# Mechanism of Sustained Release of Vascular Endothelial Growth Factor in Accelerating Experimental Diabetic Healing

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In this study, we hypothesize that local sustained release of vascular endothelial growth factor (VEGF), using adenovirus vector (ADV)-mediated gene transfer, accelerates experimental wound healing. This hypothesis was tested by determining the specific effects of $VEGF_{165}$ application on multiple aspects of the wound healing process, that is, time to complete wound closure and skin biomechanical properties. After showing accelerated wound healing *in vivo*, we studied the mechanism to explain the findings on multiple aspects of the wound healing cascade, including epithelialization, collagen deposition, and cell migration. Intradermal treatment of wounds in non-obese diabetic and db/db mice with ADV/$VEGF_{165}$ improves healing by enhancing tensile stiffness and/or increasing epithelialization and collagen deposition, as well as by decreasing time to wound closure. $VEGF_{165}$, *in vitro*, stimulates the migration of cultured human keratinocytes and fibroblasts, thus revealing a non-angiogenic effect of VEGF on wound closure. In conclusion, ADV/VEGF is effective in accelerating wound closure by stimulating angiogenesis, epithelialization, and collagen deposition. In the future, local administration and sustained, controlled release of $VEGF_{165}$ may decrease amputations in patients with diabetic foot ulcers and possibly accelerate closure of venous ulcers and pressure ulcers.

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## INTRODUCTION

It is estimated that in excess of 4.5 million patients with diseases of the skin and subcutaneous tissue visit emergency departments across the country each year (Nawar *et al.*, 2007). Of these, diabetic foot ulcers (DFUs) pose the most serious threat to patient safety. DFUs have multiple physiological impairments to healing, including impaired innervation (Gibran *et al.*, 2002), impaired cellular migration (Brem *et al.*, 2007), and inadequate angiogenesis (Cho *et al.*, 2006), that lead to extensive mortality and limb amputation in patients. Therefore, of all chronic wounds, DFUs are the most insidious, as many patients do not realize that they have a break in the skin, nor is the true extent of infection always noticed by clinicians on presentation. Foot ulcers have become one of the most common and severe complications of diabetes and are now the leading cause for hospitalization in patients with diabetes (Lavery *et al.*, 2007). The incidence of foot ulcers in the general population ranges between 2 and 5% (Abbott *et al.*, 2002; Rathur and Boulton, 2007). Persons with type 1 and type 2 diabetes have a 9.1% risk of developing a foot ulcer in their lifetime (Lavery *et al.*, 2006), and the presence of an ulcer may increase the risk of lower-extremity amputation by almost six fold, affecting hospital stay and survival rate (Valensi *et al.*, 2005; Davis *et al.*, 2006; Faglia *et al.*, 2006; Malay *et al.*, 2006).

Angiogenesis is a vital component of wound repair (Brem *et al.*, 1997; Folkman, 2007) and there is a need for a local, sustained therapy that can reverse this and other

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*Abbreviations: ADV, adenovirus vector; DFU, diabetic foot ulcer; EGF, epidermal growth factor; H&E, hematoxylin and eosin; HUVEC, human umbilical vein endothelial cell; VEGF, vascular endothelial growth factor; NOD, non-obese diabetic*

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physiological impairments. Despite multiple advances (Marston *et al.*, 2003; Veves *et al.*, 2001; Steed, 1995; Steed, 2006) and a recombinant form of growth factor with angiogenic effect—that is, PDGF, which has been approved by the FDA for use in DFUs (Smiell, 1998; Smiell *et al.*, 1999)—a sustained release formulation has yet to be approved. Vascular endothelial growth factor (VEGF) has shown positive and safe results when administered alone or as adjunctive therapy to angioplasty and surgery and in ischemic heart disease (Stewart *et al.*, 2006; Rosengart *et al.*, 1999a, b; Laitinen *et al.*, 2000; Hedman *et al.*, 2003). However, studies that further elucidate the mechanism of VEGF, which could better inform the design of clinical trial, are needed.

VEGF is one of the most vital and potent angiogenesis-stimulating growth factors, and it exists in many isoforms derived from alternative splicing (Robinson and Stringer, 2001). The most common isoform is $VEGF_{165}$. The functions of VEGF are multifold. It acts in a paracrine manner on both endothelial cells and dermal microvessels. Furthermore, it functions as an endothelial cell mitogen, a chemotactic agent, and an inducer of both vascular and skin permeability (Ferrara, 1999; Yamagishi *et al.*, 1999; Griffoen and Molema, 2000; Brkovic and Sirois, 2007; Gavard and Gutkind, 2006). One of the mediators of VEGF activity, nitric oxide, enhances collagen deposition in diabetic wounds and may restore endothelial function to improve both nerve conduction and tissue oxygenation (Witte *et al.*, 2002). Recombinant VEGF has been applied in experimental diabetic wounds *in vivo* and *in vitro* (Table 1) (Iwaguro *et al.*, 2002; Romano Di Peppe *et al.*, 2002; Shimpo *et al.*, 2002; Vajanto *et al.*, 2002; Chang *et al.*, 2003; Michaels *et al.*, 2005; Huang *et al.*, 2006; Saaristo *et al.*, 2006; Li *et al.*, 2007). However, recombinant VEGF requires frequent repeated topical applications for

**Table 1. A compilation of recent studies on therapeutic angiogenesis involving release of $VEGF_{165}$ in animal models**

| Authors | Delivery system/(species) | Assay | Endpoints |
|---|---|---|---|
| Huang *et al.* | ADV-$2 \times 10^{10}$ vp (rat) | Time to viable skin area | Increased skin flap viability. Increase in NO synthesis |
| Iwaguro *et al.* | ADV/VEGF ($1.5 \times 10^{4}$) vp (mice) | Laser Doppler perfusion imaging (LDPI): used to record blood flow measurements.<br>ELISA: used to quantify VEGF levels in plasma<br>Histology: to evaluate vascular density | Neovascularization and blood flow recovery improved. Limb necrosis/auto-amputation Reduced by 63.7% in comparison with control animals. |
| Makinen *et al.* | ADV/VEGF ($2 \times 10^{10}$) vp (human) | Digital subtraction angiography: to evaluate vascularity<br>ABI analysis | Increased vascularity in the VEGF-treated group. Improvement in ABI. |
| Chang *et al.* | Adeno-associated viral vectors ($5 \times 10^{11}$) vp (rat) | Immunohistochemistry: to determine cellular proliferation and capillary-to-muscle ratios<br>RTPCR: to detect hVEGH levels in muscles | Skeletal muscle oxygen tension normalized. Arteriogenesis induced. No significant increase in angiogenesis in rats with severe hind-limb ischemia. |
| Vajanto *et al.* | ADV-mouse VEGF—$2 \times 10^{10}$ vp (rabbit) | Angiography: for vascularization<br>Histology: to asses granulation levels<br>-ELISA: to detect VEGF concentration | Induction of angiogenesis. Hind limb and testes edema were observed. |
| Shimpo *et al.* | Adeno-associated virus with hVEGF—$2 \times 10^{13}$ vp (rat) | ELISA: to detect VEGF concentration<br>–RT-PCR: to assess gene expression<br>-Ultrasound Flowmeter: to detect Blood flow in arteries<br>Histology: to analyze capillary density. | Efficient and stable gene expression without ectopic expression. Stimulation of angiogenesis and improved blood flow. |
| Romano Di Peppe *et al.* | ADV/VEGF ($1 \times 10^{8}$) vp (mice) | Histology: check increase in granulation<br>Time to closure measurement | Reduction of time to closure of wound, and increase in granulation tissue density. |
| Saaristo A. *et al.* | ADV/VEGF ($1 \times 10^{8}$) vp (mice) | Histology: density of blood vessels<br>Time to full wound closure | Promotion of angiogenesis through increase in blood vessel density and reduction of time to closure of wound. |
| Li Y. *et al.* | ZFP-VEGF—125 μg (mice) | mRNA and protein quatification<br>Histology: capillary density | Induction of angiogenesis as observed and increase in capillary density. Increase in quantity of VEGF mRNA, protein |
| Kirchen LM. *et al.* | PEG-VEGF (1 μg) (mice) | Histology: chronic inflammation and neovascularization<br>Time to full wound closure | Promotion of angiogenesis by an increase in neovascularization and a decrease in time to full closure of wound |
| Michaels J 5th. *et al.* | Topical treatment of VEGF 10 μg (mice) | Histology:neovascularization<br>Time to complete wound closure | Increase in neovascularization by VEGF treatment. Decrease in time to full closure |

