Therapeutic Approaches to the Regulation of Wound Angiogenesis

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Significance: Re-establishment of a functional vascular network is a critical component of successful wound repair. One of the most potent pro-angiogenic agents is vascular endothelial growth factor (VEGF), which, from a basic science and pre-clinical perspective, seems ideal for the therapeutic stimulation of blood vessel growth in non-healing wounds.

Critical Issues: Current strategies to improve the dysfunctional angiogenesis that occurs in non-healing wounds are inadequate with regard to the nature and magnitude of the clinical problem. However, VEGF therapy has so far been unsuccessful in promoting healing in the clinic. More effective means of delivery to the wound, which take into account the biochemical and spatio-temporal aspects of angiogenesis, may be necessary to realize VEGF's therapeutic potential. Reviewed approaches for the regulation of wound angiogenesis include: targeting regulators of intracellular VEGF signaling, making use of collagen-binding VEGF fusion proteins for increased retention in the wound, and implantation of heterogeneous scaffold systems for spatial control of angiogenesis with simultaneous use of VEGF and its inhibitor.

Future Directions: To maximize efficacy of therapeutic VEGF, it may be necessary to also target its intracellular inhibitory mechanisms. Immobilizing VEGF to the wound matrix may increase its bioavailability and therapeutic efficacy. Gaining spatial control of angiogenesis opens up possibilities for advanced directed therapy. The reviewed studies present innovative approaches to *in vivo* directed modulation of angiogenesis utilizing VEGF biology which can, if taken further and validated in human subjects, have significant impact on clinical wound care in the future.

SCOPE

RE-ESTABLISHMENT OF A functional vascular network is one of the most important components of successful wound repair. The process by which new blood vessels sprout from pre-existing vasculature to supply the hypoxic wound bed—angiogenesis—is highly complex and tightly regulated. Angiogenesis involves endothelial cell (EC) proliferation, differentiation, migration, and organization into a branched tubular network and is controlled by specific interaction of endogenous pro- and antiangiogenic factors with

ECs.¹ One of the most potent pro-angiogenic agents is the well-characterized vascular endothelial growth factor (VEGF), a matricellular protein produced by keratinocytes, and macrophages during the early phases of physiological wound repair.^{2,3} VEGF functions by binding compatible receptors on EC membranes, which initiate and amplify signaling cascades that lead to pro-angiogenic cellular changes.⁴ Endogenous inhibitors to this process are produced to aid in the spatio-temporal control of angiogenesis during healing.^{5,6} In diabetic chronic

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Abbreviations and Acronyms

CBD = collagen-binding domain
EC = endothelial cell
ECM = extracellular matrix
FT = full-thickness
MAPK = mitogen-activated protein kinase
PBS = phosphate-buffered saline
PDGF = platelet-derived growth factor
PLGA = poly(lactic-co-glycolic) acid
Spry2 = Sprouty2
STZ = streptozocin
TAT = transactivator-of-transcription
VEGF = vascular endothelial growth factor
VEGFR = VEGF receptor

wounds, an imbalance of important angiogenic mediators may be responsible for the observed dysfunctional angiogenic response.⁷ In response, clinical therapies are being developed that aim at shifting the balance toward a desired phenotype by the exogenous directed delivery of specific angiogenic agents.

TRANSLATIONAL RELEVANCE

The temporal pattern of blood vessel growth and regression has been determined in experimental full-thickness (FT) dermal wounds. Angiogenesis begins as early as day 3 postwounding and peaks at around day 10, when blood vessel density is more than double that of unwounded tissue.⁸ Such robust growth of vascularity is caused by the cellular production and extracellular matrix (ECM) release of abundant pro-angiogenic agents, including platelet-derived growth factor (PDGF), basic fibroblast growth factor 2, and VEGF.^{1,3}

