Therapeutic application of the bone marrow derived stem cells in hindlimb ischemia of diabetic mice.

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ABSTRACT

Therapeutic application of the bone marrow derived stem cells in hindlimb ischemia of diabetic mice.

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Diabetes mellitus (DM) is one of the greatest risk factors for the cardiovascular disease. Diabetes patients have delayed wound healing and recovery from vascular injury. Coronary and peripheral occlusive disease and its complication such as acute myocardial infarction and peripheral artery disease have been an important issue among in cardiovascular medicine. The purpose of these experiments is to show the mechanism of the delayed recovery from the ischemic injury in DM and to reveal the molecular functional assessment of the bone marrow derived endothelial progenitor cells (EPCs) and mesenchymal stem cells (MSCs) as well as to study their therapeutic application with the murine hindlimb ischemia model.

Blood flow recovery from the hindlimb ischemia was delayed in the diabetic mice. This study showed the possible mechanisms for the delay : decrease vascular endothelial growth factor (VEGF)-A level in the diabetic skeletal muscle; decreased circulating EPCs number in DM; decreased the important angiogenic cytokines such as VEGF-A, insulin-like growth factor-1, angiopoietin-1 in the diabetic bone marrow derived mononuclear cells (BM-MNC) with RT-PCR; decreased VEGF-A in diabetic EPCs, and MSCs; reduced multiple angiogenesis related cytokines in diabetic BM-MNCs, EPCs and MSCs with Microarray, functional derangements of EPCs and MSCs for angiogenesis confirmed by migration, adhesion, tube formation assay. Transplantation of EPCs and MSCs cultured from normal BM-MNCs to the diabetic hindlimb ischemia showed significantly better recovery compared to the saline injection. The mechanism of these favorable effects was proposed of increased capillary density, long engraftment of the

injected stem cells up to 2 weeks in vivo, increased cytokines such as VEGF-A, HIF-1α, FGF-2, and PlGF in the cell transplanted hindlimb, and partly transdifferentiation to the newly forming vascular structures of the injected stem cells documented by co-localization of injected cells to vessels. In conclusion, DM induced delayed blood flow recovery after ischemia and showed dysfunction of bone marrow derived stem cells such as EPCs and MSCs. Normal EPCs or MSCs transplantation in to diabetic hindlimb ischemia showed significant improvement in their recovery from ischemia.

Key words: diabetes mellitus, stem cell, endothelial progenitor cell, mesenchymal stem cell, Bone marrow-mononuclear cells, hindlimb ischemia

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I. INTRODUCTION

Diabetes mellitus (DM) comprises a heterogeneous group of disorders characterized by high blood glucose levels. DM is one of the most important diseases in national health system in US in the 21st century because, as prevalence and incidence of DM have been increasing, mortality and morbidity from it also are increasing. Accordingly, its economic impact is also considered being important. From 1980 through 2003, the number of Americans with diabetes more than doubled (from 5.8 million to 13.8 million). In 2003, about 4.9% of the U.S. population reported that they had diabetes. People aged 65 years or older account for almost 40% of the population with diabetes [1, 2]

Most of the DM morbidity related its chronic complications. Disability is the major social issue resulted from DM. The major cause of DM related disabilities is peripheral artery occlusive disease (PAOD), or amputation. Center for disease control (CDC) data revealed that PAOD, foot ulcer, and neuropathy are costly and disabling conditions in diabetic patients that can lead to lower extremity amputation[2]. Among the three, PAD which resulted from artery occlusion or stenosis is the most troublesome cause of amputation. Peripheral artery occlusive disease

(PAOD) is identified clinically by intermittent claudication and/or absence of peripheral pulses in the lower legs and feet, representing decreased arterial perfusion of the extremity. Most of the lower extremity amputation level was below knee (1.6/1,000), and the highest was toe amputation(2.6 per 1000)[3]. That means severe small vessel disease of lower extremity has no optimal therapeutic option and finally lead to make the patients extremity amputed.

Among all the PAOD, critical limb ischemia refers to a condition characterized by chronic ischemic at-rest pain, ulcers, or gangrene in one or both legs attributable to objectively proven arterial occlusive disease. Critical limb ischemia implies chronicity and is to be distinguished from acute limb ischemia which is usually caused from embolic occlusion. Most of the diabetic PAOD is chronic arterial occlusive disease which means, in the end, most of the peripheral below knee arteries are diffusely occluded and there are not many options to survive the leg muscles but amputation to survive the patients themselves.

Although the strengths of surgical revascularization in severe peripheral arterial disease are well recognized, not all needed patients can have appropriate revascularization. In a large proportion of these patients, the anatomic extent and the distribution of arterial occlusive disease make the patients unsuitable for operative or percutaneous revascularization[4, 5]. In general, by-pass grafting and percutaneous interventions are reserved only for patients with critical limb-threatening ischemia caused from discrete, proximal artery disease, such as femoral or, at the most, popliteal artery stenosis[6]. However, diffuse severe infra-popliteal small arterial stenosis has no good option to improve symptoms and to survive muscle. Thus, the disease frequently follows an inexorable downhill course. Therefore, beyond the generally used surgical-medical treatment modalities, novel therapeutics is necessary to treat these severe terminal complicated diabetic peripheral artery disease patients. That is the new vessel formation which is neovascularization.

Generally speaking, the new vessel formation has three way including angiogenesis which means the sprouting of new capillaries from existing vascular structures, vasculogenesis which means the in situ formation of new blood vessels from circulating bone marrow-derived endothelial progenitor cells or other stem cells that differentiate into endothelial cells, and arteriogenesis which refers to an increase in the caliber of pre-existing arteriolar collateral connections by recruitment of perivascular cells and expansion and remodeling of the extracelluar matrix[7]. The early age of studies of therapeutic revascularization used recombinant protein or the gene that codes for angiogenic growth.

The first case report of therapeutic angiogenesis in 1996 was VEGF gene transfer in a patient with critical limb ischemiab[8], after then, there have been numerous clinical studies of different angiogenic agents in PAOD. There is no doubt that these protein or gene based therapeutic trial has opened the new era of treatment of critical limb ischemia. However, having discouraged the wonderful expectation of protein or gene therapy, they have not been totally applied to the clinics due to some not-solved major limitations such as inconsistent and inconclusive results of clinical data and some unexpected side effects including unwanted neovascularization, tumor growth, hemorrhage from fragile new vessels, and even an adverse effect on atherosclerotic plaques[8-10]. In addition to these arguing points, protein or gene base therapy has still much to learn about the optimum treatment modality, dosing frequency and route of administration. For this reason, there must be new notion to overcome these limitations.

As an alternative, what it comes down to is new techniques, stem cell based therapy. In this regard, these days, according to the development of stem cell handling techniques, critical limb ischemia have been adapted various kinds of stem cells in its studies which not only help angiogenesis with angiogenic cytokines but also may be transformed to endothelial cells, vascular smooth muscle cells, as well as skeletal muscle cells in the ischemic area.

Among the stem cells in body, bone marrow-derived stem cells such as endothelial progenitor cells (EPCs) from bone marrow or peripheral blood,[11-13] are one of the best candidates. The adult bone marrow contains hematopoietic (1–2%) and stromal (<0.05%) stem/progenitor cells. Bone marrow stromal stem cells include adult mesenchymal stem cells (MSC) and multipotent adult progenitor cells, which are capable of multilineage differentiation[14]. The mononuclear fraction of bone marrow consist of various subpopulations which have the potential to participate in the repair and regeneration of diseased myocardium in vivo through transdifferentiation into cardiomyocytes and initiate neovascularization by expressing various angiogenic cytokines [15]. Among the cells from bone marrow mononuclear fraction, MSCs, EPCs are well known for the regenerative therapy. MSCs remain undifferentiated with stable phenotype and may be reprogrammed to transdifferentiate into cardiomyocytes and increase the recovery capacity after hindlimb ischemia[16, 17]. EPCs have the capacity to initiate neovascularization to alleviate myocardial and hindlimb ischemia[12, 18]. Hence, the heterogeneous mononuclear fraction of the bone marrow is the most promising stem cell population for cellular regenerative therapy.