ABI, ankle brachinl index; ADV, adenovirus vector; NO, nitric oxide; PEG, polyethylene glycol; VEGF, vascular endothelial growth factor, ZFP, Zinc Finger DNA protein.
Recombinant VEGF has been shown to stimulate angiogenesis, but has also caused serious side effects such as edema.

sustained drug levels in the local tissue, which may be a reason it has not been successful in clinical trials for DFUs. Because sustained release of VEGF has shown promise, but has not yet benefited patients with chronic wounds, we suggest that the mechanism of VEGF in wound healing may not be fully understood.

Gene therapy with VEGF is an attractive alternative as it can provide local, sustained release without systemic toxicity. Gene therapy has already proven effective in a variety of experimental wound healing models (Eming *et al.*, 1999; Chandler *et al.*, 2000; Crombleholme, 2000; Davidson *et al.*, 2000; Yao and Eriksson, 2000; Keswani *et al.*, 2004; Lee *et al.*, 2005b; Saaristo *et al.*, 2006). For gene therapy to be effective in chronic wounds, the gene must be efficiently delivered to the specific target cell in which it must be expressed and sustained locally without significant systemic absorption (Levy *et al.*, 2001; Aoyama *et al.*, 2003). Vector-mediated gene therapy meets these criteria (Galeano *et al.*, 2003). VEGF alone or in combination with other treatment modalities may prove to be an effective treatment for diabetic vascular disease and ulcers. This possibility is confirmed by the demonstration that gene therapy with VEGF restores the impaired angiogenesis found in ischemic limbs of diabetic mice (Rivard *et al.*, 1999). In this study we show that both *in vivo*, in experimental diabetic wound models, and *in vitro*, in primary cultured cells from patients with DFUs, a local sustained delivery of VEGF using adenovirus vector (ADV) promotes epithelialization and wound closure, enhances granulation tissue formation, increases tensile stiffness, promotes wound contraction, and increases collagen deposition. We conclude that ADV/VEGF accelerates wound healing in a diabetic wound model, underscoring its potential use as topical gene therapy in patients with DFUs.

## RESULTS

### ADV/VEGF$_{165}$ accelerates time to closure in db/db mice

To determine whether ADV/VEGF$_{165}$ accelerates wound healing, time to wound closure was determined using four different doses of ADV/VEGF$_{165}$. Wounds treated with ADV/VEGF$_{165}$ healed 6.6 days sooner than controls (Figure 1). Treated wounds healed in $27.2 \pm 1.4$ days. Saline-treated wounds healed in $34.2 \pm 7.0$ days, whereas wounds that were treated with the virus vector alone healed in $33.5 \pm 6.5$ days. Statistical significance ($P<0.05$) was noted after comparison of the $5 \times 10^{11}$ vp per wound VEGF$_{165}$-treated group and control groups (Table 2). However, such high doses of VEGF$_{165}$ may have a toxic effect in the mice used in the study, as the incidence of mortality in the high-dosage VEGF$_{165}$-treated group was greater than in other groups. A minimum 10% increase in mortality relative to controls was found at ADV/VEGF doses of $5 \times 10^{9}$ vp per wound and higher.

### ADV/VEGF$_{165}$ promotes epithelialization

To determine the effects of ADV/VEGF on re-epithelialization, we performed histological analysis and found increased re-epithelialization in $5 \times 10^{8}$ ADV/VEGF-treated wounds *versus* controls ($P<0.05$) in day 10 db/db diabetic mice (Table 3). Increased re-epithelialization was qualitatively

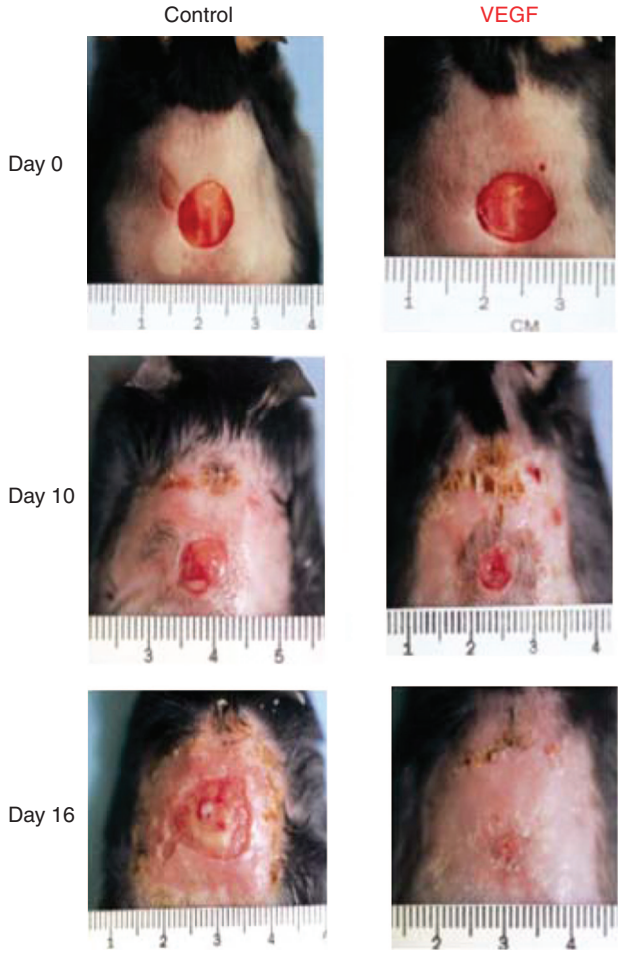


**Figure 1. ADV/VEGF$_{165}$ accelerates time to closure in db/db mice.** Photograph of the time to closure experiment showing accelerated time to closure in db/db mouse wounds treated with ADV/VEGF compared with wild-type control.

observed in samples from day 14 non-obese diabetic (NOD) mice treated with VEGF compared with the controls (data not shown). The width of the fourteenth day ADV/VEGF-treated open wounds was less than the width of the saline- and empty virus-treated wounds. In the ADV/VEGF$_{165}$-treated open wounds, wound contraction was enhanced, in that the surrounding skin was pulled into the wound, decreasing the area of open wound and increasing the area of normal skin covering the initial wound. Consistent with more rapid re-epithelialization, the volume of granulation tissue was reduced in the ADV/VEGF-treated wounds compared with the two other treated wound groups. Therefore, we conclude that in open wounds VEGF treatment enhanced re-epithelialization.

