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ENDOTHELIAL PROGENITOR CELL DYSFUNCTION CONTRIBUTES TO IMPAIRED WOUND HEALING IN DIABETES MELLITUS

presented by

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has been accepted towards fulfillment
of the requirements for the

Ph.D. degree in Cell and Molecular Biology

Major Professor's Signature

May 12, 2009

Date

ENDOTHELIAL PROGENITOR CELL DYSFUNCTION CONTRIBUTES TO IMPAIRED WOUND HEALING IN DIABETES MELLITUS

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Cell and Molecular Biology

2009

ABSTRACT

ENDOTHELIAL PROGENITOR CELL DYSFUNCTION CONTRIBUTES TO IMPAIRED WOUND HEALING IN DIABETES MELLITUS

By

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Impaired wound healing in diabetic patients is a significant clinical problem that frequently results in amputation. Inadequate angiogenesis is a key factor that contributes to poor wound healing in diabetes mellitus. Endothelial progenitor cells (EPCs) induce angiogenesis at the site of wound healing and are dysfunctional in persons with diabetes. However, the mechanisms of EPC dysfunction in diabetes are poorly understood. Normal EPCs express intrinsically high levels of manganese superoxide dismutase (MnSOD) which makes them resistant to oxidative stress. Hyperglycemia in diabetes causes an elevated systemic oxidative stress. We measured the expression level of antioxidant enzymes and found that MnSOD expression in EPCs isolated from type 2 diabetic mice (db/db) was significantly decreased with a concomitant increase in oxidative stress. Dysfunction of EPCs was significantly improved upon ex *vivo* MnSOD gene therapy both *in vitro* and *in vivo.* To determine the therapeutic effect of EPCs on wound healing in diabetic mice, EPCs from normal and diabetic animals were transplanted to full thickness excisional wounds in mice with type 2 diabetes. The EPCs from non-diabetic animals were significantly more effective at rescuing impaired wound healing than EPCs from diabetic animals. Doubling the number of transplanted EPCs from diabetic animals significantly accelerated the rate of wound healing, however, not to the

extent achieved by an equal number of normal EPCs. Transplantation of EPCs from diabetic animals transfected with MnSOD ex *vivo* resulted in significantly enhanced *in vivo* angiogenesis and accelerated wound healing rate. What is more important, there was a synergistic effect between the number and function of transplanted EPCs when used to induce regeneration in the impaired wound healing found in diabetes mellitus healing. These results indicate that the number of EPCs transplanted, as well as their functional ability, play a significant role in wound healing in diabetic animals.

ACKNOWLEDGEMENTS

First I would like to acknowledge my mentor, Dr. Alex Chen, for his guidance, insight and support throughout my graduate training. The knowledge and experience that I have gained from working with Dr. Chen will greatly help me navigate through my career as a physician-scientist.

I would also like to also thank my committee members Drs. Andrea Amalfitano, Steven Arnoczky, Justin McCormick and Karl Olson. I am grateful for their guidance throughout this study.

I would like to thank the faculty and graduates students of the Department of Pharmacology and Toxicology for the support and advice they provided to me throughout my graduate training.

I would like to thank Dr. Veronica Maher and Justin McCormick the Co-Directors of the DO/PhD program. I appreciate the guidance they have both provided to me throughout my graduate career.

Most important of all, I would like to thank my wife, Carol, and son, Andrew. I am extremely grateful for all of their love, support and patience.

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Images in this dissertation are presented in color.

LIST OF ABBREVIATIONS

Akt	Protein kinase B
Ad-GFP	Adenovirus-green fluorescent protein
Ad-MnSOD	Adenovirus-manganese superoxide dismutase
ANOVA	Analysis of Variance
β-gal	Beta-galacotsidase
BH4	(6R)-5,6,7,8-tetrahydro-L-biopterin
BrdU	5-bromo-2'-deoxyuridine
Ca	Calcium
CD14	Cluster Designation 14
CD31	Cluster Designation 31 (PECAM-1)
CD34	Cluster Designation 34/stem cell marker
Cd133	Cluster Designation 133/stem cell marker
CDC	Centers for Disease Control
c-kit	Receptor tyrosine kinase Kit
CMV	Cytomegalovirus
CO_2	Carbon dioxide
CuZnSOD	Copper zinc superoxide dismutase
db/+	Normal mice
db/+-EPC	Normal endothelial progenitor cells
db/db	Type 2 diabetic mice

db/db-EPC	Diabetic endothelial progenitor cells
DHE	Dihydroethidium
DiI-Ac-LDL	Acetylated low density lipoprotein
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
ECM	Extracellular matrix
EGF	Epithelial growth factor
EGFR	Epidermal growth factor receptor
EGM-2	Endothelial growth media-2
eNOS	Endothelial nitric oxide synthase
EPC	Endothelial progenitor cell
EPO	Erythropoietin
E-selectin	Endothelial adhesion molecule 1
FADH2	Flavin adenine dinucleotide
FCS	Fetal Calf Serum
Flk-1	Vascular endothelial growth factor-2 receptor
G-CSF	Granulocytic colony stimulating factor
GFP	Green fluorescent protein
Gpx-1	Glutathione peroxidase type 1

HBSS	Balanced Salt Solutions
HG-EGM-2	High glucose endothelial growth media-2
HIF-1α	Hypoxia-inducible factor 1 alpha
HIF-2β	Hypoxia-inducible factor 2 beta
ICAM-1	Intercellular adhesion molecule-1
IGF-1	Insulin-like growth factor 1
IL-1	Interleukin-1
IL-6	Interleukin-6
K1	Keratin1
K2	Keratin2
KGF-1	Keratinocyte growth factor-1
KGF-2	Keratinocyte growth factor-2
MAPK	Mitogen-Activated Protein Kinase
Mg	Magnesium
mkitL	Membrane-bound kit ligand
MMP	Matrix metalloproteinases
MnSOD	Manganese superoxide dismutase
MOI	Multiplicity of Infection
N_2O	Nitrous oxide
NAD(P)H	Nicotinomide adenine dinucleotide phosphate
NO	Nitric oxide

O^-_2	Superoxide
O_2	Oxygen
ONOO-	Peroxynitrite
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PECAM-1	Platelet endothelial cell adhesion molecule-1
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SAS	Statistical analysis software
Sca-1	Stem cell antigen-1
SDF-1α	Stromal derived factor-1 alpha
SHH	Sonic hedge hog
siRNA	Short interfering ribonucleic acid
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TGF-α	Transforming growth factor-alpha
TGF-β1	Transforming growth factor-beta1
Tie-2	Protein Receptor Tyrosine Kinase-Epithelial-Specific
TNF-α	Tumor necrosis factor-alpha

TSP-1	Thrombospondin-1
TXD	Transplanted
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
vWF	von Willebrand factor

CHAPTER 1

GENERAL INTRODUCTION

I. Diabetes mellitus

A. Epidemiology

It is estimated by the Centers for Disease Control that 23.6 million people in the United States have diabetes mellitus (DM) (1-3). The two most common types of diabetes are type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM) (1-3). T1DM, previously referred to as insulin dependent diabetes, accounts for 5% to 10% of patients with DM (1-3). T2DM, previously referred to as non-insulin dependent diabetes, accounts for 90% to 95% of patients with DM (1-3). The remaining 1% to 5% of DM cases are caused by infection, surgery, pancreatitis, medication and gestational diabetes (1-3).

B. Diagnosis

Diabetes mellitus is a metabolic disease in which elevated blood glucose level leads to glycemic-induced pathology (1-6). A patient with new onset DM may present clinically with lethargy, polydipsia and polyuria. To confirm the diagnosis of DM, laboratory tests are performed to measure the level of blood glucose on two separate days (1). The current standard guidelines used for the diagnosis of diabetes are as follows: a fasting elevated blood glucose level greater than or equal to 126 mg/dL, or a random non-fasting blood glucose level greater than or equal to 200 mg/dL, or a 2 hour plasma glucose level greater than or equal to 200

mg/dL during a glucose tolerance test (1, 6).

C. Type 1 Diabetes

The onset of T1DM typically occurs prior to the age of 25 years old (6). In patients with T1DM, the number of insulin-producing β-islet cells in the pancreas is significantly reduced (1, 7, 8). The subsequent decreased level of circulating insulin results in hyperglycemia. The mechanisms that cause a decrease in the number of β-islets in the pancreas in T1DM are incompletely understood. Nevertheless, T1DM is associated with the expression of human leukocyte antigen DR3, DR4, and DQ, indicating that genetics plays a role in the development of the disease (6, 7). Environmental factors may also contribute to a decrease in pancreatic β-islets. This is indicated by the fact that the concordance rate for identical twins with diabetes is 42% to 50% rather than 100% (6, 9). An autoimmune process may be the major cause of T1DM because destruction of β-islets results from lymphocytic infiltration, and autoantibodies against the β-islet cells can be found in the majority of patients with T1DM (1, 7, 8). The destruction of β-islets may progress rapidly or take several years (1). In patients with T1DM, hyperglycemia does not become evident until approximately 80% of the β-islet cells have been destroyed (1), at which point, insulin therapy is required to maintain euglycemia (1, 10).

D. Treatment of Type 1 Diabetes

Insulin therapy is the primary treatment for T1DM patients (1, 11). Ideally, a patient should maintain his or her blood glucose level within a narrow range. The overall therapeutic goal in T1DM is to maintain a preprandial glucose level of 90–130 mg/dL and a peak postprandial glucose level of less than 180 mg/dL. Glycosylated hemoglobin, hemoglobin A1C (HgbA1C), is measured to determine the average blood glucose level over a three month period. The goal of a patient with DM is to maintain his or her HgbA1C at less than 7% (1, 11). An HgbA1C of 7% correlates with an average blood glucose level of 172 mg/dL (12, 13). There are several different insulin preparations available to treat hyperglycemia. They can be classified into two main categories, short acting which last 3-6 hours and long acting which last 10-24 hours (1, 11). Short-acting insulin is used to mimic physiologic insulin release after meals; long-acting insulin is used to mimic the basal insulin physiologic levels between meals (1).

E. Type 2 Diabetes

In T2DM, insulin resistance occurs in key metabolic tissue, such as skeletal muscle, adipose tissue and the liver, additionally, the level of insulin secretion from the pancreas is insufficient to compensate for the acquired resistance found in these tissues (1, 4, 5, 8). The combination of insulin resistance and inadequate insulin secretion leads to hyperglycemia (1, 4, 5). Cells respond to

insulin by upregulation of the glucose transporter isoform (Glut 4), which leads to the subsequent uptake of glucose (4, 14, 15). The mechanisms that lead to insulin resistance are not completely understood. However, emerging evidence suggests that impairment of GLUT 4 is a key factor (4, 14, 15).

Obesity is common in T2DM patients and plays a major role in the development of this disease (1, 2, 4, 5). Calorie intake that supersedes calorie expenditure can lead to adipocytes that have undergone hyperphagic-induced hypertrophy. Adipocytes that have undergone hyperphagic-induced hypertrophy secrete monocyte chemoattractant protein-1 (MCP-1) (4, 14, 15). Monocytes respond to MCP-1 by infiltrating adipose tissue. These monocytes then secrete tumor necrosis factor (TNF-α) (4, 16, 17). TNF-α then deleteriously affects adipocytes by inhibiting triglyceride uptake and promoting free fatty acid secretion (FFA) (4, 16, 17). In addition, TNF-α causes insulin resistance in adipocytes by impairment of the GLUT 4 transporter (4, 15). The combination of hyperphagia and the release of FFA from adipocytes further lead to hyperlipidemia and subsequent accumulation of FFA in skeletal muscle (4). Accumulation of FFA leads to insulin resistance in skeletal muscle via impairment of the Glut 4 transporter (4, 14, 15), as well as the reduction of levels of the insulin receptor in skeletal muscle (4, 18). In hepatocytes, insulin normally suppresses gluconeogenesis (1, 5). In T2DM, however, hepatocytes develop insulin resistance, which leads to excessive glucose production (1, 4, 5). In addition, the increase in circulating FFA released from adipocytes leads to increased

hepatocyte production of low-density lipids (LDLs) (1, 4). At the onset of type 2 diabetes, β-islets cells respond to hyperglycemia with increased secretion of insulin, leading to hyperinsulinemia (1, 4, 5). However, with insulin resistance, the elevated insulin response is insufficient to compensate for the elevated level of glucose. Insulin resistance in the liver, adipose tissue, and skeletal muscle, combined with insufficient insulin secretion to compensate for insulin resistance, lead to T2DM (4, 5).

F. Treatment of Type 2 Diabetes

As described above, obesity plays a significant role in the development of T2DM. Therefore, nutritional therapy, exercise and weight loss are an integral part of therapy for T2DM patients (1, 6). Mechanistically, insulin resistances, combined with insufficient blood insulin levels, are the main pathophysiological processes that lead to hyperglycemia (1, 4-6). Therefore, based on these mechanisms, several classes of drugs have been developed to modulate blood glucose levels to promote normoglycemia. These include drugs that increase insulin secretion (Sulfonylureas, Repaglinide and Nateglinide), reduce glucose production (Biguanides), decrease glucose absorption (Alpha-Glucosidase Inhibitors), or increase insulin sensitivity (Thiazolidinediones) (1). In T2DM, hyperglycemia may lead to hyperinsulinemia at the onset of the disease (1, 5). T2DM is a progressive disorder and over time, insulin secretion from the beta-islet cells diminishes or fails, at which point insulin therapy is required (1, 5). As with

T1DM, the overall goal of therapy is to maintain a hemoglobin A1C (HgbA1C) of less than 7% (1).

G. Complications

T1DM and T2DM share similar signs and symptoms such as polyuria and polydipsia (1, 6). Patients with T1DM tend to be slim, whereas, patients with T2DM tend to be obese (1, 6). DM-specific complications may be present in up to 20–50% of individuals with newly diagnosed T2DM. Multiple complications commonly arise in patients with either T1DM or T2DM. These include retinopathy, chronic kidney disease, vasculopathy, cardiomyopathy, neuropathy, periodontitis and impaired wound healing (1, 2).

H. Impaired Wound Healing in Patients with Diabetes Mellitus

It has been estimated that 15-25% of patients with DM develop foot ulcers (19). A recent five-year long prospective study, performed by Boyko et al., (20) found that patients with DM who developed foot ulcers presented with an average hemoglobin A1C% (HgbA1C) of 11.8±3.4% compared to 9.5±3.0% in patients who did not develop ulcers ($P<0.001$). This correlates with an average glucose level of 315 mg/dL over a 2-3 month period for patients who developed ulcers, compared to 262 mg/dL in patients who did not (4, 5). Another factor that significantly contributes to ulcer formation is duration of the disease. Patients

who developed ulcers had been diagnosed with diabetes for 12.6±10 years, whereas patients in which ulcers did not develop had been diagnosed 10.0±9.3 years previously ($P<0.001$) (20). Whether the type of diabetes (T1DM or T2DM) contributes to wound healing is still unclear (20). However, in a study by Benotmane et al., it was found that 89.39% of diabetic patients presenting in a wound clinic were diagnosed with T2DM, whereas 10.61% of patients were diagnosed with T1DM (21). Despite aggressive therapy of patients with DM that develop ulcers, 14% to 24% will present with non-healing wounds and undergo amputation within their lifetime (19). In the United States alone, impaired wound healing in patients with DM leads to over 72,000 amputations each year (2, 3).

II. Wound Healing Phases

A. Phase I: Inflammation

Wound healing is a complex process that can be described in three overlapping phases: inflammation, proliferation and remodeling (22-24). These three different phases involve the interplay of many different cell types including: platelets, endothelial cells, neutrophils, macrophages, mast cells, lymphocytes, fibroblasts, keratinocytes and bone marrow-derived endothelial progenitor cells (EPCs) (22-27). In inflammation, the first phase of wound healing, white blood cells (WBCs), such as neutrophils and macrophages, infiltrate the injured site and clear debris and microbes. Immediately after injury, mast cells respond to both

cytokines released by injured tissue, and to mechanical force by releasing histamine (28). Histamine causes vascular permeability and subsequent extravasation of fluid into the interstitial space (23). Increased vascular permeability allows for migration of WBCs from the vascular space into the site of injury (23, 28). Infiltration by WBCs is promoted through the release of inflammation cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α), from injured tissue (23). IL-1 and TNF-α induce endothelial cell expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and platelet endothelial cell adhesion molecule (PECAM-1, CD31) (23, 24). Endothelial cell surface expression of VCAM-1, ICAM-1, and PECAM-1 allow neutrophils to bind to the endothelial cells of nearby capillaries and migrate to the site of injury (diapedesis) (23, 24). Within the wound, neutrophils digest and clear bacteria and nonviable tissue from the area. Within 48 to 96 hours, monocytes enter the wound and differentiate into macrophages (23, 24). Macrophages continue to **phagocytose** debris, bacteria, and necrotic tissue throughout the remainder of the inflammation phase (23, 24).

Regeneration of the injured tissue occurs after attenuation of the inflammatory response (23). The mechanism for inflammation attenuation is poorly understood (23). However, emerging evidence suggests that lipoxins play a role in inflammation attenuation (23, 29). As neutrophils infiltrate the wound, they release arachidonate (23). Arachidonate is subsequently converted to lipoxins

via the enzyme 15-lipoxygenase (23). Lipoxins then bind to their receptors, ALXR, and block the neutrophil influx to the site of injury, and in this way reduce inflammation (23, 29).

B. Phase II: Proliferation

The proliferation phase is initiated during the inflammation phase. In the proliferation phase, regeneration/repair of the injured site occurs through cellular proliferation, which leads to barrier formation and nascent blood vessels (23, 24). Barrier formation, which prevents microbial invasion into the wound and fluid extravasation from the wound, occurs with epithelialization and the formation of a connective tissue matrix (22-25). The coordination of keratinocyte and fibroblast proliferation and migration is initiated via cells that infiltrate the wound during the inflammation phase, such as activated platelets and macrophages. Activated platelets and macrophages release epithelial growth factor (EGF) and transforming growth factor-alpha (TGF-α), which subsequently stimulate keratinocytes at the wound edges to migrate and proliferate (23-25). Fibroblasts respond to inflammatory cytokines, such as IL-1 and TNF-α, by releasing keratinocyte growth factor-1 (KGF-1), KGF-2 and IL-6. This release of KGF-1, KGF-2, and IL-6 by fibroblasts further promotes keratinocyte migration into the wound, proliferation and differentiation (23, 30). During the proliferation phase of wound healing, fibroblasts synthesize and release type III collagen and fibronectin, in response to the TGF-β1 released by activated macrophages, an action that

contributes to barrier formation. Some fibroblasts will further differentiate into myofibroblasts, aiding in the contraction of the wound (24, 25). The complex process of wound healing involves the coordination of many different cell types, as described above, which leads to the formation of a barrier and prevents the leakage of fluid and microbe infiltration.

Nascent blood vessel formation allows for perfusion to take place in and around a healing wound, which is necessary for the delivery of nutrients, oxygen and removal of toxic metabolites (22, 31-35). This angiogenesis, which is induced by endothelial progenitor cells (described below), is initiated during the inflammation phase, and continues in the proliferation phase (23, 25, 36). Normal angiogenesis promotes wound healing, whereas impairment of angiogenesis leads to impaired wound healing (22, 31-35).

C. Phase III: Maturation

The final phase of wound healing is termed "remodeling" or "maturation" (22, 23, 25). In this phase, fibroblasts, macrophages and endothelial cells release matrix metalloproteinases (MMP) which break down type III collagen (24, 25). In addition, fibroblasts release TGF-β, as an autocrine factor, which causes them to produce type I collagen. Remodeling typically lasts for 6-12 months (24, 25). During this time, type III collagen is replaced by type I collagen, which strengthens the protective barrier.

III. Wound Healing in Diabetes Mellitus

Wound healing is a complex process, as is the pathophysiology that leads to impaired wound healing in patients with diabetes mellitus. The rate of wound healing in patients with diabetes is significantly slower than that of non-diabetic patients (37-39). Several factors lead to impaired wound healing in patients with diabetes. These include cellular dysfunction, chronic inflammation, impaired angiogenesis and vascular disease (33, 38, 39). Specific cellular dysfunction occurs in keratinocytes, fibroblasts, macrophages described below, and EPCs (described several pages further on) have been found to contribute to poor wound healing in diabetes (31, 33, 34, 36, 38, 39).

