Published in final edited form as:

J Surg Res. 2014 July ; 190(1): 367–377. doi:10.1016/j.jss.2014.02.051.

Adenoviral Mediated Gene Transfer of IGF-1 Enhances Wound Healing and Induces Angiogenesis

S. Balaji^{1,§}, M. LeSaint^{1,§}, S. S. Bhattacharya¹, C. Moles¹, Y. Dhamija¹, M. Kidd¹, L.D. Le¹, A. King¹, A. Shaaban¹, T. M. Crombleholme², P. Bollyky³, and S. G. Keswani^{1,*}

¹Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA

²Children's Hospital Colorado, Aurora, CO, USA

³Stanford University, Palo Alto, CA, USA

Abstract

Background—Chronic wounds are characterized by a wound healing and neovascularization deficit. Strategies to increase neovascularization can significantly improve chronic wound healing. Insulin like growth factor (IGF-1) is reported to be a keratinocyte mitogen and is believed to induce angiogenesis via a vascular endothelial growth factor (VEGF) dependent pathway. Using a novel *ex vivo* human dermal wound model and a diabetic impaired wound healing murine model, we hypothesized that adenoviral over expression of IGF-1 (Ad-IGF-1) will enhance wound healing and induce angiogenesis through a VEGF dependent pathway.

Methods—*Ex vivo*: 6 mm full thickness punch biopsies were obtained from normal human skin, and 3 mm full thickness wounds were created at the center. Skin explants were maintained at air liquid interface. *Db/db murine model*: 8 mm full thickness dorsal wounds in diabetic (db/db) mice were created. Treatment groups in both human *ex vivo* and *in vivo* db/db wound models include 1×10^8 PFU of Ad-IGF-1 or Ad-LacZ, and PBS (n=4–5/group). Cytotoxicity (LDH) was quantified at days 3, 5 and 7 for the human *ex vivo* wound model. Epithelial gap closure (H&E; Trichrome), VEGF expression (ELISA) and capillary density (CD 31+ CAPS/HPF) were analyzed at day 7.

© 2014 Elsevier Inc. All rights reserved.

*Corresponding author: Sundeep G. Keswani, MD, Laboratory for Regenerative Wound Healing, Division of Pediatric General, Thoracic, and Fetal Surgery, Cincinnati Children's Hospital Medical Center, 3333 Burnet Avenue, MLC 11025, Cincinnati, OH 45229-3039, USA. Phone (513) 803-2219, Fax (513) 636-7657, sundeep.keswani@cchmc.org.

§Authors contributed equally

There are no competing financial interests for any of the authors listed on the manuscript.

Author contributions: All authors approved the final version of the manuscript. SB-conception and design, analysis and interpretation, data collection, writing the article, critical revision of the article, MLS-analysis and interpretation, data collection, writing the article, critical revision of the article, SSB-data collection, critical revision of the article, CM-analysis and interpretation, data collection, critical revision of the article, YD-analysis and interpretation, data collection, critical revision of the article, MK-data collection, critical revision of the article, LDL-analysis and interpretation, critical revision of the article, AK-analysis and interpretation, critical revision of the article, AS- analysis and interpretation, critical revision of the article, TMC- analysis and interpretation, critical revision of the article, obtaining funding, PB- analysis and interpretation, critical revision of the article, SGK-conception and design, analysis and interpretation, data collection, critical revision of the article, obtaining funding.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Results—In the human ex vivo organ culture, the adenoviral vectors did not demonstrate any significant difference in cytotoxicity compared to PBS. Ad-IGF-1 over expression significantly increases basal keratinocyte migration, with no significant effect on epithelial gap closure. There was a significant increase in capillary density in the Ad-IGF-1 wounds. However, there was no effect on VEGF levels in Ad-IGF-1 samples compared to controls. In db/db wounds, Ad-IGF-1 over expression significantly improves epithelial gap closure and granulation tissue with a dense cellular infiltrate compared to controls. Ad-IGF-1 also increases capillary density, again with no significant difference in VEGF levels in the wounds compared to control treatments.

Conclusions—In two different models, our data demonstrates that adenoviral mediated gene transfer of IGF-1 results in enhanced wound healing and induces angiogenesis via a VEGF-independent pathway. Understanding the underlying mechanisms of IGF-1 effects on angiogenesis may help produce novel therapeutics for chronic wounds or diseases characterized by a deficit in neovascularization.

Keywords

wound healing; IGF-1; gene therapy; diabetes; neovascularization

Introduction

Chronic wounds, including diabetic ulcers, are characterized by the disruption of normal healing process which results in a pathological state of non-healing cutaneous inflammation and delayed wound closure^{1,2}. These wounds account for an increasingly significant economic burden as their prevalence is raising commensurate with an aging population, and an increase in obesity and diabetes^{3,4}. Chronic diabetic ulcers are responsible for more than 42,500 non-traumatic lower-limb amputations and 27% of diabetic health care costs in the United States annually^{5,6}. Despite standard treatment protocols and active investigation on several fronts including novel cell, gene and molecular therapies^{7,8}, successful treatment of diabetic ulcers is limited, and currently there is no effective therapy.

Although the pathogenesis of impaired diabetic wound healing is multifactorial, dysfunctional angiogenesis is a primary contributing factor, which is characterized by hyperglycemia-related microvascular changes and deficiencies in both endothelial cells^{9,10} and endothelial progenitor cells (EPC)^{2,11–14}, which leads to impaired diabetic wound healing. The development of novel therapeutics that promotes angiogenesis has the potential to improve diabetic tissue repair and would be a boon for patients suffering with chronic non-healing wounds. Diabetic wounds are also known to have reduced growth factor levels^{12,15–17}, some of which promote the angiogenic response to injury. Many groups have demonstrated that supplementation of exogenous growth factors via recombinant growth factor therapy or gene transfer at the cellular and molecular level has vulnerary effects and improves wound healing outcomes^{2,18–21}. Amongst the several growth factors studied, insulin-like growth factor (IGF-1) levels have been shown to be reduced in diabetic wounds and are associated with delayed wound healing^{22–24}. Streptozotocin-induced diabetic rats demonstrated reduction in wound fluid IGF-I levels. Normal induction of IGF-1 mRNA is delayed and impaired in diabetic mice²⁵. Importantly, endothelial dysfunction observed in

diabetes has been shown to be associated with a blunted insulin/IGF signaling response in endothelial cells²⁶.

