Vascular Endothelial Growth Factor Delivery via Gene Therapy for Diabetic Wounds: First Steps

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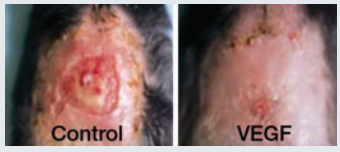

VEGF-stimulated wound healing at day 16.

Nonhealing wounds are a major complication of diabetes. Diabetic foot ulcerations (DFUs) affect 12–15% of patients with diabetes mellitus during their lifetime (Reiber *et al*., 1995). As a result, DFUs impair quality of life, cause significant morbidity, and account for 20% of all diabetes-related hospital admissions. A majority of neuropathic DFUs fail to heal even after 5 months of therapy, and foot ulcers precede 85% of nontraumatic amputations in patients with diabetes, indicating a need for better therapy (Margolis *et al*., 1999).

One growth factor—topically applied recombinant platelet-derived growth factor—is currently approved by the US Food and Drug Administration for the treatment of neuropathic DFUs (Boulton *et al*., 2004). Its clinical success is limited in part by the need for daily application and the hostile proteolytic wound environment in which the protein is applied. Sustained growth factor delivery would have advantages, and vascular endothelial growth factor (VEGF), a potent angiogenesis-stimulating growth factor, is thought to have therapeutic potential for diabetic vascular disease and ulcers.

In a study reported in this issue, Brem and colleagues (2009) sought to determine whether one isoform of VEGF, $VEGF_{165}$, delivered to wounds by an adenovirus vector (ADV), would improve healing. After the investigators had established the ability of the $ADV/VEGF_{165}$ to produce VEGF effectively, they used it to treat wounds in nonobese diabetic and db/db mice. $ADV/VEGF_{165}$-treated wounds exhibited accelerated wound healing. The authors then studied the mechanisms by which $ADV/VEGF_{165}$ works. They found that $ADV/VEGF_{165}$ enhanced tensile stiffness, epithelialization, and collagen deposition.

Through the following questions, we examine this paper in greater detail. For brief answers, please refer to the supplementary material online.

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Brem H, Kodra A, Golinko MS, Entero H, Stojadinovic O, Wang VM *et al.* (2009) Mechanism of sustained release of vascular endothelial growth factor in accelerating experimental diabetic healing. *J Invest Dermatol* 129:2275–87

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QUESTIONS

1. What was the rationale for using VEGF to treat diabetic wounds?
2. What studies were carried out to demonstrate that VEGF improved diabetic wound healing?
3. What methods were used to study the mechanisms by which VEGF improved wound healing?
4. What are the limitations of this study?
5. What obstacles may prevent the application of findings from animal wound healing studies to the treatment of human chronic wounds?

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Nonhealing wounds are a major complication of diabetes. Diabetic foot ulcerations (DFUs) affect 12–15% of patients with diabetes mellitus during their lifetime (Reiber *et al.*, 1995). As a result, DFUs impair quality of life, cause significant morbidity, and account for 20% of all diabetes-related hospital admissions. A majority of neuropathic DFUs fail to heal even after 5 months of therapy, and foot ulcers precede 85% of nontraumatic amputations in patients with diabetes, indicating a need for better therapy (Margolis *et al.*, 1999).

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Through the following questions, we examine this paper in greater detail.

1. What was the rationale for using VEGF to treat diabetic wounds?

Diabetic foot ulcers (DFUs) are one of the most common complications of diabetes and a leading cause of hospitalization for diabetic patients. For a patient living with diabetes, the lifetime risk of developing a DFU is estimated to be 25% (Boulton *et al*. 2008; Setacci *et al.*, 2009). Setacci *et al*. estimated the annual incidence of DFUs to be 1–4%, and Young *et al*. (1993) noted in their multicenter study of 6,487 diabetic patients that in patients with type 2 diabetes over the age of 60, more than 50% had peripheral neuropathy, a condition associated with the majority of DFUs. Owing to the prevalence of DFUs, significant health-care resources are spent on the management of ulcers,

including emergency room visits, antibacterial medications, amputations, and a multitude of other therapies directed at nonhealing wounds.

