Nanofiber Microenvironment Effectively Restores Angiogenic Potential of Diabetic Endothelial Cells

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Objective: The effect of chronic hyperglycemic exposure on endothelial cell (EC) phenotype, impaired wound neovascularization, and healing is not completely understood. The hypotheses are: 1) chronic exposure to diabetic conditions *in vivo* impairs the angiogenic potential of ECs and 2) this deficiency can be improved by an extracellular microenvironment of angiogenic peptide nanofibers.

Approach: Angiogenic potential of microvascular ECs isolated from diabetic (db/db) and wild type (wt) mice was assessed by quantifying migration, proliferation, apoptosis, capillary morphogenesis, and vascular endothelial growth factor (VEGF) expression for cell cultures on Matrigel (Millipore, Billerica, MA) or nanofibers under normoglycemic conditions. The *in vivo* effects of nanofiber treatment on wound vascularization were determined using two mouse models of diabetic wound healing.

Results: Diabetic ECs showed significant impairments in migration, VEGF expression, and capillary morphogenesis. The nanofiber microenvironment restored capillary morphogenesis and VEGF expression and significantly increased proliferation and decreased cell apoptosis of diabetic cells versus wt controls. In diabetic wounds, nanofibers significantly enhanced EC infiltration, neovascularization, and VEGF protein levels, as compared to saline treatment; this effect was observed even in MMP9 knockout mice with endothelial progenitor cell (EPC) deficiency.

Innovation: The results suggest a novel approach for correcting diabetes-induced endothelial deficiencies via cell interactions with a nanofiber-based provisional matrix in the absence of external angiogenic stimuli.

Conclusion: Impaired endothelial angiogenic potential can be restored by angiogenic cell stimulation in the nanofiber microenvironment; this suggests that nanofiber technology for diabetic wound healing and treatment of other diabetes-induced vascular deficiencies is promising.

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INTRODUCTION

DIABETES IS A SIGNIFICANT health concern in the United States, where 25.8 million people (8.3% of the population) have diabetes. Chronic diabetic ulcers represent a significant

health care burden and are responsible for more than 65,700 nontraumatic lower-limb amputations in the United States annually.¹ Despite standard treatment protocols and novel cell, gene, and molecular

therapies,^{2,3} successful treatment of diabetic ulcers is limited. This often results in lower-limb amputations with high morbidity and mortality.⁴

Therapeutic strategies for diabetic wound healing are limited in their effectiveness due to complex diabetes-associated changes in the wound microenvironment and simultaneous disruption of several pathways involved in the healing response. Thus, the diabetic wound environment is associated with inflammatory dysregulation,⁵ intensified by increased proteolysis,⁶ which results in insufficient accumulation of granulation tissue and fast degradation of protein-based treatment compounds. Along with a severely altered wound microenvironment, deficiencies are also seen on the cellular level in the diabetic condition. Multiple studies have focused on the effects of diabetes on wound cells, including keratinocytes, fibroblasts, macrophages, and smooth muscle and endothelial cells (ECs).⁷ Hyperglycemia-related microvascular pathology is the major contributor to impaired diabetic healing and is associated with deficiencies in both ECs^{7,8} and endothelial progenitor cells (EPC).^{9,10} In particular, hyperglycemia can affect endothelial function via several major mechanisms, including toxic effects on ECs via nitric oxide, oxidative stress and inflammation,^{11,12} and accumulation of advanced glycation end products (AGEs) that results in altered extracellular matrix and disrupted angiogenic growth factor signaling.^{5,13} Studies *in vitro* have used high-glucose culture conditions that demonstrated increased endothelial apoptosis;^{14,15} impaired proliferation, adhesion, and tube formation;^{16,17} and changes in cytokine production.¹⁸ Recent reports suggest, however, that an *in vitro* acute hyperglycemic model may not be ideal for mechanistic studies of the effects of diabetes on EC responses, due to a phenomenon called “hyperglycemic memory” (reviewed by Giacco and Brownlee¹⁹). Thus, experimental evidence suggests that oxidative stress results in sustained activation of antiangiogenic, proinflammatory pathways in ECs even after glycemia is normalized (i.e., the cell phenotype becomes altered permanently, although the exact mechanisms are not well understood). Our recent findings also supported this concept for the diabetic fibroblasts.²⁰ The important implication of these findings is that there is a need for novel therapies that can reverse hyperglycemic memory of endothelium.