IGF-1 is a 7.6 KDA molecule that has 70 amino acids in a single chain with three intramolecular disulfide bridges. In the skin, IGF-1 is produced by cells of mesenchymal origin, such as fibroblasts of the dermis and dermal papilla^{27,28}. IGF-1 has several roles in skin homeostasis. It is recognized as a proliferation and survival factor for the skin and hair morphogenesis^{29,30}. Deficiency in IGF-1 is associated with decreased epidermal thickness and sparse hair growth³¹. The importance of IGF-1 signaling in the skin is evident from the original studies with IGF-1 receptor null (*Igf-1r*^{-/-}) mice, which exhibited hypotrophic skin with reduced number and size of the hair follicles³². IGF-1 also has an important role in wound healing. It is a keratinocyte mitogen, motogen and morphogen^{33,34}. IGF-1 was shown to stimulate keratinocyte proliferation and migration, as well as collagen production by fibroblasts³⁴⁻³⁶. In addition, IGF-1 is a potent stimulator of hair follicle morphogenesis and cycling, and of re-epithelialization of skin wounds³⁷. IGF-1 has also been shown to increase angiogenesis in several different models of tissue injury, potentially through an increase in HIF-1 α and VEGF dependent mechanisms³⁸⁻⁴². Further, it has been shown that the addition of IGF-1 to diabetic wounds using non-viral gene transfer in combination with cell therapy improves diabetic wound closure significantly⁴³. However, the underlying mechanism of how IGF-1 enhances angiogenesis and wound healing are not completely understood.

The limited data available supports the concept that IGF-1 therapy has the potential to improve diabetic wound healing. One of the inherent challenges of developing novel therapeutics for wound healing clinical trials is the species specific differences between preclinical animal models and humans. To address this, we have developed a novel model that utilizes wounded human skin that is maintained in culture *ex vivo* to screen candidate therapeutics for toxicity and wound healing effects in a rapid and efficient manner. These data can then be compared to *in vivo* data in animal models to develop a more complete understanding of the translational capability of the therapeutic agent. For diabetic animal studies, the best available model for type-II diabetic wound healing is the leptin receptor deficient db/db murine model. Wounds created in db/db wounds demonstrate increases protease activity and have a significant impairment in neovascularization and wound healing^{1,2}, similar to the impaired wound healing phenotype observed in diabetic patients.

Taken together, we hypothesize that over expression of IGF-1 via a VEGF dependent pathway will enhance angiogenesis and improve wound healing. To test this hypothesis we will first screen adenoviral overexpression of IGF-1 in a novel human *ex vivo* skin organ culture wound model to assess toxicity and vulnerability effects. We will then validate this data in a known diabetic impaired wound healing murine db/db mouse for similar end points.

Materials and Methods

Human *ex vivo* model

Human skin was obtained from National Disease Research Interchange (NDRI, Philadelphia, PA). All human tissues were obtained according to the guidelines set forth by

Cincinnati Children's Hospital Medical Center and under a protocol approved by the Institutional Review Board. Surgically discarded skin from de-identified donors was immediately collected and shipped overnight in hanks buffer. The samples used in this study were obtained from Caucasian female donors between 31–46 yrs. Skin was disinfected in 70% ethanol, and several changes of DMEM buffer containing penicillin/streptomycin (Life Technologies, Carlsbad, CA, USA).

Subcutaneous fat was carefully removed with iris scissors. 6 mm full thickness punch biopsy samples were created. In the middle of each biopsy samples, 3 mm full thickness wound was created by using a 3 mm punch biopsy. The skin discs with wounds were embedded in rat tail collagen I gel matrix (2.5 mg/ml) (BD Bioscience, San Jose, California). The cell culture media consisted of serum-free Dulbecco's Modified Eagle's medium (Life Technologies, Carlsbad, CA, USA), supplemented with 10 mM HEPES, 50 μ g/ml ascorbic acid, 100 μ M adenine, 0.5 μ M hydrocortisone, 0.1 μ M cholera toxin, 100 IU/ml penicillin and 10 μ g/ml streptomycin (Sigma-Aldrich, St. Louis, MO). The *ex vivo* organ cultures were maintained at the air-liquid interface and kept in the cell culture incubator at 37 °C with 5% CO₂. The media was changed every other day.

Db/db murine model

All animal procedures were performed using protocols approved by Cincinnati Children's Hospital Institutional Animal Care and Use Committee. 8–10 weeks old BKS.Cg-m+/+Leprdb/J (db/db) mice with confirmed serum glucose levels >400 mg/dl were used. (Jackson Laboratories, Bar Harbor, ME). Under isoflurane (0.5 ml titrated) inhalational anesthesia, the dorsal skin was shaved, scrubbed with betadine, and two full-thickness excisional skin wounds were created on the back of mice using an 8 mm dermal biopsy punch, leaving the underlying panniculus carnosus muscle intact. Wounds were covered with a sterile adhesive dressing (Tegaderm™, St Paul, MN). 1×10⁸ PFU Ad-IGF-1 or Ad-LacZ (in 50 μ l total volume) or 50 μ l PBS was then injected on to the wound between the panniculus carnosus layer and the dressing using a Hamilton syringe and 30 ½ gauge needle. Analgesics were administered and the mice were monitored daily for food consumption, pain and distress and general health.

Experimental Design

To determine if adenoviral mediated overexpression of IGF-1 improves wound healing and neovascularization, we used an *ex vivo* human organ culture wound model and an *in vivo* diabetic wound healing model. Wounds from both models were measured for epithelial gap closure, neovascularization and VEGF levels.

