Cellular Dysfunction in the Diabetic Fibroblast

Impairment in Migration, Vascular Endothelial Growth Factor Production, and Response to Hypoxia

Oren Z. Lerman, Robert D. Galiano, Mary Armour, Jamie P. Levine, and Geoffrey C. Gurtner

From the Laboratory for Microvascular Research and Vascular Tissue Engineering, Institute of Reconstructive Plastic Surgery, New York University Medical Center, New York, New York

Although it is known that systemic diseases such as diabetes result in impaired wound healing, the mechanism for this impairment is not understood. Because fibroblasts are essential for wound repair, we compared the *in vitro* behavior of fibroblasts cultured from diabetic, leptin receptor-deficient (*db/db*) mice with wild-type fibroblasts from mice of the same genetic background in processes important during tissue repair. Adult diabetic mouse fibroblast migration exhibited a 75% reduction in migration compared to normal fibroblasts (*P* < 0.001) and was not significantly stimulated by hypoxia (1% O$_2$), whereas wild-type fibroblast migration was up-regulated nearly twofold in hypoxic conditions (*P* < 0.05). Diabetic fibroblasts produced twice the amount of pro-matrix metalloproteinase-9 as normal fibroblasts, as measured by both gelatin zymography and enzyme-linked immunosorbent assay (*P* < 0.05). Adult diabetic fibroblasts exhibited a sevenfold impairment in vascular endothelial growth factor (VEGF) production (4.5 ± 1.3 pg/ml *versus* 34.8 ± 3.3 pg/ml, *P* < 0.001) compared to wild-type fibroblasts. Moreover, wild-type fibroblast production of VEGF increased threefold in response to hypoxia, whereas diabetic fibroblast production of VEGF was not up-regulated in hypoxic conditions (*P* < 0.001). To address the question whether these differences resulted from chronic hyperglycemia or absence of the leptin receptor, fibroblasts were harvested from newborn *db/db* mice before the onset of diabetes (4 to 5 weeks old). These fibroblasts showed no impairments in VEGF production under basal or hypoxic conditions, confirming that the results from *db/db* fibroblasts in mature mice resulted from the diabetic state and were not because of alterations in the leptin-leptin receptor axis. Markers of cellular viability including proliferation and senescence were not significantly different between diabetic and wild-type fibroblasts. We conclude that, *in vitro*, diabetic fibroblasts show selective impairments in discrete cellular processes critical for tissue repair including cellular migration, VEGF production, and the response to hypoxia. The VEGF abnormalities developed concurrently with the onset of hyperglycemia and were not seen in normoglycemic, leptin receptor-deficient *db/db* mice. These observations support a role for fibroblast dysfunction in the impaired wound healing observed in human diabetics, and also suggest a mechanism for the poor clinical outcomes that occur after ischemic injury in diabetic patients. (*Am J Pathol 2003, 162:303–312*)

Tissue repair involves the coordinated interaction of numerous cell types in processes (inflammation, matrix deposition, and remodeling) that restore the continuity and architecture of a cutaneous or visceral defect.[1] The fibroblast is central to the processes of extracellular matrix deposition and remodeling. It functions both as a synthetic cell, depositing a collagen-rich matrix, and as a signaling cell, secreting the growth factors important for cell-cell communication during the repair process.[2,3] Any impediments to fibroblast function prevent normal wound healing and result in chronic, nonhealing wounds.

Impaired wound healing is a significant clinical problem, with an economic cost totaling billions of dollars yearly. One of the most common disease states associated with impaired tissue repair is diabetes mellitus. The mechanisms by which diabetes impedes tissue repair remain unclear. Previous studies have suggested decreased growth factor [keratinocyte growth factor, vascular endothelial growth factor (VEGF), platelet-derived growth factor] production, excess protease activity, or an increased microbial load as possible etiologies for the impaired wound healing observed in diabetes mellitus.[4–6] In addition, diabetic patients often have co-existing peripheral vascular disease and polyneuropathy that further impede wound healing.

Although many studies have correlated imbalances or deficiencies in growth factor expression or protease activity with chronic wounds *in vivo*, few studies have examined the *in vitro* behavior of cells harvested from unwounded diabetics in processes integral to tissue repair. The evidence demonstrating that diabetes does have effects on essential aspects of fibroblast biology, such as proliferation and collagen synthesis,[7–9] supports the need for further examination of more complex fibroblast functions during tissue repair. For example, fibroblasts produce matrix metalloproteinases (MMPs), which are critical for cell migration and tissue remodeling during wound healing. MMPs have been observed to be elevated in wounds of healing impaired mice and in wound fluid from chronic wounds.[10–12] However, potential mechanisms for the increased production have not been specifically examined.

Fibroblasts also secrete numerous growth factors involved in the wound healing process, including VEGF.[13–15] VEGF is absolutely essential for vascular development *in utero*, and its importance in tissue repair in the adult organism is highlighted by studies demonstrating deficiencies in tissue repair in models in which VEGF expression is experimentally reduced.[16,17] The diminished neovascularization observed in animal models of impaired wound healing is in part thought to be the result of delayed or diminished production of VEGF and other angiogenic growth factors.[18] However, the mechanism underlying this impairment is unclear.

To study the possible pathophysiological effect of the diabetic state on the effector cells of wound healing, we analyzed the behavior of early-passage fibroblasts harvested from the leptin receptor-deficient *db/db* mouse. This well-established model of type II diabetes is characterized by hyperglycemia, obesity, and well-documented impairments in wound repair.[19–21] The *db/db* fibroblasts were compared to age- and passage-matched fibroblasts cultured from nondiabetic, wild-type mice of the same background strain. We specifically examined migration, MMP production, and the production of VEGF. Because wound healing is thought to occur in a relatively hypoxic environment[22,23] we further analyzed these variables in both normoxic and hypoxic conditions. We demonstrate that fibroblasts from adult *db/db* mice maintain selective impairments in multiple cellular processes, and that these impediments to repair are accentuated by exposure to low oxygen tension. Notably, these deficits in VEGF production are acquired only after the onset of hyperglycemia and are therefore not directly attributable to the absence of the leptin receptor. These cellular abnormalities may play a role in the impaired wound healing observed in diabetics and may serve as future targets for intervention.