Production of VEGF by keratinocytes, smooth muscle cells, and macrophages is induced by hypoxia in the early phases of wound repair and peaks around day 5 postinjury in the experimental wound model.^{8–10} VEGF acts on vascular ECs through compatible receptor tyrosine kinases VEGF receptor (VEGFR)-1, -2, -3 and through co-receptor Neuropilin in a context-specific manner.² Activated receptors initiate and propagate mitogen-activated protein kinase (MAPK) signaling cascades in ECs that lead to pro-angiogenic cellular behavior (for details, please see Refs.^{4,11}). Importantly, VEGF is also a potent vasodilator, as it increases vascular permeability through the promotion of nitric oxide synthase and cyclooxygenase activities in ECs.² VEGF may, thus, promote robust growth of immature, leaky vessels, the maturation of which may be the responsibility of other endogenous angiogenic mediators, for example, PDGF, an attractant of vessel-stabilizing pericytes.³ Pruning of excess nonfunctional vasculature is hypothesized to be mediated through the action of endogenous angiogenic inhibitors that are produced during the resolving phases of healing.^{5,6}

In diabetic chronic wounds, there may exist a prolonged imbalance of endogenous pro- and anti-angiogenic factors that results in dysfunctional angiogenesis.⁷ The molecular basis for the impaired production of VEGF in diabetic tissues has recently been described.¹² Currently, an active area of research is the development of effective methods for the delivery of VEGF and other angiogenic mediators to restore blood vessel function and to correct pathological phenotypes.

CLINICAL RELEVANCE

Insufficient angiogenesis and nonfunctional vasculature are common phenotypes of nonhealing wounds and are often predictive of poor healing outcomes in diabetic foot ulcers.¹³ Current therapeutics to stimulate reparative angiogenesis, including applications of topical PDGF, tissue-engineered dressings, hyperbaric oxygen, and negative pressure,¹⁴ are often inadequate,¹⁵ and new treatments will be needed, as the magnitude of the problem grows along with increasing rates of obesity and diabetes in the population. From a basic science and preclinical perspective, VEGF is an ideal factor for the stimulation of blood-vessel growth in wounds. However, in a phase II clinical trial, topical VEGF failed to improve diabetic foot ulcer healing. More effective means of delivery to the wound, which take into account the biochemical and spatio-temporal aspects of angiogenesis, may be necessary to realize VEGF's therapeutic potential.

DISCUSSION OF FINDINGS AND RELEVANT LITERATURE

Three target articles^{16–18} reviewed here investigated aspects of experimental regulation of wound angiogenesis (Table 1). A common thread is the exogenous application of bio-active molecules to a healing wound, with each method somehow utilizing or modifying VEGF biology to achieve specific angiogenic phenotypes in animal models.

The study by Wietecha *et al.*¹⁶ characterized the expression, production, and function of an endogenous intracellular protein, Sprouty2 (Spry2), in the context of dermal wound repair. Sprouty proteins are known negative feedback loop modulators of pro-angiogenic MAPK signaling pathways in EC downstream of major growth factor stimuli such as VEGF (Fig. 1).¹⁹ To determine the function of Spry2 in the healing wound, recombinant Spry2 was engineered with a peptide (transactivator-of-transcription, TAT) which enables efficient entry of tagged proteins into EC. A dominant-negative mutant of Spry2 (Spry2^{Y55F}), which is known to inhibit wild-type Spry2 and, thus, promote angiogenesis, was engineered in the same manner. The recombinant TAT-tagged proteins were dissolved in a biocompatible controlled-release gel that is commonly used in drug delivery applications (Fig. 1). The treated gels were topically applied onto 3-mm excisional, FT murine dermal wounds 5 days postinjury. Wound samples were harvested at day 10, and it was observed that, relative to control, exogenous TAT-Spry2 significantly decreased