As the morbidity and mortality of diabetes mellitus increase, it became an important subgroup that may have definite benefit from therapeutic angiogenesis and cell therapy. However, So far, there have been few data about stem cell treatment in the diabetic hind limb ischemia.

What make DM more interesting is that it has distinctive pathophysiology and therapeutic response to ischemia and stem cell therapy compared to non-diabetic patients. So far there have been some data that showed the differences. The physiologic mechanisms leading to collateral vessel formation to ischemia is insufficient in patients with diabetes[19-21]. Especially Diabetes and raised levels of cholesterol and lipoprotein (a) are associated with a reduced angiogenic response[22, 23]. In patients with diabetic retinopathy triggering neovascularization plays an important role in new vessel formation around the optic disc and in sight-threatening macular

edema[24]. Therefore, it is important that we should focus on the diabetic ischemic limb disease with different eyes. Moreover, recently, researchers have been thinking the reason why diabetic patients have different response to therapeutic neovascularization. They are pursuing that stem cells of diabetic patients might have some serious dysfunction, and it has been documented from recent researches. It has been shown that patients at risk for coronary artery disease have decreased numbers of circulating EPCs with impaired activity[25]. Some study also suggest that vasculogenesis may be impaired in animal models of diabetes mellitus[26]. Others revealed that human endothelial progenitor cells from Type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures [9]. The others said that adverse metabolic stress factors in type 1 diabetes are associated with reduced EPC numbers and angiogenicity[27]. Not only EPC, but also BM-MNC showed a impairment in ischemia-induced neovascularization in diabetes[28]. Therefore, it is definite that diabetes has major pathophysiology different form other disease in the aspect of neovascularization and stem cell therapy. So far, there has been no data showed dysfunction of MSCs in DM.

Therefore, the hypothesis and aims of this research are followed. We hypothesized that there might be a do novo defect in the hind limb such as decreased cytokines in the diabetic skeletal muscle. Bone marrow, which may act supporters via mobilization of stem cells to the tissue, also may has dysfunction. Firstly, we focused on the mechanisms of impaired neovascularization in the diabetic hind limb in the various ways. We tried to figure out the differences between diabetic and non-diabetic BM-MNC, EPCs, and MSCs in their molecular characteristics especially in terms of the content of the important cytokines. Secondly, we studied functional differences between diabetic and non-diabetic stem cells. With the results of these data, we hypothesized that diabetic stem cells need, so called, normal non-diabetic stem cells in the ischemic condition. Therefore, thirdly, we studies if normal, not diabetic, bone

marrow-derived stem cells such as EPCs or MSCs have the therapeutic effect in the hindlimb ischemia. We also studied the difference in the therapeutic effect of EPCs and MSCs and the mechanisms incurring the therapeutic effects in the various ways.

II. MATERIALS AND METHODS

1. Induction of Diabetes Mellitus

C57Bl/6 male mice (8 weeks old, The Jackson Laboratory, USA) weighing 20 to 25grams were used. Mouse was fed standard laboratory murine chow and water ad libitum and housed 3 diabetic mice and 5 non-diabetic mice in a cage.

To induce moderate diabetes, C57Bl/6 mice were intraperitoneally injected 40 mg/kg of streptozotocin (STZ; Sigma-Aldrich, St. Louis, MO, USA) in 0.9% sterile saline daily for 5 consecutive days. In the majority of treated mice (around 85%) this procedure creates a moderate diabetic condition that allows them to survive for up to 4 months after treatment without receiving insulin supplementation. Seven days after the injection, blood glucose levels were measured using Accu-Check glucometer (Roche, Indianapolis, IN, USA). If serum glucose was more than 200 mg/dl, they were considered as a diabetic mouse. Mice with glucose levels less than 200 mg/dl were excluded from further study. Mean glucose levels were 380±50 mg/dl. Except for a gradual weight loss, the health of the mice was not overtly affected by the treatment throughout the experimental period. All diabetic mice maintained for 3 months after streptozotocin injection, after then, they were used for the experiments. Before using, glucose levels were retested again to ensure serum glucose level of greater than 200 mg/dl. In the non-diabetic groups, mice were injected intraperitoneally with 0.9% sterile saline.

2. Cell Culture

A. Bone Marrow–derived EPC Culture

Bone marrow–derived mononuclear cells isolated tibia and femur were plated on cell culture dishes coated with rat vitronectin at a density of 5×105 /cm^2 and cultured in endothelial cell basal medium-2 (EBM-2, Clonetics) supplemented with 5% fetal bovine serum (FBS), human vascular endothelial growth factor (VEGF)-A, human fibroblast growth factor-2, human epidermal growth factor, insulin-like growth factor-1, ascorbic acid, and antibiotics. After 4 days in culture, non-adherent cells were removed by washing with PBS, new medium was applied, and the culture was maintained through 7 days. The cells were used as EPC-rich cell population for real time RT-PCR analysis and cell transplantation[29, 30].

B. Bone Marrow-derived MSCs Culture

Whole bone marrow cells were plated in high-glucose Dulbecco's Modified Eagle Medium (DMEM, Cambrex Co. USA) supplemented with 10% FBS and 50units/mL penicillin/streptomycin. After 5 to 7 days of incubation in a humidified incubator at 37°C with 5% CO2, the non-adherent hematopoietic cells were discarded. The adherent MSCs were further maintained in a humidified incubator at 37°C with 5% CO^2 in the absence of any exogenous growth factor or anchoring materials such as fibronectin or collagen. MSCs were kept subconfluent and expanded in number over 3 passages by a 1:2 split on a weekly basis. Two morphologically different components of plastic adherent MSCs were identified, spindle fibroblastoid MSCs and round or satellite-shaped MSCs. Passage 3 cells are used for real time RT-PCR and cell transplantation[31].

3. Mouse Ischemic Hindlimb Surgery and Stem Cell Transplantation

A. Ischemic Hindlimb Surgery

Unilateral hindlimb ischemia was created in diabetic or non-diabetic C57Bl/6 mice as previously described[32]. The animals were anesthetized with pentobarbital (160 mg/kg intraperitoneally) following which an incision was performed in the skin overlying the middle portion of the left hindlimb. After ligation of the proximal end of the femoral artery, the distal portion of the saphenous artery was ligated and the artery, as well as all side-branches, was dissected free and excised. The skin was closed using a surgical stapler.

B. Transplantation of Ex Vivo Expanded EPCs, MSCs

The impact of EPCs, MSCs transplantation on therapeutic neovascularization was investigated in a murine model of hindlimb ischemia. Just after operative excision of one femoral artery, described above, the diabetic C57BL/6 mice received the 4 points local intramuscular injection of MSCs (1×10^6) or EPCs (1×10^6) versus saline along the path of dissected femoral artery. Cells were labeled with Ac-LDL-DiI solution (0.1-0.3 ul, Biomedical Technologies, Stoughton, MA) for the cell tracking after sacrifice on the designated day.