A. Keratinocyte Dysfunction

Proliferation and migration of keratinocytes is required for epithelialization, which contributes to barrier formation within a wound. In impaired wound healing in diabetes, keratinocyte dysfunction can lead to the formation of a callus around the wound edge, rather than barrier formation. Usui et al. found that an increased number of keratinocytes isolated from wounds of diabetics (henceforth referred to as diabetic wounds) express Ki67 (a marker of cell proliferation) compared to normal wounds (40). Keratin proteins K2 and K10 are normally expressed in mature keratinocytes. However, Usui et al. (40) also found that K2 and K10 are not expressed in keratinocytes isolated from diabetic wounds.

Several other studies have found that keratinocytes isolated from diabetic wounds (henceforth referred to as diabetic keratinocytes) exhibit decreased migration (41, 42). The response of diabetic keratinocytes to cytokines and growth factor is also impaired (43). Mechanistically, this may occur from diminished expression of receptors, such as the epidermal growth factor receptor (EGFR) (43). Taken together, these studies suggest that hyperproliferation, impaired cytokine response and synthesis, as well as diminished migration of diabetic keratinocytes, lead to impaired barrier formation and the development of a callus at the edge of a diabetic wound.

B. Fibroblast Dysfunction

The formation of newly synthesized connective tissue is necessary to form the extracellular matrix (ECM), which contributes to the nascent barrier during wound healing (44, 45). Fibroblasts are necessary for the production of the ECM (44, 45). However, fibroblasts from patients with DM (henceforth referred to as diabetic fibroblasts) have been found to produce increased levels of MMP-9, which leads to degradation of the ECM in diabetic wounds and contributes to delayed wound healing (44, 45). Fibroblasts isolated from diabetic wounds demonstrate decreased proliferation and migration and a diminished response to growth factors and cytokines such as TGF-β1 and PDGF (46, 47). Kim et al. showed that expression of the type 2 receptor for TGF-β1 is decreased in fibroblasts isolated from patients with DM (46). As with diabetic keratinocytes,

diminished expression of extracellular receptors, by diabetic fibroblast, may contribute to an impaired response to cytokines. In addition, production of vascular endothelial growth factor (VEGF) and stromal-derived factor-1 alpha (SDF-1α) by fibroblasts in DM is reduced (48). Consequently, diminished levels of VEGF and SDF-1α is thought to impair the necessary mobilization of EPCs to the site of injury and the induction of the angiogenesis needed in wound repair (48). Therefore, the dysfunction found in diabetic fibroblasts leads to both impaired barrier formation and impaired angiogenesis.

C. Macrophage Dysfunction

The dysfunction of macrophages in wound healing in diabetes also contributes to delayed wound repair (49, 50). The role of macrophages in wound healing is to release growth factors and cytokines, as well as to phagocytose debris and microbes (23, 24). In diabetes, macrophages demonstrate diminished phagocytosis (49). The number of macrophages and the level of cytokines produced by macrophages, such as VEGF, are decreased in wounds of diabetic patients compared to normal wounds (47).

IV. Angiogenesis in Diabetes

In diabetes, the overall coordination of wound healing is disrupted (25). Cell-cell interactions and the production of growth factors, cytokines, connective

tissue, and the responses of cells are all necessary for the complex process of wound healing to take place. As described above, wound healing requires multiple cell types working in concert. In diabetes, wound healing is disrupted by defects in the function of keratinocytes, fibroblasts, and macrophages (37, 40-42, 44-50). Although multiple mechanisms contribute to impaired wound healing in diabetes, impaired angiogenesis remains a critical step in this process that can lead to delayed wound healing.

Angiogenesis and subsequent blood flow (perfusion) to an injured site is the *sine qua non* of wound healing. Impaired angiogenesis in diabetic wound healing leads to reduced perfusion and subsequent impaired delivery of nutrients, a reduction in the removal of toxic metabolites, and a loss in the ability of progenitor cells and WBCs to home to the wound site (22, 31-35, 39). Impaired perfusion also leads to tissue hypoxia (22, 31-35, 39). The effects of impaired perfusion are profound and ultimately lead to tissue necrosis and colonization of the wound with microbes. The importance of the microcirculation is evident in patients with diabetes that have microvascular pathology. Diabetic patients demonstrate significant vascular disease in both the microcirculation (capillaries and arterioles) and the macrocirculation (arteries). Therapeutic re-vascularization of limbs in patients with DM is possible if the problem is in the macrocirculation. For example, if decreased blood flow is found in one of the major blood vessels in the leg, such as the popliteal artery, or in the foot, such as the pedal artery, a surgical bypass can be performed (22, 31-35, 39). This, will

return blood flow to the foot. However, in cases where microcirculation pathology exists in and around the wound, surgical bypass of a larger vessel will have no therapeutic effect, because perfusion of the wound will remain impaired, antimicrobial therapy will be ineffective, and tissue necrosis/infection will develop (22, 31-35, 39). In these "no option" patients, the only remaining clinical therapy is amputation (22, 31-35, 39). Because EPCs, the primary cells responsible for the induction of angiogenesis and vascular repair, have been found to be dysfunctional in diabetic patients, it is possible that improving the function of EPC from such patients could lead to novel therapies to enhance would healing.

V. The Discovery of Endothelial Progenitor Cells

In 1997, a landmark study by Asahara et al., entitled *Isolation of Putative Progenitor Endothelial Cells for Angiogenesis* was published in *Science* (51). Prior to this study, it was understood that new blood vessel formation occurred from the branching and extension of existing blood vessels, and that repair of the intimal layer only occurred as the result of action of pre-existing endothelial cells (36). Asahara et al. (51) reported for the first time the existence of a circulating progenitor/stem cell population that could integrate into solid tissue and induce regeneration. These investigators isolated circulating CD34 (stem cell marker) positive cells from humans and cultured them on fibronectin-coated plates. They found that these cells share similar spindle shape morphology with endothelial cells, as well as several endothelial cell markers, such as

1'-dioleyl-3,3,3',3'-tetramethylindocarbocyanin-acetylated-low-density lipoprotein (DiI-Ac-LDL) uptake, isolectin binding (BS-1 lectin), and expression of Flk-1 (vascular endothelial growth factor receptor-2); CD31 (platelet endothelial cell adhesion molecule-1), Tie-2, E-selectin (Endothelial adhesion molecule 1) and endothelial nitric oxide synthase (eNOS). To determine whether these cells integrate into the vasculature, the isolated cells were injected into immunodeficient nude mice that had undergone hind limb ischemia. The injected cells were found to be incorporated into capillaries and small arteries. These cells, first described in this publication (51), have been termed endothelial progenitor cells (EPCs), and are a heterogeneous group of bone marrow-derived cells that migrate through the circulation to the site of injury/ischemia and repair injured vessels through re-endothelization and/or promoting neo-vascularization (36, 52).

VI. The Basic Function of Endothelial Progenitor Cells

Since the original study published by Asahara et al. (51), it has become clear that EPCs are a heterogeneous group of cells that can be identified by several markers (53-65), and as a result, different methods for identifying isolated EPCs have arisen. In humans, CD34/CD133/Vascular endothelial growth factor-2 receptor (Flk-1) positive cells are commonly classified as EPCs (53, 57). Several other markers in various combinations have also been used to identify EPCs. These include CD14, vWF (von Willebrand factor), CD31, Tie-2

(angiopoietin receptor) and c-kit (stem cell factor receptor) (53, 58, 59, 61, 63-65). In mice, Sca-1 (stem cell antigen)/Flk-1 positive cells (56) or Flk-1 positive cells alone (54) are defined as EPCs. In both humans and mice, dual labeling of cells with Dil-acLDL uptake and lection binding (BS-1 lection or Isolating B_4) have been the accepted methods to confirm EPC isolation (36, 51, 55, 60, 62, 64). Regardless of the method used to identify EPCs, the most important functional characteristic is their ability to induce angiogenesis in vitro and in vivo (26, 66-70). EPCs mobilization, migration, and adhesion/integration, are critical for the induction of angiogenesis and tissue repair (26, 55, 60, 66-97).

A. Signaling of Injured/Ischemic Tissue

Upon the formation of an ischemic area or injury, several molecular mechanisms orchestrate the involvement of EPCs in regenerating the injured tissue. Within injured or hypoxic tissue, the intracellular degradation of the transcription factor Hypoxia-inducible factor-1 alpha (HIF-1α) is inhibited (77, 94), allowing it to dimerize with Hypoxia-inducible factor-1 beta (HIF-1β) (77, 94). This heterodimer then binds to the enhancers of SDF-1α and VEGF genes, upregulating SDF-1α and VEGF expression. VEGF and SDF-1α are then released into the vasculature as EPC chemoattractants (38, 54, 77, 81, 94). VEGF and other cytokines induce the formation of new vasculature in and around the wound, in part by mobilizing EPCs from the bone marrow; EPCs then play a significant role in neo-vascularization (54, 56, 59, 63-65). Mobilization, homing

and migration of EPCs from the bone marrow are elicited by the release of several cytokines, including VEGF, platelet-derived growth factor (PDGF), granulocytic colony stimulating factor (G-CSF) and SDF-1α. These cytokines are derived from several different cell types involved in wound repair such as, platelets (VEGF, SDF-1α, PDGF), fibroblasts (VEGF, SDF-1α), macrophages (VEGF, G-CSF, PDGF), keratinocytes (VEGF, SDF-1α), mature endothelial cells (VEGF) and EPCs (VEGF, SDF-1α) (25, 27, 98).

B. EPC Mobilization from the Bone Marrow

In the bone marrow, EPCs are bound to the stroma via the membrane bound mkitL (38, 54, 74, 78). An increase in SDF-1α and VEGF from the injured/ischemic site increases the activation of eNOS in bone marrow stromal cells, which causes upregulation of MMP-9 production (38, 54, 74, 78). MMP-9 cleaves mkitL, releasing EPCs from the stem cell niche of the bone marrow. Increased permeability of the vasculature through MMP-9 activity may also allow for transmigration of EPCs from the bone marrow into the vasculature (38, 54, 74, 78).

C. Homing/Localization of Endothelial Progenitor Cells to the Site of Injury/Ischemia

Upon release from the bone marrow, EPCs enter the circulation and localize

in the injured/ischemic tissue. As described above, chemoattractants (VEGF, SDF-1α, PDGF, G-CSF) aid in the localization of EPCs to the site of injury (54, 74). EPCs are also localized to an injured area via the expression of E and P selectins on endothelial cells (74). EPCs express CD31 and integrins αvβ3 and αvβ5 (74, 97), which aid in the adhesion of EPCs to the site of injury/ischemia. In addition, SDF-1α, itself, has been shown to play a significant role in localizing EPCs to the site of injury (36, 62, 74). Therefore, migration of EPCs into the injured tissue occurs as a result of the response of EPCs to VEGF and SDF-1α. After localization, EPCs express MMP-9, which degrades connective tissue and allows EPCs to migrate into the injured/ischemic tissue. At the site of injury, EPCs exhibit a paracrine function by releasing multiple cytokines and growth factors, including VEGF, SDF-1α, angiopoietin-1, EGF, IGF-1 and KGF (74, 75). These growth factors act on the surrounding cells, including keratinocytes, fibroblasts, macrophages and endothelial cells, to induce proliferation and migration within the injured tissue. Angiogenesis, the formation of new blood vessels, occurs via the migration and proliferation of both resident endothelial cells and the proliferation and differentiation of EPCs at the site of injury.

VII. Endothelial Progenitor Cell Dysfunction in Diabetes

Vascular disease is a significant complication in diabetic patients (22, 31-35, 53, 65). EPCs have been shown to play a significant role in neo-vascularization. Therefore, several studies examined whether there is a link between the quality

functional activity and quantity of EPCs as these relate to vascular disease in diabetes. Schatteman et al. (91) performed hind limb ischemia in nude type 1 diabetic (T1D) mice and non-diabetic controls, and found that transplantation of normal EPCs into the ischemic limbs of diabetic mice improved re-vascularization. In contrast, transplantation of normal EPCs into normal mice did not improve re-vascularization. Early studies continued to provide more evidence that EPCs were dysfunctional in diabetes. Tepper et al. (95) used in vitro assays to study EPC functional activity in DM. These investigators found that EPCs isolated from type 2 diabetic (T2D) patients had a diminished ability to proliferate, adhere to connective tissue, and incorporate into tubes in the tube formation assay (an in vitro measurement of angiogenesis capacity) (95). Loomans et al. (87) found that a reduced number of EPCs could be isolated from T1D patients when compared to normal individuals, and that cells exhibited a decreased ability to form tubes on Matrigel, even when they were cultured in normal media. Such studies provide strong evidence that EPC dysfunction may contribute to vascular disease in diabetes mellitus.

Several studies confirmed that EPC dysfunction occurs in both T1DM and T2DM (71, 87, 91, 95, 99) and that the number of circulating EPCs is significantly decreased in both types of diabetes (87, 99). EPCs incubated in high glucose were found to have decreased cell proliferation, nitric oxide production, MMP-9 activity and migration (84). *In vivo* functions of EPCs derived from T1DM and T2DM, including mobilization, migration, adhesion, re-endothelialization and

neo-vascularization, are also impaired (71, 82, 95, 99). These studies have demonstrated that every known functional activity of EPCs is impaired in diabetes. Studies have recently emerged that begin to describe the putative mechanisms that may contribute to EPC dysfunction in DM. Current evidence suggests that diminished expression of HIF-1α, VEGF and SDF-1α in ischemic/injured diabetic tissue contributes to impaired mobilization of EPCs in DM (38, 77).

VIII. Mechanism of EPC Dysfunction in Diabetes

A. Impaired Adhesion

EPC adhesion at the site of injury is a necessary step in regeneration (74, 82). A study published by li et al., demonstrates that increased thrombospondin-1 (TSP-1) expression of EPCs isolated from T2D mice impairs adhesion both *in vitro* and *in vivo* (82). In this study, normal EPCs transfected with a TSP-1 vector demonstrated elevated TSP-1 levels and diminished adhesion to vitronectin. In addition, both EPCs isolated from T2D mice and non-diabetic mice incubated in high glucose medium experienced elevated TSP-1 levels and impaired adhesion. This study showed that EPCs isolated from diabetic mice exhibit impaired adhesion and that impaired EPC adhesion contributes to impaired vascular regeneration (82).

B. Impaired Proliferation

At the site of injury, an important step in EPC function is proliferation and differentiation. Two separate studies have demonstrated that p38 MAPK activation is higher in EPCs isolated from patients with DM (EPCs isolated from patients or mice with DM are henceforth referred to as diabetic EPCs or dEPCs) compared to normal EPCs (85, 92). In the study performed by Seeger et al., (90), SB2303580 was used to inhibit p38 MAPK in dEPCs. They found that inhibition of p38 MAPK increased the proliferation of EPCs and the number of cells with EPCs markers. The EPC population in which p38 MAPK was not inhibited exhibited decreased proliferation and increased monocyte markers instead. In a similar study by Kuki et al., EPCs were incubated in high glucose for 7 days, after which EPCs had increased p38 MAPK activity and consequently an increase in the number of cells that stained positive for β-galactosidase (a marker of cell senescence) and decreased proliferation (85). SB2303580 treatment increased proliferation and decreased senescence in EPCs incubated in high glucose. Upregulation of p53 has also been implicated in dEPC senescence (100). Hyperlipidemia is found in many patients with type 2 diabetes (83). In a study performed by Rosso and colleagues dEPCs were incubated in medium containing low-density lipid (LDL). They found that the expression level of p53 and the number of cells that stained positive for β-galactosidase increased, indicating the cells has undergone senescence. Silencing of endogenous p53 with siRNA down-regulated p53 and decreased the number of cells that stained positive with

β-galacotsidase. These studies demonstrate the decreased proliferation and increased senescence of dEPCs is affected by both p53 and p38 MAPK. In addition, these studies provide evidence that correcting one problem, such as inhibiting p38 without inhibiting p53, will only partially correct for dEPC dysfunction. Together, these studies suggest that independent pathways impair dEPC functional activity.

C. EPCs and Oxidative Stress

A 2007 paper suggests that increased oxidative stress contributes to dEPC dysfunction (93). Multiple mechanisms contribute to increased oxidative stress in diabetes mellitus. Under hyperglycemic conditions, excessive glucose can generate reactive oxygen species (ROS) via autoxidation (101, 102). In addition, in hyperglycemia, the level of proteins that have undergone non-enzymatic glycosylation is increased (101, 102). During the process of non-enzymatic glycosylation of proteins, additional ROS are generated (101, 102). Hyperglycemia can also lead to an increase in glucose-6-phosphate, the first step in glycolysis, which increases the activity of the pentose phosphate pathway (101, 102). As part of the pentose phosphate pathway, $NADP^+$ is reduced to NAD(P)H and then NAD(P)H is recycled back to $NADP^+$ via the enzyme complex NAD(P)H-oxidase (101-103). In the process of NADPH oxidation, O_2 is converted to O_2^-, a reactive oxygen species.

As hyperglycemia induced formation of intracellular O_2^- increases, molecular pathways important for normal EPC functional activity can become deleteriously affected. One such pathway includes the production of nitric oxide (NO). Endothelial nitric oxide synthase (eNOS) converts L-arginine to L-citrulline; in this reaction nitric oxide (NO) is formed. (6R)-5,6,7,8-tetrahydro-L-biopterin (BH4) is a necessary cofactor for normal eNOS function. Several studies have demonstrated that O_2^- oxidizes and lowers intracellular BH4 levels (104, 105). When BH4 levels are insufficient, eNOS becomes "uncoupled", in this state, eNOS produces destructive the reactive oxygen species O_2^- and peroxynitrite (ONOO-) instead of NO. NO, itself, is important for normal EPC functions, including, mobilization, migration, proliferation and angiogenesis. Two separate studies have found that the impaired production of NO in dEPCs contributes to dysfunction (76, 96). In these studies intracellular NO was increased in dEPCs with a NO donor, sodium nitroprusside (76) or with improvement in eNOS function by the addition of its cofactor, BH4, to cell media (96). In these studies it was found that increased levels of NO lead to improvement of dEPC functional activities, including, mobilization, migration, proliferation and angiogenesis.

Hyperglycemia can cause a substantial level of ROS to be generated in the mitochondria as well. Hyperglycemia causes increased levels of NADH and $FADH_2$, therefore increasing the activity of mitochondrial complexes I and II and subsequently cytochrome Q activity (101, 102). As a result, an accumulation of electrons and increased activity of cytochrome Q generates excessive levels of

O_2^- (74, 97), which in turn causes oxidative damage to lipids, organelles, proteins and nucleic acids, diminishing cellular function. MnSOD removes superoxide radicals from the mitochondria. The importance of MnSOD expression for cellular function is exemplified by the fact that MnSOD knockout mice are neonatal lethal (106). Over-expression of MnSOD has been found to correct for hyperglycemia-induced oxidative stress in target cells subject to diabetic complications, including: glomerular mesangial cells, dorsal root ganglion, endothelial cells and skin (39, 101, 102). Consistent with this observation, a previous study from the laboratory of Alex F. Chen, found that skin isolated from T1D mice are deficient in MnSOD and that treating wounds of T1D mice with MnSOD gene therapy significantly improves the rate of wound healing (39).

Elevated oxidative stress has been found in EPCs isolated from T2D patients (71). This study demonstrated that one source of ROS in dEPCs was NAD(P)H oxidase. This enzyme was inhibited with siRNA to a subunit of NAD(P)H oxidase $p47^{phox}$. After this inhibition, dEPCs demonstrated decreased oxidative stress and an improved ability to promote re-endothelialization of denuded carotid arteries in nude mice after dEPC transplant. This study provides evidence that oxidative stress in dEPCs reduces the in vivo regenerative capacity of these cells.