Ex vivo organ culture—At 24 hours after implanting the skin organ cultures, the media was changed and treatments were added to the wound well. 1×10⁸ PFU of Ad5 CMV IGF-1 vector (n=4) or Ad5 CMV LacZ (n=4) (in 20 μ l total volume) or 20 μ l PBS (n=4) were added and maintained for 2 days. The Ad5 CMV LacZ served as a control for the effects of the adenoviral vector. Virus was removed by gentle washing with several changes of media. Thereafter the samples were maintained in the cell culture incubator, with the media changed every other day. Samples were harvested at 7 days, bisected on the longitudinal

diameter of the wound, and fixed in 10% neutral buffered formalin for histological analyses. In addition to epithelial gap closure, basal keratinocyte migration was quantified in the *ex vivo* samples. The media was assessed by 1) LDH assay to determine the sample viability and investigate any cytotoxic effects of the viral particles, and 2) VEGF ELISA to determine VEGF expression levels.

Db/db murine model—One wound received either Ad5 CMV IGF-1 solution (1×10^8 PFU prepared in 50 μ l PBS) or Ad5 CMV LacZ solution (1×10^8 PFU prepared in 50 μ l PBS) (n=5 animals/group). The second wound (internal control) received 50 μ l of sterile PBS. In an additional control group (n=5 animals), both wounds received 50 μ l of PBS. The animals were killed and wounds were harvested at 7 days post wounding (proliferation stages of wound healing). Wounds were bisected on the longitudinal diameter of the wound, one half was fixed in 10% neutral buffered formalin for histological and immunostaining analyses. The other half was frozen at -80°C for protein quantification.

Adenovirus production

All constructs used in this study were first generation recombinant replication defective, type 5 adenovirus vectors. All adenoviral genomes had either E1 or both E1 and E3 regions deleted and were driven by a cytomegalovirus (CMV) promoter. The parental adenovirus for Ad-IGF-I used in the db/db model was Ad-Easy and the parental adenovirus for Ad-LacZ was dl7001. Both viruses were prepared using 293 cells, purified by double cesium chloride density centrifugation, desalted using a DG-10 column (Bio-Rad Laboratories, Hercules, CA) and stored in 10% glycerol at -80°C . Viral titers were determined by measuring absorbance at 260 nm using a DU-640 spectrophotometer (Beckman Instruments, Fullerton, CA). Viral preparations were diluted at least 1:10 with phosphate buffered saline (PBS) prior to use in animals in order to prevent glycerol toxicity. The adenoviral construct for IGF-I was generously supplied by Dr. Lee Sweeney (University of Pennsylvania, Philadelphia, PA). The recombinant adenovirus containing the β -galactosidase transgene (Ad-LacZ) was obtained from Dr. James Wilson (University of Pennsylvania, Philadelphia, PA). Ad-IGF-1 and Ad-LacZ constructs used in the *ex vivo* organ culture model were obtained from Dr. Meenhard Herlyn (Institute for Human Gene Therapy, University of Pennsylvania). We have previously reported their functional ability to induce efficient gene transfer and significantly increase IGF-1 levels⁴⁴.

Cytotoxicity assay

The release of lactate dehydrogenase (LDH) into the supernatant (medium) of organ culture, which was collected every two days, was measured as an indicator of cytotoxicity. The LDH assay was executed according to manufacturer's instructions (Cytotoxicity Detection Kit; Roche, Indianapolis, Indiana).

Histological structure

5 μ m wound sections were cut from paraffin embedded blocks from both models. Epithelial gap and granulation tissue deposition were studied from hematoxylin and eosin (H&E) stained sections and morphometric image analysis using Nikon Elements (Nikon Instruments, Melville, NY). Entire wound was scanned. It is well established that open

wound re-epithelialization occurs via circumferential lateral inward movement of the keratinocytes to close the wound. Keratinocytes migrate in a leapfrog pattern at the migrating epidermal tail to establish closure⁴⁵. Therefore, on a 4X edge-to-edge scanned image of the wound, epithelial gap closure was measured as the remaining gap (in μm) between the encroaching epithelial tails. In the *ex vivo* skin wounds, the basal keratinocyte migration is measured as the length (in μm) of the migrating basal cells on either sides of the encroaching epithelial tails, which demonstrate a distinct elongated morphology compared to the spindle shaped cells at the unwounded margin. In addition, the height of the epidermis increases along this distance. Wound extra cellular matrix composition was determined using Masson's Trichrome staining as per the manufacturer's protocol (Polyscientific, Bay Shore, NY). Analysis was performed by a reviewer who was blinded to the treatment groups.

Immunohistochemistry

Serial sections were immunostained with rat CD31 (1:20; BD Pharmingen, Franklin Lakes, NJ), followed by biotinylated goat anti-rat antibodies (1:200; Vector laboratories, Burlingame, CA). In the *ex vivo* organ culture wound model, capillary lumen density was measured as the average number of CD31-positive lumens from 8 randomly chosen 20X sections across the organ culture sample. Capillary lumen density in db/db wounds was measured as the average number of CD31-positive lumens from 6 HPF (high powered fields, 40X) per section, chosen just above the panniculus carnosus and equally distributed between the epithelial margins. Analysis was performed by a reviewer who was blinded to the treatment groups.

ELISA and quantification of total protein

VEGF levels were measured in media collected from human *ex vivo* organ culture samples at day 7. All the different treatment groups in the db/db wounds were harvested/cored out similarly using a standard size punch biopsy. The wound tissue homogenates at day 7 post wounding were analyzed using a VEGF ELISA kit per manufacturer's protocol (R&D systems, Minneapolis, MN). ELISA data for each sample was normalized to total protein in the sample analyzed by Coomassie Plus protein assay (Thermo Scientific, Logan, UT).

Statistical Analysis

All data are presented as mean $\pm$ standard error. Two-way analysis of variance (ANOVA) followed by Bonferroni test was used for comparing data between all treatments. For comparing different treatments or time points Student's t-test and one-way ANOVA followed by Tukey's multiple comparison test was used. Probability values of $p < 0.05$ were interpreted to denote statistical significance.