Materials and Methods

Reagents

Dulbecco's modified Eagle's medium, fetal bovine serum (FBS), trypsin, collagenase I, fibronectin, phosphate-buffered saline (PBS), Hanks' balanced salt solution, and antibiotics for cell culture were all obtained from Life Technologies Inc. (Grand Island, NY). Type I rat-tail collagen was obtained from BD-Biosciences (San Jose, CA). Unless otherwise stated, all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA).

Diabetic Animal Model

Eight- to 12-week-old female, pathogen-free, genetically diabetic C57BLKS/J-*Lepr^db^* (*db/db*) mice and their C57BLKS/J wild-type littermates were obtained from Jackson Laboratories (Bar Harbor, ME). Heterozygote breeder mice from the C57BLKS/J-*Lepr^db^* strain were also obtained from Jackson Laboratories and bred under standard conditions to obtain neonatal *db/db* and wild-type mice. Neonatal *db/db* homozygotes were identified by polymerase chain reaction genotyping as performed according to the protocol provided by the supplier (The Jackson Laboratory, Jackson, ME) (*http://aretha. jax. org/pub-cgi/protocols/protocols. sh?objtype=protocol&protocol_id=348*). The animals were housed in a central animal care facility maintained at 24°C, 30% humidity, and a 12-hour light-dark cycle. Water and standard rodent laboratory chow were available *ad libitum*. Body weights and nonfasting blood glucose levels were recorded at 2-week intervals from 2 weeks of age until 20 weeks. All procedures were approved by the Animal Care Committee of New York University.

Cell Culture

Primary isolates of dermal fibroblasts were harvested from separate neonatal and adult mice for each experiment. The animals were sacrificed and trunk skin was removed by sharp dissection. Special care was taken to remove the underlying adipose tissue. The harvested skin was then minced and digested for 2 hours in 0.20% collagenase I solution in serum-free Dulbecco's modified Eagle's medium at 37°C. The dissociated cells were then centrifuged and resuspended in a culture medium of Dulbecco's modified Eagle's medium with 10% FBS and 1% antibiotic/antimycotic supplement. The cells were grown at 37°C with 100% humidity in 5% CO_2 in air. Medium was changed every other day; cells were passaged before they attained confluence. Experiments were performed using cultured cells at passage 3 or less and the experiments were repeated using cell lines isolated from different animals.

In Vitro *Hypoxia*

Hypoxic conditions were achieved via a custom-designed incubator with an attached hypoxic workspace (Reming BioInstruments, Redfield, NY). Hypoxia was achieved by the infusion of calibrated nitrogen gas into both the incubator as well as the attached workspace to achieve a constant oxygen concentration of 1%. The carbon dioxide concentration was maintained at 5%, and the air within the incubator was humidified and kept at

37°C. Before any experiment in the hypoxic incubator, media was placed within the incubator for 12 hours to pre-equilibrate the dissolved oxygen concentration within the media to 1% O_2.

Migration Assays

Two independent migration assays were used to quantitate migration in response to extracellular matrix molecules. The first was a gold-salt phagokinetic migration assay, which was performed according to the method of Albrecht-Buehler,[24] as modified by O'Keefe and colleagues.[25] Briefly, glass coverslips were placed into an eight-well dish and coated with a colloidal gold salt solution. They were then coated with either: type I rat tail collagen ranging in concentration from 0 to 20 μg/ml, fibronectin ranging in concentration from 0 to 40 μg/ml, or laminin ranging in concentration from 0 to 60 μg/ml. Fibroblasts ($n = 5000$) were plated onto each coverslip in Dulbecco's modified Eagle's medium with 10% FBS and incubated at 37°C for 20 hours. The coverslips were rinsed with Hanks' balanced salt solution and the cells were fixed. The cells were then visualized under dark-field optics. To quantitate migration, 10 high-power fields at ×40 magnification were digitally photographed. The migration tracks of the cells were visible as black empty spaces against the background of bright gold-salt particles. The track area of the cells was calculated in pixels using Scion image analysis software.

The second assay was a modified Boyden chamber haptotaxis assay.[26] Transwell plates (8-μm pore) (Costar; Corning, Corning, NY) were coated with either 10 μg/ml of type I collagen, 20 μg/ml of fibronectin, 30 μg/ml of laminin, or 0.5% bovine serum albumin solution. Cells ($n = 75,000$) were added to the upper chamber of each migration well and were allowed to migrate for 3 hours. After gentle removal of the nonmigratory cells from the filter surface of the upper chamber, the cells that migrated to the lower side were fixed, stained with crystal violet, and washed twice with PBS. The stain was then eluted with 10% acetic acid and the wavelength absorbance was measured at 600 nm on a Spectramax 340 plate reader. Background migration was controlled for by subtracting the absorbance value measured in the plates coated with 0.5% bovine serum albumin alone without extracellular matrix. Some experiments were conducted under hypoxic (1% O_2) conditions.

Gelatin Zymography

Diabetic *db/db* and normal dermal fibroblasts in culture conditions described above were washed twice with PBS and the medium was changed to a serum-free fibroblast basal medium (Clonetics, San Diego, CA). The cells were serum-starved for 24 hours. Tissue culture dishes were coated with type I rat tail collagen at a concentration of 10 μg/ml. The fibroblasts were then plated onto these dishes at a density of 20,000 cells/cm^2 and allowed to attach for 4 hours in fibroblast basal medium. After 4 hours the medium was removed and the cells were washed twice

with PBS. The medium was then changed to a serum-free fibroblast growth medium (Clonetics) that was supplemented with 1 μg/ml of human fibroblast growth factor and 5 mg/ml of insulin. After 20 hours of incubation the conditioned medium was harvested and phenylmethyl sulfonyl fluoride and *N*-ethylmaleimide were added to a final concentration of 1 mmol/L and 2 mmol/L, respectively. The conditioned medium was then centrifuged for 1 hour at 5000 relative centrifugal force (RCF) and the protein content was normalized between samples. Gelatinolytic activity of conditioned medium from cultured cells was analyzed by electrophoresis in 10% sodium dodecyl sulfate-polyacrylamide gels containing 1 mg/ml of gelatin (Bio-Rad Ready Gels; Bio-Rad, Richmond, CA). After electrophoresis, the gels were soaked for 1 hour in 2.5% Triton X-100 at room temperature and then incubated overnight in digestion buffer (50 mmol/L Tris-HCl, pH 7.6, 0.2 mol/L NaCl, 5 mmol/L CaCl$_2$, and 0.2% Brij-35). Subsequently the gels were stained using a single-step staining/destaining method.[27] The bands representing specific activity of both MMP-2 and MMP-9 were then quantified via densitometry using Kodak 2D image analysis software (Eastman-Kodak, Rochester, NY).