Diabetic patients are found to have many risk factors affecting wound healing, from the microenvironment to the macroenvironment. Patients with diabetes often suffer from micro- and macrovascular disease. Nerve ischemia due to thickened endoneural blood vessel walls is implicated as a cause of neuropathy in these individuals (Ceriello *et al*. 1999). Patients with peripheral neuropathies are at greater risk for ulcer formation because of decreased sensation, but other effects of neuropathy may contribute to ulcer development. Foot deformities, such as Charcot foot and hammertoes, have also been implicated in impaired wound healing (Falanga, 2005). Many factors place the diabetic patient at increased risk for developing an ulcer, but these patients also experience difficulty with wound healing. Microvascular disease not only affects nerves but also produces thickened basement membrane and arteriolar hyalinosis—conditions that undermine effective wound healing by negatively impacting physiological exchanges and leading to decreased migration of leukocytes and increased risk for infection (Falanga, 2005). Phenotypic alterations in wound cells in patients with diabetes may contribute to inhibited wound healing. Normal epithelialization of acute wounds undergoes a progression of stages, including migration, proliferation, and differentiation (Usui *et al*., 2008); the cells of diabetic wounds are described as being fixed in the proliferative stage (Falanga, 2004).

Angiogenesis is necessary for granulation tissue formation as well as for providing oxygen and nutrition to wounds (Li *et al.*, 2003). Inadequate angiogenesis in diabetic patients inhibits wound healing. By examining the role of vascular endothelial growth factor (VEGF), which promotes angiogenesis, the authors sought to promote wound healing in patients with diabetes.

VEGF is one of the most potent growth factors in stimulating angiogenesis. It triggers endothelial cell division, chemotaxis, and vascular permeability (Ferrara, 1999; Yamagishi *et al*., 1999; Griffoen and Molema, 2000; Brkovic and Sirois, 2007; Gavard and Gutkind, 2006) VEGF acts in a paracrine manner on dermal microvessels and endothelial cells. It also promotes the production of nitric oxide, which enhances collagen deposition.

VEGF's main limitation is its short duration of action. In previous experiments using VEGF to promote wound healing, frequent applications were required to maintain its efficacy; however, standard of care requires the application of compression bandages that remain in place for a week, making daily application of VEGF impractical for these patients. Researchers have dealt with this problem by using gene therapy to extend VEGF's duration of action; a vector holds and delivers VEGF to ensure continued administration for a period of 1–2 weeks.

Previous studies with VEGF demonstrated its efficacy in ischemic heart disease. In a gene therapy study by Rosengart *et al*. (1999), two groups of patients received AdVEGF121. The first group received injection during a coronary artery bypass graft procedure (CABG) to areas that could not be bypassed, and the second group, in whom CABG could not be performed, received VEGF injection as their sole therapy. The first group could not be evaluated accurately, as two interventions were made. The researchers did note a trend toward decreased ischemia compared with baseline in the second group, which received VEGF therapy only. The main contribution of this study, however, was delineating the safety profile of the therapy rather than determining its efficacy.

In 2000, Laitinen *et al*., in a randomized double-blinded control study, examined catheter-mediated VEGF plasmid versus percutaneous translumenal coronary angioplasty (PTCA). Patients received VEGF after angioplasty but prior to stent implantation. Control subjects received β-galactosidase or Ringer's lactate. The investigators concluded that catheter-mediated gene transfer therapy was safe and well tolerated for coronary heart disease. At the 6-month evaluation with

angiography, no clinical effects were noted; however, the microvascular angiogenesis expected from this therapy may not have been noted by angiography (Laitinen *et al*., 2000).

Hedman *et al*. (2003) examined patients receiving VEGF via a catheter during PTCA. In this study, participants were administered VEGF plasmid liposome, a VEGF adenovirus, while controls received only Ringer's lactate. At the 6-month follow-up, patients who had received the VEGF-adenovirus showed a significant improvement in myocardial perfusion.

More recently, a replication-deficient adenoviral vector carrying VEGF121 (AdVEGF121) was administered to patients via thoracotomy; these patients were compared with others receiving maximal medical treatment for severe angina pectoris (Stewart *et al*., 2006). Patients receiving AdVEGF121 therapy demonstrated a significant improvement in exercise capacity and symptoms. The researchers also presented data from the autopsy of a participant who died from cardiogenic shock following perioperative myocardial infarct as evidence of the benefits of therapy: robust neovascularization was evident in the areas that had been injected with AdVEGF121, whereas no new vascularization was observed in other areas (Stewart 2006).

2. What studies were carried out to demonstrate that VEGF improved diabetic wound healing?

As a model for type 2 diabetes with increase in lipids, obesity, and insulin resistance, the researchers used the db/db mouse, which is homozygous for a mutation making the mouse leptin resistant. These mice become obese by 3–4 weeks of age, with elevated blood sugar at 4–8 weeks of age. Wound healing in these mice is noted as being delayed. As a model for type 1 diabetes, the researchers chose the nonobese diabetic (NOD) mouse, which has a spontaneous phenotype of insulitis. As in humans and mice with type 1 diabetes, there is infiltration of the islets with macrophages; dendritic cells; and CD4, CD8, and B cells (Giarratana *et al*., 2007). These models allowed the authors to study wound healing in type 1 and type 2 diabetes.

