2009

Intradermal Delivery of Plasmid VEGF(165) by Electroporation Promotes Wound Healing

Bernadette Ferraro

Yolmari Cruz

Domenico Coppola

Richard Heller
Old Dominion University, rheller@odu.edu

Original Publication Citation

Ferraro, B., Cruz, Y. L., Coppola, D., & Heller, R. (2009). Intradermal delivery of plasmid vegf(165) by electroporation promotes wound healing. *Molecular Therapy, 17*(4), 651-657. doi:10.1038/mt.2009.12

Intradermal Delivery of Plasmid VEGF$_{165}$ by Electroporation Promotes Wound Healing

Bernadette Ferraro[1], Yolmari L Cruz[1], Domenico Coppola[2] and Richard Heller[1,3]

[1]Department of Molecular Medicine, University of South Florida, Tampa, Florida, USA; [2]Department of Anatomic Pathology, H. Lee Moffitt Cancer Center, Tampa, Florida, USA; [3]Frank Reidy Research Center for Bioelectrics, Old Dominion University, Norfolk, Virginia, USA

Skin flaps are extensively used in reconstructive surgeries to repair large defects and deep wounds, but severe ischemia and necrosis often results in loss of the transplanted tissue. Thus, skin flap models are often used to study the biology of healing and necrosis of acute ischemic wounds. Delivery of exogenous vascular endothelial growth factor (VEGF) to areas of ischemia has shown promise for promoting therapeutic angiogenesis, but its expression must be tightly regulated to avoid adverse effects. In this study, plasmid DNA encoding VEGF$_{165}$ (pVEGF) was delivered to the ischemic skin of a rat skin flap model by intradermal injection followed by electroporation (EP) (pVEGFE+). Treatment with pVEGFE+ significantly increased VEGF expression for 5 days after delivery compared to injection of pVEGF without EP (pVEGFE–). The short-term increase in VEGF was sufficient to mediate an upregulation of endothelial nitric oxide synthase, an angiogenic factor that increases vascular permeability. pVEGFE+ significantly increased skin flap perfusion at both days 10 and 14 postoperatively. The observed increase in perfusion with pVEGFE+ correlated with an increase in skin flap healing and survival. Our results demonstrate that pVEGFE+ is a potential nonviral noninvasive therapy to increase perfusion and healing of skin flaps and ischemic wounds.

Received 22 December 2008; accepted 12 January 2009; published online 24 February 2009. doi:10.1038/mt.2009.12

INTRODUCTION

Skin flaps are frequently used in plastic and reconstructive surgeries to repair large skin defects and deep wounds formed from injuries, surgeries, ulcerations, or congenital defects. In skin flap surgery, a piece of full thickness skin is transferred to the affected area while remaining attached to the donor site by a vascular pedicle. Skin flaps are grouped into two general categories, axial and random. Axial skin flaps are perfused by a single direct subcutaneous artery through the vascular pedicle while random skin flaps (RSFs) are perfused by small musculocutaneous or septocutaneous perforators.[1] In both axial and RSFs, insufficient arterial supply and inadequate venous drainage can lead to distal skin flap ischemia and necrosis.[2] Despite the intense use of skin flaps as a model to study the healing and necrosis of ischemic wounds, a viable clinical solution to prevent distal necrosis and enhance healing remains elusive. Gene therapy techniques delivering exogenous angiogenic growth factors and cytokines, such as vascular endothelial growth factor (VEGF), to skin flaps are currently being investigated to increase skin flap perfusion and healing.[3]

In vascular endothelial cells, VEGF induces cell proliferation, promotes cell migration, differentiation, and inhibits apoptosis. VEGF expression is potentiated in response to hypoxia, by activated oncogenes, and by a variety of cytokines. The angiogenic and blood vessel permeability effects of VEGF are mediated by binding to the tyrosine kinase receptors VEGF Receptor 1 and VEGF Receptor 2. Signaling through VEGF Receptor 1 mediates VEGF-induced migration of monocytes while signaling through VEGF Receptor 2 mediates the mitogenic and vessel permeability effects of VEGF.[4] VEGF-mediated increases in vascular permeability are largely due to an increase in the downstream angiogenic growth factor endothelial nitric oxide synthase (eNOS) that mediates an increase in the release of the vasodilator nitric oxide.[5,6] *In vivo* and *in vitro* studies demonstrated that the level of VEGF expression must be finely regulated to induce successful angiogenesis[7–10] and, more recently, that high levels of VEGF may inhibit new vessel stabilization.[11] High levels of constitutive VEGF expression can result in edema,[12] formation of hemangiomas, and unstable immature vessels.[7–11] In contrast, low levels or premature cessation of VEGF expression results in regression of the newly formed vessels.[13] Thus, the development of a gene therapy approach that allows for tight control over expression level and duration would be advantageous for VEGF-mediated therapeutic angiogenesis.

Direct injection of naked plasmid DNA has been intensively investigated as a gene delivery approach for the treatment of a variety of diseases. One drawback to this approach is that inefficient uptake of the plasmid by the cells results in low levels of gene expression.[14] *In vivo* electroporation (EP) has been successfully used to increase the uptake and expression of naked plasmid DNA in the skin and other target tissues. Electrically mediated delivery of plasmid DNA to the skin could potentially be used to deliver plasmids with therapeutic potential to treat a variety of diseases and has recently shown promise in a clinical trial.[15] In previous work, we developed a novel electrode, the four-plate electrode, and optimized conditions using this electrode for cutaneous gene delivery with minimal tissue damage.[16] Here, we show that using

Correspondence: *Richard Heller, Frank Reidy Research Center for Bioelectrics, Old Dominion University, Norfolk, Virginia 23510, USA. E-mail: rheller@odu.edu*

the four-plate electrode to deliver pVEGFE+ to the ischemic skin of a modified McFarlane[17,18] 8 cm × 3 cm RSF increases VEGF expression more than pVEGFE–. Although the increase in VEGF expression was short-term, it was sufficient to induce an endogenous angiogenic response and increase perfusion and survival of the distal area of the RSF. These results suggest that pVEGFE+ is a potential novel therapeutic approach to increase healing and decrease necrosis of ischemic wounds and RSFs.