MMP Enzyme-Linked Immunosorbent Assay (ELISA)

Conditioned medium from diabetic *db/db* and normal dermal fibroblasts in culture was harvested as described above. Total MMP-2 and MMP-9 concentration was determined using the commercially available Biotrak ELISA (Amersham Pharmacia, Piscataway, NJ). Standard curves were generated using serial dilutions of 100 ng/ml of stock standards supplied with the assays. The wavelength absorbance was measured at 405 nm on a Spectramax 340 plate reader and the total MMP concentration was determined by comparison to a standard curve.

VEGF ELISA

Production of VEGF by fibroblasts from newborn and adult, diabetic and normal, mice was measured using an ELISA technique for murine VEGF (Quantikine M Murine; R&D Systems, Minneapolis, MN). VEGF production by cells in culture was assayed in serum-free basal medium incubated in standard 21% O_2 conditions (normoxia) or in 1% O_2 (hypoxia). The hypoxic conditions were achieved as described above. Serum-free FBS that had previously equilibrated to the O_2 tension of the respective experimental conditions was used throughout the entire experiment. Conditioned medium from diabetic *db/db* and normal dermal fibroblasts in culture was harvested as described above for zymography and protease inhibitors were added. The conditioned medium was then concentrated and the protein content was normalized. The VEGF concentration was determined using the Quantikine ELISA kit according to the manufacturer's instructions.

Protein Assay

For protein quantitation the Coomassie Plus-200 Protein Assay Reagent (Pierce, Rockford, IL) was used. Protein level normalization for VEGF and MMP ELISA as well as for zymography was performed after concentration using Centricon-10 centrifugal filter devices (Amicon, Bedford, MA).

Proliferation Assay

To assess the proliferation rates of the diabetic and normal fibroblasts, the 5-bromo-2′-deoxy-uridine (BrdU) labeling and detection kit III (Roche, Indianapolis, IN) was used. The cells were plated at a density of 6400 cells/well into a 96-well plate and allowed to reach 70% confluence. BrdU was then added to the cells and incubated for 12 hours. A peroxidase labeled anti-BrdU antibody was then bound to the incorporated BrdU and the peroxidase substrate was then added. The catalysis of the peroxidase substrate by the enzyme yielded a colored reaction product. The wavelength absorbance of the samples was then measured at 405 nm on a Spectramax 340 plate reader and the absorbance of diabetic *db/db* fibroblasts was compared to that of normal fibroblasts.

Senescence Assay

β-Galactosidase staining (Cell Signaling Tech, Beverly, MA) was used to quantify senescence at a pH of 6 where β-galactosidase activity is only present in senescent cells.[28,29] Briefly, the cells were first washed with PBS and then fixed at room temperature. They were then stained with a solution containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and allowed to incubate overnight at 37°C. The next day the cells were viewed under ×200 magnification. Normal fibroblasts were compared to diabetic *db/db* fibroblasts for development of any blue color.

Statistical Analysis

All results are expressed as mean ± SEM of untransformed data. Controls for results expressed in percentile form were first averaged and then used for normalization. Statistical comparisons between groups were performed by Student's *t*-test. *P* values <0.05 were considered significant. All experiments were repeated at least three times using cell lines isolated from at least three different mice of the same strain.

Results

Migration of Normal and Diabetic Fibroblasts in Vitro

Differences in migration on type I collagen and fibronectin were observed between diabetic *db/db* and wild-type fibroblasts. Diabetic fibroblast migration was markedly impaired when compared to wild-type fibroblasts in both the gold-salt phagokinetic migration assay and the modified Boyden chamber haptotaxis assay. In the gold-salt phagokinetic migration assay (Figure 1) wild-type fibroblast migration was greater than diabetic fibroblasts at all concentrations of type I collagen or fibronectin. At peak migration, wild-type fibroblast migration was fourfold greater (pixel area, 117,915.1 ± 10,284.7 *versus* 28,293.8 ± 3841.7; *P* < 0.001) than diabetic fibroblast migration (Figure 2). Similarly, when plated onto fibronectin, wild-type fibroblast migration was more than twofold (pixel area, 107,055.1 ± 6108.4 *versus* 51,033.9 ± 2871.5; *P* < 0.001) greater than diabetic fibroblasts (Figure 2). Neither cell type migrated to a significant degree when plated on laminin (data not shown).

This impaired migration of diabetic fibroblasts was confirmed using a modified Boyden chamber haptotaxis assay. Wild-type fibroblasts migrated 77% more (OD_{600}, 0.211 *versus* 0.119; *P* < 0.05) than diabetic fibroblasts in response to type I collagen (Figure 3A). Normal fibroblasts migrated 32% more (OD_{600}, 0.469 *versus* 0.356; *P* < 0.05) than diabetic fibroblasts in response to fibronectin (Figure 3A). In hypoxic conditions (1% O_2) wild-type fibroblast migration on type I collagen was upregulated almost twofold (OD_{600}, 0.211 *versus* 0.416; *P* < 0.05) whereas diabetic fibroblast migration was not significantly elevated (OD_{600}, 0.119 *versus* 0.160; *P* = 0.1) (Figure 3B).