Table 1. *Experimental model or material: advantages and limitations*

	Target Article		
	Wietecha et al. ¹⁶	Yan et al. ¹⁷	Yuen et al. ¹⁸
Animal model	FVB mice; 3 mm FT excisional dermal wounds on dorsal surface	Sprague-Dawley rats, STZ-induced T1 diabetes; two 2.5 cm FT excisional dermal wounds on dorsal surface	SCID mice; hind-limb ischemia via ligation of external iliac and femoral arteries and veins
Treatment	Pro- or antiangiogenic recombinant proteins engineered for cell permeability	Recombinant VEGF engineered to contain a CBD	Recombinant VEGF and/or anti-VEGF antibody
Delivery vehicle	Dissolved in 25% wt/vol (in PBS) Pluronic F-127 controlled-release gel	Fusion protein dissolved in PBS	Three-layer heterogeneous, 4.2 mm diameter, 3 mm thick macroporous co-polymer of PLGA scaffold system
Treatment method	Single topical application of treated gels onto dermal wound	Solution sprayed onto wound using a syringe at multiple time points	Implantation of protein-treated scaffolds into hind-limb ligation site
Advantages	<ul style="list-style-type: none"> • Highly reproducible wound model with well-defined stages of healing • Dermal wounds highly accessible to manipulation and topical treatment • Pluronic gel is commonly used in clinical drug delivery applications 	<ul style="list-style-type: none"> • Highly reproducible wound model • Dermal wounds highly accessible to manipulation and topical treatment • Thicker epidermis in rat compared with mouse • Targeting a conserved ECM component in mammals ensures relevance in humans 	<ul style="list-style-type: none"> • Highly reproducible model of chronic ischemia with stable loss of perfusion over time • SCID model used to minimize inflammation-induced angiogenesis • PLGA is a commonly used polymer in clinical applications
Limitations	<ul style="list-style-type: none"> • Rodent wounds heal primarily by contraction • Scarring is minimal in mouse wounds compared with humans • Mouse epidermis very thin compared with humans 	<ul style="list-style-type: none"> • Rodent wounds heal primarily by contraction • Excisional wound healing in rat is less well characterized than mouse • STZ-induced diabetes is a model of delayed—not chronic—wound healing 	<ul style="list-style-type: none"> • Model doesn't share important hallmarks of tissue repair • Proof-of-concept study with minimal direct application to clinical practice

CBD, collagen-binding domain; ECM, extracellular matrix; FT, full-thickness; FVB, Friend leukemia virus, strain B; PBS, phosphate-buffered saline; PLGA, poly(lactic-co-glycolic) acid; SCID, severe combined immunodeficiency; STZ, streptozocin; VEGF, vascular endothelial growth factor.

blood vessel density as well as MAPK signaling, whereas TAT-Spry2^{Y55F} moderately increased blood vessel density and MAPK signaling in the healing wound. The results demonstrate that endogenous Spry2 downregulates angiogenesis in the healing dermal wound, potentially by inhibiting the MAPK signaling pathway.

While the previous study explored the physiological control of wound angiogenesis, Yan *et al.*¹⁷ sought to improve healing by therapeutically induced angiogenesis. The authors engineered VEGF with a CBD to produce a CBD-VEGF fusion protein that counteracts the short half-life of exogenous VEGF by specifically targeting it to a common ECM component (Fig. 1). CBD-VEGF had been shown to specifically bind collagen-I and retain pro-angiogenic activity *in vitro* and in a myocardial infarction model *in vivo*.²⁰ To assess the ability of the fusion protein to improve healing of experimental diabetic wounds, 2 cm × 2.5 cm FT excisional dermal wounds made on the dorsal surface of rats were topically treated with CBD-VEGF, native VEGF, or PBS at multiple time points post-injury. Healing rates were analyzed and, between the three groups, CBD-VEGF treated wounds were observed to heal significantly faster. Wound tissue at day 7 postinjury showed about a twofold increase in blood vessel density in the CBD-VEGF group

compared with the native VEGF group. Importantly, CBD-VEGF was retained in the tissue up to 21 days postwounding, whereas native VEGF diffused into the serum soon after administration. The results indicate that collagen-binding VEGF efficiently promotes blood vessel growth and improves healing in experimental diabetic wounds.