RESULTS

Electrically mediated intradermal delivery of pVEGF increases VEGF expression and promotes a VEGF-mediated increase in eNOS expression

Plasmids encoding a wide variety of genes with therapeutic potential, including VEGF,[19] have been successfully delivered to skeletal muscle by intramuscular injection with EP.[20] Delivery of plasmids with EP significantly increased transgene expression compared to injection of the plasmid alone. Before delivering plasmid DNA encoding $VEGF_{165}$ (pVEGF) to the RSF model, we first evaluated whether it could increase VEGF levels *in vitro* (data not shown) and *in vivo* by delivering pVEGFE+ and pVEGFE– to the rat gastrocnemius muscle. On days 2, 5, 7, and 10 after pVEGF delivery, muscle was excised from the delivery site and assayed for VEGF expression by enzyme-linked immunosorbent assay. Compared to pVEGFE–, pVEGFE+ significantly increased VEGF expression at both days 2 and 5 after delivery (**Figure 1a**) but VEGF levels returned to background levels by day 7 after delivery (data not shown). Because pVEGFE+ successfully increased VEGF expression in gastrocnemius muscle we then investigated whether the same increase could be achieved with pVEGFE+ in the skin of the RSF model.

Skin flaps were treated on day 2 postoperatively. On days 2, 5, 8, and 12 after treatment, skin samples from the delivery sites were harvested and assayed for VEGF expression using an enzyme-linked immunosorbent assay that specifically detects human $VEGF_{165}$. Necrosis prevented all sites from being harvested, especially in the pVEGFE– group at later time points. At both 2 and 5 days after delivery, VEGF expression was significantly higher in the pVEGFE+ treatment group compared to the pVEGFE– treatment group ($P < 0.05$, both time points). VEGF expression was determined for each treatment group for delivery to the proximal area, closest to the RSF pedicle, and distal area, farthest from the RSF pedicle. Interestingly, 2 days after pVEGF delivery, VEGF expression was significantly higher in the distal area of the RSF, compared to the proximal area, for the pVEGFE+ treatment group ($P < 0.05$). In the pVEGFE– treatment group, there was a twofold increase in VEGF expression in the distal area, but this increase was not statistically significant ($P = 0.157$). The level of VEGF expression achieved with pVEGFE+ in the distal area of the RSF was similar to the level and duration observed in the gastrocnemius (**Figure 1a,b**). Compared to day 2 after delivery there was an overall significant decrease in VEGF expression at day 5 after delivery ($P < 0.05$, both treatment groups in both the proximal and distal areas) but levels were still significantly higher with pVEGFE+ ($P < 0.05$) (**Figure 1b**). At day 8 after delivery there was not a significant increase in VEGF expression with pVEGFE+ in either the proximal or distal area of the RSF, and by day 12 only background levels of VEGF were detected for both pVEGFE+ and pVEGFE–. VEGF expression was not detected when the vector backbone lacking the VEGF cDNA insert was delivered to the RSF with EP (pVAXE+), in the skin of untreated