### Mechanism of VEGF and epithelialization

Our observation that ADV/VEGF accelerated time to closure and promoted epithelialization in mice prompted us to explore this finding *in vitro*. To establish whether VEGF stimulates keratinocyte migration, normal primary human epidermal keratinocytes were used in an *in vitro* scratch-wound assay. When normal human keratinocytes grown in

**Table 2. Effect of different concentrations of $VEGF_{165}$ on time to wound closure in *BKS.Cg-m+/+Lepr$^{db}$* type 2 diabetic male mice**

| | Time to closure in db/db male mice | |
|---|---|---|
| **Treatment** | **Dosage (vp per wound)** | **Mean time to closure ± SD (days)** |
| $ADV/VEGF_{165}$ | $5.0 \times 10^{11}$ | 25.4 ± 3.9 |
| | $1.6 \times 10^{11}$ | 27 ± 4.3 |
| | $5.0 \times 10^{10}$ | 28 ± 5.8 |
| | $1.6 \times 10^{10}$ | 28.6 ± 3.2 |
| Saline | Saline | 34.2 ± 7.0 |
| DL-312 ($5 \times 10^8$ vp) (vehicle control) | $5 \times 10^{11}$ | 33.5 ± 6.5 |

| ***P*-values** | **$ADV/VEGF_{165}$ ($5.0 \times 10^{11}$)** | **$ADV/VEGF_{165}$ ($1.6 \times 10^{11}$)** | **$ADV/VEGF_{165}$ ($5.0 \times 10^{10}$)** | **$ADV/VEGF_{165}$ ($1.6 \times 10^{10}$)** |
|---|---|---|---|---|
| Saline | 0.028 | 0.070 | 0.145 | 0.173 |
| DL-312 ($5 \times 10^8$ vp) | 0.029 | 0.08 | 0.125 | 0.147 |

ADV, adenovirus vector; VEGF, vascular endothelial growth factor.
Data indicate that administration of $VEGF_{165}$ leads to a decrease in the time it takes for wounds to close. On average wounds treated with $VEGF_{165}$ healed 6.6 days faster than the wounds that did not get treated with it.

**Table 3. Histological grading and scores of day 10 wounds at incision site of female *BKS.Cg-m+/+Lepr$^{db}$* type 2 diabetic mice**

| | | H&E | |
|---|---|---|---|
| **Treatment** | ***n*** | **Epithelialization (0–4) ± SD** | **# of cells/HPF ± SD** |
| Treated (ADV/hVEGF $5 \times 10^8$ vp per wound) | 8 | 3.6 ± 0.8 | 453 ± 156 |
| DL-312 $5 \times 10^8$ vp per wound | 7 | 2.1 ± 1.1 | 283 ± 104 |
| Saline | 8 | 2.6 ± 0.7 | 335 ± 57 |

| **Comparison** | ***P*-values** |
|---|---|
| *ADV/VEGF vs saline* | |
| H&E: Epith (0–4) | 0.017 |
| *ADV/VEGF vs vehicle* | |
| H&E: Epith (0–4) | 0.008 |
| *Saline vs vehicle* | |
| H&E: Epith (0–4) | 0.170 |

ADV, adenovirus vector; H&E, hematoxylin and eosin; VEGF, vascular endothelial growth factor.
Statistically significant increase in epithelialization was observed after H&E staining of $ADV/VEGF_{165}$ and control groups.

tissue culture are ''wounded,'' they migrate over the scratch area to close the gap. Data from experiments suggested that $VEGF_{165}$ accelerated cell migration after 24 hours and further after 48 hours (Figure 2a). As expected, cells incubated with epidermal growth factor, which was used as a positive control because it is a potent stimulator of keratinocyte migration and proliferation, migrated significantly faster than untreated, control cells (Figure 2a). To distinguish whether VEGF affects migration directly or indirectly, by promoting proliferation, an analogous experiment with Mitomycin C treated keratinocytes was performed and produced similar results. $VEGF_{165}$ stimulated migration of Mitomycin C treated keratinocytes, thus confirming that VEGF has direct effects on keratinocyte migration (results not shown). Taken together, our results suggest that VEGF promotes epithelialization via migration of keratinocytes.

To test which keratinocyte phenotype is susceptible to VEGF stimulation, the effects of $VEGF_{165}$ were tested on wound healing and differentiating cell phenotypes. Keratinocytes grown in culture medium containing low calcium (0.09 mM) resemble the activated keratinocyte phenotype, the cells that actively participate in re-epithelialization.

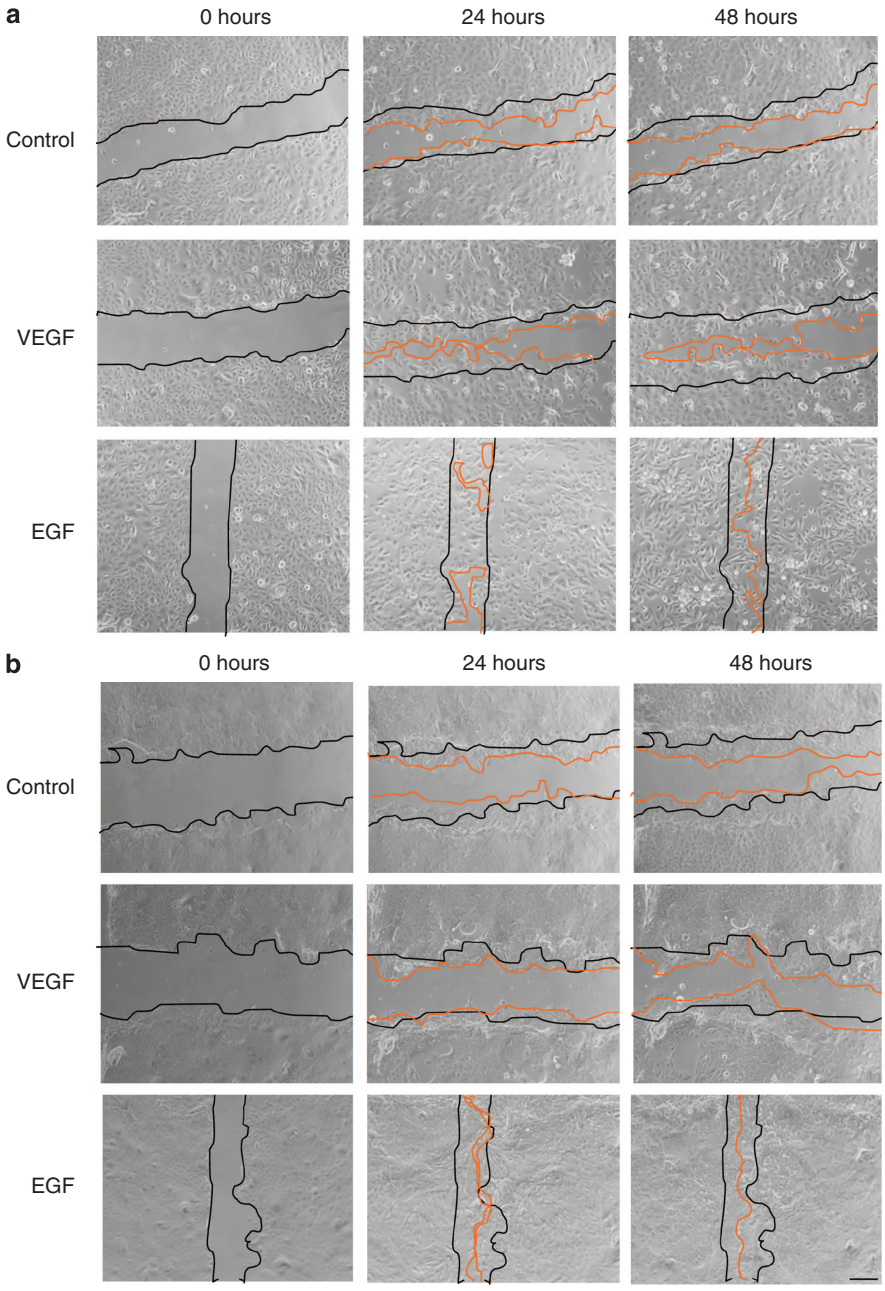


**Figure 2. VEGF promotes migration of activated, but not differentiating keratinocytes.** (**a**) Activated keratinocyte migration with the addition of VEGF and EGF (epidermal growth factor) at different time points after scrape wounding of the keratinocyte monolayer. Keratinocytes residing in low calcium are activated keratinocytes. $VEGF_{165}$ and EGF stimulated migration of activated keratinocytes over the wound area. These keratinocytes most closely resemble keratinocytes that are responsible for wound re-epithelialization. (**b**) Differentiated keratinocyte migration at different time points after release of $VEGF_{165}$ into the wound area. When maintained in high concentrations of calcium, keratinocytes become differentiated. These differentiated keratinocytes least resemble those keratinocytes that participate in wound re-epithelialization. Results show that $VEGF_{165}$ does not have a significant effect on their migration, but EGF continues to promote their migration in a scratch-wound assay. Bar = 0.05 mm.