Despite significant research efforts directed toward scaffolds and skin substitutes for treatment of diabetic wounds, there is still a paucity of successful options that are focused on correcting en-

dothelial deficiencies in the diabetic environment. One attractive *in situ* tissue engineering option is a scaffold made from synthetic peptide nanofibers, which can be used to create a suitable microenvironment for diabetic wound healing applications. *In vitro* studies have shown that RAD16-II nanofibers support multiple cell types (including cardiomyocytes, ECs, and fibroblasts), enhance capillary morphogenesis and angiogenic growth factor expression, and allow for long-term study of cell–cell interactions.^{21,22} Our recent study²³ presented a novel mechanism for nanofiber-mediated activation of angiogenic signaling via relatively weak low-affinity interactions between ECs and RAD motifs in the RAD16-II nanofibers, which can trigger phosphorylation of β_3 integrin domain. In ECs, integrin $\alpha_v\beta_3$ is the most abundant and influential receptor regulating angiogenesis.²⁴ For $\alpha_v\beta_3$ integrin-dependent function, phosphorylation of β_3 cytoplasmic domain is crucial: It regulates $\alpha_v\beta_3$ affinity, avidity, ligand-binding strength, as well as basic cellular functions such as cell spreading and survival through interactions with intracellular signaling proteins.^{25–27} Our recent findings suggest that the angiogenic cell stimulation in the nanofiber microenvironment likely results from sustained extracellular signal-regulated kinase (ERK) pathway activation and increased vascular endothelial growth factor (VEGF) expression.²³ Importantly, by design, the nanofiber scaffold may be resistant to the AGE-associated damage that may be detrimental to the native materials, such as collagen I. Overall, these studies suggest that proangiogenic RAD16-II nanofibers may be uniquely suited to improving potential deficiencies in diabetic ECs and wound healing.²⁸

CLINICAL PROBLEM ADDRESSED

Despite significant efforts, successful treatment of diabetic ulcers is limited. Hyperglycemia-related microvascular pathology is thought to be the major contributor to impaired diabetic healing, although the mechanisms are not completely understood. The long-term goal of this research is to develop a new therapeutic strategy for correcting diabetes-associated vascular deficiency in the wound using the angiogenic peptide nanofiber microenvironment as a granulation tissue substitute. The objectives of this study were to quantify the effects of chronic *in vivo* exposure to diabetes on angiogenic potential of microvascular ECs and to determine whether these deficiencies can be improved by the proangiogenic nanofiber microenvironment.

MATERIALS AND METHODS

In vitro methods

Cell isolation and culture

All animal procedures were performed using protocols approved by the University of Cincinnati and Cincinnati Children's Hospital Institutional Animal Care and Use Committees. Microvascular ECs were isolated from the lungs of 8- to 10-week-old female C57BLKS/J inbred mouse strain (BKS). Cg-m +/+ Lepr^{db}/J (db/db) mice with serum glucose levels >450 mg/dL and C57BL/6J wild type (wt) mice (Jackson Laboratories, Bar Harbor, ME) were used, as described previously.²³ Briefly, harvested tissues were washed in cold phosphate buffered saline (PBS) several times, finely chopped, and digested in Collagenase III (180 to 200 U/mL) at 37°C for 45 min. Supernatant containing cells were separated from remaining tissue using a 70 μ m disposable cell strainer (Becton Dickinson Labware, Bedford, MA) and centrifuged at 400 $\times g$ for 10 min at 4°C. ECs were immediately double sorted from the total cells isolated using CD31- and CD102-conjugated magnetic beads (0.75 μ g of primary antibody per 25 μ L of Dynabeads; Invitrogen Corporation, Carlsbad, CA) as per manufacturer's protocol to obtain a pure EC culture.

Wild type and db/db ECs were cultured in Medium 199 (HyClone, Logan, UT) containing 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 1% Antibiotic-Antimycotic (Atlanta Biologicals, Lawrenceville, GA), 10 μ g/mL heparin (Sigma-Aldrich, St. Louis, MO), and 0.2 ng/mL growth supplement (Sigma-Aldrich, St. Louis, MO). All cells were serum starved for 12 h before experiments, and for all experiments cell culture medium without growth supplement was used. Cell cultures were maintained at 37°C in 100% humidified air containing 5% CO₂. Cells of passage 4–12 were used in all experiments.

Migration: BD assay with or without VEGF stimulus

A 3- μ m pore size, 96-well migration assay system (BD Biosciences, Franklin Lakes, NJ) was used. This system consisted of a multiwell plate with lid and inserts, where the cell-seeding surface was coated with fibronectin (Abcam, Cambridge, MA). ECs stained with CellTracker (Invitrogen Corporation, Carlsbad, CA) were plated on the insert membrane at a seeding density of 3,000 cells/insert ($n=3$ per db/db and wt groups). The insert was filled with EC culture medium with no growth supplements, while the bottom plate reservoir well was filled with EC medium supplemented with 20 ng/mL of mouse VEGF (R&D Systems,

Minneapolis, MN) to create an angiogenic gradient for ECs to migrate through the membrane. At 24 h, the cell-seeded insert surface was swabbed clean and the samples were fixed and stained using a nonspecific cellular stain, Diffquick (Polysciences Inc., Warrington, PA). Ten high power images at 64X were taken of each well and the total number of cells that migrated was counted.