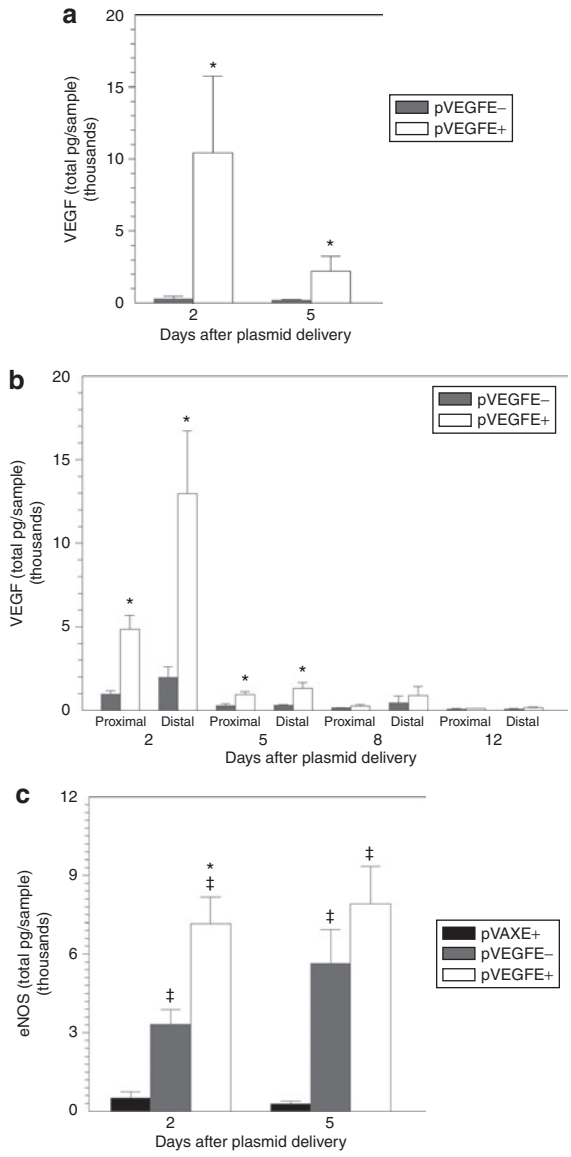

Figure 1 VEGF expression kinetics. pVEGFE+ significantly increased VEGF compared to pVEGFE–. (**a**) pVEGFE+ increased VEGF expression in gastrocnemius muscle. Day 2: $n = 6$ per group. Day 5: pVEGFE– $n = 5$; pVEGFE+ $n = 4$. (**b**) Two and 5 days after delivery VEGF expression was significantly greater with pVEGFE+ than pVEGFE– in the RSF. VEGF expression was further increased in the distal region of the RSF. pVEGFE–: day 2 proximal $n = 8$, distal $n = 6$; day 5 proximal $n = 3$, distal $n = 4$; day 8 proximal $n = 3$, distal $n = 2$; and day 12 proximal and distal $n = 2$. pVEGFE+: day 2 proximal $n = 4$, distal $n = 7$; day 5 proximal $n = 7$, distal $n = 6$; day 8 proximal $n = 3$, distal $n = 4$; and day 12 both proximal and distal $n = 3$. (*c*) pVEGFE+ significantly increased eNOS expression in the distal area of the RSF 2 days after delivery; $n = 7$ per group per time point. For groups with $n \leq 3$ the mean ± SD is reported. For groups with $n > 3$ the mean ± SEM is reported. *$P < 0.05$ compared to pVEGFE–. ‡$P < 0.01$ compared to pVAXE+. eNOS, endothelial nitric oxide synthase; pVEGFE+, mice injected with pVEGF followed by electroporation; pVEGFE–, rats injected with pVEGF without electroporation; VEGF, vascular endothelial growth factor; pVAXE+, empty vector control.

controls (P-E–), or in serum samples at any time point during the study (data not shown).

Both *in vitro* and *in vivo* studies[6,21] have shown that increases in VEGF can increase levels of eNOS. To determine whether pVEGFE+ and pVEGFE– increased eNOS in the RSF, skin samples from the distal area of the RSF were assayed for eNOS by enzyme-linked immunosorbent assay at days 2 and 5 after delivery. Two days after delivery eNOS was significantly higher with pVEGFE+ treatment compared to pVEGFE– treatment ($P < 0.05$), but by day 5 after delivery eNOS levels were similar in the pVEGFE+ and pVEGFE– treatment groups (**Figure 1c**). The application of an electric field to tissues alone can transiently induce expression of some genes[22] but pVAXE+ did not increase eNOS in the RSF model ($P < 0.01$).

Electrically mediated delivery of pVEGF increases perfusion to the distal area of the RSF

Therapeutic angiogenesis aims to increase perfusion to the affected area thereby decreasing the effects of ischemia and preventing necrosis. Because RSF survival shows little change after day 15 postoperatively[18] we monitored skin flap perfusion and healing for 14 days postoperatively. pVEGFE+ resulted in the highest level of VEGF expression in the distal area of RSFs which is more at risk for ischemia and necrosis because it is farthest from the vascular pedicle. We next evaluated whether pVEGFE+ treatment increased perfusion more than control treatments in the distal area of the RSF, corresponding to ~25% of the total skin flap area. A Laser Doppler Imager was used to measure perfusion preoperatively (baseline), immediately postoperatively (day 0) and on days 2, 4, 7, 10, and 14 postoperatively. A sharp decrease in distal perfusion was observed immediately postoperatively through day 4 postoperatively indicating that the RSF was effectively rendered ischemic. Perfusion for each treatment group is reported as the percent recovery of baseline (**Figure 2a**) and postoperative perfusion (**Figure 2b**). At days 10 and 14 postoperatively, perfusion was significantly different between all treatment groups for both recovery of baseline and postoperative perfusion ($P < 0.01$). Perfusion in the pVEGFE+ treatment group was significantly greater than all controls on both days 10 and 14 postoperatively for both recovery of baseline ($P < 0.01$) and postoperative ($P < 0.05$) perfusion.

Electrically mediated delivery of pVEGF promotes healing and decreases necrosis

Visual assessment of skin flap viability over the time course of the study showed a clear decrease in distal necrosis in the pVEGFE+ treatment group compared to control groups (**Figure 3a**). The surviving region of the distal area of the RSFs, corresponding to ~25% of the total skin flap area, was quantitated on days 7 and 14 postoperatively (**Figure 3b**). To quantitate the percent survival of the distal area, the area of skin with normal texture and color was expressed as a percent of the total distal area. Nonsurviving areas were defined as dark, rigid necrotic skin or scabbed necrotic lesions and surviving areas were defined as normal in texture and color. The pVEGFE+ treatment increased distal RSF survival at day 7 postoperatively (89.4 ± 4.3, $P < 0.05$) compared to pVEGFE– (56.6 ± 10.6), pVAXE+ (59.3 ± 9.8) and P-E– (60.2 ± 9.6). The surviving area in all treatment groups increased ~5–10% at day 14

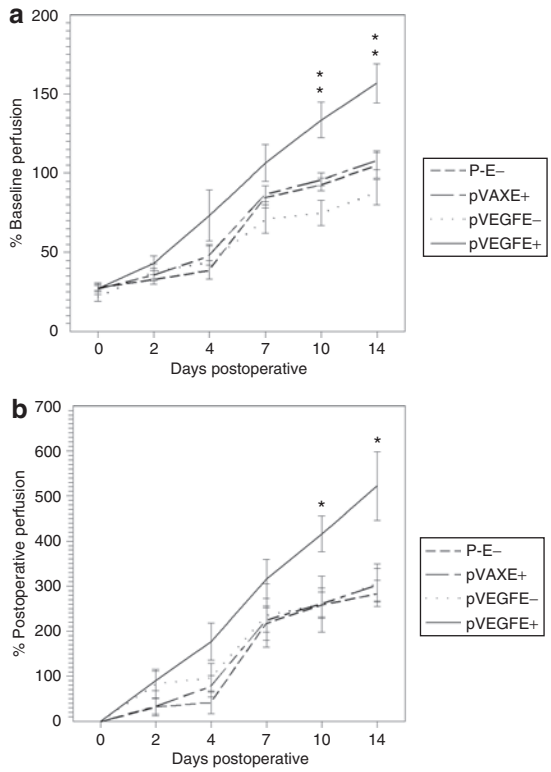

Figure 2 pVEGFE+ increases perfusion. Perfusion was measured in the distal region of the RSF by Laser Doppler Imaging at the indicated time points. pVEGFE+ significantly increased recovery of (**a**) baseline and (**b**) postoperative perfusion on days 10 and 14 postoperatively compared to all controls; $n = 7$ per group, per time point. *$P < 0.05$, **$P < 0.01$ compared to all controls. pVEGFE+, rats injected with pVEGF followed by electroporation; RSF, random skin flaps.