Results

Ex Vivo Skin Model

There are no cytotoxic effects of the adenovirus in the *ex vivo* human skin organ culture—To determine if the adenoviral vector was affecting the viability of *ex vivo* organ cultures, Ad-IGF-1 and the control vector Ad-LacZ was compared to PBS treated

controls and LDH activity was assessed in the media as a measurement of cytotoxicity. There are no significant differences between the LDH activity between the adenoviral treated and PBS treated groups at days 3, 5 or 7; demonstrating no cytotoxic effects of the viral vector or the transgene on the wound culture (Figure 1A). Similarly there are no apparent histological differences in the extra cellular matrix organization or the epidermal stratification by H&E and Trichrome staining from day 0 skin to day 7-post wounding between the three treatment groups (Figure 1B–E).

IGF-1 over expression significantly increases basal keratinocyte migration but has no effect on epithelial gap closure—

To investigate the role of Ad-IGF-1 in keratinocyte migration during wound healing in the human *ex vivo* wound model, Ad-IGF-1, Ad-LacZ or PBS were added to the wound beds. Keratinocyte migration at day 7 post wounding was assessed by Masson's Trichrome and H&E staining using computer assisted morphometric analysis. Representative images are shown in Figure 2A–C. Adenoviral overexpression of IGF-1 significantly increases the basal keratinocyte migration compared to controls (Figure 2D: Ad-IGF-1 $423.67 \pm 47.5 \mu\text{m}$ vs. Ad-LacZ $287.59 \pm 20.0 \mu\text{m}$, PBS $308.08 \pm 11.4 \mu\text{m}$, $p < 0.05$). However, there are no significant differences in wound closure between the treatments at the day 7 time point as assessed by epithelial gap (Ad-IGF-1 $2994.12 \pm 97.27 \mu\text{m}$ vs. Ad-LacZ $3141.86 \pm 77.87 \mu\text{m}$, PBS $3066.85 \pm 82.7 \mu\text{m}$, $p > 0.05$).

IGF-1 overexpression significantly increases neovascularization without increased VEGF levels at day 7—

To examine the effect of IGF-1 overexpression on neovascularization during wound healing in the *ex vivo* organ culture model, CD31-positive capillary lumen density was compared between Ad-IGF-1, Ad-LacZ or PBS treatments at day 7 post wounding. There is a significant increase in the density of CD31-positive capillary lumens in the Ad-IGF-1 treated wounds (Figure 3A–D: Ad-IGF-1 14.5 ± 0.68 CAPS/HPF vs. Ad-LacZ 9.9 ± 0.65 CAPS/HPF and PBS 10.7 ± 0.49 CAPS/HPF, $p < 0.05$). However, VEGF ELISA of the organ culture media demonstrate that there is no further effect of adding Ad-IGF-1 on VEGF protein levels compared to the control treatments at day 7 post wounding (Figure 3E: Ad-IGF-1 $163.3 \pm 24.9 \text{ pg/mg}$ vs. Ad-LacZ $170.8 \pm 57.4 \text{ pg/mg}$, PBS $161.5 \pm 12.07 \text{ pg/mg}$, $p > 0.05$).

Db/db *in vivo* wound model

Adenoviral mediated gene transfer of IGF-1 enhances diabetic impaired wound healing by accelerating epithelial gap closure and promoting granulation tissue formation—

To determine the role of Ad-IGF-1 on wound healing in the diabetic impaired murine model, we assessed epithelial gap closure and granulation tissue formation. 8 mm wounds were created and were treated with Ad-IGF-1, Ad-LacZ or PBS. Ad-IGF-1 significantly improves wound closure obtained by measuring the remaining gap between the two encroaching migrating epithelial tails shown by black arrows (Figure 4A–D: Ad-IGF-1 $3331.47 \pm 189.1 \mu\text{m}$ vs. AD-LacZ $5278 \pm 100 \mu\text{m}$ or PBS $3952.67 \pm 134.4 \mu\text{m}$, $p < 0.05$). In addition, IGF-1 treated wounds demonstrate the presence of a robust wound granulation tissue with a dense cellular infiltrate that appeared well vascularized, as compared to a very minimal and sparse granulation tissue formation in the control treated wounds. Representative wound sections are shown (Figure 4A–C).

IGF-1 over expression significantly increases neovascularization in diabetic wounds without an increase in VEGF—To determine the effect of Ad-IGF-1 on neovascularization in the diabetic impaired murine model, Ad-IGF-1, Ad-LacZ or PBS were added to the wound beds. Wound vessel density was assessed by CD31 immunohistochemical staining. At day 7, Ad-IGF-1-treated wounds demonstrate significantly increased capillary density compared to controls (Figure 5A–D: Ad-IGF-1 9.5 ± 0.64 CAPS/HPF vs. Ad-LacZ 3.8 ± 0.5 CAPS/HPF, PBS 2.9 ± 0.5 CAPS/HPF, $p < 0.01$). However, similar to the findings in the human *ex vivo* skin organ culture model, there is no significant difference in the VEGF protein levels between the treatments when measured by ELISA at day 7 (Figure 5E: Ad-IGF-1 40.3 ± 4.7 pg/ μ g vs. Ad-LacZ 37 ± 3.4 pg/ μ g, PBS 33 ± 4.6 pg/ μ g, $p > 0.05$).

Discussion

Our results demonstrate that IGF-1 increases wound healing, and increases neovascularization without an increase in VEGF. We have used the human *ex vivo* model to examine the vector toxicity and efficacy of IGF-1 gene therapy. In this model that is representative of viral vector-human cellular interactions, we demonstrate no cytotoxic effects of IGF-1 gene therapy on wound healing. Our data further demonstrate an increase in keratinocyte migration and improved neovascularization with IGF-1 treatment; however we did not see a change in wound closure or the VEGF levels. The effects on keratinocyte migration may be a precursor to improved wound closure at a time point beyond 7 days that will need to be examined. Chronic wounds are characterized by decreased epidermal cell migration over the wound bed⁴⁶; therefore being able to increase keratinocyte migration with IGF-1 over expression might be a way to accelerate the wound healing process. This *ex vivo* data is corroborated by the *in vivo* data in the db/db model, where IGF-1 overexpression significantly improves wound closure at 7 days post wounding. These results are all the more impressive in light of the fact that the addition of the adenoviral vectors alone as shown in this study and previously reported², impairs wound healing. IGF-1 overexpression overcomes this deficit and further improves the wound healing phenotype.