Gelatinase Activity and MMP ELISA

MMP-9 activity was threefold greater (3.1 ± 0.2-fold, *P* < 0.001) in diabetic fibroblasts as assayed by gelatin zymography of the conditioned medium from cultured cells (Figure 4). This elevation seemed to be specific for the latent 92-kd form of MMP-9 because there was no measurable difference in gelatinolytic activity of the active 83-kd form of MMP-9. Conversely, there was no statistically significant difference in the gelatinase activity of MMP-2 (latent 72-kd or active 62-kd forms). These results were confirmed by ELISA (Figure 5A) with significant elevation in MMP-9 concentrations demonstrated in conditioned medium from diabetic fibroblasts when compared to wild-type controls (0.816 ± 0.115 ng/ml *versus* 0.420 ± 0.024 ng/ml. *P* < 0.05). There was no difference detected by ELISA between diabetic and wild-type fibroblasts in production of total MMP-2 (Figure 5B).

VEGF Production by Wild-Type and Diabetic Fibroblasts

Differences between wild-type and diabetic fibroblasts were observed in the production of VEGF. Diabetic fibroblasts showed a severe impairment in VEGF production in all experimental conditions (Figure 6). Under standard oxygen tensions (21% O_2) diabetic fibroblasts secreted less than 13% of the total VEGF produced by wild-type fibroblasts in basal media (4.5 ± 1.3 pg/ml *versus* 34.8 ± 3.3 pg/ml, *P* < 0.001). Under hypoxic (1% O_2) conditions wild-type fibroblast production of VEGF was elevated
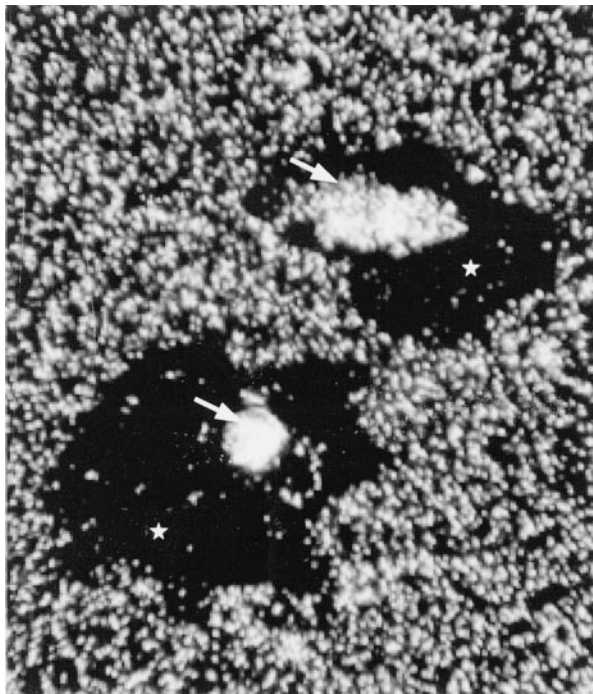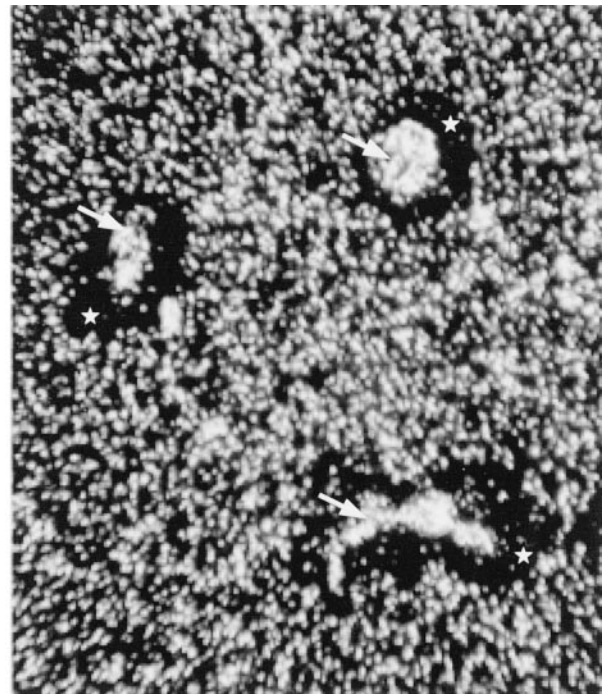
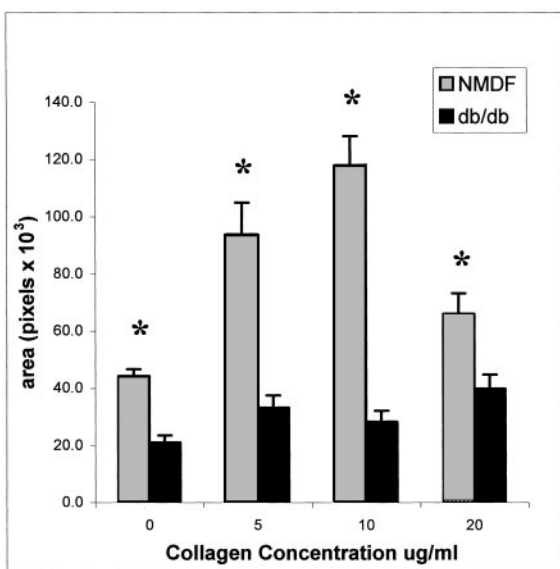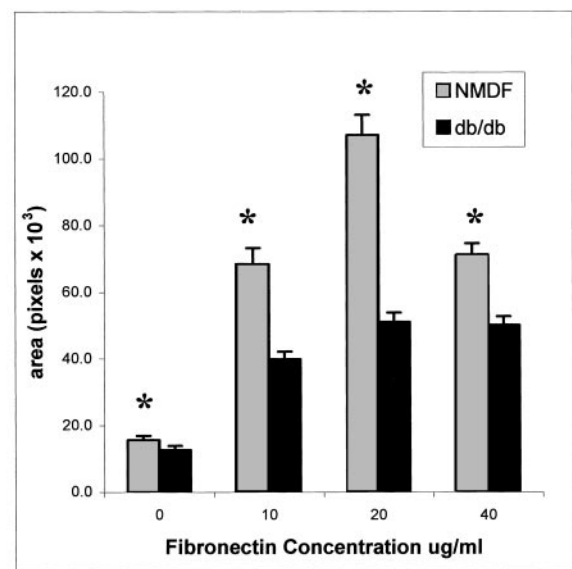A. Normal Fibroblasts   B. Diabetic Fibroblasts

Figure 1. Migration of murine dermal fibroblasts after 20 hours of culture. Coverslips coated with colloidal gold salts and extracellular matrix molecules after fibroblast incubation viewed with ×40 magnification under dark-field optics. **A:** Wild-type murine dermal fibroblasts plated on 20 μg/ml of fibronectin. **B:** Diabetic *db/db* murine dermal fibroblasts plated on 20 μg/ml of fibronectin. The migration tracks (**asterisk**) are visible as black empty spaces against the background of bright gold salt particles. The cells are indicated by **arrows**. Note how the migration tracks of the wild-type cells are clearly larger than those of the diabetic cells.

threefold (101.9 ± 8.9 pg/ml *versus* 34.8 ± 3.3 pg/ml, $P <$ 0.001). Diabetic fibroblasts, however, exhibited no significant up-regulation in VEGF production in response to the same hypoxic stimulus (11.8 ± 3.6 pg/ml *versus* 4.5 ± 1.3 pg/ml, $P =$ 0.08).