C. Monitoring of Hindlimb Blood Flow

After anesthesia, hair was removed from both legs using a depilatory cream, following which the mice were placed on a heating plate at 37^oC for 10 minutes to minimize temperature variations. The hindlimb perfusion was measured using a laser Doppler perfusion imager (LDPI) system (Lica Inc., North Brunswick, NJ, USA). The LDPI uses a beam from a 2-mW helium-neon laser that sequentially scans a 12 X 12 cm tissue surface to a depth of a few hundred microns. During the scanning procedure, the moving blood cells shift the frequency of

incident light according to the Doppler principle. A photodiode collects the back-scattered light, and the original light intensity variations are transformed into voltage variations in the range of 0 to 10 V. A perfusion output value of 0 V was set to 0% perfusion, whereas 10 V was set to 100%. When the scanning procedure is terminated and the back-scattered light collected from all of the measured sites, a color-coded image representing the microvascular blood flow distribution appears on a monitor. The perfusion signal is split into six different intervals, each displayed in a separate color. Low or no perfusion is displayed in dark blue, whereas the highest perfusion interval is displayed in red. The stored perfusion values behind the color-coded pixels are then available for analysis. Consecutive measurements were obtained after scanning the same region of interest (leg and foot) with LDPI. Color photographs were recorded and analysis performed by calculating the average perfusion of the ischemic and non-ischemic foot. To account for variables such as ambient light and temperature, the results are expressed as the ratio of perfusion in the left (ischemic) versus right (normal) limb[33].

D. Tissue Preparation

The mice were sacrificed at predetermined arbitrary time points after surgery with an overdose of sodium pentobarbital. For the in vivo perfusion capillary staining, 100 ul of Fluorescein griffonia simplicifolia lectin I (Vector laboratories, USA) was injected into the heart before sacrifice and kept circulating for 20 minutes in the blood. Whole hind limbs (thigh area) except bone were immediately frozen with liquid nitrogen and isopentan-2-methybutan (Sigma-Aldrich, St. Louis, MO, USA) and stored at -80^{o}C until use. The tissue was used for total protein, mRNA extraction, and immunohistochemistry.

4. Microarray for the angiogenic cytokines

Total RNA was extracted from tissue or cells with RNA-Stat (Tel-Test INC., Friendswood, TX, USA) according to the manufacturer's instructions and purified using the Arraygrade Total RNA isolation kit (Superarray) following the manufacturer's instructions. We quantitated RNA with spectrophotometer in 10mM Tris EDTA buffer (PH 8.0). A Oligo GEArray mouse angiogenesis series was obtained from Sueprarrray Inc. (Bethesda, MD, USA). This array includes 112 genes involved in angiogenesis, including Ephrin Family Members, fibroblast growth factors (FGF), Platelet-Derived Growth Factors, Transforming growth factors (TGF) and vascular endothelial VEGF families etc. (See www.superarray.com for details). A Total of 3ug RNA was used as a template to generate Biotin-16-dUTP-labeled cDNA probes (Roche, 11388908) according to the manufacturer's instructions. The cDNA probes were denatured and hybridized at 60 ºC with the Superarray membrane (OMM-024).

After overnight at 60 ºC, the membrane was washed twice with 2x SSC containing 1% SDS for 15min, once with 0.1x SSC containing 0.5% SDS for 15min, blocked and exposed with the use of a chemiluminescent substrate. To analysis the Superarray membrane, we scanned the X –ray film and imported it into Adobe Photoshop program as a TIFF file. The image file was inverted, and the spots were digitized with the use of Analysis software (http://www. superarray.com/ support_software.php), and normalized by subtraction of the background as the average intensity value of 3 spots. The averages of 2 GAPDH or RPL32 spots were used as positive controls and set as baseline values with which the single intensity of other spots were compared. Using these normalized data, we compared the single intensity from the membranes using the GEArray analyzer program. Genes were considered differentially expressed when logarithmic gene expression ratios in hybridization were more than 1.4 fold difference in expression level.

5. RNA Extraction and quantitative Real Time Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was extracted from tissue or cells with RNA-Stat (Tel-Test INC., Friendswood, TX, USA) according to the manufacturer's instructions. First-strand cDNA was generated using the Taqman Multiscribe Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) primed with a mix of oligo dT and Random Hexamers. Gene expression was determined by Taqman real-time quantitative PCR on the 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using Taqman PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Taqman primer/probe sets (Biosearch Technologies, Novato, CA, USA) shown in Table 1 were designed using Primer Express Software (Applied Biosystems, Foster City, CA, USA). The PCR Conditions were as follows: hold for 2 min at 50C, and 10 min at 95C followed by 2 steps PCR for 40 cycles of 95^{o}C for 15 seconds and 60C for 60 seconds with fluorescence monitoring at the end of each elongation step. Relative mRNA expression of target genes was calculated with the comparative CT method. All target sequences were normalized to GAPDH in multiplexed reactions performed in duplicate. Differences in CT values were calculated for each target mRNA by subtracting the mean value of GAPDH (relative expression = 2-DCT).

Table 1.

Oligonucleotide primers used for gene expression analysis by real-time RT-PCR

Gene	Primer Sequences (5'-3')
VEGF-A	Fwd GCGGGCTGCTGCAATG
	Rev CATAGTGACGTTGCTCTCCGAC
	Probe FAM-AGCCCTGGAGTGCGTGCCCA-BHQ
IGF-1	Fwd CAGATACAACAGAATGCGGTTCA
	Rev TGAGACAAGAGGCTGGTTCCTAT
	Probe FAM-AACCACACGGCCACCATGCTGG -BHQ
HIF-1α	Fwd TGCATCTCCATCTTCTACCCAAGT
	Rev GACTGTGAGTGCCACTGTATGCT
	Probe FAM-CTCAAGAAACGACCACTGCTAAGGCAT-BHQ
FGF-2	Fwd GTCACGGAAATACTCCAGTTGGT
	Rev CCGTTTTGGATCCGAGTTTATACT
	Probe FAM-TGTGGCACTGAAACGAACTGGG-BHQ
PlGF	Fwd TGGTGCCTTTCAACGAAGTG
	Rev TTCATCCAAGATGTACACCAGCTT
	Probe FAM-TCGCAGCTACTGTCGGCCCATG-BHQ
PDGF-β	Fwd ACCTCGCCTGCAAGTGTGA
	Rev TGCTCCCTGGATGTCCCA
	Probe FAM-AGTGACCCCTCGGCCTGTGACTAGAAGTC-BHQ
Ang-1	Fwd TGCTTCCGGAGCTGTGATCT
	Rev CGGGCTGCTTTTGTAGGCT
	Probe FAM-AGGAGACTGGAGATGTACTGTGCCCCAC-BHQ
Ang-2	Fwd TGCACAGCATTGGACACGTA
	Rev AGCATCTGGGAACACTTGCAG
	Probe FAM-CACAAAGGATTCGGACAATGACAAATGCA-BHQ
HGF	Fwd CTGACCCAAACATCCGAGTTG
	Rev TTCCCATTGCCACGATAACAA
	Probe FAM-TGCTCTCAGATTCCCAAGTGTGACGTGT-BHQ
8DF1-α	Fwd CCTCCAAACGCATGCTTCA
	Rev CCTTCCATTGCAGCATTGGT
	Probe FAM-CTGACTTCCGCTTCTCACCTCTGTAGCCT-BHQ

VEGF-A: Vascular endothelial growth factor-A
IGF-1: Insulin-like growth facot-1
HIF-1α: Hypoxia inducible factor-1α
FGF-2: Fibroblast growth factor-2
PlGF: Placenta growth factor
PDGF-β: Platelet derived growth factor-β
Ang-1: Angiopoietin-1
Ang-2: Angiopoietin-2
HGF: Hepatocyte growth factor
8DF1-α: 8tromal growth factor1-α