postoperatively, but healing was still greater and more consistent in the pVEGFE+ group (95.2 ± 2.2) ($P < 0.01$) compared to pVEGFE– (65.6 ± 9.3), pVAXE+ (70.6 ± 7.4) and P-E– (73.5 ± 4.1). Adverse side effects associated with increased VEGF expression levels, such as edema,[7–10] were not observed by gross visual assessment in any of the treatment groups. The mean percent survival of the pVEGFE– group at day 7 postoperatively in our study is similar to previously reported values delivering a plasmid encoding VEGF to a similar RSF model.[23] On day 14 postoperatively, skin samples were excised 2-cm from the distal end of the RSFs for histological analysis by hematoxylin–eosin staining (**Figure 3c**). If the skin was necrotic at 2-cm from the distal end samples were excised near the edge of the surviving border. Skin from the pVEGFE+ treatment group appeared healthy (**Figure 3c,** A,B) while skin from the pVEGFE– treatment group showed evidence of acute inflammation, necrosis, and myonecrosis (**Figure 3c,** C–F).

DISCUSSION

In addition to direct injection of plasmid DNA, the most prevalent methods of therapeutic delivery of VEGF to skin flaps documented in the literature are adenoviral-mediated gene transfer and recombinant protein injection. These methods are also promising for increasing skin flap healing[3,24,25] but have draw backs. In human clinical trials, adenoviral vectors have caused inflammatory responses, formation of antibodies to the adenoviruses,

transient fever,[26] and hepatotoxicity.[27] Also, adenoviral delivery results in long-term and high levels of gene expression increasing the potential for the occurrence of the adverse side effects associated with continuous unregulated VEGF expression.[7–10] Recombinant VEGF protein injection has also shown beneficial effects on skin flap viability, but its use in a clinical setting is not practical due to its short half-life, poor bioavailability, and consequently the need for frequent administration to sustain lasting effects.[28,29] Moreover, intravenous injection of recombinant VEGF was more effective than local administration[25] for decreasing skin flap necrosis, which again raises the concern of pathological angiogenesis that may result from systemic VEGF expression.[7–10]

Here, we have presented a novel therapeutic approach to increase VEGF expression, induce an endogenous angiogenic response, increase perfusion, and decrease necrosis of the distal area of RSFs. The skin is an attractive target for delivery of plasmid DNA because it allows for enhanced control over expression levels and aids in targeting expression to specific tissue areas. Further, if higher expression levels are needed, the area treated or number of treatments can be increased. Previous gene therapy studies delivered VEGF to skin flaps either preoperatively or intraoperatively.[3,24,25] Our study was the first to deliver pVEGF 2 days postoperatively. VEGF modulates the proliferative phase of wound healing that begins 2–4 days after the wound is created, and is characterized by angiogenesis, collagen deposition, granulation tissue formation, epithelialization, and wound contraction.[30] By administering pVEGFE+ 2 days postoperatively, we timed VEGF expression to peak during the beginning of the proliferative phase of wound healing. Also, by administering treatment 2 days postoperatively, when the distal area of the skin flap is most hypoxic,[31] we also determined that electrically mediated plasmid delivery to a large area of ischemic skin is in fact possible. Our results demonstrate that pVEGFE+ significantly increased VEGF expression, compared to pVEGFE–, and that VEGF expression further increased with distance from the vascular pedicle. Further, in the distal area of the RSF pVEGFE+ resulted in similar levels of VEGF expression as delivery to the gastrocnemius muscle (**Figure 1a, b**). Higher VEGF expression in the distal area of the RSF, compared to proximal region, could be due to hypoxia-induced stabilization of the VEGF mRNA or increased translation.[32] The current mechanisms described for increasing VEGF mRNA stability and translation involve genetic regions not contained within the plasmid used in this study. It is possible that other currently unknown mechanisms could enhance VEGF levels and may be responsible for the increase in VEGF expression in the distal area, compared to the less hypoxic proximal region. Importantly, VEGF expression was transient and not detected systemically, indicating that the increase was localized to the delivery area, which decreases the possibility of the adverse side effects that can result from high levels and/or systemic VEGF.

Increases in eNOS resulting from pVEGFE+ and pVEGFE– indicate the increase in VEGF expression resulting from these treatments successfully stimulated an endogenous angiogenic