Cell seeding

ECs (wt and db/db) were seeded on RAD16-II peptide nanofibers (RARADADARARADADA; purchased in acetate salt form with >95% purity from SynBioSci Corporation, Livermore, CA; 10 mg/mL) or Matrigel in culture plate inserts (13-mm diameter, 0.4- μ m pore size; Millipore, Billerica, MA) at a seeding density of 50,000 cells/insert. Matrigel was chosen as a control because it is an established system for capillary morphogenesis, which, similar to the nanofiber hydrogel, does not require addition of angiogenic factors (such as VEGF) to the culture medium to initiate network formation. Importantly, both Matrigel and nanofiber hydrogel have similar microstructure (discussed in detail by Mahabeshwar and colleagues²⁹).

Capillary morphogenesis

Before seeding, ECs were labeled with CellTracker Dye (Invitrogen Corporation, Carlsbad, CA). Samples were cultured for 24 h to allow capillary-like network formation. After fixation with 2% paraformaldehyde, images were taken ($n=5$ per sample) using an inverted fluorescent microscope (Olympus IX81; Olympus America Inc., Center Valley, PA). Correlation analysis with MATLAB (The MathWorks Inc., Natick, MA) was used to characterize capillary-like endothelial network size as previously described.^{21,22}

EC proliferation in vitro

Staining for proliferation marker Ki67 (Dako, Carpinteria, CA) and 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen Corporation, Carlsbad, CA) was performed to assess cell growth. ECs (wt and db/db) were seeded on RAD16-II peptide nanofibers or Matrigel in culture plate inserts) as described above at a seeding density of 25,000 cells/insert. Cells were fixed with 2% paraformaldehyde at 24 h. Samples were immunostained with rat antimouse Ki67 (1:250; Dako, Carpinteria, CA), followed by fluorescent goat antirat AlexaFluor secondary antibody (1:200; Invitrogen Corporation, Carlsbad, CA) and DAPI (Invitrogen Corporation, Carlsbad, CA), to identify proliferating cells. The average absolute number and percentages (number of positive cells to the total number of cells) of proliferating ECs

were quantified in five fields per sample at 20X magnification.

EC apoptosis in vitro

Anti-ACTIVE Caspase-3 (Promega Corporation, Madison, WI) and DAPI (Invitrogen Corporation, Carlsbad, CA) staining was performed to assess cell death. ECs (wt and db/db) were seeded on RAD16-II peptide nanofibers or Matrigel in culture plate inserts at a seeding density of 25,000 cells/insert. Cells were fixed with 2% paraformaldehyde at 24 h and immunostained with Anti-ACTIVE Caspase-3 (1:250), followed by fluorescent goat anti-rat antibody (1:500) and DAPI (Invitrogen Corporation, Carlsbad, CA). The average absolute number and percentages (number of positive cells to the total number of cells) of apoptotic ECs were quantified in five fields per sample at 20X magnification.

VEGF expression by ECs in vitro

ECs (wt and db/db) were cultured (50,000 cells/insert) on Matrigel or RAD16-II peptide nanofibers (10 mg/mL). Medium samples were collected at 24 h in culture and stored at -80°C until testing. A VEGF enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) was used per manufacturer's protocol to determine protein concentrations in medium samples. Additional controls of the cell culture medium alone (containing 10% serum) were included to confirm that growth factors in the serum would not affect protein expression and detection, with no differences observed between medium samples and the 0 pg/mL standard.

In vivo methods

Murine diabetic wound healing model and treatments

To validate the *in vitro* results, two experimental *in vivo* mouse models of impaired wound healing were used.

Db/db model (type II diabetes). For this model, 8- to 10-week old female BKS, Cg-m+/+Lepr^{db}/J (db/db) mice with serum glucose levels >450 mg/dL and C57BL/6J wt mice (controls), were used. Two full-thickness excisional skin wounds (8 mm) were created on the back of mice, leaving the underlying panniculus carnosus muscle intact, and covered with sterile adhesive dressing (TegadermTM, 3M, St. Paul, MN), as by Leslie-Barbick and colleagues.²⁸