6. Immunohistochemistry

The histological sections, 6 um thick, prepared from frozen tissue samples of the lower limbs were used for immunohistochemical analysis. Identification of endothelial cells was performed by immunohistochemical staining for platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31) using a rat monoclonal antibody directed against mouse CD31 (Pharmingen, San Diego, CA, USA). Immunofluorescence staining was performed as previously described (14). In brief, to prevent nonspecific antibody binding, sections were preincubated for 20 minutes in PBS containing 10% horse serum after cold aceton fixation for 10 minutes. Next, sections were incubated with the primary antibodies directed against CD31 at appropriate dilutions overnight at 4^{o}C. Sections were then rinsed for 15 minutes with PBS, followed by incubation with Cy2 labeled secondary antibody (Pharmingen, San Diego, CA, USA) for 1 hour at room temperature. After a 15-minute wash, Sections were rinsed with PBS. Negative control slides were prepared by substituting pre-immune rat serum for CD31. For the assessment of cell proliferation after designated stem cell transplantation, the detection of Ki67 antigen was performed. In brief, after cold aceton fixation for 10 minutes, epitope retrieval method was used with DakoCytomation target retrieval solution (DakoCytomation, Carpinteria, CA, USA). Then rabbit anti-human Ki67 (Novocastra, UK) and Cy3 goat anti-rabbit IgG (Pharmingen, San Diego, CA, USA) were used as a primary and secondary antibody, respectively.

7. Stem Cell Function Assay

A. EPC culture assay

EPC culture assay was performed as described previously (15). Briefly, mononuclear cells isolated from 500 μL of peripheral blood were cultured in EBM-2 (Clonetics, USA) on rat vitronectin (Sigma-Aldrich, St. Louis, MO, USA) with 0.1% gelatin-coated 4-well glass

chamber slides. After 4 days in culture, cells were coincubated with DiI-acLDL (Biomedical Technologies, Stoughton, MA, USA) for 1 hour, followed by FITC-BS-1 lectin (Sigma-Aldrich, St. Louis, MO, USA) staining for 2 hours at 40C. The dual-stained cells, considered EPCs, were counted in 10 randomly selected high-power fields (x200) under a fluorescent microscope[34].

B. EPC Tube formation assay

Matrigel (Becton Dickinson, San Jose, CA, USA) was coated into 2-well glass chamber and incubated in a CO2-free incubator at 37°C for 30 min. The same batch of Matrigel was used for all of the experiments. The gels were then overlaid with 2.5 x 105 cells suspended in culture medium and incubated at 37°C in an atmosphere of 5% CO2. Gels were examined by using a phase-contrast microscope equipped with a digital camera. A second observer measured the total length of the tube-like structures (defined as those exceeding 200 μm in length) per each image[35].

C. EPCs adhesion assay

Seven day-diabetic and non-diabetic EPCs were washed with PBS and gently detached with 0.25% trypsin. After centrifugation and resuspension in the EPC media with 5% FBS, identical cell numbers (2X104 cells / well) were replated onto vitronectin (Sigma-Aldrich, St. Louis, MO, USA) or laminin (Sigma-Aldrich, St. Louis, MO, USA) -coated 96-well plate in 100 ul cell solution per well and incubated for 1 hour at 37 °C. Adherent cells were counted by independent blinded investigators[36].

D. EPCs and MSCs migration assay

The day 4 cultured EPCs and passage 3 MSCs were used for the migration assay. Each assay was done separately. After cells were detached mechanically, harvested by centrifugation, resuspended in EBM with 3% FBS (without VEGF) for EPCs and in DMEM with 10 % FBS for MSCs and counted. Next, 6×10^4 of EPCs, $8x10^4$ of MSCs were placed in the upper chamber of a modified Boyden chamber which was placed in a 24-well culture dish respectively. In the lower chambers, the same medias for each cell line but included human recombinant VEGF (100 ng/mL) (Sigma-Aldrich, St. Louis, MO, USA) were used. After 16 h of incubation at 37^{o}C, the lower side of the filter, containing the migrated cells, was washed with PBS and fixed with 2 % paraformaldehyde. For quantification, cell nuclei were stained with Diff-Quick stain set (Dade Behring Inc., Newark, USA) and counted in three random microscopic fields. Measurement was performed in a duplicate manner [37](19).

8. Statistical analysis

Data were expressed as a mean value ± SE. Statistical comparisons between the groups were made with t-test and an analysis of variance test with post hoc analysis. A P value <0.05 was considered significant.

III. RESULTS

1. Physiologic data

Blood pressure did not differ between the 2 groups on the day of surgery. Significant differences were observed in body weight, plasma glucose between diabetic and non-diabetic mice (Table 2).

Table 2. Physiologic data

	Non diabetic	Diabetic	P value
Blood Pressure (mmHg)	118 ± 3	110 ± 3	Non specific
Blood Glucose (mg/dl)	110 ± 20	380 ± 50	<0.05
Body weight (g)	32 ± 5	23 ± 5	<0.05

2. Decreased neovascularization in diabetic hindlimb muscle in the absence of hindlimb ischemia surgery

To evaluate the baseline difference of neovascularization between diabetic and normal mice hindlimb without ischemia, we compared capillary density with CD31 immunochemical staining. Quantitative analysis of capillary density in ischemic adductor muscle of diabetic and

non-diabetic C57BL/6 mice was determined in histological sections harvested 3 months later of induction of DM. To confirm the reduction of muscle mass in the diabetic mice, the tendon to tendon Gastrocnemius muscle was isolated and weighted. Diabetic muscle showed significantly reduced in the muscle size and weight compared to the non-diabetic mice (Figure 1). Quantitative analysis of CD 31-positive cells revealed that the capillary density per single myofiber was essentially decreased in the diabetic mice (Figure 2) indicative of an impaired angiogenic response in these animals.

3. Decreased amount of VEGF-A in the diabetic hindlimb

To figure out the reason of the decreased capillary density in the baseline statues of DM, we measure VEGF-A , the most important cytokines among the angiogenic cytokines, Figure 3 shows that diabetic hindlimb muscle has the smaller amount of VEFG-A mRNA. This suggested the possible mechanism of impaired angiogenesis after hindlimb ischemia in the diabetic mouse.

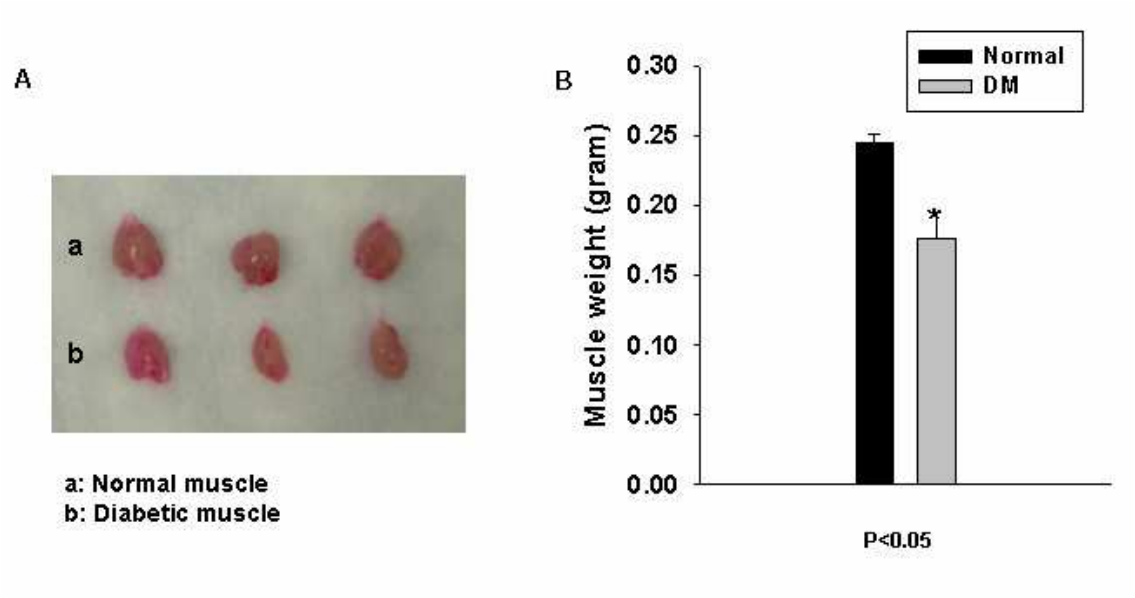

Figure 1. The Gastrocnemius muscle size was significantly reduced in the diabetic mice (b) compared to the non-diabetic normal mice (a). The weight of Gastrocnemius muscle was significantly smaller in the diabetic mice compared to the non-diabetic mice (* $p<0.03$).