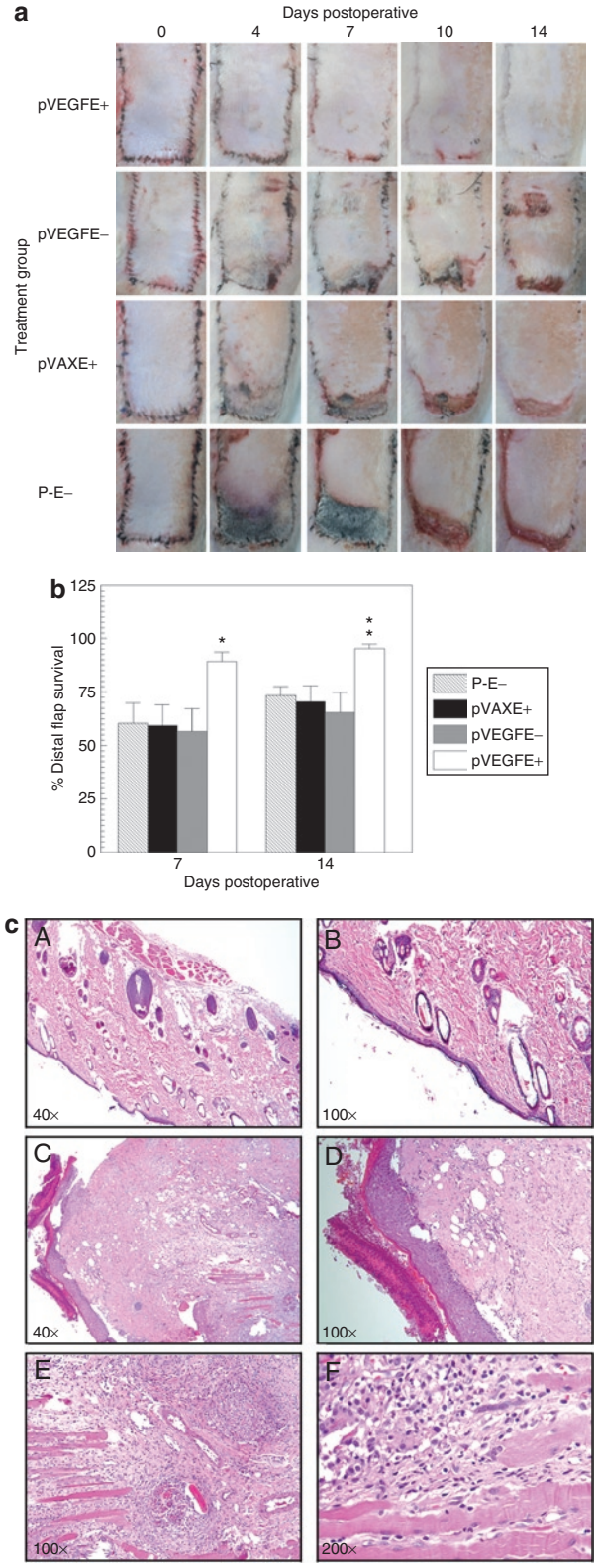

Figure 3 pVEGFE+ increases healing. (**a**) Representative images of RSFs from each treatment group over the time course of the study. (**b**) pVEGFE+ accelerated skin flap healing and decreased necrosis compared to all controls. Quantification of distal survival on days 7 and 14 postoperatively; $n = 7$ per group, per time point. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to all controls. (**c**) Representative sections of skin excised 2-cm from the distal end of RSF stained with hematoxylin–eosin from the pVEGFE+ (A,B) and pVEGFE– (C–F) treatment groups. pVEGFE+, mice injected with pVEGF followed by electroporation; pVEGFE–, rats injected with pVEGF without electroporation; RSF, random skin flaps.

response. pVEGFE+ induces an earlier and more robust increase in eNOS expression compared to pVEGFE– (**Figure 1c**). Our results are consistent with reports that *in vitro* VEGF-induced upregulation of eNOS is time and dose dependent[33] and with a study by Huang *et al.*[34] that demonstrated adenoviral delivery of $VEGF_{165}$ to RSFs increased eNOS. Relative to pVEGFE–, there was a 2.2-fold increase in eNOS protein with pVEGFE+ in the distal area of the RSF at day 2 after treatment that was mediated by a 6.6-fold increase in VEGF protein (**Figure 1b, c**). Significant progress has been made elucidating the mechanisms underlying VEGF-mediated increases in eNOS, but many aspects of the complete mechanism remain unclear despite the well-established role of this pathway in wound healing and angiogenesis.[34–36] *In vivo*[34] and *in vitro*[6,33] studies have shown that VEGF-mediated increases in eNOS protein levels are typically two- to fivefold over background levels at an optimal *in vitro* dosage. In the work presented here eNOS levels with pVEGFE+ remain similar from day 2 to day 5 after treatment, but increase with pVEGFE– over this time period, suggesting that maximal levels of eNOS has been reached in the pVEGFE+ group 2 days after treatment. The small increase in eNOS levels, relative to VEGF levels, may be due to inhibition of eNOS production by high levels of nitric oxide.[37] Also, the persistence of eNOS expression in the pVEGFE+ and pVEGFE– treatment groups at day 5 after treatment, despite a decrease in VEGF expression, may be due to a VEGF-mediated increase in eNOS mRNA stability.[6] eNOS mRNA has an normal half-life of 48 hours[38] and further extending the half-life would allow for increases in eNOS protein to persist despite a decrease in VEGF levels.

The early increase in eNOS observed with pVEGFE+ could contribute to the increased perfusion and healing observed at the later time points. eNOS production of nitric oxide is important in the early stages of skin flap healing[39] and contributes to maintaining peripheral skin flap circulation.[40] Another possible mechanism for the increased perfusion and healing of the RSF after treatment with pVEGFE+ is increased neovascularization. We did not observe a significant increase in vessel density (data not shown) on day 14 of the study in skin samples harvested 2-cm from the RSFs distal end. Other groups delivering exogenous VEGF to wound healing models have reported the same phenomenon of increased healing without an associated increase in vessel density[24,41] although other studies do not discuss changes in capillary density. To prevent ischemic necrosis there must be adequate perfusion pressure to the distal region of the skin flap.[42] In this study, increased eNOS, and subsequently increased vasodilation, could be the primary mechanism of the pVEGFE+ mediated increase in perfusion and healing, but determination of the exact mechanism will require further studies.