These experiments were repeated using fibroblasts harvested from neonatal *db/db* and wild-type mice to determine whether the differences observed were a result of the genetic absence of the leptin receptor or acquired secondary to the development of diabetes and hyperglycemia.

A   B

Figure 2. Diabetic fibroblasts show impaired migration on collagen and fibronectin compared to wild-type fibroblasts. Fibroblast migration assays were performed as described in Materials and Methods. In the gold-salt phagokinetic migration assay, fibroblasts were plated onto coverslips coated with varying concentrations of the matrix of interest and allowed to migrate for 20 hours. The figures depict mean migration track area ± SEM of fibroblasts from diabetic *db/db* and wild-type mice. Area is shown in pixels. **A:** Fibroblast migration on type I collagen. **B:** Fibroblast migration on fibronectin. *, $P <$ 0.001 compared with wild-type control.
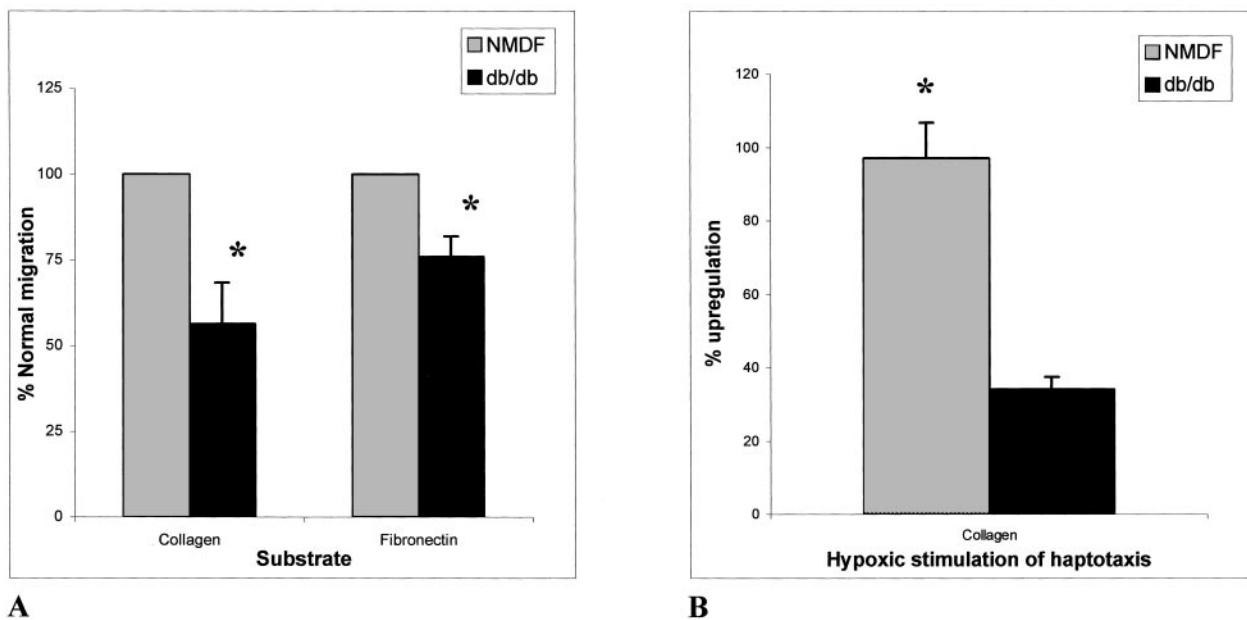
Figure 3. Haptotaxis assays confirm impaired migration of diabetic fibroblasts on collagen and fibronectin. Modified Boyden chamber haptotaxis assays were prepared as described in Materials and Methods. **A:** Haptotaxis on collagen- and fibronectin-coated inserts. **B:** Up-regulation of haptotaxis by hypoxia (1% O_2) stimulation on collagen-coated dishes. *, $P < 0.05$ compared with wild-type control.

Hyperglycemia is not present in neonatal mice and develops between 4 to 6 weeks of age as does the development of impaired wound healing. The development of these changes is demonstrated by serial weight and glucose measurements performed on *db/db* and wild-type mice (Figure 7).