MMP9 knockout (KO) and streptozotocin (STZ) model (type I diabetes). This model was used as a model of "double" deficiency in wound healing,

where effects of STZ-induced type I diabetes and impaired endothelial progenitor cell mobilization and vasculogenesis^{30,31} are combined. For this model, 8- to 10-week-old MMP9 KO (FVB.Cg-Mmp9tm1Tvuj; (Jackson Laboratories, Bar Harbor, ME) were used. Diabetes was induced by STZ (Sigma-Aldrich, St. Louis, MO) injection according to the protocol from the American Models of Diabetic Complications Consortium (available from www.diacomp.org/shared/showFile.aspx?doctypeid=3&docid=19). Briefly, mice were fasted for 4 h before injection and injected for 5 consecutive days with 50 mg/kg of STZ dissolved in 0.1 M of sodium citrate buffer (pH 4.5). The animals with serum glucose levels >300 mg/dL were considered diabetic. Two full-thickness excisional wounds (6 mm) were created on the back. Donut-shaped splints with 8-mm inner diameter were made from a silicone sheet (Grace Bio-Laboratories, Bend, OR) as suggested by Galiano and colleagues.³² The splint was fixed to skin around the wound with an adhesive (Krazy Glue[®], Elmer's Inc., Columbus, OH), followed by interrupted 6-0 nylon sutures (Ethicon Inc., Somerville, NJ), before the wounds were covered with Tegaderm. Preliminary experiment animals demonstrated that for the larger, sluggish db/db mice, the Tegaderm dressings were well maintained throughout the experiment, with the histological assessment of wounds showing little to no signs of contraction at day 7.²⁸ In contrast, in the leaner, active MMP9 KO mice, splints were required to maintain the Tegaderm dressing and prevent wound contraction, in order to obtain the wounds of similar quality to that in db/db model. In all groups, one wound received either 50 μL of RAD16-II peptide nanofiber solution (10 mg/mL) ($n=4-5$ animals/group) were used based on the power analysis that showed that at least $n=3$ animals/group were needed to achieve test power >0.8). The second wound (internal control) received 50 μL of sterile PBS. In an additional control group ($n=8$ animals), both wounds received 50 μL of PBS. The animals were sacrificed and wounds were harvested at day 7.

EC infiltration and proliferation

Serial sections were immunostained with rat anti mouse Meca-32 (to identify ECs; 1:10; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) and rat antimouse Ki67 (to identify proliferating cells; 1:250; Dako, Carpinteria, CA), followed by appropriate fluorescent antibodies (Invitrogen Corporation, Carlsbad, CA) and DAPI (Invitrogen Corporation, Carlsbad, CA). The average percentages of EC infiltration (number of

Meca-32 positive cells to the total number of cells) and proliferating ECs (number of both Meca-32 and Ki67 positive cells to the total number of Meca-32 positive cells) were quantified by counting positive cells in 3–6 high-power fields (HPFs, 40X, chosen just above the panniculus carnosus and equally distributed between wound epithelial margins) per section.

Vascular density

To determine the vascular density in the wounds, serial wound sections were immunostained either with rat antimouse CD31 (1:20; BD Pharmingen, Franklin Lakes, NJ) or rat antimouse Meca-32 (1:10; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) antibodies followed by biotinylated rabbit or goat antirat antibodies (1:200; Vector Laboratories, Burlingame, CA) to identify ECs. Vascular or capillary lumen density was measured as the average number of CD31, or Meca-32-positive lumens from 6 HPF (40X) per section, chosen just above the panniculus carnosus and equally distributed between the epithelial margins.

VEGF expression in the diabetic wound

At day 7 post wounding, wounds were harvested and homogenized in 50 mM Tris-HCl buffer containing 1% NP40, aprotinin (3.3 $\mu\text{g/mL}$), leupeptin (10 $\mu\text{g/mL}$), and pepstatin (4 $\mu\text{g/mL}$). VEGF protein expression in wound tissue homogenate was measured using an ELISA kit (R&D Systems, Minneapolis, MN) as per manufacturer's protocol.

Statistical analysis

The results are reported as average $\pm$ standard deviation. Statistical comparisons between experimental groups were performed using either one-factor or two-factor analysis of variance (ANOVA) and *post hoc* test with Bonferroni corrections.

Results were considered statistically significant at $p < 0.05$. All *in vitro* experiments were performed in triplicates and repeated at least twice.

RESULTS

Angiogenic response of ECs is impaired with diabetes

Chemotactic migration of ECs was measured using a modified Boyden Chamber assay with and without VEGF stimulus (20 ng/mL). For both experimental conditions, significant impairment in chemotactic migration was observed in diabetic ECs, as compared to wt ECs (Fig. 1). Interestingly, while wt cells demonstrated a significant increase in migration in the presence of VEGF, this effect was absent in the diabetic cells, suggesting a major impairment in functional response of diabetic endothelium to proangiogenic stimulus. Capillary morphogenesis by wt and diabetic ECs (Fig. 2ab) was assessed using Matrigel assay and network characterization with MATLAB.^{21–23} Diabetes dramatically impaired the ability of ECs to form capillary-like networks on the Matrigel (Fig. 2b, $p < 0.001$, ANOVA). Staining with Ki67 proliferation marker (Fig. 2c) demonstrated no difference in the percentage of proliferating diabetic ECs, as compared to wt ECs, when cultured on Matrigel controls. Analyses of the Caspase-3 staining showed a lower levels in cell apoptosis (Fig. 2d) in diabetic ECs ($7.8 \pm 1.6\%$ Caspase-3-positive cells), as compared to wt ECs ($13.1 \pm 1.7\%$ Caspase-3-positive cells, $p < 0.001$, ANOVA), when cultured on Matrigel controls.