Our results demonstrate that IGF-1 overexpression significantly increases neovascularization in both the *ex vivo* organ culture model and the *in vivo* db/db model. In the db/db model, we have further observed the formation of a robust granulation tissue, which is a highly vascularized matrix consisting of fibroblasts and endothelial cells. IGF-1 has been shown in several other tissue repair models to have similar angiogenic properties. In scald wounds, IGF-1 cDNA delivery increased neoangiogenesis along with increased extra cellular matrix deposition, improved keratinocyte migration and re-epithelialization⁴¹. Growth hormone-mediated increase of insulin-like growth factor 1 has been shown to correct age-dependent impairment of EPCs⁴⁷. There is further evidence that higher bioavailability of IGF-1 may protect against onset of ischemic heart disease⁴⁸, and in type 2 diabetic patients, may offer improved metabolic control and prevent vascular complications⁴⁹.

The major mechanism for IGF-1 effects on neovascularization is known to be mediated through HIF-1 and VEGF³⁸. In this study we determined if IGF-1 increases VEGF levels. In

both the *ex vivo* organ culture model and the *in vivo* db/db model, IGF-1 increases angiogenesis without a concomitant increase in the VEGF levels at the 7 day time point. It is possible that VEGF may have been up-regulated at an earlier time point. However, based on our previous work¹, 7 days post wounding in the db/db animal model is a time of robust angiogenic response, and therefore it would be reasonable to expect that VEGF levels would be increased at this time point if indeed IGF-1 angiogenic effects were mediated through a VEGF-dependent mechanism. Therefore our results suggest that in this model, IGF-1 may be increasing angiogenesis via VEGF independent mechanisms. The pathways of how IGF-1 increases angiogenesis have not been fully explained. Literature shows that IGF-1 activates a complex that includes IGF-1R to cause the initial onset of angiogenesis. In addition, once activated this complex is required for VEGF-2R activation by VEGF⁵⁰. Furthermore it has been shown that CD-147 has a positive feedback mechanism with IGF-1 and when CD 147 is blocked, IGF effects on angiogenesis can be partially blocked⁵¹. Therefore, IGF-1 may not cause a direct up regulation of VEGF but may influence angiogenesis in multiple ways, including via the activation of VEGF-2R and via CD-147. It has been further demonstrated that IGF-1 may add stability to existing endothelial cells which may in part potentiate the effect of IGF-1 on improved angiogenesis⁵². Alternatively, IGF-1 can directly oppose endothelial dysfunction in a number of ways⁵³; by interacting with high-affinity endothelial binding sites that lead to nitric oxide (NO) production⁵⁴, and by promoting insulin sensitivity⁵⁵. These are areas where further research needs be conducted to discover the alternate mechanisms of how IGF-1 may improve angiogenesis. The human *ex vivo* organ culture model and the db/db murine model are excellent systems to define these underlying molecular mechanisms.

We have shown that IGF-1 overexpression has improved wound closure in the db/db murine model, which has a major clinical significance. However, these chronic wounds have exacerbated protease activity which is detrimental to any recombinant growth factor therapy, and better approaches for sustained and targeted delivery of growth factors is critical for efficacy of treatment. Using gene therapy, it is possible to commandeer the cell machinery and allow for a more natural and sustained approach for growth factor delivery. In this study, we have used an adenoviral vector that encodes for IGF-1 to deliver the transgene. Our previous data supports that adenoviral-mediated gene therapy efficiently delivers the transgene and significantly improves neovascularization and wound healing outcomes^{2,56}. An advantage of the adenoviral mediated gene transfer is that the vector begins to work within 48 hours and has transgene expression decreasing from 2 to 7 days with no significant expression after 14 days⁵⁷. In support, several studies have shown that growth factor and/or transgene delivery during the initial critical phase of wound healing may be sufficient to correct the neovascularization and wound healing deficit and facilitate favorable wound healing progression. Further, compared to other viral therapies, the adenovirus does not integrate and the risk for oncogenic potential is abrogated.

We developed a human *ex vivo* model to be representative of a human wound that can be used as a rapid screening tool and investigate species specific differences. However the model has some limitations. Foremost, this is wound healing in culture with no systemic response. There is no influence of bone marrow stem cells or circulating precursor cells or

systemic inflammatory response on wound healing in this model. However, the advantage of this model is that it can be used to rapidly and consistently screen therapeutic candidates. Our diabetic model is also not a true model of type II diabetes. The db/db model is characterized by leptin receptor deficiency, and while it is representative of the diabetic phenotype, it also has some limitations. Therefore the findings from the *ex vivo* model when used/interpreted in conjunction with *in vivo* outcomes, yields enhanced data from these preclinical models. This will potentially decrease costs when validating these findings in large animal models, like pigs, which closely represent the human dermal phenotype⁵⁸.

In conclusion our data support that IGF-1 can be a potential vulnerary therapeutic and points to interesting IGF-1 biology in its ability to induce angiogenesis by a potential VEGF independent mechanism. This may have implications in other models, where IGF-1 has a role in angiogenesis, such as in tumors and heart diseases. Further, the two wound models can be used to understand this underlying mechanism of IGF-1 effects on neovascularization.

Acknowledgments

The authors sincerely acknowledge the technical support received from our laboratory staff members. This research is supported by K08 GM098831-03 NIH/NIGMS (SGK) and Wound Healing Society Foundation 3M Award (SGK).