When VEGF assays were performed in neonatal mice before the onset of hyperglycemia, the results demonstrated that neonatal *db/db* fibroblasts exhibited no impairment in VEGF production when compared to wild-type fibroblasts (22.7 ± 1.8 pg/ml *versus* 29.3 ± 4.7 pg/ml, $P = 0.1$). The neonatal (nondiabetic) *db/db* fibro-
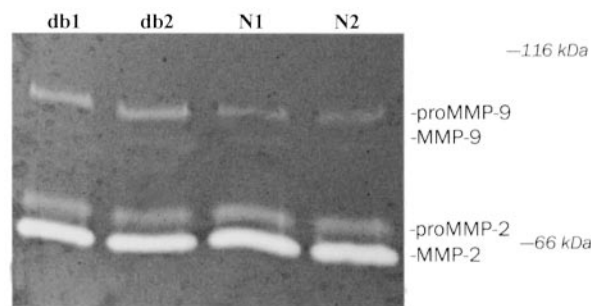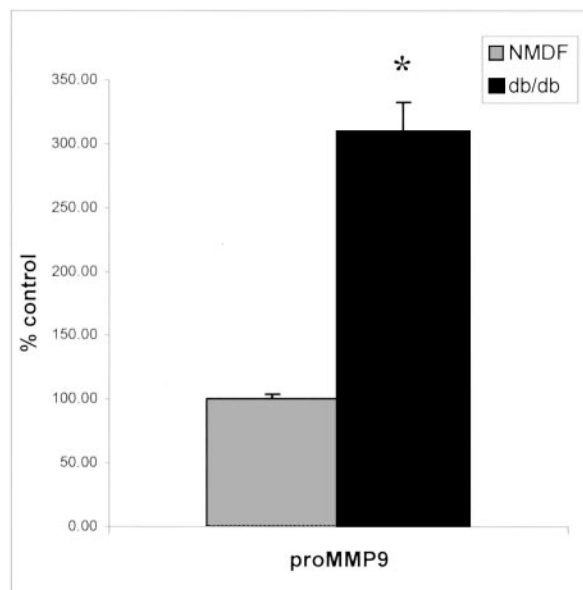
Figure 4. Gelatin zymography shows that conditioned medium from cultured diabetic *db/db* fibroblasts contains elevated levels of pro-MMP-9 compared to wild-type fibroblasts. Gelatinase activity in conditioned medium from wild-type and diabetic *db/db* fibroblasts grown in culture on 10 μg/ml of type I collagen analyzed via gelatin zymography. Positions of molecular mass standards are indicated. **A:** Gelatin zymography showing enhanced 92-kd gelatinolytic activity of diabetic fibroblasts. Results show a typical 92/83-kd MMP-9 duplex and a 72/62-kd MMP-2 duplex. **Lanes db1** and **db2**: Conditioned medium from diabetic fibroblast cultures. **Lanes N1** and **N2**: Conditioned medium from wild-type control fibroblast cultures. No significant changes were detected in 72-kd (MMP-2) gelatinolytic activity. **B:** Densitometry analysis of 92-kd (latent MMP-9) gelatinolytic activity. Histograms represent the mean ± SEM of three separate samples. Wild-type control fibroblast MMP-9 densitometry is 100%. *, $P < 0.001$ compared with wild-type control.
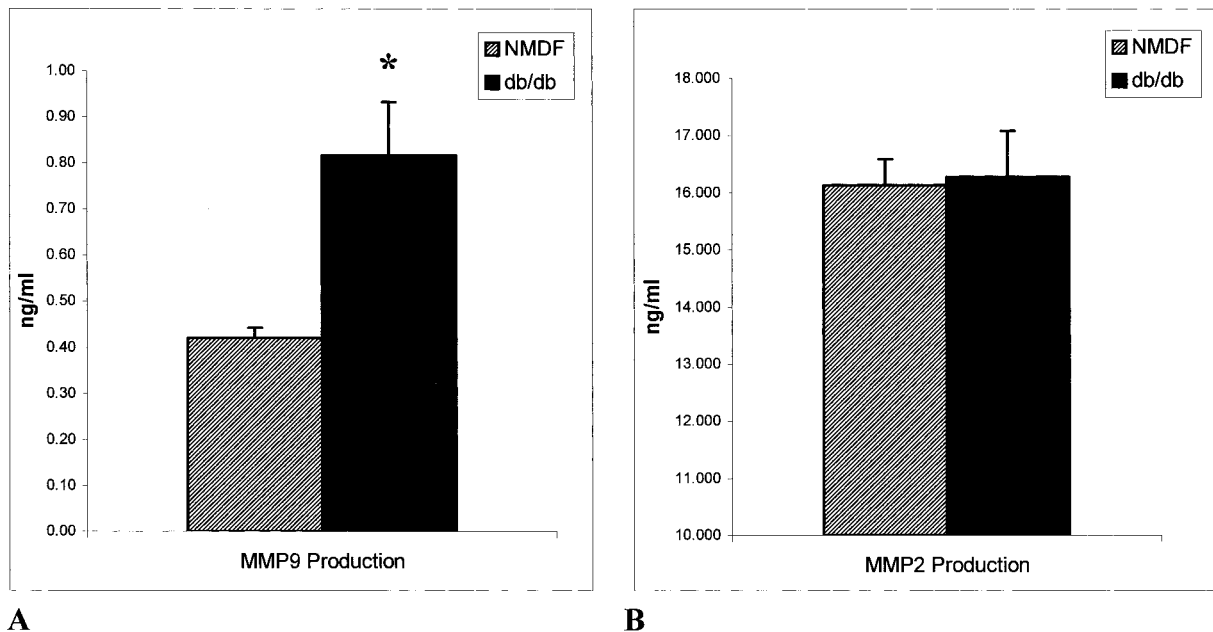
A

B

Figure 5. ELISA analysis of conditioned medium demonstrates total MMP-9 production is elevated in diabetic fibroblasts, while there is no difference in total MMP-2 production. MMP-2 and MMP-9 activity in conditioned medium from wild-type and diabetic *db/db* fibroblasts grown in culture. All experiments were repeated in triplicate. **A:** Histogram shows mean ± SEM total MMP-9 concentration in conditioned medium from three separate samples. *, *P* < 0.05 compared with wild-type control. **B:** Histogram shows mean ± SEM total MMP-2 concentration in conditioned medium from three separate samples.

blasts also exhibited a normal up-regulation in response to hypoxia (144.3 ± 5.2 pg/ml in 1% O_2 *versus* 22.7 ± 1.8 pg/ml in 21% O_2, $P < 0.05$). There were no statistically significant differences in VEGF expression and up-regulation between neonatal (nondiabetic) *db/db* and wild-type cells (Figure 8).
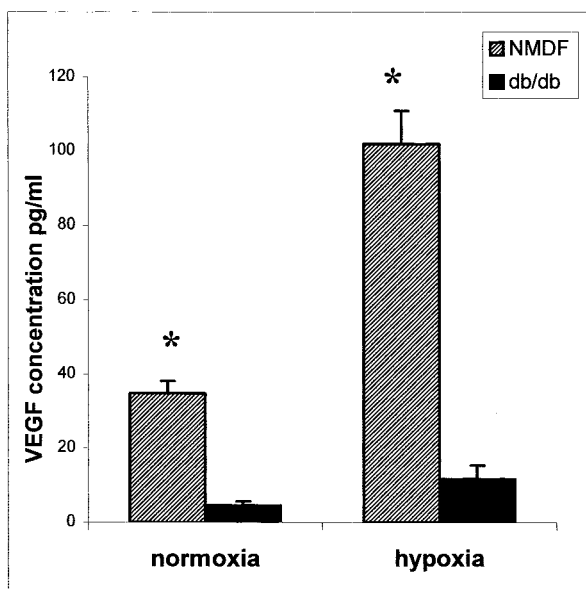