In order to assess whether a proangiogenic environment can restore diabetes-induced deficiency in angiogenic cell responses, a previously described system of angiogenic peptide nanofibers is used. In

Figure 1. *In vitro* cell migration of wt and diabetic ECs. Cell migration without any stimulus or toward a chemotactic VEGF stimulus was measured. Cell migration was significantly reduced in diabetic cells as compared to wild type cells ($p < 0.05$, ANOVA, $n = 9/\text{group}$). Interestingly, while wt cells demonstrated significant increase in migration in the presence of VEGF as compared to no stimulus, a similar response was not observed in diabetic cells. EC, endothelial cell; VEGF, vascular endothelial growth factor.

Figure 2. *In vitro* capillary-like network formation, proliferation, and apoptosis of wt and diabetic ECs cultured on Matrigel or peptide nanofiber scaffolds. **(a)** Capillary morphogenesis by wt and diabetic ECs cultured on Matrigel or nanofiber scaffolds (scale bar = 10 μm). **(b)** Capillary-like network formation (μm) was measured on angiogenic Matrigel or nanofiber scaffolds at 24 h. Analyses demonstrated significant effects of both diabetic condition and substrate on network formation ($p < 0.01$, ANOVA, $n = 9/\text{group}$), where diabetes resulted in significantly impaired capillary morphogenesis on Matrigel, which was restored in the nanofiber microenvironment. **(c)** Cell proliferation was measured using Ki67 staining. On Matrigel, no difference in cell proliferation was observed between wt and diabetic cells; however, nanofiber scaffolds resulted in significantly increased proliferation in diabetic cells as compared to wild type cells ($p < 0.05$, ANOVA, $n = 6/\text{group}$). **(d)** Cell apoptosis was measured using Caspase-3 staining. In Matrigel, the percentage of apoptotic wt ECs was significantly larger than that of diabetic cells ($p < 0.05$, ANOVA, $n = 6/\text{group}$), while no difference in apoptosis was observed between wt and diabetic cells cultured on the peptide nanofibers. EC, endothelial cell.

this system, spontaneous formation of capillary-like three-dimensional network formation has been reported following EC seeding on the peptide nanofiber hydrogel.^{21–23} The characteristic network size of capillary networks formed by diabetic ECs (Fig. 2ab) was significantly increased on nanofibers ($23.0 \pm 4.1 \mu\text{m}$) as compared to Matrigel ($11.6 \pm 4.5 \mu\text{m}$, $p < 0.001$, ANOVA), thus restoring the capillary morphogenesis in diabetic cells to near the levels of normal (wild type) cells.

Significant increase in cell proliferation was observed in diabetic ECs in response to the nanofiber microenvironment (Fig. 2c), with a significantly increased percentage of proliferating diabetic ECs ($7.8 \pm 1.6\%$ Ki67-positive cells), as compared to wt ECs ($2.6 \pm 1.6\%$ Ki67-positive cells, $p < 0.001$) or Matrigel controls ($p < 0.005$) (ANOVA). No difference was observed in wt EC proliferation between nanofibers and Matrigel controls. These results suggest that the diabetic cells are more sensitive to the extracellular cell substrate, as compared to normal (wild type) cells.

Interestingly, cell culture in the nanofiber microenvironment reduced high levels of cell apoptosis in wild-type cultures to the levels of the diabetic cells (Fig. 2d; diabetic ECs, $5.6 \pm 1.1\%$ Caspase-3-positive cells), with both diabetic and wt ECs

demonstrating a significant decrease in staining for Caspase-3 as compared to Matrigel controls (Fig. 2d; diabetic ECs, $p < 0.001$; wt ECs, $p < 0.05$).

Nanofiber microenvironment rescues deficiency in VEGF expression by diabetic ECs *in vitro*

VEGF levels in the *in vitro* cultures and diabetic wound tissue were measured using ELISA (Fig. 3).

Figure 3. *In vitro* VEGF expression was measured using ELISA. On Matrigel, VEGF expression was significantly decreased in diabetic cells as compared to wild type cells ($p < 0.05$, ANOVA, $n = 5/\text{group}$), while no difference was observed between wild type and diabetic cells in the nanofiber microenvironment. VEGF, vascular endothelial growth factor.