Resistance to oxidative stress is necessary for normal EPC functional activity. Two previous studies have demonstrated that the level of MnSOD expression is critical for normal EPC functional activity (107, 108). In the first study (107),

siRNA was used to decrease the expression of MnSOD in normal EPCs, and the cells were then cultured with an O^{-}_{2} radical generator LY83583. When the investigators compared the untreated EPCs to EPCs treated with MnSOD-siRNA, they found increased reactive oxygen species (ROS) in the siRNA group. The EPCs treated with LY83583 and siRNA demonstrated impaired migration. In another study, EPCs were compared to mature endothelial cells (108). LY83583 was used to generate ROS. EPC exposed to elevated ROS continued to form tubes on Matrigel, whereas mature endothelial cells, which had lower levels of MnSOD, did not. Taken together, these studies indicate that MnSOD is a critical enzyme for normal EPC functional activity. Further evidence that resistance to oxidative stress is important for EPC functional activity was found in glutathione peroxidase type 1 (Gpx-1) knockout mice (54). Gpx-1 takes part in an intracellular reaction to eliminate hydrogen peroxide (H_2O_2). Gpx-1 knockout mice have been shown to have increased ROS in EPCs, and a decrease in the level of circulating EPCs. EPCs isolated from Gpx-1 knockout mice also demonstrate impaired migration and diminished response to vascular endothelial growth factor (VEGF). The most significant finding of this study was the impaired ability of EPCs to induce angiogenesis *in vivo*. Tolerance of EPCs to oxidative stress in a non-disease state is necessary for normal EPC functional activity. Therefore, impairment of MnSOD expression in EPCs under pathological conditions in which ROS are generated, such as DM, could be a contributing factor for EPC dysfunction.

IX. Therapeutic use of Endothelial Progenitor Cells

Soon after the discovery of endothelial progenitor cells, investigators began to study the regenerative capacity of these cells. The majority of the research performed has been in the cardiovascular field to examine whether EPCs could be used to regenerate both the vasculature and the heart. Therapeutic cytokine induction to elevate the levels of circulating progenitor cells has been found insufficient for inducing complete revascularization (36). In contrast, several studies have demonstrated that injection of progenitor cells into a blood vessel of an ischemic area or directly into the injured tissue induced neo-vascularization, improved perfusion, and enhanced regeneration of the injured/ischemic tissue (109-111). Previous studies have found that EPCs interact with, and differentiate into cardiomyocytes. The exact mechanism of cardiac regeneration remains controversial. However, recently it has become clear that progenitor cell therapy of injured/ischemic myocardium decreases the infarct size and improves both cardiac perfusion and function (109-114). Although human trials with EPCs are currently underway, the efficacy of using EPCs isolated from patients with diseases that are known to deleteriously affect EPC functional activity has not yet been fully elucidated.

Patients with DM can develop significant vascular disease. One possible contribution to this vascular disease is the dysfunction found in the cells that repair the vasculature, i.e. EPCs. Recent studies have found that dEPCs

therapy is not as effective in inducing regeneration as normal EPCs therapy (72, 73, 112). For example, a 2008 study by Jiménez-Quevedo et al., demonstrated that treatment of diabetic patients with autologous progenitor/stem cells is not as effective in regenerating injured/ischemic tissue as is autologous progenitor/stem cell therapy of non-diabetic patients (112). A study published by Caballero et al., determined that "ischemic vascular damage can be repaired by healthy but not diabetic, endothelial progenitor cells" (73). The research finding that dEPCs were not effective in regeneration of injured tissue was also found by Award et al. (72). In these studies, the same number of normal EPCs or dEPCs was used therapeutically. Taken together, these studies demonstrate that non-diabetic EPCs are more effective in induction of regeneration of the injured tissue. Furthermore, the evidence provided in these studies suggests that reversal of dEPC dysfunction may be necessary for dEPC therapy to become clinically feasible.

Preclinical studies have started to emerge in which EPC therapy is explored as a treatment for impaired wound healing in DM (henceforth referred to as diabetic wound healing). In a study published by Sivan-Loukianova et al., normal EPCs were isolated from humans and then transplanted into the wounds of T1D nude (immunodeficient) mice (113). This study demonstrated that transplantation of normal EPCs could improve the rate of diabetic wound healing. However, from this study it was not clear if transplantation of EPCs would still be effective in a mouse with an intact immune system. In a similar study, normal

human EPCs were transplanted onto the wounds of T2D nude mice, Suh et al., found that transplanted human EPCs were incorporated into the vasculature and released VEGF and PDGF (114). In T2D mouse studies, it was found that transplantation of dEPCs is not as effective in inducing angiogenesis as transplantation of normal EPCs. However, in these studies, the rate of wound healing was not measured (113, 114). In comparing transplantation of different cell type, a study performed by Wu et al. found that transplanted normal bone-marrow derived progenitor cells were more effective at improving the rate of wound closure then transplanted normal mouse fibroblast (115). In addition to EPC transplantation, other researchers have examined the use of EPC chemoattractants such as, PDGF, SHH, and combined therapy with SDF-1α and hyperbaric oxygenation to improve diabetic wound healing (27, 38, 71). The therapeutic use of these cytokines improved the rate of wound healing as well as angiogenesis (27, 38, 71). These studies demonstrate that increasing the number of EPCs at the site of wound healing with either transplantation or cytokine therapy ameliorates wound healing in diabetes. The optimal number of EPCs needed to induce wound repair has yet to be determined. In addition, a comprehensive study which compares the number and functional activity of EPCs transplanted in diabetic wound healing is lacking.

X. Current Therapy Regarding Wound Healing in Diabetes Mellitus

A combination of medical and surgical therapy is used to improve wound

healing in patients with DM (22, 31-35). Diabetic wound infections are typically polymicrobial. Both gram positive and gram negative, as well as aerobic and anaerobic bacteria can be found in diabetic wounds. At the start of therapy, a culture is taken from the wound to determine the type of bacteria within the wound as well as the sensitivity of bacteria to antibiotics. Initially patients will be given a broad- spectrum antibiotic, which may be adjusted to a more specific antibiotic upon determination of antibiotic sensitivity of the bacteria. Surgically, the wound is treated with debridement to remove the callus that forms from keratinocyte hyperplasia and any infected tissue including bone. The key to successful debridement is removal of unperfused tissue. Wide debridement past the wound well into skin with an intact microvasculature is necessary for revascularization to take place. The wound is then kept clean, moist and bandaged with the patient keeping pressure on the wound. Intravenous or oral antibiotics may then be given for several weeks while the wound heals. Delayed wound closure may lead to further colonization of bacteria and worsening of the infection. As noted above, the key to wound healing is blood flow and angiogenesis. Clinically, perfusion of the wound and/or limb can be measured by several methods including dye studies via interventional radiology or Laser Doppler studies. Amputation becomes necessary when inadequate perfusion of the microvasculature and/or perfusion of the macrovasculature cannot be surgically corrected, as antibiotic therapy is ineffective without perfusion to the wound site. In addition, inadequate perfusion leads to tissue hypoxia and necrosis. In patients with impaired perfusion, development of a procedure in which the

microvasculature can be re-established in and around the wound would allow for effective antibiotic therapy and prevent tissue hypoxia, subsequent necrosis, and amputation.

A. Adjunct Therapies for Diabetic Wound Healing

As described above, adjunctive therapy for foot ulcers in DM should be initiated if the wound has not achieved greater than or equal to 10% closure in 3 weeks or 50% closure within four weeks after the start of standard therapy (22, 116-118). There are several adjunct therapies available that have demonstrated clinical efficacy for improving the rate of wound healing in patients with DM. These include, Regranex (recombinant PDGF), negative pressure therapy, hyperbaric oxygen (HBO) and bioengineered skin (22, 117, 118). PDGF is the first and only cytokine to be approved by the Food and Drug Administration as a therapy to promote wound healing in patients with diabetes (22, 119-121). The application of PDGF to wounds improves migration of fibroblast and macrophages to the site of injury (120). These cells release growth factor and cytokines which promote wound healing (120).

HBO therapy involves placing the patient into a hyperbaric chamber in which the patient breathes 100% oxygen at 2.5 atmospheres. The mechanism by which HBO therapy promotes wound healing is not completely understood (120). However, previous studies have demonstrated that HBO therapy increased tissue

oxygenation and the production of NO and VEGF (38, 120, 122). Although HBO therapy can be used to treat ischemic wounds, the wounds must be well perfused (119).

Negative pressure wound therapy, also known as vacuum assisted closure (VAC) uses a vacuum to induce negative pressure on the wound. VAC promotes wound healing through several possible mechanisms. First, VAC decreases edema at the site wound healing (119, 120). This is believed to improve oxygenation of the wound by decreasing the area of diffusion for oxygen from the vasculature to the site of injury (119, 120, 123, 124). Second, micromechanical force may promote cell proliferation at the wound site and bring the wound edges closer together (119, 120, 123, 124). Third, micromechanical force may upregulate the release of growth factors and chemotactic factors at the wound site (119, 120, 123, 124). Forth, VAC may increase perfusion at the wound site, however, with this therapy the surrounding vasculature must be intact and the wound may not be ischemic or necrotic (119, 120).

Bioengineered skin can also be used to promote wound healing (120, 125). Bioengineered skin is derived from neonatal foreskin (120, 125). Once transplanted, it provides extracellular matrix molecules, such as collagen, laminin and fibronectin (120, 125). In addition, the graft supplies growth factors, such as, TGF-α, PDGF, TGF-β and G-CSF. The combination of growth factors and extracellular matrix stimulates cell proliferation and angiogenesis within the

wound (120, 125).

All of the adjunct therapies described above improve wound healing and promote angiogenesis. PDGF and HBO therapy have both been shown to improve EPC migration to the site of wound healing (27, 38). The secretion of growth factors and cytokines found with the use of negative pressure therapy (119, 120, 123, 124) and bioengineered skin (120, 125), may promote EPC migration as well. Although the use of adjunct therapies may promote EPC migration to the site of injury and promote angiogenesis, none of these therapies have been shown to correct for diabetes-induced EPC dysfunction. In addition, despite the use of adjunct therapies to promote wound healing, as previously described, over 72,000 amputations occur each year from impaired diabetic wound healing in the United States alone (2, 3, 23).

XI. Research Goals

Multiple factors are known to contribute to impaired wound healing in diabetes. These include, increased ROS, impaired cellular function and impaired perfusion. Angiogenesis is a critical step in wound healing and is impaired in diabetic wound healing. EPCs, the primary cells responsible for the induction of angiogenesis, are dysfunctional in DM. The focus of the present study is to determine a putative cause for EPCs dysfunction in diabetic wound healing.

The *central hypothesis* of the present study is, diminished antioxidant protection is a mechanism that contributes to EPC dysfunction in diabetic wound healing. In the present study, evidence is presented in the next four chapters that support the hypothesis that diminished expression of MnSOD in EPCs contributes to impaired EPC functional activity in diabetic wound healing.

The level of circulating EPCs, as well as that of ROS in EPCs, was explored in T2D mice. Blood mononuclear cells were isolated from both normal and diabetic mice. The level of EPCs was then determined by using flow cytometry using established assays. These studies provide physiological evidence that a key mechanism, namely EPC protection against ROS, is impaired in diabetes.

A putative cause of elevated ROS in EPCs isolated from T2D mice (db/db-EPCs) was also explored. The level of mRNA expression of catalase, CuZnSOD and MnSOD are explored in db/db-EPCs. Furthermore, the protein level of MnSOD in db/db-EPCs is explored as well. The results of these studies indicate that the expression level of MnSOD, a critical enzyme for EPC functional activity, is lower in EPCs isolated from T2D mice.

The question, "Does the diminished expression of MnSOD found in db/db-EPCs contribute to dysfunction?" was then asked. Gene therapy was used to increase the level of expression MnSOD in EPCs isolated from T2D mice. *In vitro* functional assays were then used to determine whether gain of function

occurred with increased MnSOD expression in db/db-EPCs. The results of these *in vitro* studies indicate that MnSOD deficiency impairs the ability of EPCs to induce angiogenesis.

Finally, EPC functional activity in wound healing is explored. Wound healing is a complex process that requires the interplay of many different cells types. The ability of EPCs to induce angiogenesis is impaired in diabetes. EPCs isolated from syngeneic mice were transplanted to wounds of T2D mice. Transplantation of both normal EPCs and db/db-EPCs was performed. In a separate group, gene therapy was used to increase MnSOD expression in db/db-EPCs prior to transplantation. Furthermore, it was determined whether increasing the number of EPCs transplanted would improve wound healing. Angiogenesis and wound healing were measured. The results of these *in vivo* studies indicated that increasing db/db-EPC MnSOD expression level improves the ability of these cells to induce angiogenesis and accelerates the rate of wound healing. The results also demonstrate that increasing the number of EPCs transplanted to the site of the wound from $1x10^6$ cells to $2x10^6$ cells further accelerates wound repair. These results suggest that both the number and the functional activity of transplanted EPCs work synergistically to induce regeneration.

Collectively, the results of the studies, referred to above, suggest that diminished MnSOD expression (antioxidant protection) is a mechanism that contributes to EPC dysfunction in diabetic wound healing. Furthermore, ROS is

elevated in circulating EPCs. Consistent with this finding, MnSOD expression is decreased in EPCs isolated from T2D mice. When MnSOD expression levels were increased in db/db-EPCs, gain of function was found both *in vitro*, with the improved functional activity of dEPCs to form tubes on Matrigel and accelerate wound repair *in vivo*. The novel findings described in the following chapters, could lead to autologous therapeutic use of EPCs derived from patients with diabetes to improve wound healing.

The most important results of my studies have been prepared as a manuscript, which was submitted to the Journal of Clinical Investigation. Chapters 2 through 5 contain data presented in that manuscript as well as additional data. This has allowed me to provide additional explanatory material.

CHAPTER 2

Determination of the circulating level and physiologic ROS of EPCs in type 2 diabetic mice.

Abstract

Endothelial progenitor cells (EPCs) are bone marrow derived cells that can be found in the circulation at low levels. In humans with DM, the circulating level and functional activity of EPCs are diminished. In the present study, T2D mice (db/db) were used to explore EPC dysfunction in DM. In T2DM hyperglycemia manifests in elevated systemic oxidative stress. Resistance to oxidative stress is important for normal EPC functional activity. In this chapter, the circulating level of EPCs in normal db/+ and T2D db/db was measured. In addition, the level of reactive oxygen species (ROS) in circulating EPCs was compared between normal and diabetic mice. The results summarized in this chapter demonstrate that, similar to humans with diabetes mellitus, db/db mice demonstrate a decreased level of circulating EPCs with an increase in the level of intracellular ROS.

Background

EPCs, first described by Asahara et al. in 1997 (51), are a bone marrow–derived cell population that circulates and participates in both vasculogenesis and vascular homeostasis (36, 52, 74). Recent studies suggest that EPC dysfunction contributes to impaired endothelial regeneration, angiogenesis, and subsequent poor wound healing in diabetes (71, 73, 87, 99). Studies have demonstrated that the circulating number of EPCs is decreased in patients with diseases that affect the vasculature, such as cardiovascular disease, peripheral vascular disease, hypertension and diabetes mellitus (36, 79, 88). It is not clear whether a disease causes the decrease in EPCs or whether the decrease in circulating EPCs leads to a disease (57, 79, 88, 126). However, studies have demonstrated that the number of circulating EPCs correlates with the severity of vascular disease (57, 99, 126, 127). For example, after a myocardial infarction, patients with a lower number of circulating EPCs have been found to demonstrate poorer outcomes (126, 127). Similarly, Fadini et al. (99), showed that in patients with diabetes, there is a correlation between number of circulating EPCs and the severity of peripheral vascular disease.

EPC tolerance of oxidative stress has been shown to be important for normal EPC functional activity (107, 108). Several studies have demonstrated that ROS are elevated in tissue isolated from humans or animal models with DM. If the level of ROS is found to be elevated in EPCs isolated from diabetic mice, this could indicate that ROS tolerance/resistance has been deleteriously affected. In

the first part of the present study, the level of circulating EPCs and the level of ROS in EPCs was determined. Blood mononuclear cells were isolated from normal and T2D mice and were then stained with Sca-1/Flk-1 (56) to determine circulating the EPC level or with DHE/Flk-1 to determine the level of ROS.

To determine whether there is similarity between humans and the selected mouse model used in this study, the level of circulating EPCs was measured in T2D mice. In the present study, an established T2DM mouse model, db/db (19, 116, 128, 129), was used. In these mice, a defect of the leptin receptor (OB-Rb) found in the hypothalamus leads to hyperphagia, and subsequent obesity, hyperglycemia, hyperlipidemia, and insulin resistance (19, 128). Db/db mice demonstrate many of the same complications found in humans with T2DM, including hypertension, cardiac disease, renal disease, diabetic neuropathy, impaired angiogenesis and impaired wound healing (19, 71, 116, 129). These mice (db/db) have become an established model to investigate diabetes induced wound healing impairment (27, 71, 116, 129).

Methods

Animal Model. All animal procedures were performed according to Michigan State University Institutional Animal Care and Use Committee (IACUC) guidelines. Mice used in this study were adult normal male db/+ (C57BLKS/J, Jackson Labs) and T2D db/db (BKS.Cg-*m* +/+ *Lepr*^{db}/J, with a C57BLKS/J background, Jackson Labs) 10-14 weeks old (27, 71, 116, 129). The criteria used for inclusion were blood glucose <200 mg/dl (normal, db/+) and blood glucose >300 mg/dl (T2D, db/db).

Isolation of mouse EPCs from blood. EPCs were isolated from the bone marrow and blood in both normal db/+ and T2D db/db mice according to established methods with minor modifications (71, 82). Briefly, mice were anesthetized with gas anesthesia (O_2 1L/min, N_2O 0.4 L/min, Halothane 0.5-1L/min), their hair was clipped, and their skin was prepared with Betadine and allowed to dry. Mouse peripheral blood (0.5-1 mL) was collected from a cardiac puncture into a 1 mL syringe containing 100 µL of heparin (1,000 units/mL, Sigma). Mononuclear cells were isolated from blood using Histopaque 1083 (Sigma) density centrifugation gradient (800 g, 4°C) for 30 minutes. The mononuclear fraction was removed, centrifuged (400 g, 4°C), and red blood cells were lysed with ammonium chloride solution (Stemcell Technologies), washed 3 times (400 g, 4°C) in PBS with 1% albumin, and then used for testing in the various assays.

Measurement of circulating EPCs. Blood-derived mononuclear cells in 100 µL PBS with 1% albumin were placed in polypropylene tubes. Mouse mononuclear cells were then stained with Sca-1 (FITC) and Flk-1 (PE) (BD) for 1hr at 4°C, washed 3 times in PBS with 1% albumin, and fixed in 2% paraformaldehyde (56). Quantification of Sca-1/Flk-1 double-positive cells was performed with a BD Vantage Flow cytometer.

Detection of Reactive Oxygen Species. Dihydroethidium (DHE, Invitrogen) at a concentration of 5 µmol/L in 100 µL PBS was placed in polypropylene tubes containing blood-derived mononuclear cells. Cells were incubated at 37°C for 10 min in a dark, humidified chamber, and then washed 3 times in PBS. Cells were then stained with Flk-1 (BD) for 1 hr at 4°C and washed 3 times in PBS with 1% albumin and 2% paraformaldehyde. Quantification of Flk-1/DHE double-positive cells was performed using a BD Vantage Flow cytometer.

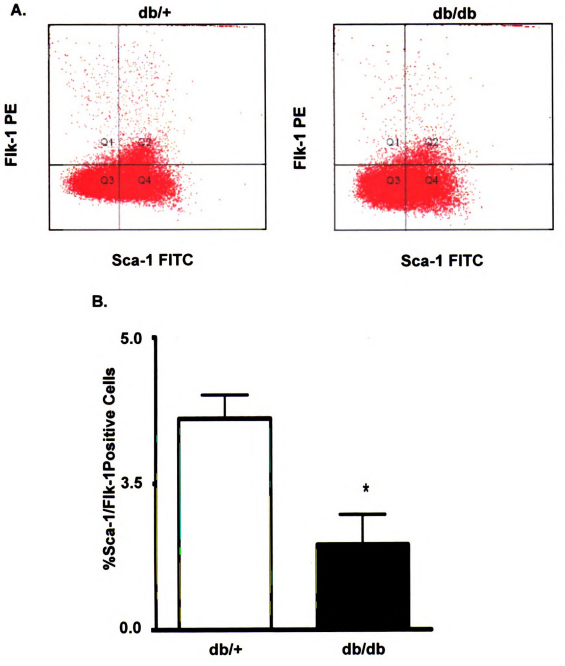
Statistical Analyses. Unpaired t-test was used to compare groups, a two-tailed value of $P<0.05$ was taken as evidence of a statistically significant finding. The above statistical analyses were carried out using GraphPad (Version 4.03).