The studies just described experimentally regulated the angiogenic response by topically treating healing tissue with modified exogenous mediators. In contrast, Yuen *et al.*¹⁸ used scaffold-directed delivery of angiogenic mediators in a unique way. To mimic the tight regulation of morphogenesis, which is often achieved by the combined use of stimulatory and inhibitory factors in nature, the goal was to establish, *in vivo*, a spatially restricted zone of stable blood vessel growth via co-delivery of VEGF and its direct inhibitor, anti-VEGF antibody (Fig. 1). A three-layer biodegradable polymer scaffold system was developed, where a central layer containing VEGF was sandwiched in-between two layers containing anti-VEGF or blank scaffold. *In vitro* and mathematical models of protein release kinetics were utilized to establish ideal dosage ratios and predicted outcomes. To test the system *in vivo*, a SCID mouse hind-limb ischemia model was developed via unilateral ligation of hind-limb blood vessels, and scaffolds were implanted in an orientation

Figure 1. Approaches to the specific regulation of angiogenesis in the context of VEGF biology. Pro-angiogenic strategies include scaffold-directed delivery of VEGF and treatment with collagen-binding (CBD-modified) VEGF, both of which act to increase functional VEGF in the ECM, where it can bind to VEGFR on ECs and initiate pro-angiogenic MAPK signaling; another strategy is to target endogenous negative feedback loop inhibitor of MAPK signaling, Spry2, by treating with EC-permeable (TAT-modified) dominant-negative mutant of Spry2 (Spry2^{Y55F}), which ultimately acts to promote pro-angiogenic signaling. Anti-angiogenic strategies include scaffold-directed delivery of anti-VEGF antibody, which binds and disables free VEGF in the ECM, and increasing the amount of Spry2 in EC by treating with EC-permeable (TAT-modified) Spry2, which then acts to inhibit pro-angiogenic MAPK signaling. CBD, collagen-binding domain; EC, endothelial cell; ECM, extracellular matrix; MAPK, mitogen-activated protein kinase; Spry2, Sprouty2; STZ, streptozocin; TAT, transactivator-of-transcription; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

that was parallel to the direction of the severed vessels such that the anti-VEGF layers were on either side of the desired angiogenic zone. At 4 weeks postimplantation, it was observed that delivery of VEGF in all scaffold types resulted in about a twofold increase in blood vessel density in the scaffold and underlying muscle. Notably, vessel formation also increased in blank scaffold layers adjacent to the central layer containing VEGF, whereas scaffold layers containing anti-VEGF inhibited vessel growth into these layers as well as into the muscles underlying the anti-VEGF scaffolds. Thus, a spatially restricted zone of stable blood vessel growth was established in the hind-limb ischemia model.

INNOVATION

Wietecha *et al.*¹⁶ elucidated a novel VEGF signaling control mechanism during wound healing, which may be exploited in therapy. Topical treatment of wounds with intracellular mediators of this mechanism was achieved by innovative engineering of the proteins with a peptide that enabled cell

entry. The use of a controlled-release gel as a delivery system prolonged the exposure of target tissue to the single topical treatment (Fig. 1).

The study by Yan *et al.*¹⁷ is innovative in its consideration of the wound microenvironment during treatment, as the ECM and its remodeling are crucial in the control of blood vessel dynamics. Importantly, the study shows that the ECM can be exploited in wound therapy for the increased retention of bio-active proteins. By immobilizing VEGF in the wound ECM, the fusion protein was able to counteract the short half-life of and function more effectively than native VEGF as a pro-angiogenic mediator in diabetic dermal wounds (Fig. 1).

The innovative study by Yuen *et al.*¹⁸ considered the spatial control of angiogenesis during healing. The study showed that by using an inhibitor simultaneously with VEGF, the spatio-temporal aspects of angiogenic mediator release from the scaffold were more predictably controlled and would lead to a restricted and highly functional angiogenic zone in a hind-limb ischemia model (Fig. 1).