When cultured on Matrigel controls (Fig. 3a), VEGF expression was significantly reduced in diabetic ECs (5.9 ± 2.2 pg/mL), as compared to wt ECs (19.5 ± 5.2 pg/mL, $p < 0.001$, ANOVA). NF microenvironment rescued this deficiency, with the levels for VEGF expression by diabetic ECs significantly increased in the nanofiber culture (32.0 ± 8.6 pg/mL), as compared to Matrigel controls (5.9 ± 2.2 pg/mL, $p < 0.001$) and restored to levels of wt ECs cultured on NFs (26.9 ± 6.6 pg/mL for wt ECs). Interestingly, similar to the results for the apoptosis, no effect of NFs on VEGF expression by wt EC controls was observed.

NF microenvironment improves endothelial deficiency and restores impaired vascularization in the diabetic wound

A db/db mouse (type II diabetes) model of diabetic wound healing was used to study endothelial deficiencies and vascularization in the diabetic wound (Fig. 4a), as previously described.^{23,28} Wound sections were double stained with Meca-32 and Ki67 proliferation marker to identify EC infiltration and the number of proliferating ECs in both wt and diabetic wounds treated with PBS and diabetic wounds treated with nanofibers (Fig. 4b to 4d). In PBS-treated wounds, there was a significant decrease in the level of EC infiltration in diabetic wounds ($12.9 \pm 1.3\%$ EC infiltration), as compared to wt wounds ($23.4 \pm 4.7\%$ EC infiltration, $p < 0.05$). This deficiency in EC infiltration in diabetic wounds was restored by treatment with the NFs ($22.6 \pm 1.7\%$ EC infiltration), with no observed difference as compared to wt wounds treated with PBS (Fig. 4c). There was a similar, although not significant, trend in the effect of nanofibers on the percentage of proliferating ECs (Fig. 4d; wt wounds treated with PBS, $6.9 \pm 2.6\%$; diabetic wounds treated with PBS, $5.2 \pm 1.9\%$; diabetic wounds treated with NFs, $6.5 \pm 2.6\%$; proliferating ECs/total ECs).

Wound sections were stained with endothelial marker CD31 to quantify lumen formation in both wt and diabetic wounds treated with PBS and diabetic wounds treated with NFs (Fig. 5ab). In PBS-treated wounds, there was a significant decrease in the number of lumens (Fig. 5c) in diabetic animals (6.8 ± 2.1 lumens/HPF), as compared to wild type animals (18.2 ± 2.1 lumens/HPF, $p < 0.001$). Treatment of diabetic wounds with peptide nanofibers partially rescued this deficiency, with the number of lumens in NF-treated diabetic wounds (13.1 ± 2.0 lumens/HPF) significantly increased as compared to PBS-treated diabetic wounds ($p < 0.01$), although the number of lumens was still less than that of PBS-

treated wt wounds ($p < 0.001$). VEGF levels were also measured in wound tissue from wt and diabetic wounds treated with PBS and diabetic wounds treated with NFs (Fig. 5d). In PBS-treated wounds, VEGF expression was significantly decreased in diabetic wounds (32.2 ± 11.2 pg/mL), as compared to wt wounds (232.4 ± 32.0 pg/mL, $p < 0.001$). Treatment of diabetic wounds with peptide nanofibers partially rescued this deficiency, with VEGF expression in nanofiber-treated diabetic wounds (53.4 ± 12.1 pg/mL) significantly increased as compared to PBS-treated diabetic wounds ($p < 0.05$), although expression was still less than that of PBS-treated wt wounds ($p < 0.001$).

In order to determine whether nanofiber wound treatment can improve vascular deficiency associated with diabetes under conditions of impaired endothelial progenitor cell mobilization, preliminary studies were performed using MMP9 KO mice model with STZ-induced diabetes (Fig. 6). Neovascularization was measured as EC infiltration and lumen density in the wounds using wound tissue staining with the mature EC marker Meca-32. In NF-treated wounds, a significant increase in EC infiltration was observed in both nondiabetic ($p < 0.05$) and diabetic ($p < 0.0005$) MMP9 KO mice, as compared with wounds treated with PBS. Similarly, wound treatment with the nanofibers resulted in significantly greater lumen density in both nondiabetic ($p < 0.05$) and diabetic ($p < 0.05$) MMP9 KO mice, when compared with PBS-treated wounds.