However, keratinocytes grown in a high calcium (1.2 mM) medium resemble differentiating keratinocytes (Bikle *et al.*, 2004). As their proliferation rate decreases, they form desmosomal junctions and start stratifying in culture, contributing minimally to re-epithelialization. Interestingly, $VEGF_{165}$ did not stimulate migration of differentiated keratinocytes in scratch-wound assays (Figure 2b). This suggests that $VEGF_{165}$ specifically targets only keratinocytes that participate in re-epithelialization, and differentiating keratinocytes are not affected.

**ADV/VEGF increases tensile stiffness in db/db mice at day 21**

To determine how VEGF affects the quality of healing, mechanical properties such as load to failure and stiffness were measured for incisional wounds on female BKS. Cg-m +/+ $Lepr^{db}$ mice. Measurements were taken 10 and

**Table 4. Effect of different concentrations of $VEGF_{165}$ on tensile strength and stiffness in db/db mice**

| Group (Vol: 200 µl) | | Testing day | Age (wks) | Pre-wounding BW (g) ± SD | Pre-testing BW (g) ± SD | Mice (*n*) | Stiffness (N/mm) ± SD | Max load (*n*) |
|---|---|---|---|---|---|---|---|---|
| I | ADV/$VEGF_{165}$ ($5 \times 10^{10}$ vp) | 10 | 9 | 30 ± 1.7 | 30.1 ± 2.8 | 6 | 0.50 ± 0.2 | 0.83 ± 0.4 |
| II | ADV/$VEGF_{165}$ ($5 \times 10^{8}$ vp) | 10 | 9 | 30.8 ± 2.2 | 31.5 ± 3.1 | 6 | 0.59 ± 0.2 | 1.06 ± 0.4 |
| III | Saline (control) | 10 | 9 | 30.6 ± 4.1 | 34.1 ± 4.7 | 6 | 0.42 ± 0.1 | 0.78 ± 0.2 |
| IV | DL-312 ($5 \times 10^{8}$vp) (vehicle control) | 10 | 9 | 31.5 ± 3 | 33.2 ± 3.5 | 6 | 0.32 ± 0.2 | 0.54 ± 0.3 |
| I | ADV/$VEGF_{165}$ ($5 \times 10^{10}$vp) | **21** | 11 | 30 ± 1.8 | 32.9 ± 2.5 | 6 | 1.70 ± 0.2 | 4.27 ± 0.7 |
| II | ADV/$VEGF_{165}$ ($5 \times 10^{8}$vp) | **21** | 11 | 32 ± 3.0 | 34.1 ± 4.3 | 6 | 1.53 ± 0.4 | 4.46 ± 0.9 |
| III | Saline (control) | **21** | 11 | 31.2 ± 1.9 | 34.2 ± 4.3 | 6 | 1.17 ± 0.15 | 3.57 ± 0.6 |
| IV | DL-312 ($5 \times 10^{8}$vp) (vehicle control) | **21** | 11 | 31.1 ± 2.3 | 33.2 ± 1.6 | 6 | 1.13 ± 0.2 | 3.72 ± 0.6 |

ADV, adenovirus vector; VEGF, vascular endothelial growth factor.
Results indicate that release of $VEGF_{165}$ at the wound area leads to an increase in the tensile stiffness as compared with controls.
Bold text indicates day at which significant difference in tensile stiffness was observed.

**Table 5. Effect of different concentrations of $VEGF_{165}$ on skin stiffness in NOD mice**

| Group (Vol: 200 µl) | | Test day | Age (wks) | HbA1c (%) ± SD | Pre-wounding BW (g) ± SD | AM glucose (mg per 100 ml) ± SD | Glucose (2 hours-post insulin) DAY 0 ± SD | Glucose (5 hours-post insulin) DAY 7 ± SD | Pre-testing BW (g) ± SD | AM Glucose (mg per 100 ml) ± SD |
|---|---|---|---|---|---|---|---|---|---|---|
| A | ADV/$VEGF_{165}$ ($5 \times 10^{8}$ vp) | 14 | 30 | 7.8 ± 2.1 | 26.6 ± 1.1 | 553 ± 110 | 179 ± 126 | 237 ± 149 | 27.6 ± 1.8 | 519 ± 164 |
| B | Saline (control) | 14 | 30 | 7.5 ± 2.2 | 26.4 ± 1.5 | 469 ± 168 | 209 ± 114 | 125 ± 101 | 27.5 ± 2.7 | 498 ± 157 |
| C | DL-312 ($5 \times 10^{8}$vp) (vehicle control) | 14 | 30 | 7.6 ± 2.1 | 26.4 ± 1.3 | 502 ± 170 | 228 ± 121 | 164 ± 142 | 27.9 ± 1.5 | 526 ± 157 |

| | | Mice (*N*) | Mortality (*n*) | Stiffness (N/mm) ± SD | Load to failure (*N*) ± SD |
|---|---|---|---|---|---|
| A | ADV/$VEGF_{165}$ ($5 \times 10^{8}$ vp) | 10 | 0 | 1.47 ± 0.4 | 3.03 ± 0.8 |
| B | Saline (control) | 10 | 0 | 1.50 ± 0.3 | 3.26 ± 1.0 |
| C | DL-312 ($5 \times 10^{8}$vp) (vehicle control) | 10 | 0 | 1.49 ± 0.3 | 3.62 ± 0.8 |

ADV, adenovirus vector; NOD, non-obese diabetic; VEGF, vascular endothelial growth factor.
Results indicate that delivery of $VEGF_{165}$ at the wound site in mice does not lead to a significant increase in tensile strength and stiffness as compared with controls.

21 days after wounding of treatment groups, which received $5 \times 10^{8}$ or $5 \times 10^{10}$ vp per wound, and saline and vehicle controls. On postoperative day 10, the loads to failure (mean ± SD) were 0.83 ± 0.4 and 0.78 ± 0.2 N for groups I and III. Groups II and IV had maximum loads at 1.06 ± 0.4 and 0.54 ± 0.3 N, respectively (Table 4). Stiffness (mean ± SD) values were 0.50 ± 0.2, 0.59 ± 0.2, 0.42 ± 0.1, and 0.32 ± 0.2 N/mm for groups I, II, III, and IV, respectively (Table 4). No significant mechanical changes had appeared by 10 days, comparing the ADV/VEGF-treated groups and the control groups. On postoperative day 21 in the BKS.Cg-m +/+ $Lepr^{db}$ full-thickness incisional wounds, the loads to failure (mean ± SD) were 4.27 ± 0.70, 4.46 ± 0.90, 3.57 ± 0.60, and 3.72 ± 0.60 N (Table 4). No statistically significant changes were obtained between the groups. Stiffness values (mean ± SD) were 1.70 ± 0.20, 1.53 ± 0.40, 1.17 ± 0.15, and 1.13 ± 0.20 N/mm for ADV/$VEGF_{165}$ $5 \times 10^{8}$ vp per wound, ADV/$VEGF_{165}$ $5 \times 10^{10}$ vp per wound, vehicle, and saline groups (Table 4). A statistically significant ($P<0.05$) change was observed between the ADV/$VEGF_{165}$ $5 \times 10^{10}$ vp per wound and the vehicle control group. There was no statistically significant difference in tensile stiffness at day 21 in the NOD mice (Table 5).

