dysfunctional capillaries may improve healing outcomes.^{8,13}

The current study adds to our understanding of the regulatory role of PEDF in angiogenesis and collagen maturation. Using a well-described model of HS, the results show that injection of either rPEDF or PEDF peptide PEDF-335 into rabbit ear wounds inhibited angiogenesis. The treatments also led to impaired collagen mRNA expression and deposition in the scar. While rPEDF/PEDF-335 treatment decreased collagen density, the treatment did not affect scar thickness at the time points measured. Nevertheless, the decreased density of collagen in PEDF/NU-355 treated wounds may predict a reduced final scar. The amount of collagen production has been identified as a predictor of final scarring outcomes.⁴⁹ Moreover, previous studies suggest that maximal collagen content in wounds occurs at around day 21.⁵⁰ Thus the decrease in collagen at day 28 in the rPEDF/PEDF-335 treated wounds may represent an important change in the progression toward an improved long-term outcome. Extremely long-term observations (eg, 6 months or more) are needed to fully understand the final effect of PEDF/PEDF-335 treatment.

The mechanism by which rPEDF treatment may lead to decreases in collagen content is likely to be multifactorial. rPEDF treatment reduces capillary content in the wound, and this is expected to improve tissue perfusion and oxygenation. PEDF targets nascent vessels

as they form, and in wounds, PEDF treatment allows for the rapid generation of a refined network of well-perfused vessels.^{8,39} Indeed, prior studies do suggest that well-perfused wounds often exhibit decreased total capillary content.⁵¹ Fibroblasts have been described to become profibrotic under hypoxia, a situation that may be reversed by PEDF treatment if the refinement of the capillary bed leads to improved oxygenation.⁵² PEDF may also directly influence fibroblast function in wounds. While not conclusive, the data here (Figure S1) suggest that PEDF treatment may inhibit the fibroblast to myofibroblast conversion in wounds. This possible mechanism, along with other direct cellular effects of PEDF on fibroblasts, should be examined in future studies. For example, a recent study shows that PEDF can inhibit endothelial to mesenchymal transition, an affect that reduces fibrosis in myocardial disease.⁵³ PEDF is also known to trigger fibroblast quiescence; this effect may be at least partially responsible for the reduced collagen levels in PEDF treated wounds.⁵⁴

Interestingly, as compared with injection, topical administration of the PEDF-335 peptide did not affect either angiogenesis, collagen deposition or wound closure. We speculate that poor absorption might have limited the efficacy of the topical application. Several prior studies have described pharmacologic treatments that inhibit hypertrophic scars in the rabbit model used here. Approaches to scar reduction that have been shown to be effective in the rabbit model include neutralization of pro-fibrotic factors, such as TBGF- β and CTGF, and the use of pharmacologic agents such as oxandrolone and statins.^{24,55–57} Some studies demonstrate that the reduction in the hypertrophic scar is associated with a decrease in angiogenesis, and a few have directly explored the use of antiangiogenic therapies, including anti-VEGF and endostatin, to reduce scar formation in the rabbit model.^{58–60} Together with the current study, then, a growing body of data supports the concept that robust angiogenesis spurs fibrosis in skin wounds, and that inhibition of angiogenesis, at least with the subgroup of agents that decreases VEGF activity, can reduce scar formation.

Taken together, our study demonstrates that angiogenesis is a prominent feature of rabbit HS model, a feature that is consistent with human HSs. Intradermal injection of PEDF significantly inhibits angiogenesis and reduces collagen deposition, suggesting that anti-angiogenic treatment with PEDF may be a novel potential therapeutic strategy for HSs. Multiple studies now suggest that antiangiogenic treatments do not impair wound closure, yet may be beneficial in restoring a more normal, nonscarring ECM. The study here, performed in a highly relevant model with good concordance to human HS treatment,¹⁴ provides new information to suggest that antiangiogenic approaches may be therapeutically sound.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