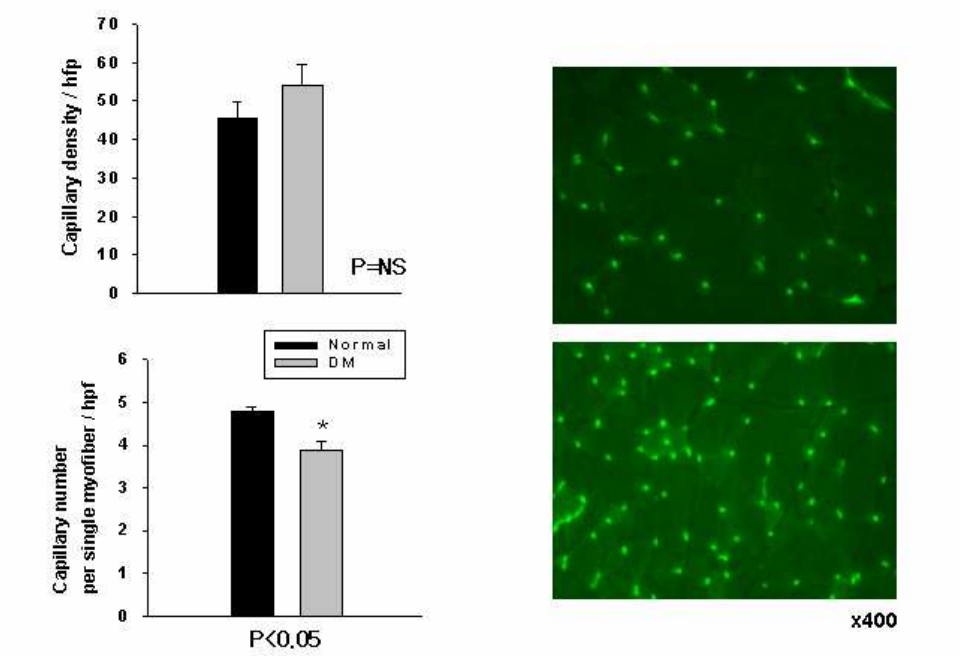

Figure 2. Baseline capillary density of hindlimb muscle with CD-31 immunofluorescence staining. A: Representative picture from diabetic (a) and non-diabetic normal (b) hindlimb. Because of the atrophy of single muscle fiber in the diabetic mice, the number of CD-31 positivity looks higherr in the diabetic muscle with high power field magnification. B: However, when counting quantitatively for each myofiber, the number of capillary was significantly less in the diabetic hindlimb compared to the normal.

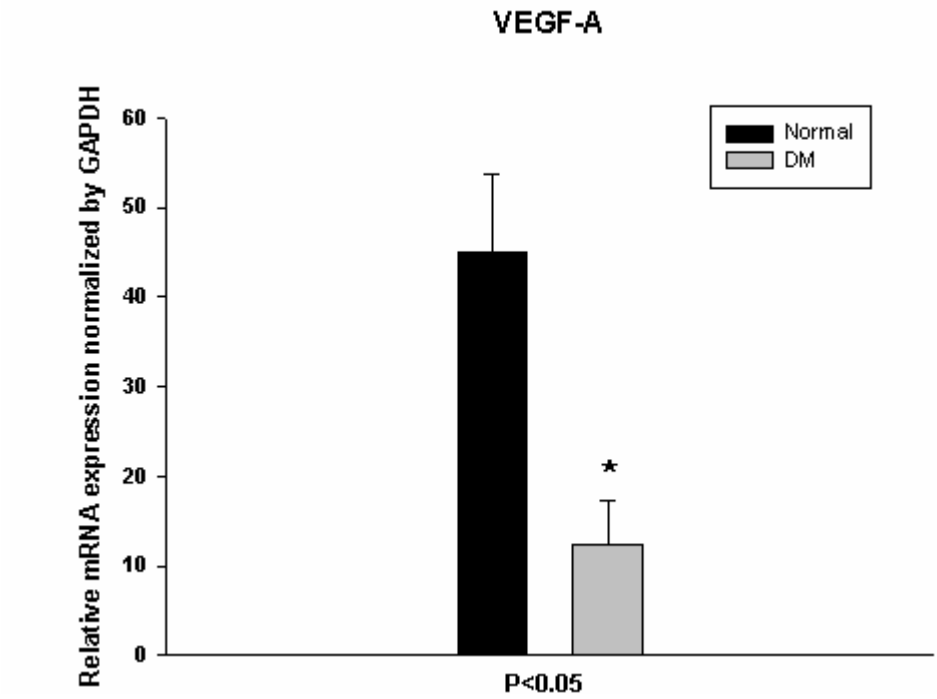

Figure 3. Real time RT-PCR of hindlimb for vascular endothelial growth factor-A (VEGF-A) revealed that diabetic hindlimb had smaller amount of mRNA for VEGF-A compared to that of the normal non-diabetic hindlimb.

4. Impairment of angiogenic property of bone marrow-derived mononuclear cells, EPCs, and MSCs: qualitative change (Microarray, RT-PCR, and cell biology study)

Microarray was performed to compare the difference of angiogenic cytokines between diabetic and non-diabetic bone marrow-derived mononuclear cells, EPCs, and MSCs. There were a total of 12 cytokines among 112 angiogenesis related cytokines that showed more than 1.4 fold decreases in the diabetic cells (Table 3). Microarray for EPCs showed a total of 11 cytokines reduced in the diabetic EPCs compared to normal EPCs (Table 4). Only 3 cytokines were reduced in the diabetic MSCs compared to normal MSCs with Microarray (Table 5).

Real time RT-PCR was performed with the bone marrow-derived mononuclear cells (MNC), EPCs, MSCs. Diabetic bone marrow derived MNCs showed significantly decrease amount of the mRNA for angiopoietin-1, and also showed smaller amount of mRNA for the vascular endothelial growth factor(VEGF)-A and insulin-like growth factor (IGF)-1 without significance (Figure 4). Diabetic bone marrow derived EPCs and MSCs showed smaller amount of mRNA for the VEGF-A compared to non-diabetic stem cells (Figure 5). When it comes to say the recovery after injury, bone marrow derived stem cells have an important role in their mobilization into the injury sites. However, in the diabetic animals the bone marrow MNC itself are deficient of some important angiogenic cytokines. Therefore, these finding also give rise to the mechanism of the impaired angiogenicity in the diabetic mice.

We assessed the EPCs MSCs angiogenic property with several functional studies. We did firstly compare the adhesion activity between diabetic EPCs, MSCs and normal cells because adhesion to the extracellular matrix is believed to be important during new blood vessel growth and recovery after ischemia. Diabetic EPCs were found to be significantly impaired in their ability to adhere to Laminin and Vitronectin compared to normal EPCs (Table 3). A Matrigel tubule assay was performed to investigate the ability of EPCs to make vessel like structures.