The end goal of exogenous VEGF delivery to ischemic tissue is to increase the amount of nutrients supplied to the affected area and the rate of waste product removal by increasing vasodilation, vascular permeability, and angiogenesis. The strategy developed here to deliver VEGF to ischemic skin offers an attractive alternative to viral and recombinant protein approaches because it allows for control of localized expression level and duration. Further, direct injection of pVEGF with EP significantly increased VEGF expression levels, compared to direct injection of pVEGF without EP, and circumvents the adverse side effects and practicality issues associated with viral and recombinant protein delivery approaches. In summary, the pVEGFE+ treatment significantly increased VEGF expression for 5 days after delivery and this short-term increase was sufficient to significantly increase eNOS, compared to pVEGFE– treatment 2 days after delivery. Further, pVEGFE+ significantly increased recovery of baseline and postoperative perfusion as well as healing compared to all control treatment groups. In conclusion, electrically mediated intradermal delivery of pVEGF is a potential noninvasive therapeutic approach to increase perfusion and healing of ischemic wounds.

MATERIALS AND METHODS

Cloning of the VEGF165 plasmid. The human $VEGF_{165}$ plasmid was cloned by subcloning the hEF1–HTLV–hVEGF165 sequence from pBLAST49–hVEGF (Invivogen, San Diego, CA) into the backbone of pVAX1 (Invitrogen, Carlsbad, CA). The cytomegalovirus promoter was previously removed from pVAX to create a promoterless pVAX1. The empty vector control (pVAXE+) is the pVAX1 backbone containing the hEF1–HTLV promoter.

Animal model. All procedures were approved by the Animal Use and Care Committee of the University of South Florida College of Medicine. A total of 51 male Sprague–Dawley rats (275–300 g) were used in this study. Eleven rats were used to determine whether delivery of pVEGF to the gastrocnemius muscle resulted in increased VEGF expression. Twelve rats were used to evaluate VEGF and eNOS expression kinetics in the RSF and 28 rats were used for analysis of distal skin flap perfusion and survival. Prior to surgery and all procedures, animals were anesthetized with 2.5–3.0% isoflurane in oxygen. Surgeries were performed using standard aseptic technique. To create the rostral-based single pedicle RSF the left lateral flank of the rat was shaved and an 8 cm by 3 cm template was traced with a surgical marker. Full thickness incisions were made along the traced template lines. The flap was then elevated and sutured back to its bed using simple interrupted and running stitches with 4-0 nonabsorbable sutures.

Plasmid delivery. Delivery of pVEGF to the gastrocnemius muscle. The gastrocnemius muscle was exposed using a 1-cm incision. Animals received one 50-μL injection of pVEGF (2 mg/ml) in sterile saline using a 25 gauge, 5/8-inch length needle. The injection site was marked with one stitch using a 4-0 nonabsorbable suture to later identify the delivery site to be excised for protein extraction and assaying VEGF expression. For pVEGFE+ a four-needle electrode array with 6-mm gap width was then placed around the delivery site and eight 20-ms pulses were delivered at 200 V/cm. *Delivery of pVEGF to the RSF.* Two days after surgery animals were randomized into one of four treatment groups (pVEGFE+, pVEGFE–, pVAXE+ or P-E–) and received 4–50 μL intradermal injections of plasmid DNA (2 mg/ml) in sterile saline (except for P-E– which did not receive any treatment). All injections were performed using a 25 gauge, 5/8-inch length needle and were centered along the midline of the skin flap at 1, 3, 5, and 7 cm from the RSF pedicle. Injection sites were marked with a surgical marker if they were to be excised later for protein extraction. Where noted proximal delivery sites are those at 1 and 3 cm from the pedicle and distal delivery sites are those at 5 and 7 cm from the pedicle. After injection of plasmid DNA, the four-plate electrode[16] was used to deliver 8–20 ms electric pulses at 200 V/cm to pVAXE+ and pVEGFE+ treatment groups.

VEGF and eNOS protein expression. At the indicated time points the delivery sites were excised and snap frozen on dry ice. Tissue samples were homogenized in 1–2 ml of lysis buffer (150 mmol/l NaCl, 20 mmol/l sodium phosphate pH 7.4, 10% glycerol, 1% NP-40 and protease inhibitors (Roche, Indianapolis, IN)). Homogenates were then centrifuged at 4 °C for 10 minutes at 10,000 rpm. The supernatants were removed and assayed for protein expression using commercially available enzyme-linked immunosorbent assays for

human $VEGF_{165}$ (DY293B, R&D Systems, Minneapolis, MN) and eNOS (DEN00, R&D Systems) according to the manufacturer's instructions.

Skin flap perfusion. A Laser Doppler Perfusion Imager (Moor Instruments, Devon, UK) was used to record perfusion at indicated time points. A low intensity (2 mW) laser light beam (λ = 632.8 nm) scanned the surface of the skin without contact at a standardized working distance of 28 cm. Scan modus was set at 10 ms/pixel and resolution at 256 × 256 pixels. Three scans were completed per time point for each animal and average perfusion in arbitrary units (flux) for the distal region of the skin flap (5-cm^2 area) was determined for each time point. Baseline perfusion was assessed immediately before surgery. Perfusion results are reported as the percentage of perfusion units relative to baseline or postoperative perfusion (normalized to 100%).

Skin flap survival. The percent survival of the distal 5-cm^2 area of the skin flaps was quantitated on days 7 and 14 after surgery. Areas dark in color and/or covered in scabs were defined as necrotic/nonsurviving areas. Areas with normal skin texture and color were defined as surviving areas. The surviving areas were calculated using the Moor imaging software (Moor Instruments) and pictures were recorded using a digital camera (Nikon, Melville, NY).

Histology. Two weeks postoperatively animals were humanely euthanized. Tissue samples were excised from the center of the skin flap, 2 cm from the distal end, fixed in 10% neutral buffered formalin for at least 12 hours at room temperature, embedded in paraffin and sectioned (4 µm). If the tissue was necrotic at 2 cm from the distal end, samples were excised at the border of the necrotic and surviving tissue. Sections were then stained with hematoxylin and eosin using standard protocols. Stained sections were analyzed using a standard light microscope (Olympus BX51; Olympus, Center Valley, PA) and representative fields photographed with a Spot Insight 2 digital camera (Diagnostic Instruments, Sterling Heights, MI).