Results

Circulating levels of EPCs were significantly decreased with a concomitant increase of oxidative stress in T2D (db/db) mice. Peripheral blood mononuclear cells were isolated from both normal (db/+) and T2D (db/db) mice, and the circulating EPCs were identified by Sca-1/Flk-1 double-positive staining as previously described (56). The flow cytometric analysis (Figure 1A and 1B) revealed a decrease in circulating EPCs in T2D mice (db/+: 3.61 ± 0.40% vs. db/db: 1.45 ± 0.50%, $P<0.05$, n=4-5). In addition, the level of circulating EPCs with elevated oxidative stress was increased (db/+: 2.08 ± 0.36% vs. db/db: 5.04 ± 1.14%, $P<0.05$, n=5) in T2D mice, as measured by flow cytometry as Flk-1/dihydroethidium (DHE) double-positive cells compared to normal controls (Figure 2A and 2B). These data indicate that the level of circulating EPCs of T2D mice is decreased with a concomitant increase in their level of oxidative stress.

Figure 1. The circulating level of EPCs was significantly decreased in T2D (db/db) mice. **A.** Representative histograms of Sca-1/Flk-1 analysis for normal (db/+) and T2D (db/db) circulating EPCs. **B.** T2D mice (db/db) have lower levels of circulating Sca-1/Flk-1 positive cells compared normal db/+ mice (*P<0.05, db/+ vs. db/db, n=4-5).

Figure 1.


A.
db/+
db/db
Flk-1 PE
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Sca-1 FITC
Sca-1 FITC
B.
5.0
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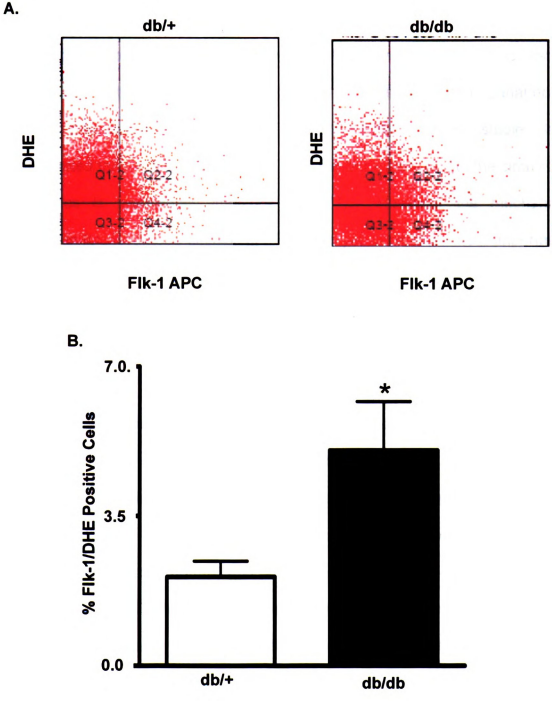
db/+
db/db

Figure 2. Type 2 diabetic mice demonstrate increased oxidative stress in circulating EPCs. **A.** Representative histogram of Flk-1/DHE analysis for normal (db/+) and T2D (db/db) circulating EPCs. **B.** T2D mice have elevated levels of Flk-1/DHE positive cells (*P<0.05, db/+ vs. db/db, n=5).

Figure 2.


A.
db/+
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DHE
DHE
Q1-2
Q2-2
Q3-2
Q4-2
Q1-3
Q2-3
Q3-3
Q4-3
Flk-1 APC
Flk-1 APC
B.
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3.5
0.0
% Flk-1/DHE Positive Cells
*
db/+
db/db

Discussion

The level of circulating EPCs is diminished in patients with vascular disease. In this study, the level of Sca-1/Flk-1 positive cells in peripheral blood was measured to determine the level of circulating EPCs in db/db mice. Consistent with previous human studies, the study described in this chapter found that db/db mice also posses a diminished level of circulating EPCs. Several studies have demonstrated that mobilization of EPCs from the bone marrow into the circulation in diabetes is significantly impaired (38, 71). However, the mechanism responsible for decreased circulating EPCs has yet to be elucidated. Importantly the decreased levels of circulating EPCs correlate with severity of vascular disease (99). This decrease in circulating EPCs may contribute to vascular dysfunction and impaired wound healing, in diabetes (38, 71).

Patients with diabetes and diabetic animal models have both demonstrated increased oxidative stress in mature endothelial cells of the vasculature (130). Such studies have found that increased oxidative stress impairs EPC functional activity (54, 93). In the present study, the level of oxidative stress was measured in EPCs isolated from normal and T2D mice. The results demonstrate that diabetic mouse EPCs have elevated levels of ROS compared to normal mice EPCs. Because these cells were isolated and tested for ROS directly, without cell culture, the results indicate that the physiologic level of ROS in EPCs isolated from diabetic animals is elevated. These data also indicate that the mechanistic basis leading to EPC tolerance of ROS may be impaired. In the next chapter,

anti-oxidant enzymes that eliminate ROS from cells are measured to determine whether decreased expression of one or more enzymes leads to increased ROS in db/db-EPCs.

CHAPTER 3

MnSOD expression is diminished in EPCs isolated from type 2 diabetic mice.

Abstract

Protection from ROS is important for normal EPC functional activity. Normal EPCs express intrinsically high levels of manganese superoxide dismutase (MnSOD), compared to mature endothelial cells, and are thus resistant to oxidative stress. In this chapter, the expression levels of antioxidant enzymes in normal and diabetic mice were measured. It was found that MnSOD expression in EPCs isolated from T2D mice (db/db) was significantly decreased compared to normal EPCs. Consistent with these findings, they exhibited a concomitant increase in oxidative stress.

Background

MnSOD and CuZnSOD remove superoxide radicals that may cause cellular damage and subsequent dysfunction (130-132). MnSOD is predominantly found in the mitochondria, whereas, CuZnSOD is predominantly found in the cytoplasm (132). NADPH oxidase is a major source of cytoplasmic O^{-}_{2} which is eliminated by CuZnSOD (132, 133). In the mitochondria, the electron transport chain produces O^{-}_{2}, which is removed by MnSOD (134). Both MnSOD and CuZnSOD catalyze the dismutation of O^{-}_{2} to H_2O_2. Catalase subsequently catalyzes the conversion of H_2O_2 to O_2 (130-132).

$$O_2^{\cdot} \xrightarrow{\text{SOD}} H_2O_2 \xrightarrow{\text{Catalase}} H_2O$$

- MnSOD
- CuZnSOD

The expression of MnSOD may be more critical for cellular function than is CuZnSOD. This is exemplified by the fact that MnSOD knockout mice are neonatal lethal, whereas CuZnSOD knockout mice survive into adulthood (135, 136). EPCs normally express intrinsically high levels of MnSOD that eliminate intracellular ROS, which allows EPCs to maintain their regenerative capacity in the unfavorable environment of injury/inflammation-induced oxidative stress (107, 108). Other studies have demonstrated the importance of increased expression of MnSOD in diabetic tissue. These studies have shown that induced overexpression of MnSOD corrects for hyperglycemia-induced oxidative stress in traget cells affected by diabetic complications, including glomerular mesangial cells, dorsal root ganglion, endothelial cells and skin (39, 101, 102). The data

summarized in chapter 2, show that circulating EPCs in diabetic mice demonstrate increased ROS, compared to normal EPCs. Similarly, Sorrentino et al., (93) found that ROS levels are elevated and impair functional activity of EPCs isolated from humans with T2DM. However, it remains to be seen whether EPCs isolated from diabetics express a diminished expression level of MnSOD, which contributes to dysfunction.

In the present study, bone marrow-derived EPCs were isolated from both diabetic and normal mice. The mRNA expression level of MnSOD, CuZnSOD and catalase were measured. Previous studies have demonstrated that ROS is elevated in both T1DM and T2DM (39, 101, 102), suggesting that hyperglycemia is cause of increased ROS. Therefore, ROS levels were measured in EPC after incubation in normal EGM-2 (100 mg/dL glucose) and high glucose EGM-2 (500 mg/dL glucose). MitoSOX was then used to measure mitochondrial ROS. Furthermore, MnSOD gene therapy was performed on EPCs to determine whether increasing MnSOD expression in hyperglycemic conditions would decrease the level of ROS in EPCs. As described in this chapter, it is also determined whether the expression level of anti-oxidant enzymes is affected in EPCs isolated from diabetic mice.

Methods

Animal Model. Described in Chapter 2.

Isolation of mouse EPCs bone marrow. EPCs were isolated from the bone marrow and blood from both normal db/+ and T2D db/db mice, according to established methods with minor modifications (71, 82). Briefly, mice were anesthetized with gas anesthesia (O_2 1L/min, N_2O 0.4 L/min, Halothane 0.5-1L/min), hair was clipped; skin was prepared with Betadine and allowed to dry. The femur and tibia were isolated and placed into endothelial growth media-2 (EGM-2 contains human epidermal growth factor (hEGF), hydrocortisone, Fetal Bovine Serum, VEGF, Long Recombinant Analog of Human Insulin-Like Growth Factor type 1 (R3-IGF-1), ascorbic acid and heparin, Cambrex) at 4°C. Bone marrow was flushed out of the tibiae and femurs with EGM-2, using a 25 gauge needle, and separated with vigorous pipetting and then centrifuged for 5 minutes (400 g, 4°C). RBCs were lysed with ammonium chloride solution (Stem Cell Technologies), and washed 3 times in EGM-2 (400 g, 4°C). The cells were plated in a vitronectin-coated (Sigma) six well plates at $8.5x10^5$ cell/cm^2 in EGM-2 at 37°C, 5% CO_2; and after four days in culture, non-adherent cells were removed. EGM-2 was changed daily. Assays were performed after day seven (on day 8, unless otherwise specified). Bone marrow-derived EPCs were characterized by endocytosis of acetylated low-density lipoprotein (Dil-acLDL) and isolectin binding (67, 82, 108). Cells were incubated for 4 hours with acLDL (Molecular Probes) at

a concentration of 10 μg/mL. After washing twice with EGM-2, cells were incubated with 5 μg/mL isolectin (Isolectin B_4) (Sigma) for 1 hr, and then with Hoechst 33258 (Sigma) 5μM for 20 min. The cells were then washed with PBS, and examined using a fluorescence microscope (Nikon Eclipse TE2000-U).

Real-time PCR for MnSOD, CuZnSOD and Catalase. EPCs were lysed and RNA was isolated with TRIZOL (Invitrogen). For each sample, 2 μg of RNA (measured with a NanoDrop ND-100) was reverse transcribed, using SuperScript II RT, Oligo (dT) (Invitrogen), dNTP Mix (Promega) with a GeneAmp PCR System 9700 (PE Applied Biosystems). Real-time PCR was carried out, using SYBR Green PCR Master mix, on a 7500 Real-time PCR system (Applied Biosystems). The primer sequences were: "MnSOD forward 5'-CACATTAACGCGCAGATCATG-3',
reverse 5'-CCAGAGCCTCGTGGTACTTCTC-3';
CuZnSOD forward 5'-ACCAGTGCAGGACCTCATTTTAA-3',
reverse 5'-TCTCCAACATGCCTCTCTTCATC-3';
Catalase forward 5'-ATGGCTTTTGACCCAAGCAA-3',
reverse 5'-CGGCCCTGAAGCTTTTTGT-3';
18S forward 5'-CGGGTCGGGAGTGGGT-3',
reverse 5'-GAAACGGCTACCACATCCAAG-3' " (71, 82, 137). Variation in mRNA levels were calculated using $2^{-\Delta CT}$ (71, 138).

Western blot for MnSOD. EPCs were lysed in Cell Lytic MT lysis buffer (Sigma) with Protease Inhibitor Cocktail (Sigma), (100 µL Protease Inhibtior/10 mL lysis buffer). 50 µg of protein were separated on a 10% SDS-polyacrylamide gel, electro-transferred to nitrocellulose membranes (Bio-Rad) and incubated with a primary antibody against MnSOD (1:1,000 (mouse), BD Transduction Laboratories), loading control anti-Actin (1:10,000 (mouse), Santa Cruz Biotech). The secondary antibodies used were IR Dye 800 conjugated anti-mouse (1:4,000, Rockland). The blot was read with an Odyssey imager (Li-Cor) and molecular band intensity was determined using Odyssey 2.1 software (Li-Cor).

Gene transfer of MnSOD or GFP into expanded EPCs. *Ex vivo* gene transfer experiments were conducted with replication-incompetent adenoviral vectors, driven by a cytomegalovirus promoter (CMV) as described (39), with minor modifications. Briefly, adenoviral vectors were used to deliver MnSOD (Ad-MnSOD) or green fluorescent protein (GFP) marker gene (Ad-GFP) to the expanded EPCs in culture at a titer of 500 MOI for 24 hrs, and EPCs were used 72 hrs after the initial infection.

Mitochondrial superoxide. Mitochondrial superoxide anion was detected as previously described (139). After seven days of culturing bone marrow-derived EPCs in high glucose EGM-2 (glucose 500 mg/dl), on day 8, EPCs were incubated with MitoSOX 5 µM (Invitrogen) for 20 min at 37°C in 5% CO_2. Cells were collected by trypsinization, washed 3 times with 1% albumin HBSS (with

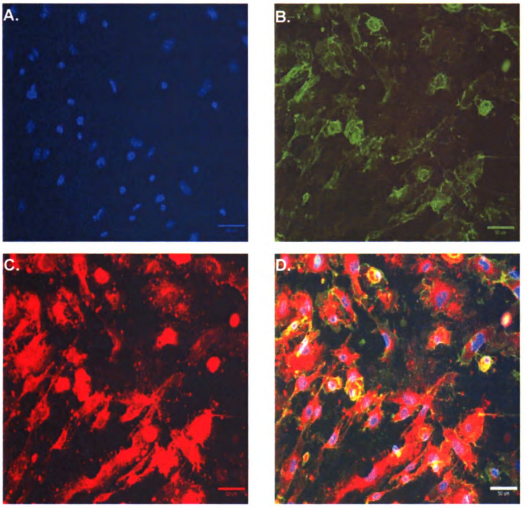
Ca/Mg), and their mean fluorescent intensity was measured by flow cytometry (BD LSRII).

For measurement of ROS after MnSOD gene therapy, bone marrow-derived EPCs were cultured in high glucose EGM-2 (glucose 500 mg/dl), on day 10 of culture, EPCs were then incubated with MitoSOX 5 μM (Invitrogen) for 20 min at 37°C in 5% CO_2. Cells were collected by trypsinization, washed 3 times with 1% albumin HBSS (with Ca/Mg), and examined using fluorescence microscopy (Nikon Eclipse TE2000-U).

Results

Isolation and identification of bone marrow derived EPCs. EPCs were isolated from mouse bone marrow as previously described (71). EPCs uptake of DiI-Ac-LDL and ability to bind isolectin was carried out as described in the methods section. The data in Figure 3 A-D demonstrate that the cells isolated from mouse bone marrow are endothelial progenitor cells. Bone marrow derived EPCs were used for all functional assays and transplant studies throughout the remainder of this study.

Figure 3.



Scale=50μm

Figure 3. Qualitative analysis of bone marrow-derived cells demonstrating they have the characteristics of EPC isolation. A. Hoechst nuclear staining **B.** Isolectin-FITC **C.** DiI-Ac-LDL **D.** Merge of A, B and C (Scale = 50 μm).

MnSOD expression was diminished in EPCs isolated from db/db mice. To investigate the expression of antioxidant enzymes, bone marrow-derived EPCs were isolated (71), and mRNA expression of MnSOD, CuZnSOD, and catalase, was quantified by real-time PCR. No significant difference was found in the expression of CuZnSOD (db/+: $1.20x10^{-4}$ ± $4.52x10^{-5}$ vs. db/db: $2.21x10^{-4}$ ± $1.28x10^{-4}$, P=N.S., n=5) or catalase (db/+: $6.00x10^{-6}$ ±$2.45x10^{-6}$ vs. db/db: $5.14x10^{-6}$ ±$2.19x10^{-6}$, P=N.S., n=5) between normal (db/+-EPCs) and dEPCs (db/db-EPCs) (Figure 4 A and B). However, MnSOD mRNA expression was significantly decreased in db/db-EPCs (db/+: $3.89x10^{-5}$ ± $6.15x10^{-6}$ vs. db/db: $1.9x10^{-5}$ ± $3.36x10^{-6}$, P<0.05, n=4-5) (Figure 4C) accompanied by a significant reduction of MnSOD protein level (db/+: 0.48 ± 0.02 vs. db/db: 0.19 ± 0.01, P<0.05, n=4, Figure 5). Normal EPCs possess a high intrinsic level of MnSOD, compared to mature endothelial cells, which is essential for normal function (107, 108). Therefore, these findings demonstrate that an essential antioxidant enzyme is deficient in EPCs isolated from diabetic mice.

Gene transfer of MnSOD and GFP into EPC with an adenoviral vector. Isolated db/db-EPCs were incubated with various amounts of an adenoviral vector containing green fluorescent protein (GFP) or MnSOD. Photos taken 48 and 72 hours after the initial infection demonstrated that 72 hours after infection at 500 MOI adenovirus GFP infection results in elevated expression levels of GFP (Figure 6A). This experiment was repeated with an adenoviral vector containing MnSOD Figure 6B. The results demonstrate 500 MOI is sufficient to induce

elevated MnSOD gene expression in bone marrow derived EPCs 72 hours after infection.

Figure 4. Expression of MnSOD mRNA is diminished in EPCs isolated from T2D mice. **A.** Catalase (P=N.S., n=5) **B.** CuZnSOD (P=N.S.) and **C.** MnSOD, the data shows that only MnSOD RNA levels were diminished in T2D (db/db) EPCs compared to normal EPCs (*P<0.05, db/+ vs. db/db, n=5).

Figure 4.

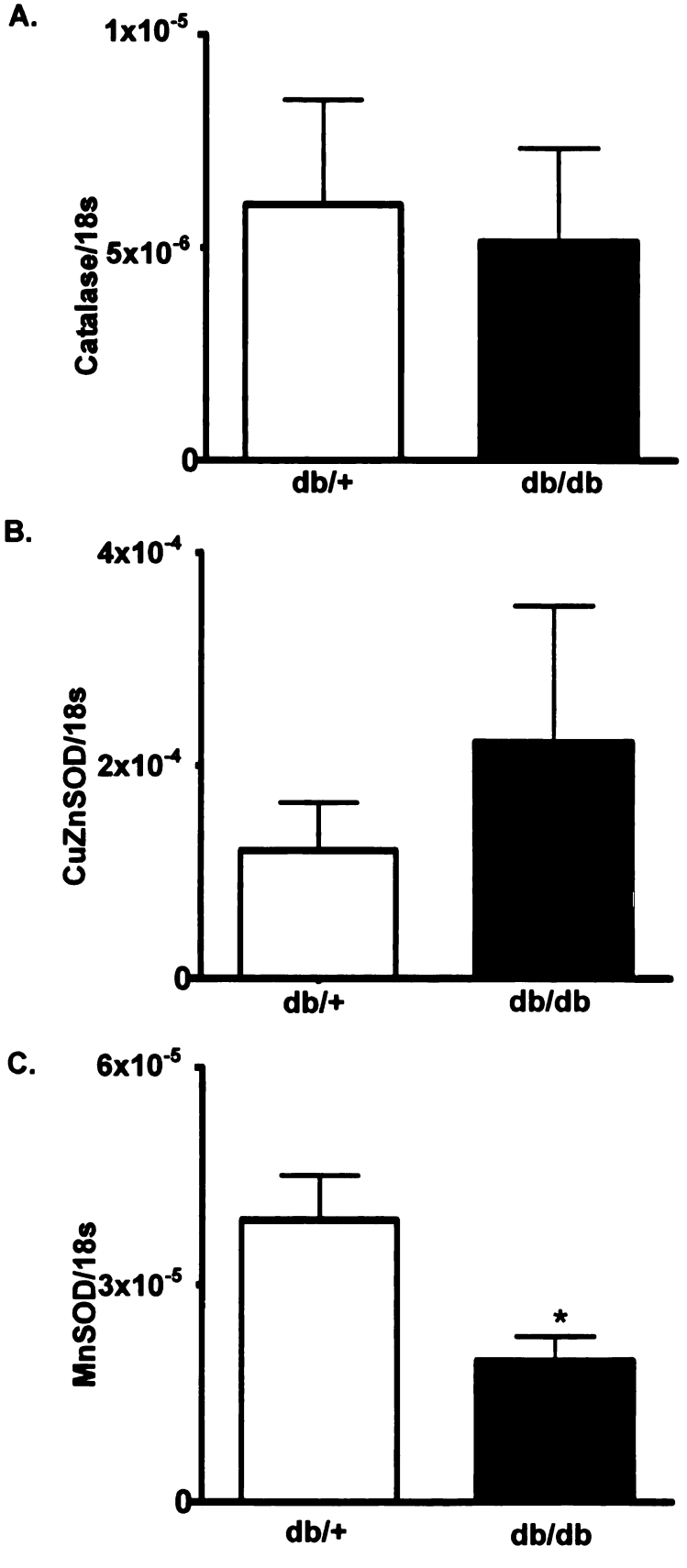

A.
1x10-5
5x10-6
0
Catalase/18s
db/+
db/db
B.
4x10-4
2x10-4
0
CuZnSOD/18s
db/+
db/db
C.
6x10-5
3x10-5
0
MnSOD/18s
*
db/+
db/db

Figure 5.