### ADV/$VEGF_{165}$ promotes wound contraction through collagen deposition

Differences in the cellular density of ADV/$VEGF_{165}$-treated wounds compared with saline and empty virus-treated wounds were clearly shown histologically (Figure 3). Saline-treated, control full excisions showed complete re-epithelialization and contained typical granulation tissue with a moderate cell density and prominent blood vessels

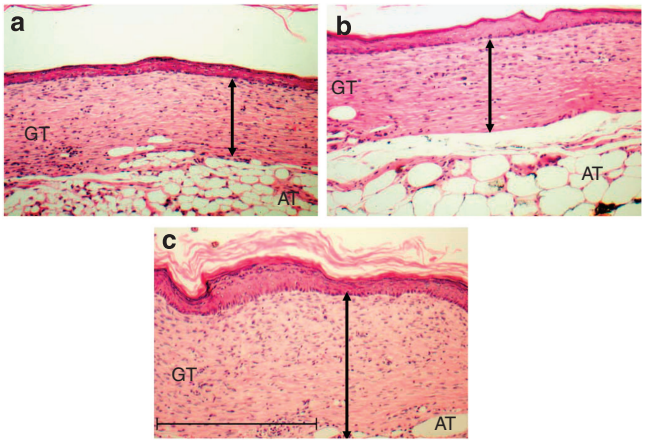


**Figure 3. ADV/VEGF$_{165}$ enhances granulation tissue deposition.** Typical H&E-stained histological sections from non-obese diabetic (NOD) mice showing healing open wounds at 14 days. Panel (**a**) originates from a saline-treated wound with an intact epidermal cell layer covering the granulation tissue (GT), which is associated with the underlying adipose tissue (AT) layer. Panel (**b**) originates from an empty virus particle-treated wound that was covered by an intact epidermal cell layer above GT that covers the adipose tissue. Panel (**c**) originates from an ADV/VEGF165-treated wound, in which the epidermal layer and GT layer are thicker in depth and the granulation tissue contains greater cell density with numerous large blood vessels. Arrows indicate granulation tissue deposition. Bar = 0.08 mm.

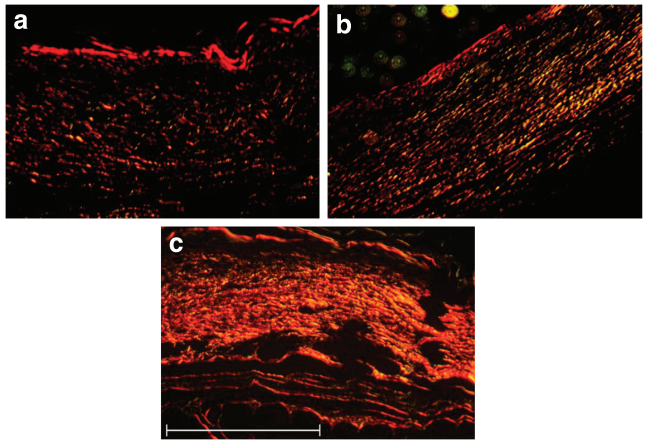


**Figure 4. ADV/VEGF$_{165}$ enhances collagen organization.** Sirius red-stained histological sections from healing open wounds in non-obese diabetic (NOD) mice were viewed with polarized light. Panel (**a**) is from a control, saline-treated full excision wound showing fragmented spots of birefringence with little intensity, due to minimal organization of collagen fiber bundles. The intense red birefringence on the upper surface of the section is from the keratin associated with a new epidermal surface layer. Panel (**b**) is from an empty viral particle-treated full excision wound that shows a more continuous birefringence pattern of greater intensity compared with the control. This birefringence pattern is a consequence of longer collagen fiber bundles that are arranged in parallel arrays. Panel (**c**) is from an ADV/VEGF165-treated full excision wound with very long collagen fiber bundles showing an intense red, continuous birefringence pattern, which is consistent with more uniform packing of newly deposited collagen fiber bundles. Bar = 0.08 mm.

(Figure 3a). No neutrophils were identified and few macrophages were present. Most of the cell populations in these saline-treated wounds were mesenchymal cells (fibroblasts and myofibroblasts). The empty virus particle-treated mouse wounds, like the saline controls, were completely re-epithelialized. The granulation tissue was similar to the saline controls (Figure 3b). The integration of granulation tissue with the subdermal adipose tissue was similar to that of the saline controls, and most cells within the granulation tissue were mesenchymal cells. The ADV/VEGF$_{165}$-treated wounds were completely re-epithelialized and the granulation tissue showed thicker pink staining in the deeper regions of the granulation tissue. The number and luminal size of the blood vessels in the ADV/VEGF$_{165}$-treated wounds were greater than in the other treatment groups (Figure 3c). In addition the cell density was greater throughout in the epidermal layer compared with the saline and empty viral particle controls. By histological evaluation, ADV/VEGF$_{165}$-treated wounds showed enhanced granulation tissue deposition, containing greater numbers of larger vessels, compared with the non-VEGF-treated wounds. In addition, the epidermal layer covering the VEGF-treated wounds was thicker and had a greater basal cell density (Figure 3c), which is consistent with the *in vitro* findings of VEGF$_{165}$ effects on human keratinocytes (see Figure 1).

To evaluate the granulation tissue collagen fiber orientation and density in the matrix deposited in the three treatment groups, Sirius red staining viewed with polarized light microscopy was employed (Figure 4). Saline-treated control wounds showed modest birefringence intensity, which was consistent with new immature collagen fiber bundles (Figure 4a). These collagen fiber bundles were short, thin, and arranged in a random pattern. This birefringence pattern would be typical of young immature granulation tissue. The red birefringence on the surface of the granulation tissue is from the keratin laid down by keratinocytes from the epidermal layer. In these saline-treated mice, there is a modest amount of collagen deposited and the newly deposited collagen is poorly organized. Empty viral particle-treated wounds showed an increase in the birefringence intensity, in which the fragments of collagen fiber bundles were longer in size and arranged in a parallel manner compared with saline-treated controls (Figure 4b). The increase in collagen deposition and organization may have resulted from viral particles causing a more intense acute inflammatory response early in the repair process. This response would have occurred soon after viral treatment and may have promoted a modest increase in connective tissue deposition. The ADV/VEGF165-treated wounds stained with hematoxylin and eosin (H&E) (Figure 3c) showed thicker granulation tissue, and when viewed by polarized light the Sirius red-stained sections (Figure 4c) showed greater amounts of collagen laid down in a more organized manner (Figure 4c). The intense, thick continuous bands of birefringence were in contrast to the fragmented birefringence patterns of the saline- and empty viral particle-treated wounds. The intense birefringence of the ADV/VEGF$_{165}$-treated wounds was consistent with the more intense pinkish staining shown by H&E (Figure 3c), and both histological methods showed greater amounts of collagen deposition, laid