Statistical analyses. All values are reported as the mean ± SEM unless otherwise noted. Analysis of VEGF expression was completed using a two-tailed Student's *t*-test. Analysis of eNOS expression and distal perfusion was completed by ANOVA with a *post hoc* Dunnett's test to adjust for multiple comparisons to one control group (pVEGFE+). Statistical significance was assumed at $P < 0.05$. Analysis of distal RSF survival was completed using the Mann–Whitney–Wilcoxon test. For distal survival, a *post hoc* Bonferroni–Dunn correction was used to correct for multiple comparisons. All statistical analysis was completed using the Statistical Package for the Social Sciences (SPSS, Chicago, IL).

ACKNOWLEDGMENTS

We thank William Marshall (University of South Florida, College of Medicine) for his insight into vascular biology and skin flaps, Margaret Baldwin (University of South Florida, Division of Comparative Medicine) for technical advice in creating the skin flap model and Mark Jaroszeski (University of South Florida, College of Engineering) for construction of the four-plate electrode. This work was supported by the Florida Center of Excellence for Biomolecular Identification and Targeted Therapeutics. Richard Heller and Bernadette Ferraro are co-inventors on a patent application, which covers the technology that was used in the work reported in this article. Richard Heller is also an inventor on other patents that was used in this work. These additional patents have been licensed to RMR Technologies, LLC and Inovio Biomedical Corporation. Richard Heller has ownership interest in RMR Technologies and owns stock and stock options of Inovio Biomedical Corporation.