Cultures of EPCs within Matrigel led to the formation of an extensive tubule network in normal non-diabetic EPCs. However, diabetic EPCs showed significantly reduced length of the tube compare to non-diabetic EPCs (Figure 6). Migration assay of EPCs to VEGF-A revealed decreased migration in normal EPCs compared to diabetic EPCs. Migration assay to MSCs to VEGF-A showed the same pattern of result (Figure 7).

Table 3. Angiogenesis related cytokines that showed more than 1.4 fold changes between diabetic and non-diabetic normal bone marrow-derived mononuclear cells (BM-MNC) among the 112 tested cytokines with Microarray.

Position	Symbol	DM BM-MNC	Normal BM- MNC	Fold change
1	*Gapdh*	56411.86111	58804.33333	1.042410801
5	Akt1	26627.13889	46176.16667	1.734176806
24	Edg1	24039.05556	34662.88889	1.441940546
26	Efna2	13853.52778	21483.94444	1.550792317
50	Il12a	30754.66667	48790.94444	1.586456617
53	Il6	16273.77778	22885.11111	1.406256828
68	Npr1	15065	23004.41667	1.527010731
69	Nrp1	20011.97222	30859.61111	1.542057463
79	Pofut1	14979.22222	23071.11111	1.540207547
84	Ptn	25696.75	36512.75	1.420909259
88	Sphk1	19410.25	32736.83333	1.686574533
97	Tgfb3	17460.5	26731.52778	1.530971494
106	Tnfaip2	15559.52778	26483.22222	1.702058224

GAPDH: Glyceraldehye-3-phosphate dehydrogenase, Akt1: Thymoma viral proto-oncogen1, Edg1: Endohelial differentiation shingolipid G-protein coupled receptor1, Efna2: Ephrin A2, Il12a: Interleukin 12A, Il6: Interleukin 6, Npr1 : Natriuretic peptide receptor1, Nrp1: Nerophilin1, Pofut1: Protein O-fucosyltransferase1, Ptn: Pleiotrophin, Sphk1: Shingosine kinase1, Tgfb3: Transforming growth factor beta3, Tnfaip2: Tumor necrosis factor, alpha-induced protein2

Table 4. Angiogenesis related cytokines that showed more than 1.4 fold changes between diabetic and non-diabetic normal endothelial progenitor cells (EPCs) among the 112 tested cytokines with Microarray.

Position	Symbol	DM EPC	Normal EPC	Fold change
1	*Gapdh*	60810.31651	62988.26812	1.035815495
22	Cxcl9	20260.49312	30389.67754	1.499947576
26	Efna2	23058.86927	50360.68841	2.184005114
27	Efna3	20690.78211	30970.80435	1.496840679
49	Il10	21515.05505	32951.01449	1.531532893
68	Npr1	24622.74541	42710.26449	1.734585798
73	Pdgfb	23612.04128	44656.44565	1.891257309
79	Pofut1	24464.25917	39698.10145	1.622697878
84	Ptn	26607.14679	57343.2971	2.155184002
97	Tgfb3	41597.68119	61167.10145	1.470444979
127	BAS2C	23825.3211	37485.57609	1.573350299
128	BAS2C	38893.57339	63106.12319	1.622533434

GAPDH: Glyceraldehye-3-phosphate dehydrogenase, Cxcl9: Chemokine (C-X-C motif) ligand 2, Efna2: Ephrin A2, Efna3: Ephrin A3, Il10: Interleukin 10, Npr1 : Natriuretic peptide receptor1, Pdgfb: Platelet derived growth facotor B polypeptide, Pofut1: Protein O-fucosyltransferase1, Ptn: Pleiotrophin, Tgfb3: Transforming growth factor beta3, BAS2C: Biotinylated artificial sequence 2 complementary sequence

Table 5. Angiogenesis related cytokines that showed more than 1.4 fold changes between diabetic and non-diabetic normal mesenchymal stem cells (MSCs) among the 112 tested cytokines with Microarray.

Position	Symbol	DM MSC	Normal MSC	Fold change
1	*Gapdh*	65243.86111	64839.33333	0.993799757
5	Akt1	22519.47222	41452.33333	1.840732897
69	Nrp1	40282.13889	56765.41667	1.409195694
72	Pdgfa	21168.72222	33754.61111	1.594551185

GAPDH: Glyceraldehye-3-phosphate dehydrogenase, Akt1: Thymoma viral proto-oncogen1, Nrp1: Nerophilin1, Pdgfa: Platelet derived growth factor alpha

Table 6. Adhesion assay of endothelial progenitor cells (EPCs) to Laminin and Vitronectin revealed that diabetic EPCs showed the decreased adhesion to laminin and vitronectin compared to normal non-diabetic EPCs.

Adhesion assay	Normal EPC (/mm^2)	Diabetic EPC (/mm^2)	P
Laminin	78	42	<0.05
Vitronectin	320	140	<0.05

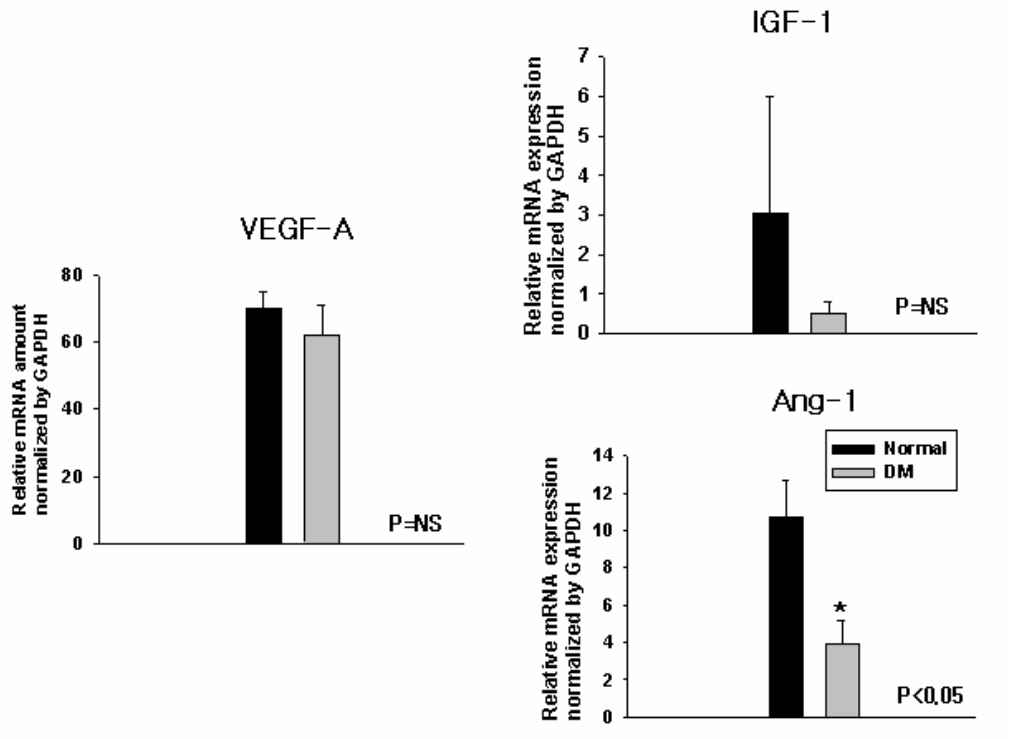

Figure 4. Real time RT-PCR of bone marrow-derived mononuclear cells (BM-MNCs) for vascular endothelial growth factor (VEGF-A), insulin-like growth factor-1 (IGF-1), angiopoietin-1 (ANG-1) revealed that diabetic BM-MNCs had smaller amount of mRNA for ANG-1 compared to normal cells.