Figure 6. Diabetic fibroblasts production of VEGF is severely impaired compared to wild-type fibroblasts in normoxic and hypoxic conditions. Serum from wild-type and diabetic *db/db* fibroblasts examined for VEGF production after 20 hours in 21% O_2 (normoxia) or 1% O_2 (hypoxia). **A:** VEGF production by fibroblasts harvested from adult mice. Diabetic fibroblasts demonstrate severe impairments in VEGF production and VEGF up-regulation in response to hypoxia. Histogram represents the mean VEGF production ± SEM of three separate experiments. *, *P* < 0.001 compared with wild-type control.

## Cellular Proliferation and Senescence Assay

The proliferation of diabetic and wild-type fibroblasts was measured using BrdU incorporation. No significant differences were detected in the proliferation rate of diabetic and wild-type fibroblasts (OD_{450}, 0.254 ± 0.022 *versus* 0.260 ± 0.024; $P = 0.85$). This assay measures the relative rate of DNA synthesis at a particular instant and is not averaged throughout time as with a growth curve. Therefore, it does not take into account growth variability that may be seen in culture throughout time.

Senescence was also examined and the results supported the proliferation data, revealing that the diabetic and wild-type fibroblasts were both nonsenescent in culture at the passages used. Less than 1% staining was detected in both cell types. No significant difference in senescence was noted (data not shown).

Discussion

Diabetic wounds represent a significant health care burden in the United States. Foot ulcerations are the most frequent cause of hospitalization in patients with diabetes, accounting for up to 25% of all diabetic admissions. Of the 18 million people in the United States with diabetes, 20% will at some point develop these ulcers.[30,31] Diabetics are also plagued by a high incidence of vascular disease that, when combined with foot ulceration, often results in lower-extremity amputation. Currently, the mainstays of therapy for diabetic wounds are meticulous wound care and revascularization. Often these interventions are ineffective and amputation is ultimately required.

Although it is well accepted that diabetic wounds heal poorly, the mechanisms underlying this phenomenon are
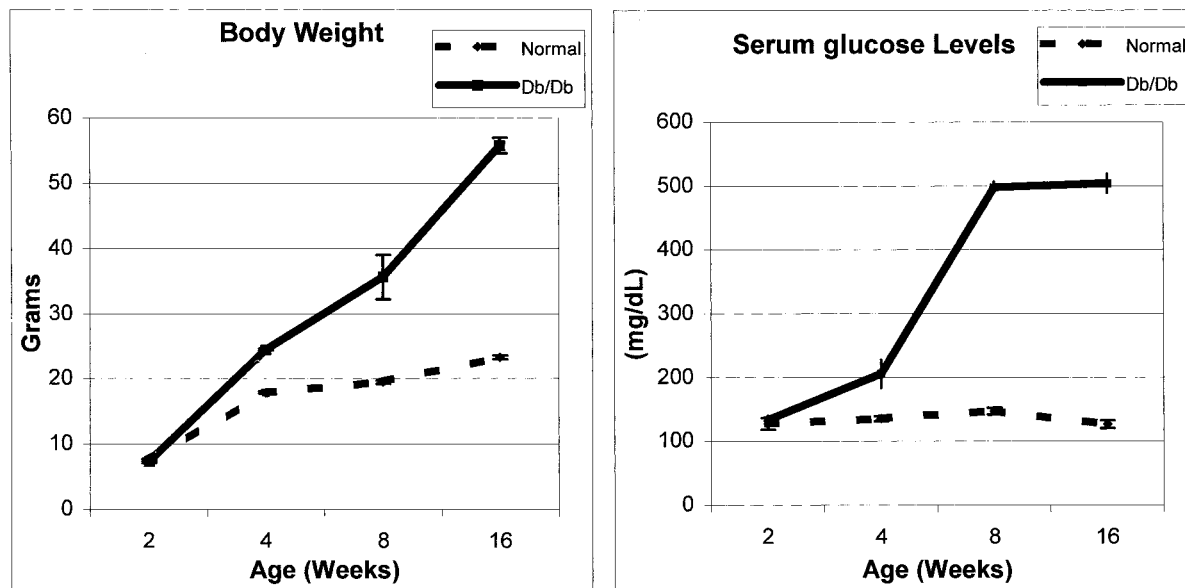
Figure 7. The diabetic phenotype is not apparent in neonatal mice and only begins to develop at 4 to 8 weeks of age. Weight and nonfasting blood glucose measurements were taken every 2 weeks until 16 weeks of age. Neonatal mice have none of the phenotypic changes observed in adult *db/db* mice. **A:** Weight in grams of diabetic and wild-type mice throughout time. **B:** Nonfasting blood glucose levels of diabetic and wild-type mice throughout time. Impaired wound healing begins between 4 to 8 weeks. Data points represent mean weight or blood glucose level ± SEM of four separate mice.

not well understood. Descriptive observations of intact wounds have shed some light on possible mechanisms, but no single etiology has been proven to be responsible.[32–34] Previous studies demonstrate that diabetic wounds have a deficiency of proangiogenic growth fac-

Figure 8. Fibroblasts harvested from neonatal leptin receptor-deficient *db/db* mice show no impairment in production of VEGF or response to hypoxia compared to wild-type fibroblasts. Wild-type and diabetic *db/db* neonatal fibroblasts were grown in serum-free basal medium in either normoxic (21% O_2) or hypoxic (1% O_2) conditions. Conditioned medium was collected from the cells after 20 hours of incubation. There was no difference between VEGF production by neonatal *db/db* fibroblasts and wild-type neonatal fibroblasts in normoxic conditions. Furthermore, VEGF up-regulation by both cell types in hypoxia was equal and similar to that of adult fibroblasts. Histogram represents the mean VEGF production ± SEM of three separate experiments.

tors, a prolonged inflammatory state, and impaired cell migration and wound contraction. Several molecular mechanisms have been proposed to account for these disparate pathophysiological effects.[35] Two in particular have relevance to the present study: the accumulation of advanced glycosylation end-products in cells exposed to chronic hyperglycemia, and oxidative damage resulting from overproduction of mitochondrial oxidative stressors.[35] Both of these would likely result in permanent damage to cells, even when returned to a normoglycemic environment. As such, cells removed from chronically hyperglycemic animals should continue to exhibit abnormalities through early passages *in vitro*.