Abbreviations

Ad vectors	Adenoviral vectors
BGS	Bovine growth serum
CMV	Cytomegalovirus
DMEM	Dulbecco's modified Eagle's Media
EC	Endothelial cells
ELISA	Enzyme linked immune sorbent assay
EPCs	Endothelial Progenitor cells
PFU	Particle forming units
HIF-1α	Hypoxia inducible factor-1 alpha
IGF-1	Insulin like growth factor 1
IGF-1R	Insulin like growth factor 1 receptor
LDH	Lactate dehydrogenase
NO	Nitric oxide
PBS	Phosphate buffered saline
VEGF	Vascular endothelial growth factor
VEGF-2R	Vascular endothelial growth factor 2 receptor

References

1. Balaji S, Vaikunth SS, Lang SA, et al. Tissue-engineered provisional matrix as a novel approach to enhance diabetic wound healing. Wound repair and regeneration: official publication of the Wound Healing Society [and] the European Tissue Repair Society. Jan-Feb;2012 20(1):15–27.
2. Keswani SG, Katz AB, Lim FY, et al. Adenoviral mediated gene transfer of PDGF-B enhances wound healing in type I and type II diabetic wounds. Wound repair and regeneration: official publication of the Wound Healing Society [and] the European Tissue Repair Society. Sep-Oct;2004 12(5):497–504.
3. Richmond, NA.; Lamel, SA.; Davidson, JM., et al. Wound repair and regeneration: official publication of the Wound Healing Society [and] the European Tissue Repair Society. Oct 17. 2013 US-National Institutes of Health-funded research for cutaneous wounds in 2012.
4. Sen CK, Gordillo GM, Roy S, et al. Human skin wounds: a major and snowballing threat to public health and the economy. Wound repair and regeneration: official publication of the Wound Healing Society [and] the European Tissue Repair Society. Nov-Dec;2009 17(6):763–771.
5. Gordo A, Scuffham P, Shearer A, Oglesby A, Tobian JA. The health care costs of diabetic peripheral neuropathy in the US. Diabetes Care. Jun; 2003 26(6):1790–1795. [PubMed: 12766111]
6. CDC. Centers for Disease Control and Prevention. National diabetes fact sheet: general information and national estimates on diabetes in the United States, 2007. Vol. 2008. Atlanta, GA: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention; 2008.
7. Shen JT, Falanga V. Innovative therapies in wound healing. J Cutan Med Surg. May-Jun;2003 7(3): 217–224. [PubMed: 12704530]
8. Brem H, Sheehan P, Boulton AJ. Protocol for treatment of diabetic foot ulcers. Am J Surg. May; 2004 187(5A):1S–10S. [PubMed: 15147985]
9. Pirola L, Balcerzyk A, Okabe J, El-Osta A. Epigenetic phenomena linked to diabetic complications. Nat Rev Endocrinol. Dec; 2010 6(12):665–675. [PubMed: 21045787]
10. Berlanga-Acosta J, Schultz GS, Lopez-Mola E, Guillen-Nieto G, Garcia-Siverio M, Herrera-Martinez L. Glucose toxic effects on granulation tissue productive cells: the diabetics' impaired healing. Biomed Res Int. 2013; 2013:256043. [PubMed: 23484099]
11. Capla JM, Grogan RH, Callaghan MJ, et al. Diabetes impairs endothelial progenitor cell-mediated blood vessel formation in response to hypoxia. Plast Reconstr Surg. Jan; 2007 119(1):59–70. [PubMed: 17255657]
12. Galiano RD, Tepper OM, Pelo CR, et al. Topical vascular endothelial growth factor accelerates diabetic wound healing through increased angiogenesis and by mobilizing and recruiting bone marrow-derived cells. The American journal of pathology. Jun; 2004 164(6):1935–1947. [PubMed: 15161630]
13. Tepper OM, Galiano RD, Capla JM, et al. Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures. Circulation. Nov 26; 2002 106(22):2781–2786. [PubMed: 12451003]
14. Loomans CJ, de Koning EJ, Staal FJ, et al. Endothelial progenitor cell dysfunction: a novel concept in the pathogenesis of vascular complications of type 1 diabetes. Diabetes. Jan; 2004 53(1):195–199. [PubMed: 14693715]
15. Brem H, Tomic-Canic M. Cellular and molecular basis of wound healing in diabetes. J Clin Invest. May; 2007 117(5):1219–1222. [PubMed: 17476353]
16. Falanga V. Wound healing and its impairment in the diabetic foot. Lancet. Nov 12; 2005 366(9498):1736–1743. [PubMed: 16291068]
17. Liu ZJ, Velazquez OC. Hyperoxia, endothelial progenitor cell mobilization, and diabetic wound healing. Antioxid Redox Signal. Nov; 2008 10(11):1869–1882. [PubMed: 18627349]
18. Singla S, Kumar A, Singla M. Role of Epidermal Growth Factor in Healing of Diabetic Foot Ulcers. The Indian journal of surgery. Dec; 2012 74(6):451–455. [PubMed: 24293898]
19. Hou Z, Nie C, Si Z, Ma Y. Deferoxamine enhances neovascularization and accelerates wound healing in diabetic rats via the accumulation of hypoxia-inducible factor-1alpha. Diabetes research and clinical practice. May 28.2013