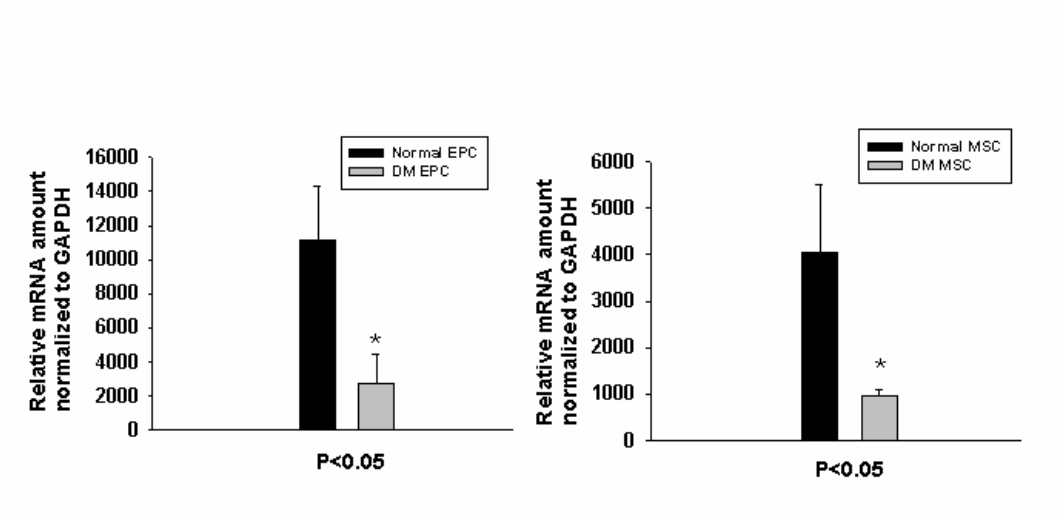

Figure 5. Real time RT-PCR of endothelial progenitor cell (EPCs) (A) and mesenchymal stem cells (MSCs) for vascular endothelial growth factor-A (VEGF-A) (B) showed that diabetic endothelial progenitor cells (BM-EPCs), mesenchymal stem cells (MSCs) had smaller amount of mRNA of VEGF-A compared to normal cells.

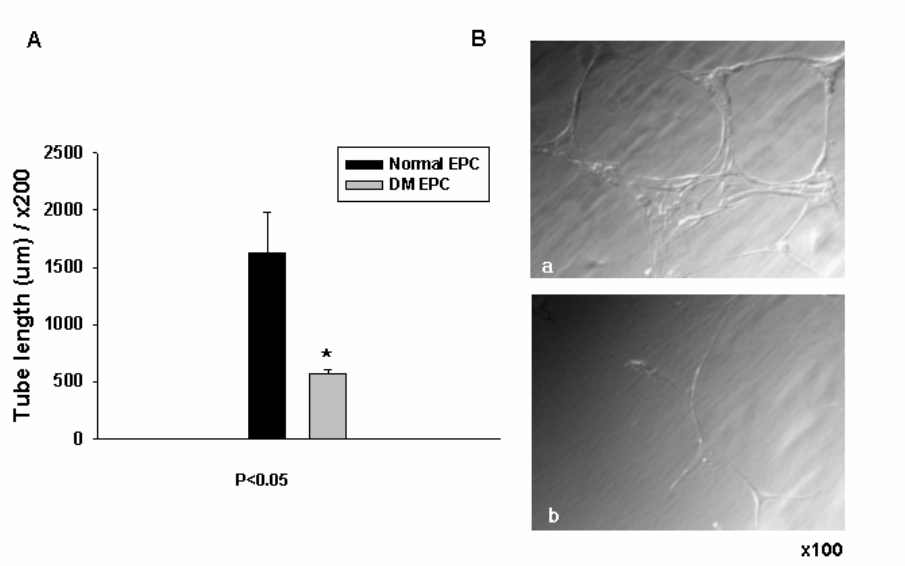

Figure 6. Matrigel tube formation assay for EPC. EPCs were plated within Matrigel to form tubule structures. A: Quantification of EPC tube formation revealed that significantly smaller tube length in the diabetic EPCs. B: Non-diabetic normal EPCs made a substantial contribution to tubule network (B-a). In contrast, diabetic EPCs showed nearly absent tube formation (B-b).

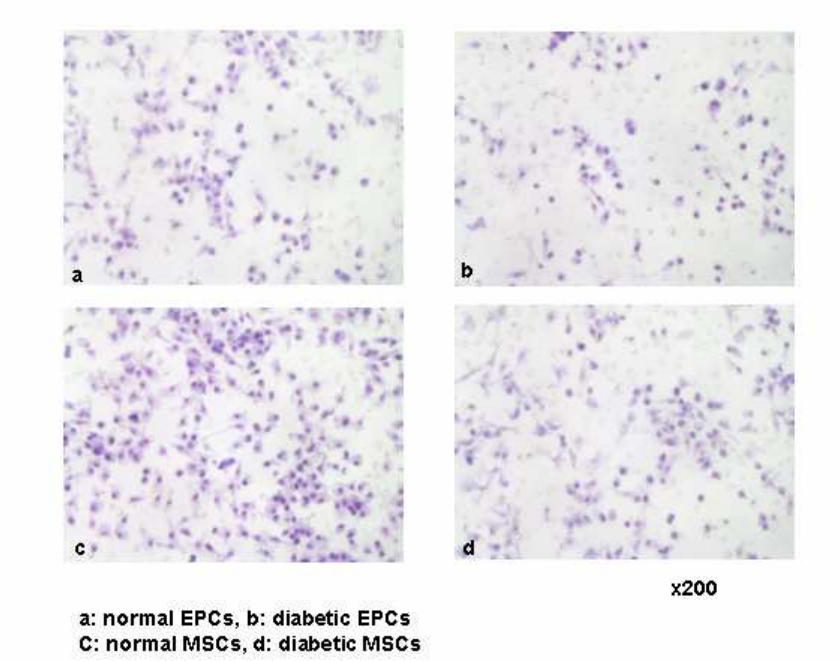

Figure 7. Migration assay showed decreased migration toward VEGF-A in the normal EPCs and normal MSCs compared to diabetic EPCs and MSCs respectively.

5. Decreased number of peripheral circulating EPCs in the diabetic mice: quantitative change

To further reveal the mechanism of the impaired angiogenic response in the diabetic mice, we guessed that the circulating EPCs probably reduced in the non-ischemic diabetic mice. As a functional index of baseline circulating EPCs, the EPC culture assay was employed. The number of cultured EPCs, confirmed by acLDL uptake and BS-1 lectin reactivity (Figure 8), were lower in the peripheral blood of diabetic mice than in control non-diabetic mice.