CAUTION, CRITICAL REMARKS, AND RECOMMENDATIONS

Currently, no animal model exists that adequately mimics the pathology of human chronic wounds. There are already many animal studies showing improvement to wound repair, including diabetic impaired wound repair, with various therapies. However, translation of these therapies to the bedside has been inadequate to make significant improvements in clinical care. Although the reviewed studies are exciting, the findings require validation in human subjects.

It is unknown whether the angiogenic control mechanisms described by Wietecha *et al.*¹⁶ involving Spry2 are altered in cases of delayed wound repair in animals and humans. If such intracellular mechanisms that limit the potentiation of pro-angiogenic signaling pathways are found in humans, then it is likely that they counteract growth factor mediated therapies, thus limiting the pro-angiogenic effects of topical PDGF and VEGF in the clinic. It may prove necessary to target these inhibitory mechanisms in conjunction with growth factor therapy so as to maximize treatment efficacy.

Additional exploration of treatment with CBD-VEGF fusion proteins described by Yan *et al.*¹⁷ is needed, especially with regard to time, method of delivery, and dose. Potential side effects of possible over-stimulation with VEGF should be considered, that is, increased permeability of vessels leading to edema and inflammation. All things considered, efforts should be made to localize such fusion proteins to specific areas of a healing wound. For example, it is known that the provisional matrix of early wounds differs in composition from the ECM of more mature wounds; therefore, targeting VEGF to the provisional matrix of chronic wounds may be more effective in the clinic and limit potential side effects in mature, healthy tissue.

Heterogeneous scaffold systems, such as those described by Yuen *et al.*,¹⁸ should be assessed in an experimental model of wound repair to evaluate whether it is capable of controlling blood vessel growth in a more complex system that includes inflammation and ECM remodeling. Given the controversy regarding anti-VEGF in cancer therapy, different combinations of endogenous angiogenic mediators should be studied. Such experiments would provide novel data about how diverse pro- and anti-

angiogenic factors interact to control angiogenesis *in vivo*.

FUTURE DEVELOPMENT OF INTEREST

Future studies are needed to explore other important factors and pathways regulating blood vessel regression so as to understand how it occurs during physiological wound healing. Exploration into what aspects of this process may be dysregulated in pathological wounds will lead to better, more directed therapies to correct affected angiogenic phenotypes.

Engineering of more versatile fusion proteins that can participate in multiple aspects of wound repair while maximizing retention in the constantly changing wound microenvironment promises to be a worthwhile effort for future development.

Another exciting area is the further development of bioengineered drug delivery systems such as scaffolds that can release important factors in a time- and spatially-controlled fashion depending on the specific needs of the clinical wound. To this end, biosensors need to be integrated that can monitor

TAKE-HOME MESSAGES

Basic science advances

- Identification of a novel endogenous inhibitor further adds to the knowledge of physiological control mechanisms during wound angiogenesis.¹⁶
- Development of fusion proteins with enhanced biological functions is an exciting new trend in wound healing research.¹⁷
- Proof of concept that spatial control of blood vessel growth can be achieved *in vivo* using pro- and antiangiogenic agents simultaneously.¹⁸

Clinical science advances

- Topical application of cell-permeable modulators of pro-angiogenic cell signaling pathways may serve as a new therapeutic approach in the clinic.¹⁶
- Increasing retention of exogenously applied growth factors via ECM binding mechanisms may lead to more efficient topical therapies.¹⁷
- Use of common biocompatible polymer to make a complex scaffold system for directing a complex biological process *in vivo* opens up possibilities for advanced therapy.¹⁸

Relevance to clinical care

- Targeting important signaling pathways may be a robust method of correcting dysfunctional angiogenesis in patients alone or in conjunction with current therapies.¹⁶
- Binding important bioactive proteins to components of wound microenvironment may overcome major obstacles in current topical therapy of chronic wounds.¹⁷
- The ability to direct therapeutic angiogenesis using a scaffold with predictable release kinetics of angiogenic mediators may be of great value in the clinic.¹⁸

levels of important mediators in clinical wounds so that therapy can be more directed and case-specific.

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