VEGF accelerates time to wound closure. Wounds were created in obese diabetic mice (db/db). Wounds in mice treated with VEGF were found to heal 6.6 days sooner than those in controls. Some mice in this group that received high concentrations of VEGF had an increased mortality.

VEGF promotes re-epithelialization, defined as either wound contraction or decreased area of open wound and increased area of normal skin pulled into wound. The NOD mice treated with VEGF were found to have increased re-epithelialization compared with controls.

VEGF increases tensile stiffness. On day 21, when tensile strength in obese diabetic mice was examined, no significant differences were noted in load to failure or in stiffness in the NOD mice.

3. What methods were used to study the mechanisms by which VEGF improved wound healing?

Mechanism of VEGF and epithelialization: after determining that VEGF accelerated the time to closure and epithelialization, Brem *et al.* attempted to establish whether this was attributable to expedited keratinocyte migration. A migration assay was performed comparing VEGF-treated keratinocytes with nontreated keratinocytes. The VEGF-treated keratinocytes showed increased cell migration after 24 hours. To ensure that the results of this study represented migration of the keratinocytes and not proliferation, the researchers repeated the experiment, treating the keratinocytes with mitomycin-C, which inhibits proliferation. The migration results remained significant in the VEGF-treated cells. The investigators characterized the keratinocytes affected by VEGF by repeating the scratch migration assays using different levels of calcium (which induces keratinocyte differentiation) in the medium. VEGF was found to specifically target only activated keratinocytes

that participate in re-epithelialization (lower calcium environment). Differentiating keratinocytes (higher calcium environment) were not affected by VEGF.

The investigators sought to determine how VEGF promotes collagen deposition (which in turn promotes wound contraction). On histological examination, the investigators noted that VEGF-treated wounds had increased granulation tissue with larger and more abundant blood vessels than wounds treated with saline or empty virus particles. Sirius red staining viewed with polarized light microscopy revealed that VEGF-treated wounds demonstrated greater amounts of and more organized collagen compared with controls, which had short, random collagen bunches. Interestingly, a third group treated with empty virus particles demonstrated longer collagen that was parallel. It was suggested that this could be attributed to a more intense inflammatory response in these wounds, prompted by the empty virus particles.

Mechanism of VEGF and collagen deposition: in an effort to determine the effects of VEGF on fibroblasts, the investigators performed scratch migration assays using primary fibroblasts grown from patients' wounds. VEGF had the greatest effect on fibroblasts from the healing edge, whereas fibroblasts from the nonhealing edge were unaffected by VEGF.

In summary, a well-established proangiogenic growth factor, VEGF, may stimulate wound healing via multiple mechanisms: by promoting epithelialization, inducing granulation tissue formation and collagen deposition, and promoting angiogenesis.

4. What are the limitations of this study?

One of the limitations of this study is the lack of other control groups, such as a nondiabetic control. The authors may have had the opportunity to further elucidate VEGF in wound healing by using such controls. In addition, the wounds studied were not chronic nonhealing wounds, but wounds created in mice by the researchers immediately prior to the experiments. While an accepted model, these healing-impaired acute wounds do not accurately represent human DFUs, which are often nonhealing—as opposed to slow-healing—ulcers.

The investigators inadequately address the high mortality in the mice treated with high-dose adenovirus vector (ADV)-VEGF (the dose that achieved the best results). The authors attribute the high mortality of these mice to intolerance of anesthesia. The high mortality is inconsistent with previous studies, which demonstrated a positive safety profile in humans.

5. What obstacles may prevent the application of findings from animal wound healing studies to the treatment of human chronic wounds?

Animal models do not duplicate human conditions exactly. For example, the wounds in these mice were on the back instead of the extremities (feet), where most diabetic wounds occur. The mouse models of diabetes are not exact replications of diabetes in humans, and the exact mechanism of diabetes has not been delineated. In other words, these mouse models may represent alternative mechanisms of acquiring diabetes. As stated before, the study examined impaired acute wounds rather than chronic wounds. Acute and chronic wounds respond differently to treatment, with differing attendant growth factors, cytokines, and inflammatory mediators. Therefore, results from an acute wound study may not translate directly to chronic wounds. A noncontracting wound model on the tail of the mouse exists, but it is unclear whether this is a better model. In the future, Brem *et al*. may wish to explore the use of VEGF in acute wounds in humans by applying ADV-VEGF to punch biopsy sites. Issues related to the increased mortality observed in higher-dose groups should also be addressed. A clinical trial is nearing completion that utilizes ADV gene delivery (ADV-PDGF) for diabetic neuropathic foot ulcers. Thus, the safety profile of this delivery mechanism may soon be established in humans.

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