20. Losi P, Briganti E, Errico C, et al. Fibrin-based scaffold incorporating VEGF- and bFGF-loaded nanoparticles stimulates wound healing in diabetic mice. *Acta biomaterialia*. Aug; 2013 9(8): 7814–7821. [PubMed: 23603001]
21. Kwon MJ, An S, Choi S, et al. Effective healing of diabetic skin wounds by using nonviral gene therapy based on minicircle vascular endothelial growth factor DNA and a cationic dendrimer. *The journal of gene medicine*. Apr; 2012 14(4):272–278. [PubMed: 22407991]
22. Velander P, Theopold C, Hirsch T, et al. Impaired wound healing in an acute diabetic pig model and the effects of local hyperglycemia. *Wound repair and regeneration: official publication of the Wound Healing Society [and] the European Tissue Repair Society*. Mar-Apr; 2008 16(2):288–293.
23. Bitar MS. Insulin-like growth factor-1 reverses diabetes-induced wound healing impairment in rats. *Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et métabolisme*. Aug; 1997 29(8):383–386. [PubMed: 9288575]
24. Blakytyn R, Jude EB, Martin Gibson J, Boulton AJ, Ferguson MW. Lack of insulin-like growth factor 1 (IGF1) in the basal keratinocyte layer of diabetic skin and diabetic foot ulcers. *The Journal of pathology*. Apr; 2000 190(5):589–594. [PubMed: 10727985]
25. Brown DL, Kane CD, Chernausk SD, Greenhalgh DG. Differential expression and localization of insulin-like growth factors I and II in cutaneous wounds of diabetic and nondiabetic mice. *The American journal of pathology*. Sep; 1997 151(3):715–724. [PubMed: 9284820]
26. Rask-Madsen C, King GL. Mechanisms of Disease: endothelial dysfunction in insulin resistance and diabetes. *Nature clinical practice. Endocrinology & metabolism*. Jan; 2007 3(1):46–56.
27. Tavakkol A, Elder JT, Griffiths CE, et al. Expression of growth hormone receptor, insulin-like growth factor 1 (IGF-1) and IGF-1 receptor mRNA and proteins in human skin. *The Journal of investigative dermatology*. Sep; 1992 99(3):343–349. [PubMed: 1324963]
28. Little JC, Redwood KL, Granger SP, Jenkins G. In vivo cytokine and receptor gene expression during the rat hair growth cycle. Analysis by semi-quantitative RT-PCR. *Experimental dermatology*. Aug; 1996 5(4):202–212. [PubMed: 8889467]
29. Sadagurski M, Yakar S, Weingarten G, et al. Insulin-like growth factor 1 receptor signaling regulates skin development and inhibits skin keratinocyte differentiation. *Molecular and cellular biology*. Apr; 2006 26(7):2675–2687. [PubMed: 16537911]
30. Weger N, Schlake T. Igf-I signalling controls the hair growth cycle and the differentiation of hair shafts. *The Journal of investigative dermatology*. Nov; 2005 125(5):873–882. [PubMed: 16297183]
31. Lange M, Thulesen J, Feldt-Rasmussen U, et al. Skin morphological changes in growth hormone deficiency and acromegaly. *European journal of endocrinology/European Federation of Endocrine Societies*. Aug; 2001 145(2):147–153. [PubMed: 11454509]
32. Liu JP, Baker J, Perkins AS, Robertson EJ, Efstratiadis A. Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). *Cell*. Oct 8; 1993 75(1):59–72. [PubMed: 8402901]
33. Rudman SM, Philpott MP, Thomas GA, Kealey T. The role of IGF-I in human skin and its appendages: morphogen as well as mitogen? *The Journal of investigative dermatology*. Dec; 1997 109(6):770–777. [PubMed: 9406819]
34. Haase I, Evans R, Pofahl R, Watt FM. Regulation of keratinocyte shape, migration and wound epithelialization by IGF-1- and EGF-dependent signalling pathways. *Journal of cell science*. Aug 1; 2003 116(Pt 15):3227–3238. [PubMed: 12829742]
35. Granot I, Halevy O, Hurwitz S, Pines M. Growth hormone and insulin-like growth factor I regulate collagen gene expression and extracellular collagen in cultures of avian skin fibroblasts. *Molecular and cellular endocrinology*. Sep; 1991 80(1–3):1–9. [PubMed: 1659542]
36. Ando Y, Jensen PJ. Epidermal growth factor and insulin-like growth factor I enhance keratinocyte migration. *The Journal of investigative dermatology*. May; 1993 100(5):633–639. [PubMed: 8491986]
37. Semenova E, Koegel H, Hasse S, et al. Overexpression of mIGF-1 in keratinocytes improves wound healing and accelerates hair follicle formation and cycling in mice. *The American journal of pathology*. Nov; 2008 173(5):1295–1310. [PubMed: 18832567]