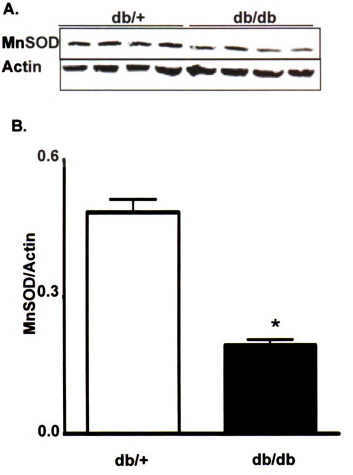


Figure 5. MnSOD protein level is diminished in EPCs isolated from T2D mice.

A and B MnSOD expression was determined by Western blot, demonstrating diminished expression of MnSOD in db/db-EPCs (*P<0.05, db/+ vs. db/db, n=4).

Figure 6.

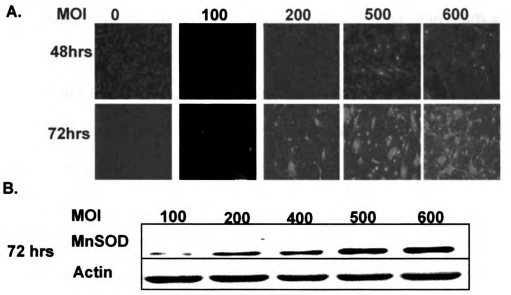


Figure 6 Adenoviral transduction of bone marrow derived EPCs. **A** To determine the optimal MOI, db/db-EPCs were incubated with Ad-GFP for 24 hrs, the media was then changed, and the cells were observed at 48 and 72 hrs for GFP expression. **B** Ad-MnSOD was incubated with EPCs for 24 hrs. Then the media was changed. A western blot was then performed 72 hours after the initial infection.

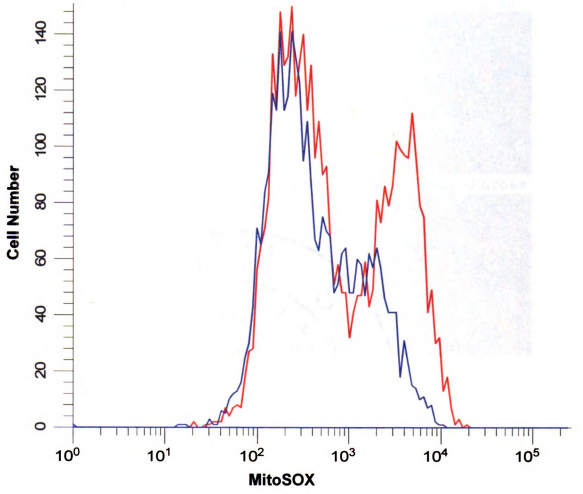
Hyperglycemia increases mitochondrial oxidative stress in EPCs. MnSOD is found predominantly in the mitochondria (130, 132, 134, 136, 140) and plays a key role in its redox balance. To determine if the diminished expression of MnSOD in EPCs renders them susceptible to mitochondrial oxidative stress, both db/+-EPCs and db/db-EPCs were exposed *in vitro* to normal and high glucose EGM-2 (HG-EGM-2). After seven days in culture (day 8), the level of mitochondrial ROS was determined by staining EPCs with MitoSOX followed by flow cytometry. This method specifically detects mitochondrial oxidative stress (139). After culture of EPCs in HG-EGM-2, db/+ (db/+-NG: 2874.0±47.0 vs. db/+-HG: 3890.8±164.8, $P<0.01$, n=5) demonstrated significantly elevated levels of mitochondrial ROS. Elevated levels of mitochondrial ROS were also found in T2D mice db/db-EPCs as well (db/db-NG: 3188.0±79.9 vs. db/db-HG: 4670.2±190.3, $P<0.01$, n=5). However, EPCs isolated from T2D mice (db/db-EPCs) possessed significantly higher levels of mitochondrial ROS compared to EPCs isolated from normal db/+ mice (db/+-HG: 3890.8±164.8 vs. db/db-HG: 4670.2±190.3 $P<0.01$, n=5) (Figure 7 A-B). These results suggest hyperglycemia contributes to mitochondrial ROS in db/db-EPCs. In addition these data suggest that the diminished level of MnSOD found in db/db-EPCs (Figure 4 and 5) may contribute to elevated ROS in db/db-EPC mitochondrial in hyperglycemic conditions. To determine if MnSOD gene therapy of EPCs was effective in lowering hyperglycemia-induced ROS, EPCs were incubated in HG-EGM-2 for 7 days then infected with Ad-MnSOD. Normal EPCs were continually incubated in HG-EGM-2. On day 10, the level of ROS was examined

using MitoSOX staining and microscopy. The results in Figure 8 demonstrate that MnSOD gene therapy of EPCs can lower hyperglycemia induced ROS formation in EPCs.

Figure 7. Hyperglycemia increases mitochondrial ROS levels in EPCs. A. Representative histogram of MitoSOX analysis (db/+-HG blue curve, db/db-HG red curve). EPCs were incubated in normal glucose EGM-2 (NG) or high glucose EGM-2 (HG). **B.** Incubation of normal (db/+) or in db/db-EPCs in HG increased mitochondrial ROS (*P<0.01, NG-db/+ vs. HG-db/+, NG-db/db vs. HG-db/db, n=5). Mitochondrial ROS was further increased in db/db-EPCs incubated in HG-EGM-2, compared to normal db/+-EPCs ($^{\#}$P<0.01, HG-db/+ vs. HG-db/db, n=5).

Figure 7.

A.


Cell Number
0
20
40
60
80
100
120
140
10^0
10^1
10^2
10^3
10^4
10^5
MitoSOX

B.

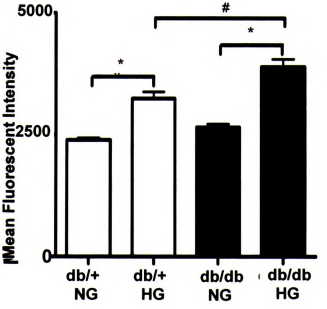

Mean Fluorescent Intensity
5000
2500
0
#
*
*
db/+ NG
db/+ HG
db/db NG
db/db HG

Figure 8.

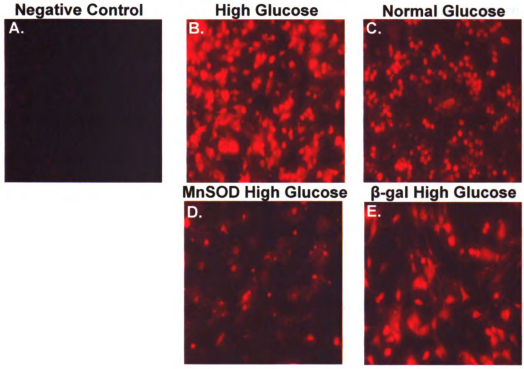


Figure 8. MnSOD gene transfer decreased ROS levels in EPCs incubated in high glucose EGM-2. Normal EPC incubated in high glucose EGM-2 demonstrate increased levels of ROS. **A.** Negative control, EPCs incubated in HG-EGM-2 that were not stained with MitoSOX. **B.** Normal EPCs incubated in HG-EGM-2 for 10 days demonstrated elevated ROS compared to **C.** EPCs incubated in normal glucose EGM-2 for 10 days. **D.** EPCs incubated in HG-EGM-2 that underwent MnSOD gene therapy demonstrate decreased ROS compared to **E.** Ad-β-gal control or **B.** the HG control.

Discussion

Marginalized EPCs (not found in the circulation) reside in the bone marrow and spleen (54, 82). However, EPCs may also reside in other tissues, such as the vasculature (74). EPCs make up a very small population of the circulating mononuclear cells, (2-4%) as demonstrated in chapter 2. Previous studies that have performed EPC experimentation using mouse models acquired EPCs from several locations, including the bone marrow, spleen and blood (71-73, 82, 87, 99). In order to isolate a sufficient number of EPCs from mice for *in vivo* and *in vitro* assays, bone marrow was selected as the source of EPCs in this study. Bone marrow cells were plated at a high density, on a tissue culture plate coated with vitronectin (71-73, 82, 87, 99). After 4 days in culture in EGM-2, the non-adherent cells were removed and the adherent cells were allowed to proliferate (71-73, 82, 87, 99). EGM-2 contains several growth factors including, human epidermal growth factor (hEGF), hydrocortisone, Fetal Bovine Serum, VEGF, Long Recombinant Analog of Human Insulin-Like Growth Factor type 1 (R3-IGF-1), ascorbic acid and heparin, which have been demonstrated to support and promote EPC growth and proliferation (54, 64, 67, 68, 71, 108, 109, 141). In this study, bone marrow-derived EPCs were used in all experiments described in this chapter and all of the following chapters.

Real-time PCR was performed to measure mRNA expression level of CuZnSOD, MnSOD and Catalase. The results demonstrate that MnSOD mRNA was decreased in db/db-EPCs compared to normal EPCs. In addition, the

protein level of MnSOD was also decreased in EPCs isolated from T2D mice. Previous studies have shown that the level of MnSOD expression in EPCs is important for angiogenesis (107, 108). MnSOD is predominantly localized to the mitochondria (132). However, others have demonstrated that MnSOD can also be found in the cytoplasm (142, 143). In this, and in previous studies in which it was found that the level of MnSOD expression is important for normal EPC functional activity, the localization of MnSOD was not determined (107, 108). However, He et al., found that MnSOD expression is important for EPC mitochondria integrity (108). MnSOD removes superoxide radicals from the mitochondria. Hyperglycemia increases the level of ROS in diabetes through increased levels of NADH and $FADH_2$, therefore increasing the activity of mitochondrial complexes I and II and consequently cytochrome Q activity (51, 101, 102, 144). As a result, an accumulation of electrons and increased activity of cytochrome Q generates excessive levels of O_2^-(51, 101, 102, 144). In this study, MitoSox was used to measure mitochondrial ROS. The mechanism in which MitoSOX detects mitochondrial ROS is incompletely understood (139, 145). In MitoSox, hydroethidine is covalently linked to a triphenylphosphonium cation (145). This moiety will target the negatively charged mitochondrial membrane (145), and can be used to detect mitochondrial ROS (139, 141). The results in Figure 7 demonstrate that EPCs from T2D mice incubated in HG-EGM-2 express elevated mitochondrial ROS. In addition, MnSOD gene therapy decreased ROS levels of EPCs incubated in HG-EGM-2. Although no study has established whether or not the level of MnSOD expression found in EPCs directly affects the

mitochondria or cytoplasmic ROS, this evidence and other studies suggest that intrinsic level of MnSOD expression protects the mitochondria in EPCs. What is even more important, this study found that MnSOD, a critical enzyme for EPC functional activity, demonstrates decreased expression in EPCs isolated from diabetic mice. The next chapter describes determination of the role of decreased expression of MnSOD on db/db-EPCs functional activity will be determined.

Previous studies have found that MnSOD expression is important for the angiogenic function of EPCs (139, 141). Further study is needed to determine if the level of MnSOD found necessary for normal EPC functional activity affects cytoplasmic or mitochondrial ROS. This information would provide further mechanistic insight to how MnSOD relates to the angiogenic function of EPCs. In addition, the use of MnSOD gene therapy to decrease ROS level in EPCs incubated in HG-EGM-2 was only demonstrative (Figure 8). Further investigation needs to be performed on multiple samples to demonstrate reperducibility of the results (greater than n=1 for each group). In addition, osmotic controls should be used to confirm the ROS found in EPCs incubated in HG-EGM-2 is induced by hyperglycemia.

CHAPTER 4

Diminished MnSOD expression contributes to endothelial progenitor cell dysfunction in type 2 diabetes.

Abstract

Endothelial progenitor cells (EPCs) induce angiogenesis at sites of injury, and are dysfunctional in diabetes. The mechanisms of EPC dysfunction in diabetes are not yet fully understood. Normal EPCs express intrinsically high levels of MnSOD compared to mature endothelial cells, and are thus resistant to oxidative stress. In the previous chapter it was found that EPCs isolated from T2D mice demonstrated decreased expression of MnSOD compared to normal mice. To determine whether diminished expression of MnSOD *in vitro* plays a role in dEPC dysfunction, dEPC functional activity was studied after *ex vivo* MnSOD gene therapy. The results demonstrate that dysfunction of diabetic EPCs was significantly improved upon *ex vivo* MnSOD gene therapy.

Background

EPC dysfunction occurs in both T1DM and T2DM (27, 36, 38, 71-73, 82, 84, 87, 95, 99). *In vitro* studies of normal EPCs exposed to high glucose demonstrate decreased cell proliferation and migration, nitric oxide (NO) production, and MMP-9 activity (84, 146). EPCs *in vivo* are impaired in T1DM and T2DM, including migration, adhesion, re-endothelialization and neo-vascularization (38, 71, 82, 95, 116). In the studies described in Chapter 3, it was found that EPCs isolated from T2D mice demonstrated diminished expression of MnSOD compared to normal EPCs. In this section, *in vitro* functional assays are used to determine if the diminished expression of MnSOD found in db/db-EPCs plays a role in their dysfunction.

Since the discovery of EPCs *in vitro* assays have been developed which allow investigators to gain a better understanding of EPC functional activity. These assays measure: adhesion, migration and tube formation and allow investigators to compare normal EPCs to EPCs isolated from patients and/or animals models with a disease, such as diabetes or cardiovascular disease. The adhesion assay, is carried out by measuring the ability of EPCs in culture to rapidly adhere (within 2 hours to connective tissue surrogates such as vitronectin or fibronectin) (82). The non-adherent cells are removed and the adherent cells are counted. The migration assay is carried out by measuring the ability of EPCs in culture to move towered a chemokine such as VEGF, in a modified Bodyen Chamber (54). The tube formation assay is carried out by measuring the ability of EPCs in culture to

from a network of tubes on growth factor-reduced Matrigel (54, 71, 108). Although the composition of Matrigel, an extract from the Engelbreth-Holm-Swarm sarcoma has not been well characterized (147), its use in studying angiogenesis is well established (54, 71, 108, 148). In the present chapter it is determined if diminished MnSOD expression in dEPCs, discussed in the previous chapter, plays a role dEPC dysfunction. Elucidation of mechanisms underlying EPC dysfunction in diabetes could lead to practical use of EPCs for autologous cell therapy of wound healing in such patients.

Methods

Animal Model. This model is described in described in Chapter 2.

Isolation of bone marrow derived EPCs and gene transfer of MnSOD or GFP into expanded EPCs is described in Chapter 3.

In vitro adhesion assay. Ninety six well plates were coated with 2.5 µg/ml of rat vitronectin (Sigma) for 2hrs at 37°C. Bone marrow-derived EPCs were plated on the coated plates at $1x10^4$ cells/well, in triplicate. After 2hrs of incubation at 37°C, 5% CO_2 in EGM-2, non-adherent cells were removed by washing 3 times with PBS, cells were fixed with 2% paraformaldehyde and stained with Hoechest (Sigma). Five random fields were counted in each well at 200x (82).

In vitro network formation assay. Twenty four-well plates were coated with growth factor-reduced Matrigel (250 µL, Becton Dickinson). Bone marrow-derived EPCs (7.5×10^4 cells/well) were plated in 500 µL of EGM-2 medium and incubated at 37°C 5% CO_2 for 24 hrs. Tube formation was counted in 15 random fields at 100x with a Nikon Eclipse TE2000-U microscope (108).

EPC migration assay. A 24 well 6µm Transwell was used to determine the ability of EPCs to migrate (54). VEGF (100 ng/ml) (BD) in EBM-2 medium supplemented with 20% FCS placed in the lower chamber. After 5 days of

culture, 5.0 x 10^4 EPCs were suspended in EBM-2 medium supplemented with 20%FCS and placed in the upper chamber. After 24 hours of incubation at 37°C, 5% CO_2, EPCs that had migrated into the lower chamber were counted (54).

Results

Ex vivo MnSOD gene therapy dose not rescue the db/db-EPC adhesion function. In the process of repairing injured tissue, circulating EPCs adhere to connective tissue at the site of injury. EPCs isolated from db/db mice have previously been found to have a diminished ability to adhere to vitronectin (82). MnSOD gene therapy was performed on db/db-EPCs to determine if EPC adhesion could be improved with MnSOD gene therapy. The results in Figure 9 demonstrate that the adhesion of db/db-EPCs to vitronectin was significantly lower than normal EPCs (db/+:78.3±1.6 vs. db/db: 50.3±3.2, n=5, $P<0.05$, Figure 9). The adhesion of db/db-EPCs to vitronectin, which underwent MnSOD gene therapy, was significantly lower than normal EPCs (db/+:78.3±1.6 vs. db/db-AdMnSOD-EPCs: 52.4±3.3, n=5, $P<0.05$, Figure 9) and equivalent to db/db-EPCs (P=N.S., Figure 9). Thus MnSOD gene therapy does not improve the adhesion function of db/db-EPCs.

Ex vivo MnSOD gene therapy rescued db/db-EPC angiogenic function. *In vitro* angiogenic capacity of EPCs was examined by their network formation on Matrigel (108). Bone marrow-derived EPCs were plated on Matrigel for 24hrs

and the number of networks formed was quantified. EPCs isolated from T2D mice formed significantly fewer networks than EPCs isolated from normal mice (db/+: 37.3±2.7 vs. db/db: 12.4±1.1, $P<0.05$, n=5 Figure 10 A and B). Adenoviral vectors were used for gene transfer of MnSOD (Ad-MnSOD) or green fluorescent protein (Ad-GFP) to db/db-EPCs *ex vivo*. Ad-MnSOD gene therapy significantly improved the ability of db/db-EPCs to form networks on Matrigel compared to unmodified db/db-EPCs or the db/db-AdGFP-EPC controls (db/db-MnSOD: 23.3±0.7 vs. db/db: 12.4±1.1 or db/db-GFP: 15.1±0.4, $P<0.05$, n=5, Figure 10 B-E). These *in vitro* data correlate with the decreased level of MnSOD found in db/db-EPCs and suggest that it is responsible for the diminished capacity of db/db-EPCs to induce angiogenesis.

Migration of db/db-EPCs is impaired. The circulating number and functional activity of EPCs in diabetes has been found to be diminished (71). EPCs isolated from T2D mice demonstrated a diminished migration in response to VEGF ((db/+:21.3±1.1 vs. db/db:5.6167±1.1, $P<0.05$, n=5, Figure 11, (The data for MnSOD gene transfer to db/db-EPCs are not shown because of technical difficulty with the migration assay)). Migration of EPCs from the bone marrow into the circulation, then to the site of injury, is one of the functional impairments of dEPCs (38, 73). The inability of dEPCs to migrate from the bone marrow to the site of wound healing could contribute to the decreased level of angiogenesis and poor wound healing.