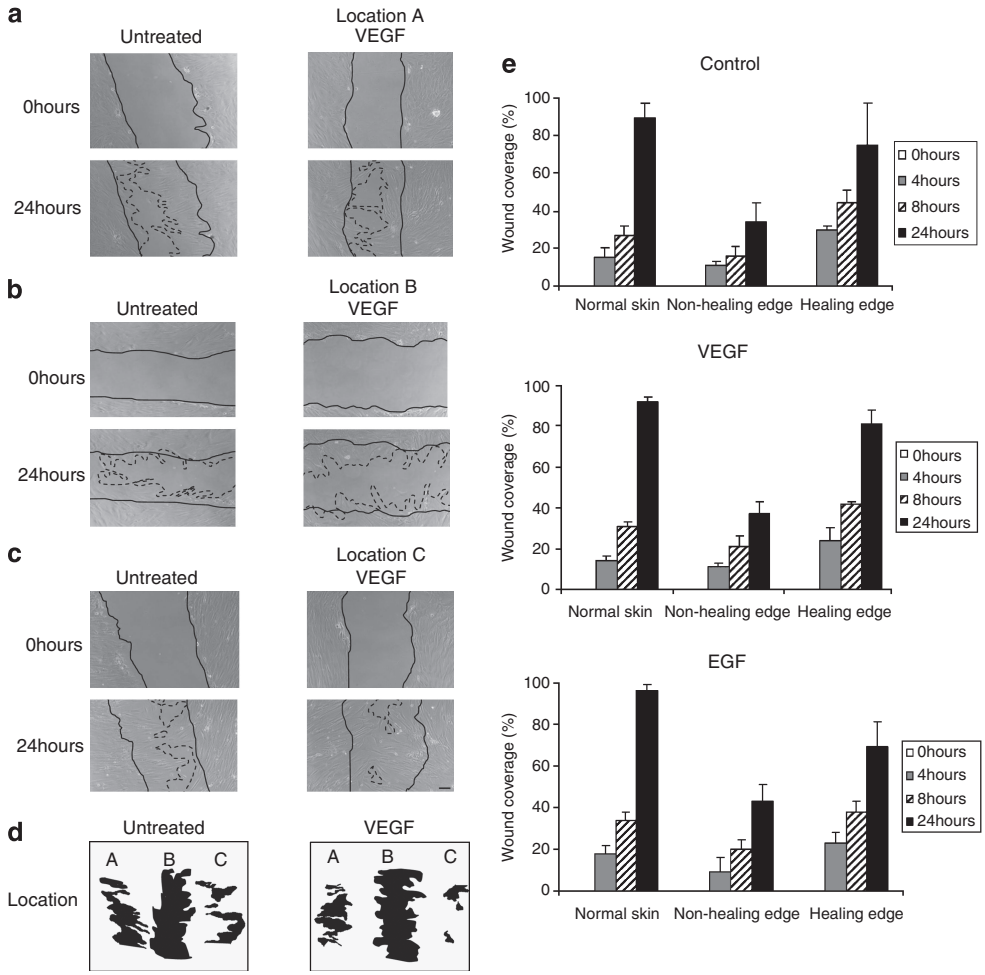


**Figure 5. VEGF promotes migration of fibroblasts isolated from a specific wound location in patients.** Human recombinant VEGF accelerates migration of fibroblasts deriving from Location C. (Full lines indicate initial wound area; dotted lines demarcate the migrating front of cells.) VEGF stimulates migration of fibroblasts deriving from Location C in a scratch-wound assay compared with untreated cells (**c**). VEGF treatment of fibroblasts deriving from Location A (**a**) and Location B (**b**) did not stimulate migration significantly. Fibroblasts derived from these wound locations migrate at a rate similar to that of untreated cells. (**d**) Uncovered surface areas from scratch wounds are shown. VEGF markedly reduced the wound area of fibroblasts from Location C. (**e**) Quantification of fibroblast cell migration from the scratch assay. Epidermal growth factor was used as a positive control. The bar graph presents the percentage of wound area coverage at 0, 4, 8, and after 24 hours. VEGF stimulated migration of fibroblasts even compared with the positive control at the healing edge of the wound (Location C), whereas migration was significantly reduced compared with normal skin at the non-healing edge (Location B). Bar = 0.025 mm.

down in a more organized manner compared with the controls.

### Mechanism of VEGF and collagen deposition

To further elucidate the mechanism underlying these *in vitro* findings in migrating fibroblasts in culture, we specifically tested VEGF using *in vitro* scratch-wound assays with human fibroblasts. Cells derived from distinct human wound locations were incubated in the presence and absence of human recombinant VEGF. Their response to wound healing stimuli was location specific. VEGF was most effective in stimulating migration of fibroblasts deriving from Location C, the healing edge, followed by those from Location A, the base of the wound. Fibroblasts from the non-healing edge (Location B) were not responsive (Figure 5a–d). These results were quantified and the percentage of wound area coverage is shown graphically in Figure 5e.

## DISCUSSION

Results show that $VEGF_{165}$ significantly enhances healing as defined by an increase in re-epithelialization, production of a more vascularized granulation tissue, and more organized collagen fiber bundles. These effects are consistent with an increase in tensile stiffness. Furthermore, experiments on the effect of $VEGF_{165}$ on time to complete wound closure suggest that $VEGF_{165}$ accelerates wound re-epithelialization rather than promoting wound contraction, as defined by more rapid reduction in open wound area over time.

The increase in re-epithelialization by $VEGF_{165}$-treated wounds has been reported by others (Romano Di Peppe *et al.*, 2002; Michaels *et al.*, 2005; Saaristo *et al.*, 2006; Li *et al.*, 2007). Data from this study confirm by histology the improvement in re-epithelialization. The rationale for the *in vitro* portion of this study was that restoration of an intact epidermal barrier through wound re-epithelialization is the

essential feature of wound closure. The directed migration of keratinocytes is critical to wound re-epithelialization and defects in this function are associated with the clinical problem of chronic non-healing wounds (Raja *et al.*, 2007).

The possible mechanism of enhanced wound closure is substantiated *in vitro* by the specific effect of $VEGF_{165}$ on enhanced keratinocyte migration. Moreover, $VEGF_{165}$ stimulated the migration of activated keratinocytes over the wound area. The activated keratinocyte is the phenotype that participates in wound closure and that is permissive to therapeutic modalities, including ADV/$VEGF_{165}$. This indicates that $VEGF_{165}$ therapy promotes epithelialization, independent of its role in recruiting and stimulating endothelial cells in the repair process. Re-epithelialization is a crucial component of wound closure. Retarded re-epithelialization is one of the main causes of delayed wound closure in diabetic patients.

The more rapid maturation of granulation tissue in the $VEGF_{165}$-treated wounds was shown by more and better organized collagen fiber bundles deposited in granulation tissue, which was confirmed histologically by H&E and Sirius red staining. Collagen is the major connective-tissue component of granulation tissue, scar, and dermis. Collagen synthesis and deposition are critical for wound closure and the development of wound tensile stiffness. The collagen fiber bundles in granulation tissue become more organized as the connective tissue in wounds matures and increases in tensile stiffness. The greater cell density in the granulation tissue of $VEGF_{165}$-treated wounds, also observed through histology, is believed to be due to an increase in the number of fibroblasts at the wound site by enhanced cell migration.

*In vitro* $VEGF_{165}$ has been shown to stimulate the migration of primary fibroblasts deriving from patients with chronic ulcers (Stojadinovic *et al.*, 2007). $VEGF_{165}$ specifically targets fibroblasts in the post-debrided wound, whereas it has no effects on the migration of fibroblasts within the non-healing edge. Further study is necessary to elucidate the correlation of fibroblast migration and collagen synthesis and deposition.