DISCUSSION

In this study, we investigated the effect of chronic *in vivo* exposure to diabetic conditions on the angiogenic responses of microvascular ECs. While previous studies have focused on the effects of diabetes on keratinocytes, fibroblasts, macrophages, and smooth muscle cells^{18,33–36} or acute *in vitro* endothelial response to hyperglycemia,^{14–18} limited information is available with regards to long-term diabetic exposure on ECs. The results of this study are consistent with the concept that ECs retain the memory of the *in vivo* diabetic environment^{19,36} even after prolonged culture in normoglycemic conditions, with evidence of significant impairment of endothelial angiogenic responses. Thus, diabetic ECs show significantly impaired cell migration, capillary morphogenesis, and VEGF expression *in vitro* as compared to wt ECs. Further, the murine diabetic wound healing model showed significant impairments in EC infiltration, lumen formation, and VEGF expression as compared to wt mouse controls.

Figure 4. Effects of the nanofiber microenvironment on the *in vivo* EC infiltration and proliferation in a db/db wound healing model ($n=5$ /group). **(a)** Picture of the mouse model of wound healing immediately after the surgery. Excisional skin wounds (8mm) were created on the back of wt or db/db mice and treated with either nanofibers or PBS. **(b)** Wound sections were stained with endothelial marker Meca-32 (green), proliferation marker Ki67 (red), and DAPI (blue). White arrow heads show proliferating ECs where the red, green, and blue staining overlap. *Asterisks (on the middle image: diabetic, PBS) show presence of red blood cells (scale bar = 10 μ m). **(c)** EC infiltration into the wound environment was measured using staining for endothelial marker Meca-32. In PBS-treated wounds, there was a significant decrease in the percentage of EC infiltration in diabetic wounds as compared to wild type wounds. In diabetic wounds treated with nanofibers no difference was observed, as compared to wt wounds treated with PBS. **(d)** EC proliferation was measured using staining for endothelial marker Meca-32 and proliferation marker Ki67. No changes in the percentage of proliferating ECs were observed between wt wounds treated with PBS, diabetic wounds treated with PBS, and diabetic wounds treated with NFs. EC, endothelial cell; PBS, phosphate buffered saline. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

The second goal of this study was to investigate the potential of proangiogenic peptide nanofibers to augment any observed deficiencies in the angiogenic response of ECs both *in vitro* and *in vivo*. Our results demonstrate that the nanofiber microenvironment did improve endothelial angiogenic potential both *in vitro* and in two murine diabetic wound healing models. When cul-

tured on peptide nanofibers, diabetic ECs showed significantly increased capillary morphogenesis and VEGF expression as compared to diabetic ECs cultured on Matrigel controls. Furthermore, a nanofiber microenvironment restored levels of capillary morphogenesis and VEGF expression by diabetic ECs to that near wild type ECs, indicating the potential of NFs to significantly

Figure 5. Effect of the nanofiber microenvironment on the *in vivo* lumen formation and VEGF expression in a db/db wound healing model ($n=5/\text{group}$). **(a)** Representative hematoxylin-eosin stain sections are shown. **(b)** Lumen formation in the wound environment was measured using staining for endothelial marker CD31 (scale bar = 10 μm). **(c)** In PBS-treated wounds, there was a significant decrease in number of lumens in diabetic wounds as compared to wild type wounds. In diabetic wounds treated with peptide nanofibers, the number of lumens significantly increased as compared to PBS-treated diabetic wounds. **(d)** VEGF expression in the wound environment was measured in wound tissue from wild type and diabetic wounds treated with PBS and diabetic wounds treated with nanofibers. In PBS-treated wounds, VEGF levels were significantly decreased in diabetic wounds as compared to wild type wounds. VEGF expression in nanofiber-treated diabetic wounds was significantly increased as compared to PBS-treated diabetic wounds. HPF, high-power field; PBS, phosphate buffered saline; VEGF, vascular endothelial growth factor. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

augment angiogenic deficiencies due to diabetes. In the db/db murine diabetic wound healing model, treatment with peptide nanofibers resulted in a restoration in EC infiltration to the level of wt wounds treated with PBS, increased lumen formation, and VEGF expression as compared to wound treatment with PBS. The absence of significant nanofiber effect on the endothelial proliferation in the wound suggests that it is EC migration and homing, rather than proliferation, that drives the observed improve-

ment in *in vivo* capillary morphogenesis and lumen formation.

Previous studies have demonstrated improvements in the angiogenic response of normal (wt) ECs in response to biomaterial microenvironments other than that of peptide nanofibers,³⁷ including a variety of polyethylene glycol-based hydrogels and native matrices such as fibrin and collagen. The information regarding how the scaffolds may affect behavior of the diabetic cells is limited, however. Our recent study²⁸ presented a