REFERENCES

1. Pearl, R and Johnson, D (1983). The vascular supply to the skin: an anatomical and physiological reappraisal—part I. *Ann Plast Surg* **11**: 99–105.
2. Robinson, J, Arndt, K, Le Boit, P and Wintroub, B (1996). Atlas of Cutaneous Surgery. Vol. 1. Saunders Co: Philadelphia. p. 400.
3. Waller, W, Lee, J, Zhang, F and Lineaweaver, W (2004). Gene therapy in flap survival. *Microsurgery* **24**: 168–173.
4. Ferrara, N, Gerber, H-P and LeCouter, J (2003). The biology of VEGF and its receptors. *Nat Med* **9**: 669–676.
5. Kroll, J and Waltenberger, J (1999). A novel function of VEGF receptor-2 (KDR): rapid release of nitric oxide in response to VEGF-A stimulation in endothelial cells. *Biochem Biophys Res Commun* **265**: 636–639.
6. Bouloumie, A, Schini-Kerth, VB and Busse, R (1999). Vascular endothelial growth factor up-regulates nitric oxide synthase expression in endothelial cells. *Cardiovasc Res* **41**: 773–780.
7. Carmeliet, P (2000). VEGF gene therapy: stimulating angiogenesis or angiomagenesis? *Nat Med* **6**: 1102–1103.
8. Ozawa, C, Banfi, A, Glazer, N, Thurston, G, Springer, M, Kraft, P *et al.* (2004). Microenvironmental VEGF concentration, not total dose, determines a threshold between normal and aberrant angiogenesis. *J Clin Invest* **113**: 516–527.
9. Lee, RJ, Springer, ML, Blanco-Bose, WE, Shaw, R, Ursell, PC and Blau, HM (2000). VEGF gene delivery to myocardium: deleterious effects of unregulated expression. *Circulation* **102**: 898–901.
10. von Degenfeld, G, Banfi, A, Springer, ML, Wagner, RA, Jacobi, J, Ozawa, CR *et al.* (2006). Microenvironmental VEGF distribution is critical for stable and functional vessel growth in ischemia. *FASEB J* **20**: 2657–2659.
11. Greenberg, JI, Shields, DJ, Barillas, SG, Acevedo, LM, Murphy, E, Huang, J *et al.* (2008). A role for VEGF as a negative regulator of pericyte function and vessel maturation. *Nature* **456**: 809–813.
12. Masaki, I, Yonemitsu, Y, Yamashita, A, Sata, S, Tanii, M, Komori, K *et al.* (2002). Angiogenic gene therapy for experimental critical limb ischemia: acceleration of limb loss by overexpression of vascular endothelial growth factor 165 but not of fibroblast growth factor-2. *Circ Res* **90**: 966–973.
13. Dor, Y, Djonov, V and Keshet, ELI (2003). Induction of vascular networks in adult organs: implications to proangiogenic therapy. *Ann NY Acad Sci* **995**: 208–216.
14. Vogel, J (2000). Nonviral skin gene therapy. *Hum Gene Ther* **11**: 2253–2259.
15. Daud, AI, DeConti, RC, Andrews, S, Urbas, P, Riker, AI, Sondak, VK *et al.* (2008). Phase I trial of interleukin-12 plasmid electroporation in patients with metastatic melanoma. *J Clin Oncol* **26**: 5896–5903.
16. Heller, LC, Jaroszeski, MJ, Coppola, D, McCray, AN, Hickey, J and Heller, R (2006). Optimization of cutaneous electrically mediated plasmid DNA delivery using novel electrode. *Gene Ther* **14**: 275.
17. McFarlane, R, DeYoung, G and Henry, R (1965). The design of a pedicle flap in the rat to study necrosis and its prevention. *Plast Reconstr Surg* **35**: 177–182.
18. Hurn, I, Fisher, J, Arganese, T and Rudolph, R (1983). Standardization of the dorsal rat flap model. *Ann Plast Surg* **11**: 210–213.
19. Jiang, J, Jiang, N, Gao, W, Zhu, J, Guo, Y, Shen, D *et al.* (2006). Augmentation of revascularization and prevention of plasma leakage by angiopoietin-1 and vascular endothelial growth factor co-transfection in rats with experimental limb ischaemia. *Acta Cardiol* **61**: 145–153.
20. Wells, DJ (2004). Gene therapy progress and prospects: electroporation and other physical methods. *Gene Ther* **11**: 1363–1369.
21. Khan, A, Ashrafpour, H, Huang, N, Neligan, PC, Kontos, C, Zhong, A *et al.* (2004). Acute local subcutaneous VEGF165 injection for augmentation of skin flap viability: efficacy and mechanism. *Am J Physiol Regul Integr Comp Physiol* **287**: R1219–R1229.
22. Peng, B, Zhao, Y, Xu, L and Xu, Y (2007). Electric pulses applied prior to intramuscular DNA vaccination greatly improve the vaccine immunogenicity. *Vaccine* **25**: 2064–2073.
23. Liu, PY, Kan, L, Xiao Tian, W, Badiavas, E, Rieger-Christ, KM, Jin Bo, T *et al.* (2005). Efficacy of combination gene therapy with multiple growth factor cDNAs to enhance skin flap survival in a rat model. *DNA Cell Biol* **24**: 751.
24. O'Toole, G, MacKenzie, D, Marucci, D, Poole, M, Lindeman, R, Buckley, MF *et al.* (2002). Vascular endothelial growth factor gene therapy in ischaemic rat skin flaps. *Br J Plast Surg* **55**: 55.
25. Kryger, Z, Zhang, F, Dogan, T, Cheng, C, Lineaweaver, WC and Buncke, HJ (2000). The effects of VEGF on survival of a random flap in the rat: examination of various routes of administration. *Br J Plast Surg* **53**: 234–239.
26. Ylä-Herttuala, S and Alitalo, K (2003). Gene transfer as a tool to induce therapeutic vascular growth. *Nat Med* **9**: 694–701.
27. Muruve, D, Barnes, M, Stillman, I and Libermann, T (1999). Adenoviral gene therapy leads to rapid induction of multiple chemokines and acute neutrophil-dependent hepatic injury *in vivo*. *Hum Gene Ther* **10**: 965–976.
28. Khan, TA, Sellke, FW and Laham, RJ (2003). Gene therapy progress and prospects: therapeutic angiogenesis for limb and myocardial ischemia. *Gene Ther* **10**: 285.
29. Vincent, KA, Jiang, C, Boltje, I and Kelly, RA (2007). Gene therapy progress and prospects: therapeutic angiogenesis for ischemic cardiovascular disease. *Gene Ther* **14**: 781–789.
30. Nissen, N, Polverini, P, Koch, A, Volin, M, Gamelli, R and DiPietro, L (1998). Vascular endothelial growth factor mediates angiogenic activity during the proliferative phase of wound healing. *Am J Pathol* **152**: 1445–1452.
31. Roman, S, Poole, M and Lindeman, R (2004). Vascular endothelial growth factor (VEGF) expression and the effect of exogenous VEGF on survival of a random flap in the rat. *Br J Plast Surg* **57**: 174.
32. Bates, DO and Pritchard Jones, RO (2003). The role of vascular endothelial growth factor in wound healing. *Int J Low Extrem Wounds* **2**: 107–120.
33. Hood, JD, Meininger, CJ, Ziche, M and Granger, HJ (1998). VEGF upregulates ecNOS message, protein, and NO production in human endothelial cells. *Am J Physiol Heart Circ Physiol* **274**: H1054–H1058.
34. Huang, N, Khan, A, Ashrafpour, H, Neligan, PC, Forrest, CR, Kontos, CD *et al.* (2006). Efficacy and mechanism of adenovirus-mediated VEGF-165 gene therapy for augmentation of skin flap viability. *Am J Physiol Heart Circ Physiol* **291**: H127–H137.
35. Namba, T, Koike, H, Murakami, K, Aoki, M, Makino, H, Hashiya, N *et al.* (2003). Angiogenesis induced by endothelial nitric oxide synthase gene through vascular

endothelial growth factor expression in a rat hindlimb ischemia model. *Circulation* **108**: 2250–2257.
36. Murohara, T, Asahara, T, Silver, M, Bauters, C, Masuda, H, Kalka, C *et al.* (1998). Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. *J Clin Invest* **101**: 2567–2578.
37. Vaziri, ND and Wang, XQ (1999). cGMP-mediated negative-feedback regulation of endothelial nitric oxide synthase expression by nitric oxide. *Hypertension* **34**: 1237–1241.
38. Yoshizumi, M, Perrella, MA, Burnett, JC Jr. and Lee, ME (1993). Tumor necrosis factor downregulates an endothelial nitric oxide synthase mRNA by shortening its half-life. *Circ Res* **73**: 205–209.
39. Furuta, S, Vadiveloo, P, Romeo-Meeuw, R, Morrison, W, Stewart, A and Mitchell, G (2004). Early inducible nitric oxide synthase 2 (NOS 2) activity enhances ischaemic skin flap survival. *Angiogenesis* **7**: 33–43.
40. Um, S, Suzuki, S, Toyokuni, S, Kim, B, Tanaka, T, Hiai, H *et al.* (1998). Involvement of nitric oxide in survival of random pattern skin flap. *Plast Reconstr Surg* **101**: 785–792.
41. Zhang, F, Lei, MP, Oswald, TM, Pang, Y, Blain, B, Cai, ZW *et al.* (2003). The effect of vascular endothelial growth factor on the healing of ischaemic skin wounds. *Br J Plastic Surg* **56**: 334–341.
42. Pavietic, MM (2002). Pedicle grafts. In: Slatter, D (ed). Textbook of Small Animal Surgery. Saunders: Philadelphia, PA. pp. 314–317.