Validation of the rabbit hypertrophic scar model. Four 8 mm full thickness excisional wounds were made on the ventral side of the rabbit ears. A, Representative photomicrographs of wound closure and scar formation at time points following wound placement. Scale bar: 8 mm. B, The scar elevation index (SEI) was calculated by dividing the measured value for the highest point of fibrous tissue ("A") by the height of normal dermis ("B"). C, SEI measurements over the time course of healing. N = 8 wounds per time point. $\#P < .01$, Wilcoxon test. D-G, Quantification of mRNA for CTGF, TGF- β 1, collagen IA, and collagen IIIA over the time course of healing. Total RNA was extracted

from unwounded skin and wounds/scars, and relative mRNA expression of the target genes determined using RT-PCR. N = 8 wounds/time points. * $P < .05$, # $P < .01$, Wilcoxon test. For all graphs, columns and lines represent mean and SEM, respectively

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FIGURE 2.

Apoptotic cells and caspase-3 in rabbit hypertrophic scars. A, Representative images of apoptotic cells (green) as detected by TUNEL staining. Scale bar = 100 μ m. B, Numbers of apoptotic cells in wound bed, measured as apoptotic cells per $\times 20$ field. C, Relative caspase 3 mRNA expression in normal skin and wounds, determined by RT-PCR. N = 8 wounds/time points. * $P < .05$, # $P < .01$, Wilcoxon test. For all graphs, columns and lines represent mean and SEM, respectively

FIGURE 3.

Pattern of angiogenesis in rabbit hypertrophic scars. Capillary vessel density in the wound bed was examined using indirect immunofluorescent staining of CD31. A, Representative images of CD31 staining of unwounded skin and wounds at different time points after injury. Scale bar: 100 μ m. B, Representative images of double staining of CD31 and VWF in rabbit ear skin wounds. Left to right, DAPI, CD31, vWF, merged (DAPI +CD31+ vWF) staining. White arrows = Circular vascular structures. Gold arrows point to enlarged photos of these vascular structures. Scale bar: 100 μ m, top panels; 35 μ m, bottom enlarged panels. C, Capillary vessel density as quantified by ImageJ analysis of CD31 staining. N = 8 wounds/time point. # P < .01, Wilcoxon test. Columns and lines represent mean and SEM, respectively

FIGURE 4.

mRNA levels for pro- and antiangiogenic factors in hypertrophic scars. Total RNA was extracted from unwounded skin and wounds/scars, and relative mRNA expression of the target genes was determined using RT-PCR. N = 8 wounds/time point. * $P < .05$, Wilcoxon test. For all graphs, columns and lines represent mean and SEM, respectively

FIGURE 5.

Effect of intradermal injection of PEDF on wound closure, angiogenesis, apoptosis in rabbit hypertrophic scars. Eight milliliter diameter excisional wounds were treated by intradermal injection of rPEDF (15 $\mu\text{g}/\text{wound}$), the PEDF peptide PEDF-335 (120 $\mu\text{g}/\text{wound}$) or saline control every 3 days beginning at day 4 post-wounding through day 19. Capillary vessel density in the wound bed was examined using indirect immunofluorescent staining of CD31. A, Representative external photomicrographs of scar formation at different time points. Scale bar: 8 mm. B, Quantification of wound size by image analysis of external photos. Points = mean, lines = SEM. $P > .05$ between the treatment groups, Two-way ANOVA followed by Bonferroni's posttest. C, Representative images of CD31 staining at day 28 after injury in wounds of each experimental group and unwounded skin. Scale bar: 100 μm . D, Capillary vessel density as quantified by ImageJ analysis of CD31 staining presented as percent area that was CD31+. Columns and lines represent mean and SEM, respectively. $*P < .05$, Wilcoxon test

FIGURE 6.