A recognized limitation of this analysis is the exclusive focus on VEGF. VEGF alone may not be responsible for the observed effects, as it is well known that VEGF upregulates the expression of various cytokines and PDGF. Moreover, the receptors for VEGF in different locations of the wound may vary in expression, as might those for other growth factors. Although we have shown decreased VEGFR at the non-healing edge using microarrays (Stojadinovic *et al.*, 2008), expression levels at multiple locations in the wound are a subject of future research. Identification of the role of other growth stimulators and their receptors may also be beneficial.

A sporadic finding was the mortality of some of the mice that were given higher doses of ADV/VEGF (up to $5 \times 10^{11}$) relative to controls. No outward signs of abnormalities were observed in the mice that died. We are not aware of a direct link between VEGF administration and mortality. The safety record of administration of adenovirus gene therapy is quite good. A large study of low dose (<5e9) and intermediate dose of viral particles ($10^9$ to $10^{11}$) in 90 individuals using six different routes of administration drew results from multiple clinical trials. The longitudinal study reported no mortalities related to the administration of gene therapy and a major adverse event rate of 0.7% (Harvey *et al.*, 2002).

On the basis of the safety record of ADV and VEGF in animal studies and human clinical trials, we can say with reasonable certainty that the observed mortality was an anomaly, probably due to the fragility of the mice and/or intolerance of anesthesia, but which was unlikely to have stemmed from the administration of ADV/VEGF. It is necessary that further studies be performed regarding the optimal effective dose of ADV/$VEGF_{165}$ (in viral particles) in type 1 and type 2 diabetic models to increase the efficacy of the treatment while avoiding toxicity.

Taken together, these results show that VEGF increases granulation tissue and promotes keratinocyte migration, and both are expected to stimulate wound closure in diabetic wound models *in vivo*. Knowledge about the effects of $VEGF_{165}$ on epithelialization, collagen deposition, and cell migration may bring to fruition more gene therapy studies involving the administration of $VEGF_{165}$ as a treatment. Such trials would be exceptionally important, particularly to target patients with DFUs to decrease amputations. In addition, ischemic ulcers could be targeted with ADV/VEGF.

## MATERIALS AND METHODS

### Construction of an ADV expressing bioactive human VEGF for murine study

Human umbilical vein endothelial cells (HUVECs) were homogenized and total RNA was extracted. The full-length human $VEGF_{165}$ cDNA was amplified by PCR with appropriate primers containing restriction sites (*Hin*dIII and *Xba*I) for subcloning into pBluescript (Stratagene, La Jolla, CA). After sequence confirmation, the human VEGF-165 cassette was cloned into the multiple cloning site of an adenovirus shuttle vector (pXC1) containing adenovirus type 5 sequences (bp 22–5,790) and a Rous sarcoma virus promoter. This same vector was used as the positive control Dl-312 in the experiments. For the rescue of the recombinant adenovirus, we successfully used the two-plasmid co-transfection system (Microbix Biosystems, Inc., Toronto, Ontario, Canada). Virus particle titer was determined by optical absorbance at 260 nm, and plaque-forming unit titer (pfu $ml^{-1}$) was quantified by standard agarose overlay plaque assay on 293 cells. Plaque-forming unit (pfu) determination can vary up to one order of magnitude when the same batch of virus is used in different assays, causing a significant variation in particle measurement. To prevent this problem and to keep the viral loads constant, the same batch of virus was used for all *in vitro* and *in vivo* experiments.

JC cells, a VEGF-negative murine breast cancer cell line that is easily transducible by adenoviruses, were infected with a multiplicity of infection of 100 pfu per cell of several different viral plaques (plaques 11-1, 11-2, 11-3, 11-9, 13-1, 16-1) of ADV/$VEGF_{165}$ or ADV/β Gal (negative control). $VEGF_{165}$ was measured after 48 hours in the conditioned supernatant of these virally infected cells by hVEGF ELISA (R&D Systems, Minneapolis, MN). Plaques 11-1, 13-1, and 16-1 were then selected to test for the functional activity of the secreted $VEGF_{165}$ in an *in vitro* bioassay

(HUVEC proliferation assay), in which HUVEC proliferation was induced by serial dilutions of conditioned supernatant of ADV/$VEGF_{165}$- or ADV/β Gal-transduced JC cells. HUVECs were seeded in flat-bottom 96-well plates that were precoated with 1% gelatin (in PBS) at $2 \times 10^3$ cells per well and serum deprived overnight (M13 medium without FBS). Conditioned supernatant was then added in several dilutions and cells were cultured for an additional 48 hours in M13 supplemented with 20% FBS. HUVEC proliferation was measured by a tetrazolium-based assay at an absorption of 450 nm, according to the manufacturer's instructions (Roche, Pleasanton, CA). Plaque 11-1 was selected for the large-scale amplification and virus purification used in our experiments.

### BKS.Cg-m +/+ Lepr$^{db}$ and NOD mice

All animal procedures were performed with approval from the Institutional Animal Care and Use Committee. All the mice were acclimatized in individual cages for at least 2 weeks before wounding. Glucose and body weight measurements were taken before acclimation, wounding, and mechanical testing.

Wounding and testing were initiated at the ages of 24 and 27 weeks in female NOD mice because the type 1 diabetes peak incidence in female NOD mice is attained at 16–20 weeks. Weekly glucose monitoring was initiated by measuring plasma glucose levels at 12 weeks of age and then bi-weekly, starting at 16 weeks of age, as insulin-dependent diabetes mellitus is usually indicated when plasma glucose levels reach ⩾200 mg per100 ml for two consecutive weeks. A mixture of intermediate-acting and long-acting insulin (Novolin 70/30, Novo Nordisk Pharmaceuticals, Inc., Princeton, NJ) was injected subcutaneously daily to control blood glucose. Before the experiment was begun, a portable monitor was used to measure hemoglobin A1c (DCA2000 + Analyzer, Bayer, Terrytown, NY). A glucometer (Ascencia Elite, Bayer, Terrytown, NY) was also used to ensure that the groups had comparable glucose control.

Male and female BKS.Cg-m +/+ Lepr$^{db}$ type 2 diabetic mice (8 weeks old) were obtained from The Jackson Laboratory (Bar Harbor, ME). They were housed in a temperature-controlled facility with a 12-hour light/dark cycle. Water and standard rodent diet were given *ad libitum*. When the animals were fasted for 12 hours, only water was supplied.

### Time to 100% closure using excisional wounds on diabetic mice

In preparation for all experiments, mice were anesthetized by intraperitoneal injection of a ketamine and xylazine mixture. Mice were shaved the day before experimentation. Full-thickness excisional wounds, 1.4 cm in diameter, were created on the dorsum of 53 male BKS.Cg-m +/+ Lepr$^{db}$ type 2 diabetic 8-week-old mice. The mice were divided into six groups: group I ($n=12$), ADV/$VEGF_{165}$ $5 \times 10^{11}$ vp per wound; group II ($n=8$), ADV/$VEGF_{165}$ $1.6 \times 10^{11}$ vp per wound; group III ($n=8$), ADV/$VEGF_{165}$ $5 \times 10^{10}$ vp per wound; group IV ($n=8$), ADV/$VEGF_{165}$ $1.6 \times 10^{10}$ vp per wound; group V ($n=7$), saline (negative control); group VI ($n=10$), DL-312 (positive control) $5 \times 10^{11}$ vp per wound. Each injection of ADV/$VEGF_{165}$ or controls was administered intradermally around the wound edge at the time of wounding. The marked areas were excised with scissors to include the epidermis, dermis, and panniculus carnosus. The wounds were digitally photographed at a fixed distance on the day of wounding and then every fourth day until the day of closure. A ruler was placed in the field of the photograph, labeled with the mouse's identification number and the date. Time to closure was clinically evaluated and defined as the day of 100% epithelialization with no drainage.