38. Fukuda R, Hirota K, Fan F, Jung YD, Ellis LM, Semenza GL. Insulin-like growth factor 1 induces hypoxia-inducible factor 1-mediated vascular endothelial growth factor expression, which is dependent on MAP kinase and phosphatidylinositol 3-kinase signaling in colon cancer cells. *The Journal of biological chemistry*. Oct 11; 2002 277(41):38205–38211. [PubMed: 12149254]
39. Yu DH, Mace KA, Hansen SL, Boudreau N, Young DM. Effects of decreased insulin-like growth factor-1 stimulation on hypoxia inducible factor 1-alpha protein synthesis and function during cutaneous repair in diabetic mice. *Wound repair and regeneration: official publication of the Wound Healing Society [and] the European Tissue Repair Society*. Sep-Oct;2007 15(5):628–635.
40. Aghdam SY, Eming SA, Willenborg S, et al. Vascular endothelial insulin/IGF-1 signaling controls skin wound vascularization. *Biochemical and biophysical research communications*. May 4; 2012 421(2):197–202. [PubMed: 22503682]
41. Jeschke MG, Herndon DN. The combination of IGF-I and KGF cDNA improves dermal and epidermal regeneration by increased VEGF expression and neovascularization. *Gene therapy*. Aug; 2007 14(16):1235–1242. [PubMed: 17538636]
42. Jiang F, Tang YT, Guo L, Jiao XY. The role of insulin-like growth factor I and hypoxia inducible factor 1alpha in vascular endothelial growth factor expression in type 2 diabetes. *Annals of clinical and laboratory science*. Winter;2013 43(1):37–44. [PubMed: 23462604]
43. Hirsch T, Spielmann M, Velandar P, et al. Insulin-like growth factor-1 gene therapy and cell transplantation in diabetic wounds. *The journal of gene medicine*. Nov; 2008 10(11):1247–1252. [PubMed: 18767030]
44. Jones HN, Crombleholme T, Habli M. Adenoviral-mediated placental gene transfer of IGF-1 corrects placental insufficiency via enhanced placental glucose transport mechanisms. *PloS one*. 2013; 8(9):e74632. [PubMed: 24019972]
45. Laplante AF, Germain L, Auger FA, Moulin V. Mechanisms of wound reepithelialization: hints from a tissue-engineered reconstructed skin to longstanding questions. *FASEB journal: official publication of the Federation of American Societies for Experimental Biology*. Nov; 2001 15(13):2377–2389. [PubMed: 11689463]
46. Morgan C, Nigam Y. Naturally derived factors and their role in the promotion of angiogenesis for the healing of chronic wounds. *Angiogenesis*. Jul; 2013 16(3):493–502. [PubMed: 23417553]
47. Thum T, Hoeber S, Froese S, et al. Age-dependent impairment of endothelial progenitor cells is corrected by growth-hormone-mediated increase of insulin-like growth-factor-1. *Circulation research*. Feb 16; 2007 100(3):434–443. [PubMed: 17234973]
48. Juul A, Scheike T, Davidsen M, Gyllenberg J, Jorgensen T. Low serum insulin-like growth factor I is associated with increased risk of ischemic heart disease: a population-based case-control study. *Circulation*. Aug 20; 2002 106(8):939–944. [PubMed: 12186797]
49. Janssen JA, Lamberts SW. Circulating IGF-I and its protective role in the pathogenesis of diabetic angiopathy. *Clinical endocrinology*. Jan; 2000 52(1):1–9. [PubMed: 10651746]
50. Rapraeger AC, Ell BJ, Roy M, et al. Vascular endothelial-cadherin stimulates syndecan-1-coupled insulin-like growth factor-1 receptor and cross-talk between alphaVbeta3 integrin and vascular endothelial growth factor receptor 2 at the onset of endothelial cell dissemination during angiogenesis. *The FEBS journal*. May; 2013 280(10):2194–2206. [PubMed: 23331867]
51. Chen Y, Gou X, Ke X, Cui H, Chen Z. Human tumor cells induce angiogenesis through positive feedback between CD147 and insulin-like growth factor-I. *PloS one*. 2012; 7(7):e40965. [PubMed: 22844419]
52. Shigematsu S, Yamauchi K, Nakajima K, Iijima S, Aizawa T, Hashizume K. IGF-1 regulates migration and angiogenesis of human endothelial cells. *Endocrine journal*. Mar; 1999 46(Suppl):S59–62. [PubMed: 12054122]
53. Conti E, Carrozza C, Capoluongo E, et al. Insulin-like growth factor-1 as a vascular protective factor. *Circulation*. Oct 12; 2004 110(15):2260–2265. [PubMed: 15477425]
54. Schini-Kerth VB. Dual effects of insulin-like growth factor-I on the constitutive and inducible nitric oxide (NO) synthase-dependent formation of NO in vascular cells. *Journal of endocrinological investigation*. 1999; 22(5 Suppl):82–88. [PubMed: 10442576]

55. Conti E, Andreotti F, Sestito A, et al. Reduced levels of insulin-like growth factor-1 in patients with angina pectoris, positive exercise stress test, and angiographically normal epicardial coronary arteries. *The American journal of cardiology*. Apr 15; 2002 89(8):973–975. [PubMed: 11950439]
56. Gordon A, Kozin ED, Keswani SG, et al. Permissive environment in postnatal wounds induced by adenoviral-mediated overexpression of the anti-inflammatory cytokine interleukin-10 prevents scar formation. *Wound repair and regeneration: official publication of the Wound Healing Society [and] the European Tissue Repair Society*. Jan-Feb;2008 16(1):70–79.
57. Hirsch T, von Peter S, Dubin G, et al. Adenoviral gene delivery to primary human cutaneous cells and burn wounds. *Mol Med*. Sep-Oct;2006 12(9–10):199–207. [PubMed: 17225867]
58. King AJ. The use of animal models in diabetes research. *British journal of pharmacology*. Jun; 2012 166(3):877–894. [PubMed: 22352879]

Figure 1.

(A) Cytotoxicity analyzed by measuring the levels of LDH activity released into *ex vivo* organ culture media demonstrates no differences between the three treatments at day 3, 5 or 7. (B) H&E stained human skin from day 0 demonstrates an undulated epidermis, and a crimped pattern of collagen in the dermis. (C–E) Wound sections from the three different treatments at day 7 post wounding in organ culture appears similar to the normal skin in the dermal and epidermal organization, demonstrating no significant effect of the treatment groups on the skin histology.

Figure 2.

(A–C) Masson's trichrome stained human *ex vivo* wound sections at day 7 post wounding demonstrates increased keratinocyte migration in Ad-IGF-1 treated wounds compared to Ad-LacZ or PBS controls. Dashed line represents the length of basal keratinocyte migration. (D) Statistical analysis demonstrates a significant difference in keratinocyte migration between the three treatments. Bar plots represent average $\pm$ SEM. P values by ANOVA.

Figure 3.

(A–C) CD31-stained human *ex vivo* wound sections at day 7 post wounding demonstrates increased capillary lumen density in Ad-IGF-1 treated wounds compared to Ad-LacZ or PBS controls. (D) Statistical analysis demonstrates a significant difference in capillaries per high powered fields (20X, CAPS/HPF) between the three treatments. (E) There was no difference in the VEGF protein levels between the three treatments measured by ELISA in the organ culture media at day 7 post wounding. Bar plots represent average ± SEM. P values by ANOVA.

Figure 4.

(A–C) H&E stained db/db murine wound sections at day 7 post wounding demonstrates increased wound closure (distance between arrowheads represents epithelial gap) in Ad-IGF-1 treated wounds compared to Ad-LacZ or PBS controls. (D) Statistical analysis demonstrates a significant difference in epithelial gap between the three treatments. Bar plots represent average $\pm$ SEM. P values by ANOVA.

Figure 5.

(A–C) CD31-stained db/db wound sections at day 7 post wounding demonstrates increased capillary lumen density in Ad-IGF-1 treated wounds compared to Ad-LacZ or PBS controls. (D) Statistical analysis demonstrates a significant difference in capillaries per high powered fields (40X, CAPS/HPF) between the three treatments. (E) There was no difference in the VEGF protein levels between the Ad-IGF-1-treated and control-treated wounds measured by ELISA in the wound homogenates at day 7 post wounding. Bar plots represent average $\pm$ SEM. P values by ANOVA.