Effect of intradermal injection of PEDF on mRNA expression of collagen I/III, collagen deposition, scar thickness, and scar elevation index in rabbit hypertrophic scars. A,B, mRNA expression of collagen IA and IIIA, respectively. $N = 8$ wounds. * $P < .05$, # $P < .01$, Wilcoxon test. C, Collagen density in wounds, quantified by ImageJ and expressed as percent area occupied by collagen. $N = 8$ wounds * $P < .05$, # $P < .01$, Wilcoxon test. D) Representative images of Masson's trichrome staining at day 28 after injury in wounds of each experimental group and unwounded skin. Scale bar: 100 μ m. E, Scar thickness measured by external slide caliper. $P > .05$ between the treatment groups, two-way ANOVA followed by Bonferroni's posttest. F, SEI at day 28 in wounds of each experimental group. $P > .05$ between the treatment groups, Wilcoxon test. $N = 8$ wounds. For all graphs, columns and lines represent mean and SEM, respectively

FIGURE 7.

Effect of topical treatment with PEDF-335 on wound angiogenesis, collagen deposition, wound closure, scar thickness, and scar elevation index in rabbit hypertrophic scars. Eight milliliter diameter excisional wounds were treated topically with PEDF-335 (120 $\mu\text{g}/\text{wound}$) or saline control every 3 days beginning at day 4 post-wounding through day 19. A, Capillary vessel density at day 28 after wounding, quantified by ImageJ analysis of CD31 staining and presented as percent area that was CD31+. $P = .32$ between control and PEDF-335 treated group, $N = 8$ wounds, Wilcoxon test. B, Collagen density in wounds, quantified by ImageJ analysis of Masson's trichrome staining, and expressed as percent area occupied by collagen. $N = 8$ wounds, $P = .87$ between control and PEDF-335 treated group, Wilcoxon test. C, Wound closure over time, quantified by image analysis of external photos. $N = 8$ wounds/time points, $P > .05$ between groups, Two-way ANOVA followed by Bonferroni's posttest. D, Scar thickness measured by an external slide caliper. $N = 8$ wounds/time points, $P > .05$ between the treatment groups, Two-way ANOVA followed by Bonferroni's posttest. E, SEI at day 28 post-wounding in each group. $P > .05$, Wilcoxon test. $N = 8$ wounds. For all graphs, columns and lines represent mean and SEM, respectively

TABLE 1

Primer sequences for real-time PCR

Standard gene abbreviation	Protein name abbreviation	Forward (5'-3')	Reverse (5'-3')
<i>Gapdh</i>	GAPDH	TCCTGGCAAAAGTGGGATGTTGT	GTGGGTGGAATCATACTGGA
<i>Vegfa</i>	VEGF-A	GGCTGCTGCAATGATGAAAG	GTGCTGTAGGAAGCTCATCTC
<i>Spry2</i>	Sprouty 2	GTGGCAAGTGCAAAATGTAAAG	GCACACACAAAGTCCCATAGT
<i>Serpint1</i>	PDF	GAGAGGAAAGCTCGGATAAA	GTTGAGCTCTGAAAGGTCTAAG
<i>Thbs1</i>	TSP-1	GACAAAGGATGGAATCGGAGATG	ATACTGGGCTGGGTTGTAAATG
<i>Ccn2</i>	CTGF	CAAGGGACTCTTCTGTGACTTC	AGGCAAGTGCACCTGGTAITTT
<i>Tgfb1</i>	TGF-β1	CTTCAGCTCCACAGAGAAAGAAC	TGTCCAGGCTCCAGATGTA
<i>Col1a</i>	Col 1A	CAAGGGAGAGAGTGTAAACAAG	GGGAACCTCTCTTTCTCTCTTC
<i>Col3a</i>	Col 1IIIA	AGGATGGCTGCTCTAAACATAC	CTTGATCAGGACCACCAATATCA
<i>Casp3</i>	Casp 3	CACGGTGATTGAAGGAGTC	GCAAGCCTGAATAATGAA