Figure 9.

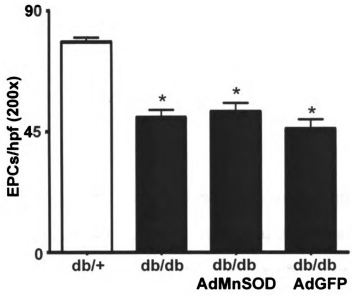


Figure 9. EPCs isolated from type 2 diabetic mice demonstrated impaired adhesion. EPCs adhesion to vitornectin after 2hrs of incubation was found to be impaired for db/db-EPCs. MnSOD gene therapy of db/db-EPCs did not ameliorate impaired adhesion (*P<0.05, control vs. all other groups, n=5).

Figure 10. MnSOD gene therapy improves the functional activity of EPCs isolated from T2D mice. Representative photographs of network formation on Matrigel for **A.** (db/+) normal EPCs, **B.** T2D (db/db-EPCs), **C.** T2D db/db-EPCs with MnSOD gene therapy, **D.** T2D db/db-EPCs transfected with the GFP marker gene. Representative photographs of **B-D** were of EPCs isolated from the same db/db mouse. **E.** MnSOD gene therapy, but not the GFP control, improved the ability of db/db-EPCs to form networks on Matrigel (*$P<0.05$, db/+-EPC vs. all other groups, #$P<0.05$ db/db-MnSOD vs. db/db or db/db-GFP, n=5, Scale = 650 μm).

Figure 10.

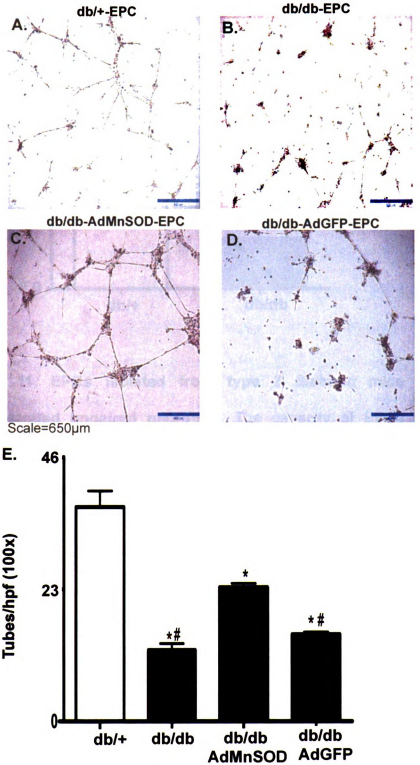

db/+-EPC
A.
db/db-EPC
B.
db/db-AdMnSOD-EPC
C.
db/db-AdGFP-EPC
D.
Scale=650μm
E.
46
23
0
Tubes/hpf (100x)
*#
*
*#
db/+
db/db
db/db AdMnSOD
db/db AdGFP

Figure 11.

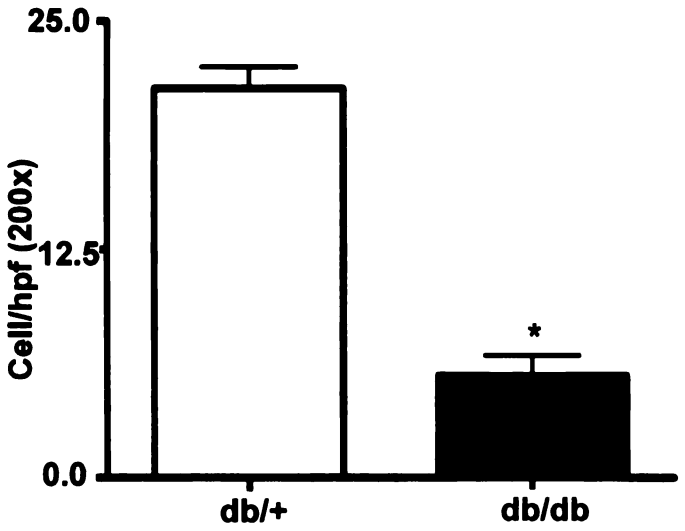


Figure 11. EPCs isolated from type 2 diabetic mice demonstrated impaired migration. The capacity of EPCs isolated from T2D (db/db) mice to migrate in response to VEGF is diminished compared to normal db/+-EPCs (*P<0.05, db/+ vs. db/db, n=5).

Discussion

The assays described in this chapter examined EPC functional activity *in vitro*, including adhesion, tube formation, and migration. Adhesion is an important function of EPCs (74, 82). In order for EPCs to induce angiogenesis EPCs they must first adhere to the site of injury (74, 82). The results of the adhesion assay demonstrated that the function of adhesion of db/db-EPCs is impaired. However, db/db-EPCs did not completely lose the ability to adhere to connective tissue. Gene therapy of db/db-EPCs was performed to determine if increasing the expression of MnSOD in db/db-EPCs (as demonstrated in chapter 3) would improve db/db-EPC adhesion. The outcome of this assay was that that MnSOD gene therapy did not improve db/db-EPC adhesion. This result suggests that the diminished level of MnSOD expression in db/db-EPCs does not contribute to impaired adhesion of db/db-EPCs.

Several recent studies have demonstrated the impaired ability of dEPCs to induce angiogenesis (27, 36, 71, 93). One *in vitro* measure of angiogenesis is the ability of EPCs to form networks on Matrigel (54, 71, 108). In this study, the ability of MnSOD gene therapy to improve network formation of db/db-EPCs was determined. The results show that db/db-EPCs, which underwent MnSOD gene therapy, demonstrate a gain of function with improved network formation on Matrigel, whereas the GFP control group did not. The results of this functional assay suggest that MnSOD plays a key role in tube formation and therefore angiogenesis. Importantly, this novel finding demonstrates that MnSOD deficiency contributes to the pathophysiology of EPCs in T2DM.

Other molecular mechanisms that have been found to contribute to EPC dysfunction in diabetes include elevated TSP-1 expression (82), impaired NO production (76), activation of the Akt/p53/p21 signaling pathway (100), and activation of p38 (85, 92), in addition to increased oxidative stress (93). A novel finding in this study is that, despite these other findings, restoration of dEPC angiogenic function with MnSOD gene therapy is a feasible approach to partially compensate for EPC dysfunction.

Migration of db/db-EPCs was also found to be impaired. Migration is an important step in mobilization of EPCs from the bone marrow (38). In addition, migration from the vasculature to the site of injury is required for EPCs to integrate into a wound site (38, 74). Transplantation of EPCs directly to the site of injury may compensate for both the decreased number of circulating db/db-EPCs and impaired migration. In the next chapter, transplantation of normal EPCs or db/db-EPC is explored as a potential therapy for diabetic wound healing.

CHAPTER 5

Partial Restoration of endothelial progenitor cell function with manganese superoxide dismutase gene transfer in type 2 diabetic wound healing

Abstract

Impaired wound healing in patients with DM is a significant clinical problem that can result in amputation. Inadequate angiogenesis is a key factor that contributes to poor diabetic wound healing. EPCs induce angiogenesis at the site of wound healing and are dysfunctional in diabetes. In the previous chapters, EPCs isolated from diabetic mice have been found to have diminished MnSOD expression compared to EPCs isolated from normal mice. Dysfunction of diabetic EPCs was significantly improved upon *ex vivo* MnSOD gene therapy *in vitro*. In the present study, the therapeutic effect of EPCs on diabetic wound healing was assessed by transplanting normal and diabetic EPCs to full thickness excisional wounds of T2D mice. Normal EPCs were significantly more effective at rescuing impaired wound healing then diabetic EPCs. Transplantation of diabetic EPCs transduced with MnSOD *ex vivo* resulted in significantly enhanced *in vivo* angiogenesis and accelerated wound healing rate. In addition, doubling the number of diabetic EPCs transplanted further accelerated the rate of wound healing, however, not to the extent achieved by normal EPCs. The present study demonstrates that ex vivo MnSOD gene therapy improves the diabetic EPC angiogenic function and the rate of diabetic wound healing after transplantation.

Background

Despite advances in wound care, refractory wound healing in patients with DM results in over 72,000 amputations each year in the US alone (22). Vascular complications in patients with DM, such as impaired angiogenesis, contribute to poor blood flow at the site of wound repair, resulting in defective endogenous inflammatory and regenerative responses (22, 34, 120). Wound healing is a complex process that involves the interplay of many different cell types including platelets, neutrophils, macrophages, lymphocytes, fibroblasts, keratinocytes and EPCs (23, 36). EPCs, first described by Asahara et al. in 1997 (35), are a bone marrow–derived cell population that circulate and participate in both vasculogenesis and vascular homeostasis (27, 36, 52, 74). Recent studies suggest that EPC dysfunction in diabetes contributes to impaired angiogenesis and subsequently; poor wound healing (38, 71, 72, 87, 99).

Progenitor cell therapy for ischemic/injured tissue is currently being studied in several clinical trials (110, 112, 149, 150). However, autologous progenitor/stem cell therapy of patients with diabetes is not as effective in regenerating injured/ischemic tissue as autologous progenitor/stem cell therapy of non-diabetic patients (112). Several basic unanswered questions regarding the use of diabetic progenitor cell therapy still remain. In this study, we attempt to provide mechanistic insights to answer some important questions as follows. When EPCs are transplanted for therapy, is there a significant difference in the regenerative capacity between normal EPCs and those EPCs isolated from T2D

mice? Is efficacious reversal of dEPC dysfunction feasible in developing cell therapy for wound healing? Would increasing the number of transplanted dEPCs compensate for their dysfunction? Determining whether the functional activity and number of transplanted cells influences the regenerative capacity of EPCs is an important step in developing clinically effective cell therapy for refractory diabetic wounds.

In the previous chapters EPCs were isolated from T2D (db/db) mice and their functional activity was studied *in vitro.* In the studies described in Chapter 4 it was found that MnSOD gene therapy improved tube formation in EPCs isolated from diabetic mice. This indicates that diminished MnSOD expression plays a role in the angiogenic dysfunction of db/db-EPCs and that MnSOD gene therapy of these cells is a putative therapy to improve functional activity. In this part of the study, db/db-EPCs are transplanted onto the wounds of T2D mice, with and without MnSOD gene transfer. The rates of wound closure were then measured. The primary role of EPCs is to induce angiogenesis (26, 54, 59, 67, 71, 74). In addition to determining the rate of wound closure, angiogenesis is measured on days 3, 6 and 16.

In the present chapter, the hypothesis that diminished MnSOD expression contributes to EPC dysfunction in diabetic wound healing was explored. These data suggest that db/db-EPCs *ex vivo* gene therapy with MnSOD prior to transplantation improve db/db-EPC functional activity *in vivo.* In addition,

evidence is provided that both the number and the functional activity of transplanted EPCs play significant roles in rescuing their regenerative capacity during wound healing and are important considerations for developing novel clinical EPC therapy strategies. Current evidence suggests that patients with diabetes may not be able to benefit from emerging therapy to improve vascular repair (72, 73, 82, 84, 87, 99, 112). In this study, evidence is provided that partial restoration of EPC functional activity prior to transplantation may allow for therapeutic EPC use in patients with diabetes.

Methods

Animal Model. Described in Chapter 2.

Isolation of bone marrow derived EPCs and described gene transfer of MnSOD or GFP into expanded EPCs. Described in Chapter in Chapter 3.

Punch biopsy wound model. A full-thickness excisional wound model was performed as previously described (39, 129). Briefly, mice were anesthetized with gas anesthesia (O_2 0.6 L/min, N_2O 0.4 L/min, Halothane 0.5-1L/min), the dorsum was clipped and depilatory cream (Nair) was used to remove hair. The skin was prepared with Betadine and let dry. A full-thickness 6 mm skin punch biopsies (Acuderm) was created for each mouse. Wounds were dressed and changed every other day with a transparent oxygen permeable wound dressing (Bioclusive, Johnson & Johnson). Wound closure rates were measured by tracing the wound area every other day onto an acetate paper. The tracings were digitized, and the areas were calculated with the use of a computerized algorithm and converted to percent wound closure (Sigma Scan Pro 5; Jandel Scientific).

EPC therapy for diabetic wounds. For EPC therapy of wounds, bone marrow-derived EPCs from normal mice and MnSOD-transduced EPCs from db/+ or db/db mice were trypsinized after seven days in culture (day 8) and re-suspended in 25 µL of PBS. $1x10^6$ cells or $2x10^6$ cells were injected onto and under the wound beds and approximately 0.5 cm past the wound edge. A

Bioclusive dressing was placed over the wound and mice remained under anesthesia for 15 min. Control mice were treated with PBS in the same fashion (39). Wound closure rates were compared between control and treatment groups, as described previously (39).

Wound Angiogenesis. Wounds were recovered from mice on days 3, 6 and 16, and capillary density in the healing wounds was quantified by histological analysis as previously described (27, 54). Briefly, wound samples were fixed with zinc chloride fixative (BD) for 24 hrs and embedded in paraffin and sectioned at 4-μm interval. Slides were deparaffinized, hydrated, and placed in Tris Buffered Saline pH 7.5 for 5 min for pH adjustment. Endogenous peroxidase was blocked utilizing a 3% hydrogen peroxide/methanol bath for 20 mins followed by distilled H_2O rinses. Slides were blocked with normal rabbit serum (Vector Labs – Burlingame, CA) for 30 min, incubated for 60 mins at room temperature with rat anti–mouse CD31 0.31 μg/mL (BD Bioscience), and the biotinylated rabbit anti–rat IgG H+L (Mouse Absorbed, Vector) 5 μg/mL for 30 minutes. Next the slides were incubated with Vectastain Elite ABC Reagent (Vector) for 30 minutes and Nova Red (Vector) for 15 minutes. Slides were counter stained with Gill (Lerner) 2 Hematoxylin (VWR Scientific) for 10 seconds, differentiated in 1% aqueous glacial acetic acid (removal of excess hemotoxylin), and rinsed in running tap water. Ten random microscopic fields (200x) were counted to determine the number of capillaries per wound (54). Pictures were taken on a Nikon Eclipse TE2000-U microscope using Metamorph software.

EPC Integration. Normal db/+ EPCs were isolated as described above and cultured in EGM-2. BrdU staining was performed on EPCs (121, 151, 152). Beginning on day 5, in culture, EPCs were labeled with 5-bromo-2'-deoxyuridine and 5-fluro-2'deoxyuridine (BrdU labeling reagent, Invitrogen). BrdU labeling reagent was diluted 1:100 in EGM-2, filtered through a 0.2 μm filter, and warmed to 37°C. 1 mL of BrdU/EGM-2 was added to cells in a 6-well plate, media was changed daily with BrdU/EGM-2 until day 7. On day 7, the wells were washed 3x with PBS followed by trypsinization to re-suspend the cells. The cells were then washed 3x via centrifugation EGM-2 (400g, 4°C). $2x10^6$ EPCs were then transplanted to a T2D mouse wound (6 mm punch biopsy) as described above. On day 6 of wound healing, the mouse was euthanized; the wound and surrounding skin were recovered and fixed in 10% formalin for 24 hrs. The skin was then stained with an anti-BrdU (0.63 μg/mL, BD) antibody, with R.T.U. Elite Avidin Peroxidase Complex (Vector), and Nova Red staining. Pictures were taken on a Nikon Eclipse TE2000-U microscope using Metamorph software.

Statistical Analyses. For data comparing multiple groups, Analysis of Variance (ANOVA) was used, followed by a post hoc pair-wise comparisons using Tukey's procedure to control for type I error (153). Statistical analysis for the rate of wound healing between groups was performed via SAS (Version 9.1) mixed procedure, followed with Tukey's correction (154-156). In all the tests, a two-tailed value of $P<0.05$ was taken as evidence of a statistically significant

finding. The above statistical analyses were carried out using Statistical Analysis System package and GraphPad (Version 4.03).

Results

EPC cell therapy improved wound healing in T2D mice. Mobilization of EPCs from the bone marrow to the site of injury is impaired in diabetes (38). Transplantation of EPCs to the site of injury could increase their number at the site of injury, and may thus be used as a therapeutic strategy to compensate for both the decreased EPC number and impaired mobilization in diabetes. The wound healing baseline for both normal and T2D mice was compared first. Each mouse underwent a single dorsal caudal 6 mm punch biopsy (day 0) as an established full-thickness excisional wound model, and the rate of wound closure was then measured every other day until day 16. The rate of wound closure in diabetic mice was markedly delayed compared to normal mice ($P<0.0001$, n=10, Figure12A). To determine the functional effect of cell therapy, bone marrow-derived EPCs were transplanted directly onto the wounds of diabetic mice after 7 days of culture in EGM-2. Immediately following 6 mm punch biopsies, mice underwent transplantation of either $1x10^6$ normal (db/+-EPCs) or $1x10^6$ diabetic (db/db-EPCs). The overall wound healing rate was significantly improved in diabetic mice that received transplantation of either db+-EPC or db/db-EPCs. However, the rate of wound healing was significantly faster in diabetic mice that received db/+-EPCs as compared to those that received the db/db-EPCs ($P<0.0001$, n=10, Figure 12A). Diabetic mice transplanted with db+-EPCs demonstrated improved wound healing over untreated diabetic mice starting on day 4 ($P<0.05$, n=10), whereas diabetic mice that received db/db-EPC transplantation did not show a significant improvement until day 10 over untreated

diabetic mice ($P<0.05$, n=10). Thus, transplantation of db/db-EPCs was not as effective in improving wound closure as transplantation of normal EPCs.

MnSOD gene therapy of db/db-EPCs rescued in vivo functional activity when transplanted onto diabetic wounds. To determine the functional effect of MnSOD gene therapy of dEPCs on wound healing, *ex vivo* infection of the MnSOD gene into db/db-EPCs (db/db-AdMnSOD-EPCs) was performed prior to their *in vivo* transplantation. Diabetic mice receiving db/db-AdMnSOD-EPC therapy demonstrated significantly improved wound healing starting on day 6 ($P<0.05$, n=10, Figure 13). In contrast, diabetic mice receiving $1x10^6$ db/db-EPCs per wound with Ad-GFP gene transfer (db/db-AdGFP-EPC) did show improvement in the rate of wound healing until day 10 ($P<0.05$, n=10), an effect that was consistent with db/db-EPCs controls (Figure 12A and 15). The overall rate of wound healing was significantly accelerated in diabetic mice that received the db/db-AdMnSOD-EPCs as compared to those that received the control db/db-AdGFP-EPCs ($P<0.007$, n=10, Figure 13A). Thus, *ex vivo* MnSOD gene therapy of db/db-EPCs partially restored their functional activity in diabetic wound healing, whereas db/db-EPCs controls transfected with GFP did not demonstrate improved functional activity *in vivo.*

Increasing the number of db/db-EPCs transfected with MnSOD further accelerated the rate of diabetic wound healing. To study the possible synergistic effect of the EPC number and functional activity on diabetic wound

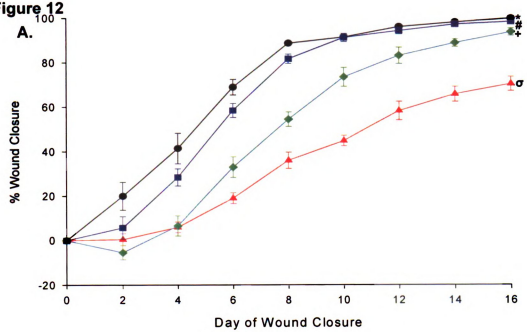
healing, the number of EPCs transplanted was doubled (i.e. db/db-AdGFP-EPCs or db/db-AdMnSOD-EPCs) from $1x10^6$ to $2x10^6$ EPCs/6mm punch wound. First, transplantation of $2x10^6$ db/db-AdGFP-EPCs significantly accelerated the rate of wound healing compared to $1x10^6$ db/db-AdGFP-EPCs ($P<0.001$, n=10) or $1x10^6$ db/db-EPCs ($P<0.001$, n=10), compare Figures 12A, 13A and 14A. Second, transplantation of $1x10^6$ db/db-AdMnSOD-EPCs was equivalent to transplantation of $2x10^6$ db/db-AdGFP-EPCs for improving the rate of diabetic wound healing ($P=0.46$, n=10, compare Figures 13A and 14A. Third, transplantation of $2x10^6$ db/db-AdMnSOD-EPCs further accelerated the rate of wound healing of diabetic mice compared to $2x10^6$ control db/db-AdGFP-EPC ($P<0.05$, n=10, Figure 14). In addition, the results demonstrate that transplantation of two million db/db-EPC-AdMnSOD significantly improved the rate of diabetic wound healing compared to transplantation of $1x10^6$: db/db-EPC-AdMnSOD ($P<0.001$, n=10), db/db-EPC-AdGFP ($P<0.001$, n=10), db/db-EPC ($P<0.001$, n=10) with no EPC-gene therapy and the db/db control mice ($P<0.001$, n=10) with no EPC therapy. Most importantly, transplantation of $2x10^6$ db/db-AdMnSOD-EPCs caused complete recovery of diabetic wound healing equivalent to the same rate as normal mice ($P=0.99$, n=10, Figure 14). No difference was found in the rate of wound healing between transplantation of $2x10^6$ db/db-MnSOD-EPCs, $1x10^6$ normal db/+-EPCs ($P=0.37$, n=10) or normal db/+ mice ($P=0.99$, n=10) Figure 15. These data indicate that both the functional activity and number of EPCs at the site of injury each play a significant role in wound healing.