Figure 6. Effect of the nanofiber microenvironment on wound neovascularization in diabetic and nondiabetic MMP9 KO mouse model with EPC deficiency ($n=4-5$ /group). **(a)** Representative hematoxylin-eosin stain sections are shown. **(b)** Neovascularization was measured as EC infiltration and lumen density in the wounds and determined by staining with the mature EC marker Meca-32. Representative 40X images are shown. Arrows represent lumens. The scale bars represent 50 μ m. **(c)** In nanofiber-treated wounds, significant increase in EC infiltration was observed in both nondiabetic ($p<0.05$) and diabetic ($p<0.0005$) MMP9 KO mice as compared with wounds treated with PBS. **(d)** Lumen density, measured by number of vascular lumens/HPF, was significantly increased in the wounds treated with nanofibers in both nondiabetic ($p<0.05$) and diabetic ($p<0.05$) MMP9 KO mice when compared with PBS-treated wounds. HPF, high-power field; NF, nanofiber; STZ, streptozotocin. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

novel mechanism for nanofiber-mediated activation of angiogenic signaling via relatively weak low-affinity interactions between ECs and RAD motifs in the RAD16-II nanofibers, which can trigger phosphorylation of β_3 integrin and stimulate angiogenesis and VEGF expression via the mitogen-activated protein kinases/ERK pathway. A novel positive finding of the present study is that this mechanism for sustained angiogenic activation appears to work for the ECs with diabetic phenotype, where chronic *in vivo* exposure to hyperglycemic conditions results in altered cell responses on the native substrates. These results suggest, therefore, that the nanofiber microenvironment can play an important role in correcting diabetes-induced endothelial deficiency.

Interestingly, nanofiber wound treatment was able to significantly increase neovascularization in

both nondiabetic and diabetic MMP9 KO models. MMP9 plays a critical role in the mobilization of EPCs from the bone marrow, and mice lacking MMP9 have impaired EPC mobilization.^{30,31} An important implication of our findings is that the use of nanofiber microenvironment as a granulation tissue substitute in the wound may be a viable treatment option for diabetic patients with a recognized deficiency in EPCs.³⁸

In summary, the results of this study indicate that diabetes does impair the angiogenic potential of microvascular ECs, with the cells retaining the memory of the physiological environment in the body for months after removal from the body. Whether this memory is related to the recently described “hyperglycemic memory” of oxidative stress and resulting activation of antiangiogenic, proinflammatory pathways in ECs remains to be

elucidated. Our results also demonstrate the promise of the proangiogenic peptide nanofiber microenvironment that may attenuate diabetes-induced deficiencies, both *in vitro* and *in vivo*, and which by design may not be susceptible to AGE-related damage. Importantly, our findings suggest that although wound treatment with the nanofibers can be used in conjunction with other strategies to increase angiogenic factor levels in the wound, such as viruses, gene therapy, and exogenous delivery using biomaterials,^{39,40} it may not necessitate it. Future studies will provide important insights into how the wound healing process in the chronic state can be directed toward healing progression, potentially benefiting a large population of patients suffering from chronic diabetic ulcers.

KEY FINDINGS

- Chronic exposure to diabetic conditions *in vivo* results in significant impairments in EC migration, VEGF expression, and capillary morphogenesis, even after normal glucose conditions have been restored.
- *In vitro*, the nanofiber microenvironment significantly increases capillary morphogenesis and VEGF expression to the control, nondiabetic levels; it also results in significantly increased proliferation and decreased cell apoptosis of diabetic cells (vs. wt controls).
- In the diabetic mouse (db/db) wounds, nanofibers significantly enhanced EC infiltration, neovascularization, and VEGF protein levels, as compared to saline treatment.
- In the mouse model of double vascular deficiency (i.e., MMP9 KO mice with endothelial progenitor cell deficiency and STZ-induced diabetes), wound treatment with the nanofibers was still effective in correcting impaired neovascularization, which resulted in increased endothelial infiltration and lumens as compared to saline treatment.
- These results suggest that using the nanofiber microenvironment as a granulation tissue substitute in the wound may be a viable treatment option for diabetic patients with a recognized microvascular deficiency.

INNOVATION

The results suggest a novel approach for correcting diabetes-induced endothelial deficiencies via cell interactions with nanofiber-based matrix without the need for the additional external angiogenic stimuli. The potential technological innovation of this research is in the development of a novel engineered microenvironment capable of directing angiogenic responses of microvascular ECs in the specific setting of a diabetic wound.

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Abbreviations and Acronyms

AGE = advanced glycation end product
 ANOVA = analysis of variance
 BKS = C57BLKS/J inbred mouse strain
 DAPI = 4',6-diamidino-2-phenylindole
 db/db = Cg-m +/+ Lepr^{db}/J mouse model
 EC = endothelial cell
 ELISA = enzyme-linked immunosorbent assay
 EPC = endothelial progenitor cells
 ERK = extracellular signal-regulated kinase
 HPF = high-power field
 KO = knockout
 PBS = phosphate buffered saline
 STZ = streptozotocin
 VEGF = vascular endothelial growth factor
 wt = C57BL/6J wild type mouse model