In this study, we have demonstrated that dermal fibroblasts harvested from diabetic mice exhibit abnormalities even when grown in an *ex vivo* culture environment that has been optimized for nutrient, growth factor, and glucose concentrations. We have demonstrated using two different *in vitro* assays of cell migration that diabetic fibroblasts do not migrate as well as wild-type fibroblasts. Because hypoxia is a known stimulus for increased migration in keratinocytes and fibroblasts,[36,37] we repeated these assays under hypoxic conditions. We demonstrated that hypoxia is unable to increase diabetic fibroblast migration, implying that the hypoxic response may be impaired in these cells.

We examined gelatinase activity as a possible mechanism for the decreased migration in the *db/db* fibroblasts. We were surprised to find only a selective increase in pro-MMP-9 in *db/db* fibroblasts, with no difference in MMP-2 levels or active MMP-9. This seems unlikely to be responsible for the observed impairments in migration. However, MMPs have physiological roles in tissue repair other than cell migration and, indeed, patho-
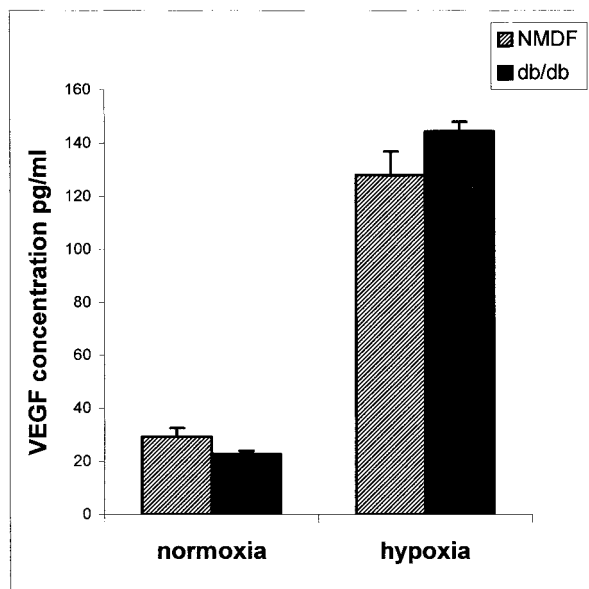
logically elevated levels seem to impair normal wound healing. Evidence for this comes from previous studies that have demonstrated increased MMP-9 in chronic wounds,[11,38] which is felt to produce the prolonged inflammatory state present in diabetic animal wound-healing models.[39,40] Previously, it has been proposed that this excess proteolytic enzyme production is secondary to an increased microbial burden *in vivo*. However, we have demonstrated that elevated MMP-9 production by *db/db* fibroblasts can occur in the absence of infection. This pattern of increased pro-MMP-9 expression in settings of elevated glucose levels has also been described in bovine aortic endothelial cells,[41] suggesting that elevated glucose levels specifically enhance MMP-9 secretion in certain cell types. The mechanism underlying the increased level of pro-MMP-9 levels remains unclear and is an area of ongoing investigation.

The most striking result in the present study is the markedly diminished production of VEGF in diabetic fibroblasts. Previous studies have described diminished levels of VEGF *in vivo* during wound healing in the *db/db* mouse,[18] and the present study suggests that an etiology for the diminished VEGF level observed is a blunted fibroblast release of VEGF in response to injury. Further support for this hypothesis is that *db/db* fibroblasts are unable to up-regulate VEGF production in hypoxic (1% O₂) conditions, whereas wild-type fibroblasts exhibited a threefold increase in VEGF production. To determine whether this impaired VEGF response was because of the diabetic state or resulted from the absence of the leptin receptor, we examined dermal fibroblasts harvested from neonatal *db/db* mice (before the onset of hyperglycemia), and compared these to their wild-type littermates. This was necessary because leptin has been implicated in processes including tissue repair and angiogenesis.[42–48] Our results demonstrated no abnormalities or difference in VEGF production or response to hypoxia in *db/db* mouse fibroblasts obtained before the development of diabetes. We conclude that the differences observed in the adult *db/db* fibroblasts are because of development of the diabetic phenotype of these animals (hyperglycemia, insulin resistance, and obesity), occurring between 4 and 8 weeks of life. Clearly, the significant abnormalities in VEGF production at baseline and in response to hypoxia cannot solely be attributed to aberrant leptin signaling.

The abnormal VEGF activity and response to hypoxia may have a profound effect on the healing process in diabetic wounds, particularly because many diabetic wounds are located in ischemic extremities. Without an appropriate angiogenic response, the subsequent phases of cell proliferation and matrix deposition are delayed because the neovasculature in healing wounds is required for the deposition of matrix.[14,49] This suggests that it may be possible to enhance wound healing in diabetic patients by reversing these discrete deficits, most obviously by supplementing them with exogenous VEGF. It is also interesting to consider whether diminished VEGF production may underlie the decreased tolerance of diabetic patients to ischemic events in a variety of other tissues, including heart, brain, and muscle.[50–54]

In conclusion, we have demonstrated selective impairments in aspects of fibroblast behavior important for tissue repair in fibroblasts harvested from diabetic *db/db* mice. These include impaired cell migration and MMP-9 overexpression and an inability of diabetic fibroblasts to produce normal levels of VEGF at baseline or in response to hypoxia. This suggests that cells are persistently altered in response to prolonged hyperglycemia, at least with respect to VEGF production. Studies are underway to elucidate how glucose and hypoxia interact to diminish angiogenic growth factor expression in fibroblasts and other cell types.

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