Figures 12-15. After a 6 mm punch biopsy was made, the area of each wound was traced onto an acetate paper and measured every other day until day 16. Control groups: normal mice wound healing db/+ (●) and T2D wound healing db/db (▲), are taken as baseline wound healing and are the same for **Figures 12, 13** and **14**. The experimental groups are separated for clarity (n=10 for all groups). **Figure 15** shows all of the groups in one plot. Representative photographs of wound healing quality are shown below each wound healing plot (no scale present) as **Figures 12B, 13B** and **14B.** All groups in **Figures 12, 13** and **14** demonstrated an improved rate of wound healing over untreated db/db mice alone ($P<0.001$, db/db vs. all other groups, n=10).

Figure 12. EPC cell therapy of diabetic wounds improves the rate of wound healing in T2D mice. **A**. Normal mice (db/+) and T2D mice transplanted (TXD) with $1x10^6$ db/+-EPCs (■) improved the rate of wound healing faster than T2D mice TXD with $1x10^6$ db/db-EPCs (◆) (*$P<0.001$, db/+ vs. db/db TXD $1x10^6$ db/db-EPCs, n=10). (#$P<0.001$, db/db TXD with $1x10^6$ db/+-EPCs vs. db/db TXD $1x10^6$ db/db-EPCs, n=10). Normal db/+ mice and T2D mice TXD with $1x10^6$ db/+-EPCs healed at equivalent rates (*$P=0.185$ db/+ vs. db/db TXD with $1x10^6$ db/+-EPCs, n=10). **B.** Representative photographs of healing wounds.

Figure 12

A.



(●) db/+
(▲) db/db
(■) db/db with $1x10^6$ db/+-EPCs
(◆) db/db with $1x10^6$ db/db-EPCs

B.

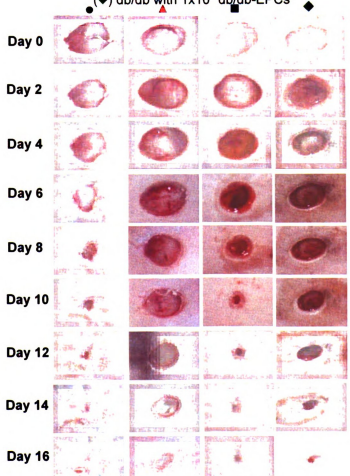
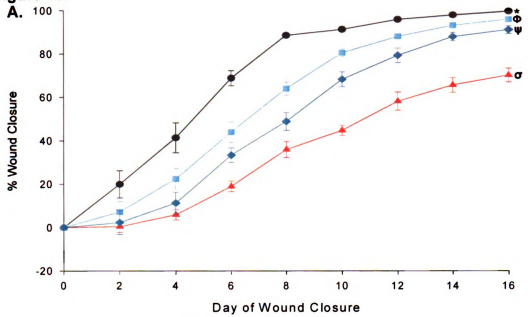


Figure 13. MnSOD gene therapy of db/db-EPCs prior to transplantation improved that ability of db/db-EPC to induce wound healing. A. MnSOD gene therapy of $1x10^6$ db/db-EPCs (db/db-AdMnSOD-EPC (■)) improved db/db-EPCs ability to enhance wound closure vs. transplantation of $1x10^6$ control green fluorescent protein db/db-EPCs (db/db-AdGFP-EPCs (◆)) ($^{\Phi}P<0.01$, db/db TXD with $1x10^6$ db/db-AdMnSOD-EPC vs. db/db TXD with $1x10^6$ db/db-AdGFP-EPCs n=10). **B.** Representative photographs of healing wounds.

Figure 13.

A.



(●) db/+
(▲) db/db
(■) db/db with $1x10^6$ db/db-MnSOD-EPCs
(◆) db/db with $1x10^6$ db/db-AdGFP-EPCs

B.

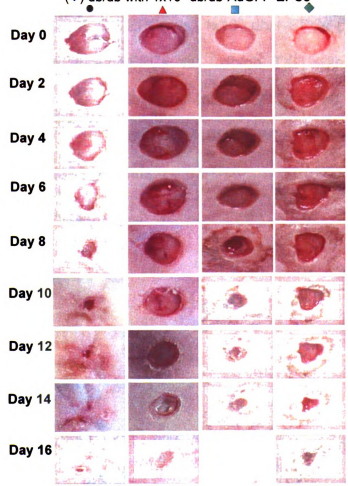
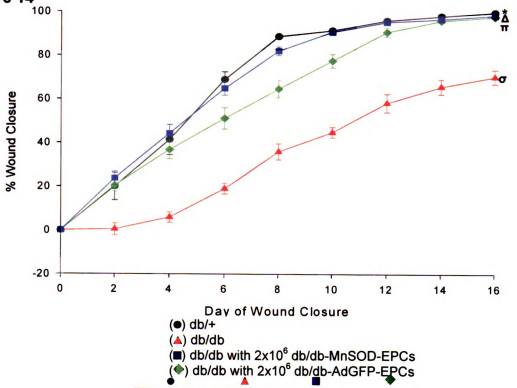


Figure 14. The number and functional activity of EPC work synergistically to improve wound healing. **A.** Transplantation of $2x10^6$ db/db-AdMnSOD-EPCs (■) improved the rate of wound healing vs. $2x10^6$ db/db-AdGFP-EPCs (◆) ($^{\Delta}P<0.05$, db/db TXD with $2x10^6$ db/db-AdMnSOD-EPCs vs. db/db TXD with $2x10^6$ db/db-AdGFP-EPCs n=10). No difference was found between the rate of wound healing of $2x10^6$ db/db-AdMnSOD-EPCs vs. $1x10^6$ db/+-EPCs ($^{\#}P=0.3$, db/db TXD with $2x10^6$ db/db-AdMnSOD-EPCs vs. db/db TXD with $1x10^6$ db/+-EPCs n=10) or db/+ mice vs. $2x10^6$ db/db-AdMnSOD-EPCs (*P=0.9, db/+ mice vs. db/db TXD with $2x10^6$ db/db-AdMnSOD-EPCs n=10). **B.** Representative photographs of healing wounds.

Figure 14

A.


% Wound Closure
100
80
60
40
20
0
-20
0
2
4
6
8
10
12
14
16
Day of Wound Closure
(●) db/+
(▲) db/db
(■) db/db with 2x10^6 db/db-MnSOD-EPCs
(◆) db/db with 2x10^6 db/db-AdGFP-EPCs
π
σ

B.

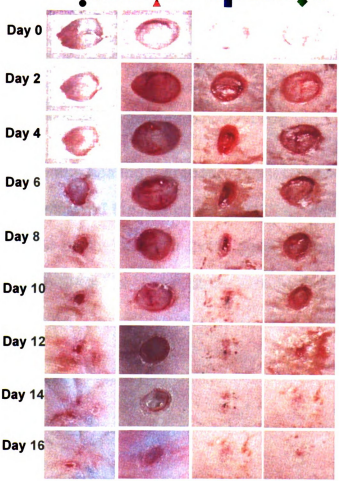

Day 0
Day 2
Day 4
Day 6
Day 8
Day 10
Day 12
Day 14
Day 16

Figure 15.

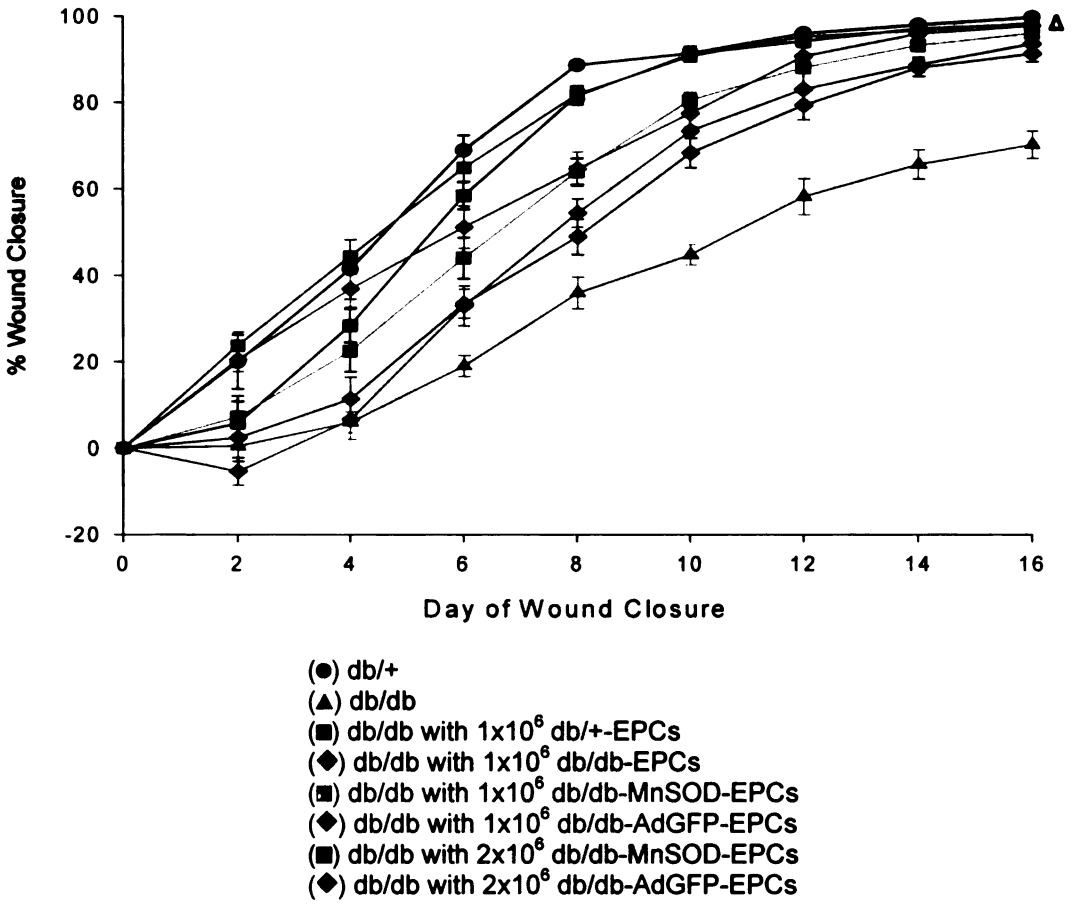


Figure 15. All wound healing groups. Transplantation of $2x10^6$ db/db-AdMnSOD-EPCs improved the rate of wound in db/db mice vs. other cell therapy groups ($^{\Delta}P<0.05$, db/db TXD with $2x10^6$ db/db-AdMnSOD-EPCs vs. db/db TXD $1x10^6$ db/db-EPCs, or $1x10^6$ db/db-AdMnSOD-EPC, or $1x10^6$ db/db-AdGFP-EPCs, n=10).

EPC therapy resulted in improved in vivo angiogenesis in diabetic wound healing. Wounds and surrounding skin were recovered and stained for CD31 as a marker of *in vivo* angiogenesis (i.e. capillary formation), on days 3, 6 and 16 of wound healing as described (27). On day 3 of wound healing, capillary formation was significantly greater in normal mice compared to all other groups ($P<0.05$, n=4-5, Figure 16A). On day 6, normal mice continued to have significantly more capillary formation compared to diabetic mice as well as diabetic mice with db/db-EPC therapy or db/db-AdGFP-EPC ($P<0.05$, n=4-5, Figure 16B). Diabetic mice treated with either $1x10^6$ normal EPC, $1x10^6$ db/db-AdMnSOD-EPCs, $2x10^6$ db/db-AdMnSOD-EPCs or $2x10^6$ db/db-AdGFP-EPCs showed significantly more capillary formation compared to control diabetic mice on day 6 ($P<0.05$, n=5, Figure 16B). On day 16, diabetic mice continued to show significantly less capillary formation compared to all other groups ($P<0.05$, n=5, Figure 16S). Correspondingly, groups with accelerated wound healing over control diabetic mice showed significantly more capillary formation on day 6 (Figure 16B and 16C-R). Furthermore, either MnSOD-transfected db/db-EPCs or doubling the EPC number from $1x10^6$ to $2x10^6$ (db/db-AdGFP-EPCs) increased capillary formation. These data demonstrate that either restoration of EPC functional activity by MnSOD gene therapy prior to transplant or increasing the number of dEPCs is effective in improving *in vivo* angiogenesis of diabetic wounds.

Figure 16. EPC therapy improves angiogenesis in mouse skin after wounding. **A.** On day 3 after wounding, capillary formation was significantly greater in db/+ mice compared to all db/db groups (black bars) (#P<0.05, db/+ vs. all other groups, n=4-5). **B.** On day 6 after wounding, angiogenesis was increased in normal (db/+) and in T2D (db/db) mice treated with $1x10^6$ EPCs isolated from: normal mice ($1x10^6$ db/+-EPCs), T2D mice after MnSOD gene therapy ($1x10^6$ db/db-AdMnSOD-EPCs), treated with $2x10^6$ EPCs from T2D mice after MnSOD gene therapy ($2x10^6$ db/db-AdMnSOD-EPCs) or the GFP control ($2x10^6$ db/db-AdGFP-EPCs) compared to T2D mice (db/db) without treatment (*P<0.05, db/db vs. db/+, db/db, $1x10^6$ db/+-EPCs, $1x10^6$ db/db-AdMnSOD-EPCs, $2x10^6$ db/db-AdMnSOD-EPCs, $2x10^6$ db/db-AdGFP-EPCs n=5). T2D (db/db) mice without cell therapy and db/db mice treated with $1x10^6$ EPCs from db/db mice (db/db-EPCs) or the GFP control ($1x10^6$ db/db-AdGFP-EPCs) continued to demonstrate diminished capillary formation compared to the normal (db/+) mice (#P<0.05 vs. db/+, n=5). Representative photographs of CD31 staining on day 6 after wounding for normal db/+ mice (**C** 40x, **D** 200x), db/db mice (**E** 40x, **F** 200x), and db/db with EPC cell therapy of $1x10^6$: normal db/+-EPCs (**G** 40x, **H** 200x), db/db-EPCs (**I** 40x, **J** 200x), db/db-AdMnSOD-EPCs (**K** 40x, **L** 200x), db/db-AdGFP-EPCs (**M** 40x, **N** 200x) and $2x10^6$ EPCs: db/db-AdMnSOD-EPC (**O** 40x, **P** 200x), db/db-AdGFP-EPCs (**Q** 40x, **R** 200x). Green arrows point to CD31 positive capillaries (Scale for 40x = 500 μm, 200x = 100 μm) (W=wound, Dm=dermis, Ep= epidermis). **Figure 16S.** On day 16 after wounding, T2D mice (db/db) angiogenesis was diminished compared to normal mice db/+ (#P<0.05

db/+ vs. db/db, n=5).

Figure 16.

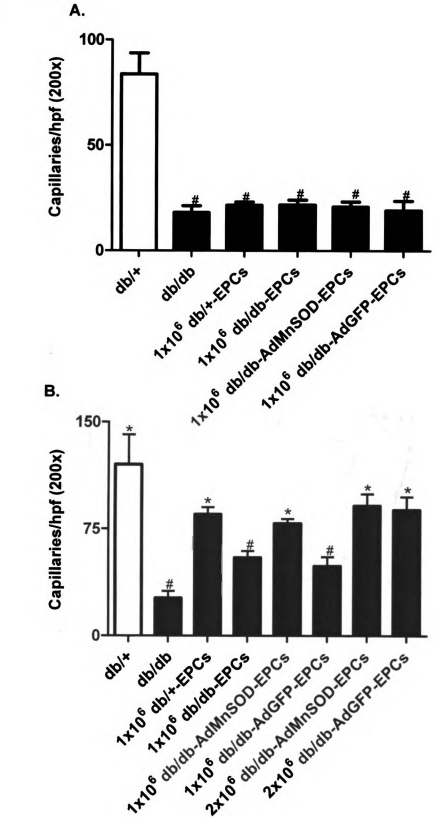
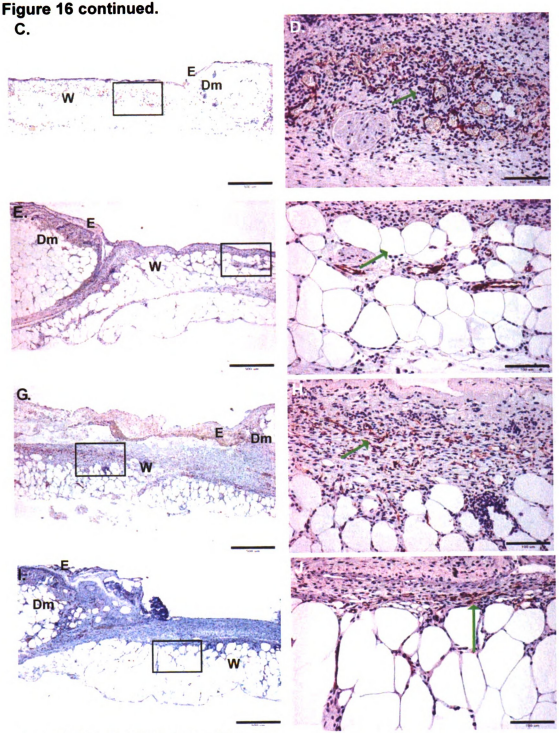
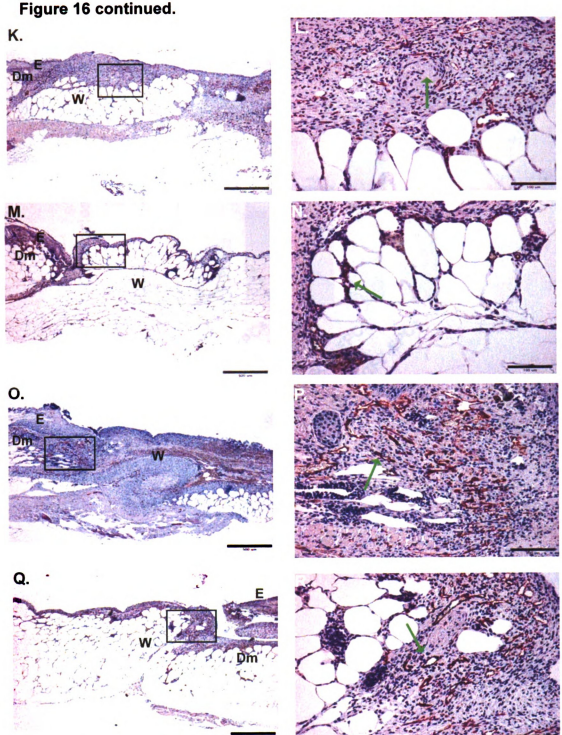

A.
Capillaries/hpf (200x)
100
50
0
#
db/+
db/db
1x10^6 db/+-EPCs
1x10^6 db/db-EPCs
1x10^6 db/db-AdMnSOD-EPCs
1x10^6 db/db-AdGFP-EPCs
B.
Capillaries/hpf (200x)
150
75
0
*
#
db/+
db/db
1x10^6 db/+-EPCs
1x10^6 db/db-EPCs
1x10^6 db/db-AdMnSOD-EPCs
1x10^6 db/db-AdGFP-EPCs
2x10^6 db/db-AdMnSOD-EPCs
2x10^6 db/db-AdGFP-EPCs

Figure 16 continued.