### Tensile strength analysis using full-thickness incisional wounds on diabetic mice

Linear incisional wounds were created on the dorsum of 57 female BKS.Cg-m +/+ Lepr$^{db}$ type 2 diabetic mice and in 42 female NOD type 1 diabetic mice. Before being wounded, the animals were acclimatized for 2 weeks by being placed in individual cages. They were prepared for surgery as described earlier. A 30-mm linear incision was initiated 5 mm below the last cervical vertebra on the dorsum of each animal in a longitudinal direction. Intradermal injections of ADV/$VEGF_{165}$ were administered at both sides of the incision at the third suture location only. The BKS.Cg-m +/+ Lepr$^{db}$ mice were divided into four treatment groups: group I ($n=12$), ADV/$VEGF_{165}$ $5 \times 10^{10}$ vp per wound; group II ($n=15$) ADV/$VEGF_{165}$ $5 \times 10^{8}$ vp per wound; group III ($n=15$), DL-312 vehicle (positive control) $5 \times 10^{8}$ vp per wound; and group IV ($n=15$), saline (negative control). Similarly, NOD mice were divided into three treatment groups: group I ($n=14$) ADV/$VEGF_{165}$ $5 \times 10^{8}$ vp per wound; group II ($n=14$) DL-312 vehicle (positive control) $5 \times 10^{8}$ vp per wound; group III ($n=14$) saline (negative control). The incision was then closed with six 5–0 nylon sutures at 5-mm intervals. Sutures were removed at seven days after wounding. The BKS.Cg-m +/+ Lepr$^{db}$ mice were killed at days 10 and 21 after wounding for mechanical property analyses of healed wounds. Tensile strength and tensile stiffness analyses were performed on excised wounds. Histological evaluation was made on day 10 wounds to correlate with tensile strength/stiffness analysis. On postoperative day 14, all NOD mice were killed, and the mechanical properties and histology of the wounds were assessed and compared.

With BKS.Cg-m +/+ Lepr$^{db}$ mice, the mechanical properties of linear incisional wounds were assayed 10 and 21 days after wounding using tensiometry. NOD mice were tested on day 14 after wounding. A 30 mm × 8 mm skin strip perpendicular to the third incision was excised, placed in 1 × PBS in an ice-water bath to prevent desiccation, and subjected to tensiometry assays. Each skin sample was clamped between sandpaper-covered plates with a consistent grip-to-grip gauge length of 15 mm. Samples were centered relative to the clamps by using a custom-made alignment apparatus. The samples were attached by the clamps to the actuator of an Instron Servohydraulic Materials Testing System (model 8872, Canton, MA). Tensile loads were measured using a 2.5 lb (11.1 N) load transducer (Transducer Techniques, Temecula, CA). A preload of 0.02 N was then maintained for 2 minutes to define the initial length needed for engineering strain computations. For the preloaded skin sample, the grip-to-grip distance (that is, specimen length) was measured with precision calipers, and the width was similarly measured along the length of the sample. Immediately after preloading, the sample was loaded to failure at 0.1 mm/second. Two measurements were taken from each skin sample: stiffness (N/mm), the amount of force required to deform the skin, and breaking load (N), the amount of force required to break (rupture) the skin sample until it no longer possesses its integral function.

### Histology analysis on skin strips from full-thickness incisional wounding

Skin strips from day 10 after wounding in the BKS.Cg-m+/+Lepr$^{db}$ mice and from day 14 after wounding in the NOD mice were used for histology analysis. The skin strips were fixed overnight in 4% paraformaledehyde, and then the specimens were transferred into 70% ethanol for an additional 24 hours of fixation. The bisected tissue was processed for paraffin embedding and 5-μm sections were cut and stained with (1) H&E for evaluation of re-epithelialization and (2) Sirius red for assessment of collagen deposition. Investigators blinded to the treatment protocol performed the assessments. Stained specimens were graded at the incisional site of the healed wound. The following categories were used to grade the levels of epithelialization: 4 = thick and intact epithelial layer; 3 = thick epithelium; 2 = complete epithelialization; 1 = minimal epithelialization; 0 = incomplete epithelium. H&E staining was also used to quantify the number of cells per high-powered field at ×40 magnification.

### Human keratinocyte cultures and scratch-wound assay

To establish keratinocyte cultures, primary human keratinocytes were grown to 80% confluence as described earlier (Lee *et al.*, 2005a; Stojadinovic *et al.*, 2006; Jho *et al.*, 2005). At 24 hours before the experimentation, cells were transferred to basal KBM medium (Gibco-BRL, Grand Island, NY) (Stojadinovic *et al.*, 2006) and incubated in either 0.09 or 1.2 mM $CaCl_2$ to induce differentiation. All cells were cultured from routinely discarded surgical specimens of normal skin and cells from different wounds were not pooled in culture. Before being wounded, cell cultures were incubated in the presence or absence of 8 μg/ml Mitomycin C (ICN, Emeryville, CA) for 1 hour and washed with PBS to inhibit proliferation. Scratch wounding of cell monolayers was performed as described earlier (Lee *et al.*, 2005a). Cultures were incubated in the presence or absence of $VEGF_{165}$ or 25 ng/ml of epidermal growth factor for 24 and 48 hours and re-photographed, and cell migration was quantified as described earlier (Lee *et al.*, 2005a). Thirty measurements were taken for each experimental condition and the distance coverage by cells moving into the scratch-wound area was quantified. Three images were analyzed per condition, per time point, and averages and standard deviations were calculated.

### Human fibroblast migration assays

We grew fibroblasts from the wound base (Location A), the non-healing edge (Location B), and the healing edge (Location C) and compared their migration capacity with that of normal primary dermal fibroblasts (obtained from mammoplasty). Biopsies of ischial pressure ulcers from three different patients were used in this experiment. The area of a pressure ulcer was prepared with Betadine (Purdue, Stamford, CT). A sterile No. 10 blade was used to biopsy the wound base, Location A. Then Location B was identified at the boundary of the wound bed and the rim of necrotic or infected tissue to be removed. After biopsy of Location B, a sharp excision was performed to remove the entire circumferential ring of necrotic, non-viable scar or infected tissue. Finally, a fresh blade was used to biopsy several millimeters of adjacent non-wounded tissue, Location C. Cells from Location B were those surgically removed and cells from Location C were the cells left behind after surgery. Cells were grown in DMEM (Bio Whittaker, Walkersville, MD) containing 10% calf bovine serum and 2% antibiotic–antimycotic (Gibco, Grand Island, NY). Twenty-four hours before the experiments, cells were switched to basal medium—phenol red-free DMEM (Bio Whittaker) supplemented by 2% charcoal-pretreated bovine serum as described earlier (Radoja *et al.*, 2000), 1% antibiotic–antimycotic (Gibco), and 1% L-glutamine (Cambrex Bio Science, Rockland, ME). Before the scratch, cells were treated with 8 μg/ml Mitomycin C (ICN Biomedicals, Emeryville, CA) for 1 hour (to inhibit cell proliferation) and washed with basal medium. Scratches were performed as described earlier (Stojadinovic *et al.*, 2005). Cells were incubated in the presence or absence of 100 ng/ml recombinant VEGF (R&D Systems) or 25 ng $ml^{-1}$ epidermal growth factor (Gibco) for 24 and 48 hours and re-photographed 24 hours after the scratch.

### Statistical analysis

All data were analyzed by ANOVA. The results were expressed as mean ± SD. The level of statistical significance for the comparison between treatment with ADV/$VEGF_{165}$ and treatment with saline or just the adenovirus without $VEGF_{165}$ was set at a *P*-value of $<0.05$.

## CONFLICT OF INTEREST

The authors state no conflict of interest.

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