Scale for C, E, G, I is 40x = 500 µm,
Scale for D, F, H, J is 200x = 100 µm

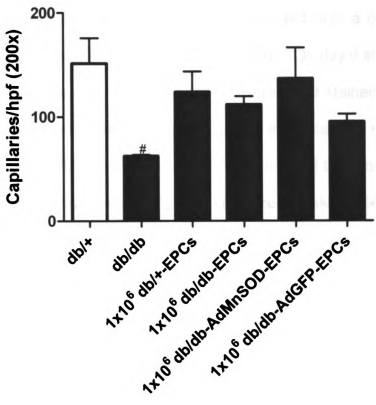
Figure 16 continued.



Scale for K, M, O, Q is 40x = 500 µm,
Scale for L, N, P, R is 200x = 100 µm

Figure 16 continued.

S.



Transplanted EPCs integrated into wound vasculature. To determine the fate and location of transplanted EPCs *in vivo*, normal EPCs isolated from db/+ mice were incubated with BrdU in EGM-2 starting on day 5 of cell culture. After day seven of culture, $2x10^6$ cells were transplanted onto a 6mm punch biopsy wound of a db/db mouse at the time of wounding. On day 6 after wound healing, the wound and surrounding skin were recovered and stained for BrdU positive EPCs. BrdU-positive EPCs were integrated into vascular structures and the dermis (Figure 17 A-D). Previous studies have found that bone marrow-derived progenitor cells integrate into multiple structures in skin (154, 156), and these data provide further evidence for these findings.

Figure 17.

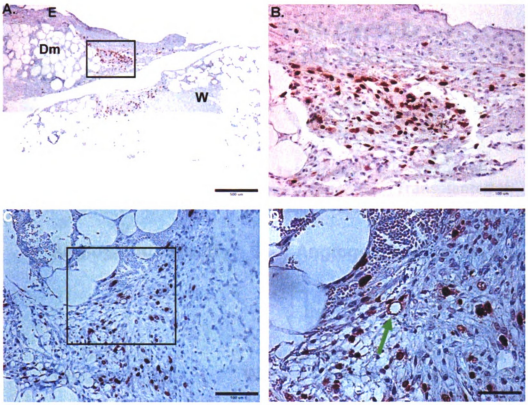


Figure 17. Transplanted EPCs integrate into the skin and vasculature. $2x10^6$ BrdU labeled db/+-EPCs were transplanted onto 6mm punch biopsy wounds of db/db mice at the time of wounding. On day 6 after wounding, tissue was isolated, fixed and stained for BrudU (Brown staining cells). Representative photographs demonstrated EPC integrating into the dermis **A** (40x), **B** (200x), **C** (200x) and **D** vascular structure, green arrow (400x), (Scale for 40x =500 μm, for 200x = 100 μm, for 400x =50 μm, W=wound, Dm=dermis, Ep= epidermis).

Figure 18.

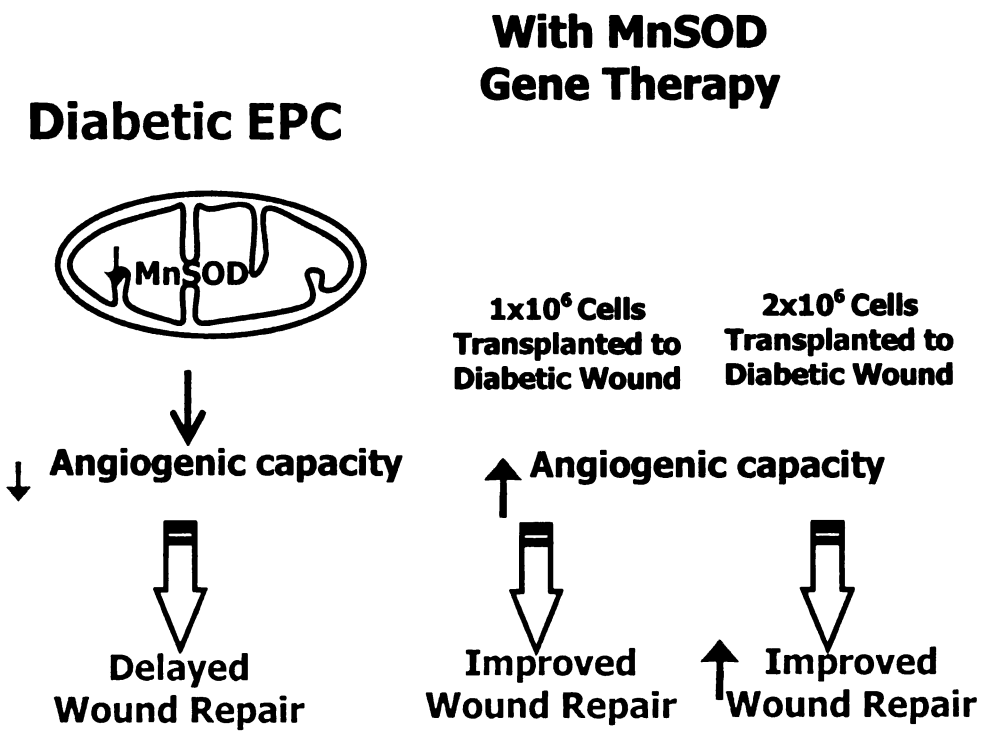


Figure 18. Model of EPC therapy for diabetic wound healing. Both the functional ability and number of transplanted EPCs contribute to accelerated wound healing. MnSOD gene therapy of dEPCs, in addition to increasing transplanted EPC number, results in synergistic improvement of wound closure.

Discussion

In this study, the transplantation of EPCs was explored as a form of cell therapy for diabetic wound healing, which compensates for db/db-EPCs migration impairment. Transplantation of $1x10^6$ normal EPCs to diabetic wounds was first performed. This strategy restored the markedly delayed wound healing of diabetic mice to the normal control level. The same number ($1x10^6$) of db/db-EPCs was transplanted to diabetic wounds. The results indicate both normal and db/db-EPCs significantly improved the rate of wound healing in diabetic mice. However, while transplantation of db/db-EPCs improved wound healing, the rate of wound healing was significantly less compared to normal EPC transplantation. This result strongly suggests that the dysfunction found in dEPCs diminishes their ability to accelerate wound repair.

EPCs isolated from T2D mice demonstrate impaired functions such as, adhesion, migration and network formation on Matrigel (described in chapters 3-4). MnSOD gene therapy of db/db-EPCs improved network formation on Matrigel, but did not improve the ability of db/db-EPCs to adhere in vitronectin in culture. These data indicate that restoration of db/db-EPC angiogenic function by MnSOD gene therapy is a feasible approach to at least partially compensate for dysfunction. Given these findings, the use of MnSOD gene therapy to improve db/db-EPC functional activity prior to transplantation was explored.

In comparison to the GFP marker gene group, transplantation of

MnSOD-transfected db/db-EPCs significantly improved wound healing. However, this improvement did not match that of normal EPCs with the same cell number (i.e. $1x10^6$ EPCs), this was consistent with previous studies that showed transplanted db/db-EPCs exhibit diminished regenerative capacity compared to normal EPCs (72, 73, 112).

We then asked the question: could increasing the number of db/db-EPCs alone compensate for their dysfunction? In other words, does a "dosage effect" exist with EPC cell therapy? By doubling the cell number from $1x10^6$ to $2x10^6$, we found that transplantation of db/db-EPCs significantly improved the rate of wound healing. Interestingly, the dosage effect of doubling the db/db-EPCs to $2x10^6$ was similar to MnSOD-transfected db/db-EPCs at $1x10^6$ in promoting wound healing *in vivo*. This highlights the importance of MnSOD for EPC functional activity. Furthermore, when the number of MnSOD-transfected db/db-EPCs was also increased to $2x10^6$, it resulted in complete restoration of wound healing equivalent to what normal EPCs achieved. Together, these observations suggest that the number and angiogenic function of EPCs work synergistically in wound healing.

The findings in this study have broad implications for the use of progenitor/stem cell therapy to induce regeneration in injured/ischemic tissue. The number of transplanted progenitor/stem cells needed for a given area of injury has yet to be optimized. In addition, isolated progenitor/stem cells from

patients or animal models with systemic disease may negatively impact affect progenitor/stem cell regenerative ability in individuals with diabetes, hypertension and cardiovascular disease. In this study, the evidence provided demonstrates that both the number and functional activity of progenitor cells used for therapy are important considerations in development of novel progenitor/stem cell therapies.

Despite current clinical therapies and the large number of studies that have been performed to improve diabetic wound healing, refractory diabetic wound healing remains a significant clinical problem that can lead to limb amputation. The pathophysiology that leads to impaired wound healing is complex, and therefore multiple approaches may be necessary in order to significantly impact care of diabetic wound healing. Here immunocompetent syngeneic mice were used to isolate and transplant EPCs, which results in an autograft. The results in chapter 4 demonstrate that the angiogenic function of EPCs isolated from T2D mice can be improved with MnSOD gene therapy. The data acquired in this study suggest that both the number and functional activity of transplanted EPCs are important for outcome. Improving the functional activity of dEPCs via MnSOD gene therapy prior to transplantation or increasing the number of dEPCs transplanted improves angiogenesis and accelerates the rate of diabetic wound healing. In developing novel cell therapies for human patients with DM, improving cell functional activity through gene therapy prior to transplantation, in combination with, determining the optimal number of progenitor cells needed for

transplantation may compensate for EPC dysfunction in DM.

Technical Acknowledgements

I would like the following people for their participation in this project:

Jeff Hakim for assistance with several EPC recoveries and for his help with RNA isolation and RT-PCR.

Dan-Dan Chen for her assistance with several EPC recoveries and her part in characterizing EPCs with Dil-Ac-LDL and isolectin staining.

The Michigan State University Histology laboratory for their part in preparing and staining of tissue sections for CD31 and Brdu positive cells.

Jiemei Wang for running the Western Blot in which the MOI for MnSOD viral transfection was determined.

Paramita Chakraborty of C-Stat and Dr. Carrie Northcott of the Department of Pharmacology for their assistance with the statistical analysis of the rate of wound closure.

CHAPTER 6

SUMMARY AND PERSPECTIVE

Novel findings in this study may lead to the practical therapeutic use of diabetes-derived EPCs for diabetic wound healing. The results of this study demonstrate that diminished MnSOD expression contributes to EPC dysfunction in T2DM. EPCs from T2D mice underwent MnSOD gene therapy and demonstrated improved network formation on Matrigel. In addition, after *ex vivo* MnSOD gene therapy, EPCs were transplanted onto diabetic wounds. The outcome of these *in vivo* experiments demonstrated that MnSOD gene therapy improved the ability of diabetes-derived EPCs to promote acceleration of wound healing and to induce angiogenesis. Together the results of the *in vitro* and *in vivo* experiments suggest that MnSOD deficiency in T2DM contributes to EPC dysfunction.

Future studies should further characterize the findings in this study. In the present study, DiI-AcLDL and isolectin binding were used to characterize mouse bone marrow derived-EPCs. The use of cell surface markers, such as Sca-1, Flk-1, and CD31, may also be useful in characterizing mouse bone marrow derived-EPCs (54, 56, 64). In the appendix, a histogram (Figure 19), demonstrates that the auto-fluorescence of bone marrow derived-EPCs is very high in both the red (PE) and green (FITC) spectrum. In order to characterize these cells with immunostaining and flow cytometry, future studies should re-examine the cells on a flow cytometer, and determine the spectrum in which the least autofluorescence is found. The information provided from such experiments may allow for the use of fluorochromes other than PE or FITC.

Additionally, the activity of MnSOD needs to be further characterized both *in vitro* and *in situ.* If a difference in the activity of MnSOD between db/+ EPCs and db/db-EPCs is found, it would provide further evidence that the diminished level of MnSOD found in db/db-EPCs plays a role in dysfunction. Additionally, isolation of mitochondrial and cytoplasmic MnSOD from both db/+ and db/db EPCs, followed by determination of MnSOD activity would provide information about the localization of MnSOD. Studies (107, 108) that determined that elevated levels of MnSOD, compared to mature endothelial cells, are required for normal EPC activity did not localize MnSOD. In this study it was found that MnSOD expression level is important for angiogenesis (Figure 10). Knowing whether MnSOD in EPCs is predominantly localized to the mitochondria or cytoplasm would be a step in characterizing the mechanism by which MnSOD aids in the induction of angiogenesis. Also, the result in Figure 8, in which gene therapy decreases ROS in EPCs, was only carried out once, with an n=1. Further studies are obviously needed to determine reproducibility of this finding.

The db/db model is a well-established T2D mouse model in which EPC functional studies have been previously performed (50, 71, 82). These mice have a defect in the leptin receptor (OB-Rb) found in the hypothalamus, which leads to hyperphagia, and subsequently obesity, hyperglycemia, hyperlipidemia, and insulin resistance (19, 128). Patients with T2D can develop leptin resistance, which is believed to be, in part, caused by hyperleptinemia (155, 157, 158).

Similar to patients with T2DM, db/db mice also express hyperleptinemia (19, 128). Additionally, patients with impaired wound healing secondary to diabetes have demonstrated a lack of OB-Rb leptin receptor expression in the wound (146). This supports the use of db/db mice as a suitable model for wound healing. However, this study should be repeated in a model that does not have a defect in the leptin receptor, such as the Tallyho mouse (159, 160), to confirm that EPC dysfunction in this study is strictly related to diabetes.

The integration of transplanted EPCs into a diabetic wound in Figure 17 is also demonstrative (n=1). This should be repeated to confirm the results. In addition, further immunohistochemistry such as CD31 staining should be performed. Combination CD31 and BrdU labeling will provide stronger evidence that the vascular like structures in fact are capillaries.

Another novel finding in this study is that when using progenitor cells for therapy, there is a synergistic effect between the number and functional activity of progenitor cells for the induction of regeneration. Impaired angiogenesis in diabetic wound healing is a significant clinical problem that can lead to tissue necrosis and subsequent amputation. This study provides proof of principle that, despite previous findings (73, 112), dEPCs can be used therapeutically to induce angiogenesis and diabetic wound healing, when both the functional activity and the number of cells transplanted are considered.

In previous studies, investigators have reported additional putative mechanisms that lead to EPC dysfunction in diabetes. These studies have demonstrated that EPC dysfunction was caused by elevated TSP-1 expression (82), impaired NO production (76, 96), activation of the Akt/p53/p21 signaling pathway (100), activation of p38 (85, 92), increased oxidative stress (93) and in the present study, decreased MnSOD expression. Given the finding in this study, that reversal of dEPC dysfunction can improve the therapeutic capacity of these cells, future studies should be performed to determine which mechanism or mechanisms, if reversed, would best restore the EPC functional activity in diabetes.

This and other studies have found that EPCs isolated from diabetics do not regain normal functional activity after incubation in euglycemic cell media. This was found to be true for both human and animal EPCs isolated from diabetics (71, 82, 96, 161, 162). In addition, this and other studies found that this dysfunction is heritable (71, 82). Currently no term exists to describe persistent dysfunction of disease induced cellular pathophysiology. Therefore, I suggest using the term cytomneosis (derived from Greek: cell memory of disease). Several possible mechanisms may cause cytomneosis. Because this persistent dysfunction is heritable, mechanisms that cause alterations in gene expression should be explored, such as DNA mutation, change in epigenetic regulation (such as DNA methylation) and change in micro-RNA expression. The present study and one earlier study (93) have found that ROS is increased in dEPCs. The possibility

that hyperglycemia or elevated ROS causes altered gene expression, and that the change in gene expression is then maintained from parent to daughter cells, should also be explored. Unclear is whether one mechanism or "multiple-hits" lead to persistent dysfunction in dEPCs. Characterization of cytomneosis could lead to a possible drug target or gene therapy target that if reversed fully, could restore EPC functional activity in diabetes. This in turn would increase the efficacy of autologous EPC therapy in diabetic wound healing.

This study also has broad implications for progenitor/stem cell therapy in general. Currently there are many clinical trials underway for the use of progenitor/stem cells to regenerate ischemic/injured tissue. The finding in this study that there is a synergistic effect between the number and the functional activity of EPCs when used as a cell therapy could aid in the planning of future investigations. A recent study, for example, in which autologous progenitor cells from patients with diabetes were used to regenerate ischemic hearts in humans, found that these progenitor cells were not as effective as non-diabetic progenitor cells (112). In this study performed by Jiménez-Quevedo et al., the same number of cells was used for autologous transplants in both the diabetic and the non-diabetic groups (112). In developing future clinical studies, rapid, reliable functional assays should be developed that will allow investigators to determine the regenerative capacity of the progenitor cells from each individual. Investigators should then determine, given a specific progenitor cell dysfunction

and an area/volume of ischemic/injured tissue, the optimal number of progenitor cells needed to induce regeneration.

In this study, a previously unknown mechanism that contributes to dysfunction in diabetes was discovered, MnSOD deficiency. Gain of function was demonstrated with MnSOD gene therapy of dEPCs *in vitro.* These cells exhibited with improved network formation on Matrigel and *in vivo* with increased angiogenesis and rate of wound healing after EPC transplantation in diabetic mice. This study also found that both the functional activity and number of EPCs at the site of wound healing synergistically play a role in wound healing. The novel findings acquired in this study, including partial reversal of dEPC dysfunction and a greater mechanistic understanding of progenitor cell therapy, have broad implications and provide new therapeutic insight for the use of progenitor cells in the field of regenerative medicine.

Appendix

Flow Cytometry Analysis of Bone Marrow Derived EPCs

In this study, bone marrow derived-EPCs were identified with DiI-Ac-LDL/isolectin staining (Figure 3). An attempt was made to further characterize bone marrow derived-EPCs with immunostaining for Sca-1, Flk-1, c-kit and CD31. EPCs were isolated and cultured as described in Chapter 3, pages 55-56. After seven days in culture, cells were removed from 6 well plates with Versene (0.5 mM EDTA, Invtrogen). The EPCs were then placed in PBS with 2% albumin. Staining was performed with primary antibodies against Sca-1-FITC, Flk-1-PE, c-kit-PE, CD31-PE and run on a BD Vantage Flow cytometer.

The results in Figure 19 show flow cytometric analysis of bone marrow derived EPCs of **A.** cells only **B.** Flk-1-PE, **C.** CD31-PE, **D.** Sca-1-FITC, and **E.** c-kit-PE. Cells without staining, **A.**, demonstrate significant autofluorescence in the same spectra as both FITC and PE. In order to acquire an accurate analysis of bone marrow derived EPCs (BM-EPCs) future studies must use a fluorochrome that has minimal spectral overlap with the autofluorescence of the cells. Accurate analysis of BM-EPCs will allow for a quantitative analysis and comparison of both db/+ and db/db EPCs.

Figure 19.

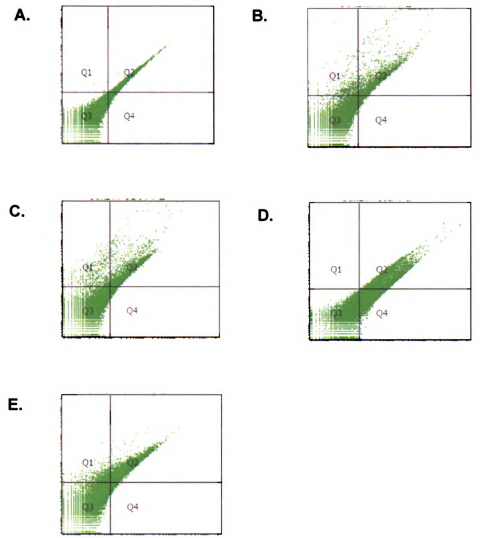


Figure 19. Flow cytometric analysis of bone marrow derived EPCs of **A.** cells only **B.** Flk-1-PE, **C.** CD31-PE, **D.** Sca-1-FITC, and **E.** c-kit-PE.

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