6. Impaired ischemia-induced neovascularization in the diabetic mice

Because of the aforementioned bone marrow-derived EPCs and MSCs dysfunction and de novo defect of skeletal muscle such as the reduced VEGF-A, we hypothesized that neovascularization after hindlimb ischemia surgery would be hampered in the diabetic mice. To figure out this hypothesis, left hindlimb ischemia surgery was conducted. All mice survived after surgical induction of left hindlimb ischemia. Figure 9A showed the representative LDPI images of hindlimb blood flow before surgery, immediately after surgery, and at 28 days after surgery in the diabetic and non-diabetic mice. Immediately after left femoral artery and vein resection, the ratio of blood flow between the ischemic and non-ischemic hindlimb decreased to 0.11±0.006 and 0.12±0.008 in the diabetic mice and non-diabetic mice, respectively, indicating that the severity of the induced ischemia was comparable in the 2 experimental groups. In the normal non-diabetic C57BL/6 mice, hindlimb blood flow perfusion recovered to 84% (ratio=0.84±0.006) of the non-ischemic limb by the day 28 (Figure 9). In contrast, flow recovery in the diabetic mice was impaired, and the deficits in flow were statistically significant at 14, 21, 28 days after surgery

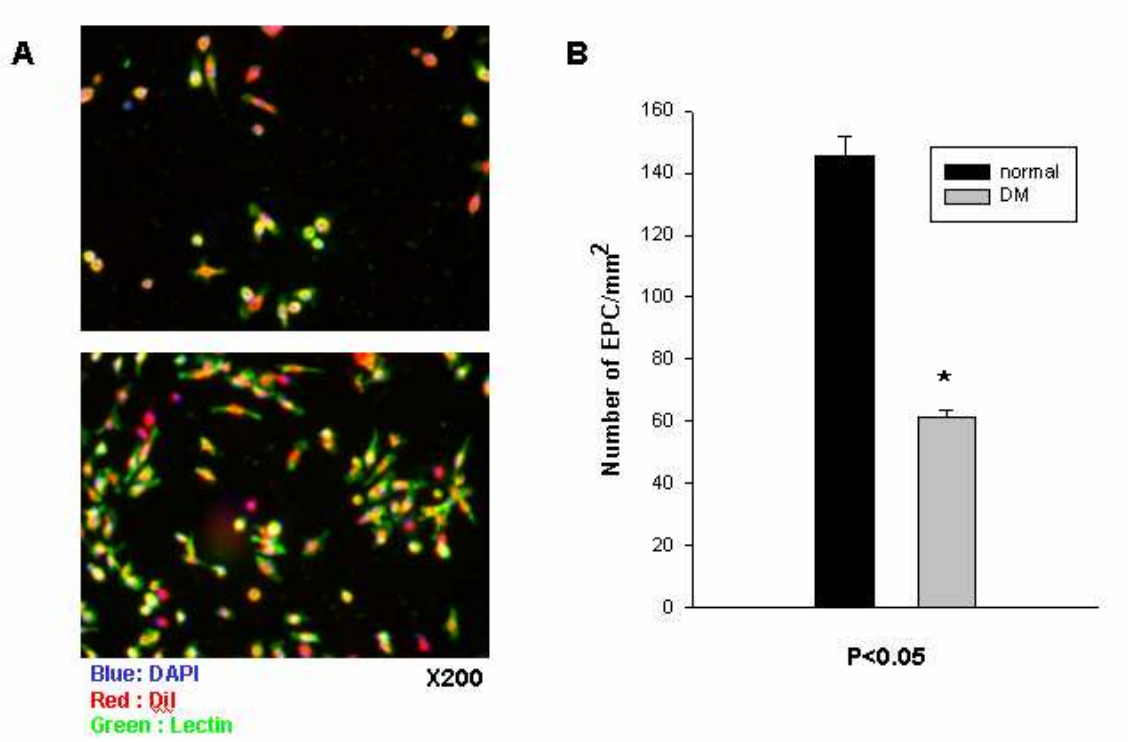

Figure 8. Circulating EPCs culture assay. EPCs were characterized as adherent cells with double-positive staining for AcLDL-Dil and BS-1 lectin. A: Representative immunofluorescence image with positive AcLDL-Dil and BS-1 lectin showed double positive spindle-shaped attaching cells. B: Quantification of cells in normal non-diabetic and diabetic mice. The number of the circulating EPCs in the diabetic nice was much less than that of the normal non-diabetic mice. Values are means± SEM, n= 5 per group ($P<0.05$).

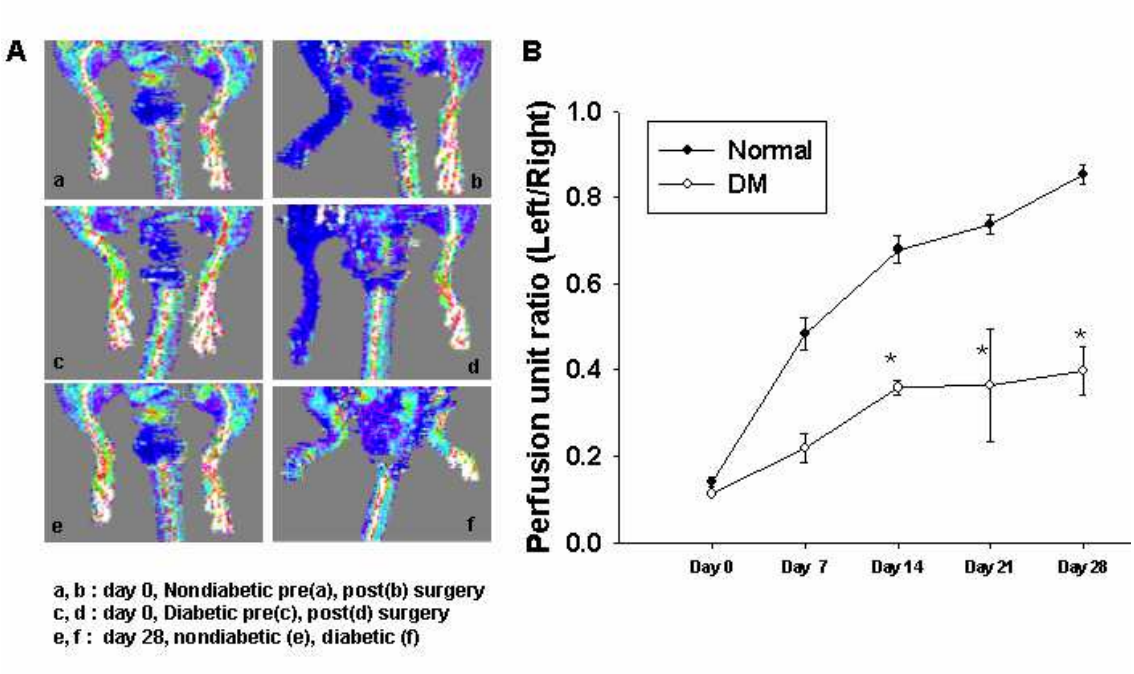

Figure 9. Laser Doppler flow image. A. Ischemia-induced changes in hindlimb blood flow monitored in vivo by laser Doppler perfusion imaging performed for a month weekly after ischemia in diabetic and non-diabetic mice. In color coded images, normal blood flow is depicted in red. A marked reduction in blood flow of ischemic hindlimb is depicted in blue. The figure a, b shows the pre and post image from the non-diabetic mice at day 0. The figure c, d shows pre and post image from the diabetic mice at day 0. The figure e, f shows LDPI from non-diabetic and diabetic at day 28, respectively. At day 0, pre- and post-image reveals significant blood flow reduction immediately after surgery. However, at day 28, normal non-diabetic mice recovered almost the same color scale (e) but still bluish in the diabetic mice (f). B: Quantitative evaluation of blood flow expressed as a ratio of blood flow in ischemic foot to that in non-ischemic one. Values are means ± SEM, n= 7 per group. *P <0.05.

7. Therapeutic effect of bone marrow-derived EPCs or MSCs

Blood flow of the ischemic hindlimb was measured by a non-invasive method using a laser Doppler perfusion imaging system. After hindlimb ischemia with stem cell transplantation for a month weekly, we found that the blood flow of the ischemic hindlimb in the diabetic mice was remarkably better in the EPCs or MSCs injected group than in the saline group (Fig. 10). However, there was no significant difference between EPCs and MSCs transplantation.

8. Mechanisms mediating therapeutic effects

A. Histopathology with capillary density

To evaluate the extent of vascular remodeling at the level of the microcirculation in diabetic and non-diabetic mice after stem cell treatment, quantitative analysis of capillary density in ischemic adductor muscle of diabetic and non-diabetic C57BL/6 mice was determined in histological sections harvested on the postoperative day 14. Quantitative analysis of CD31-positive cells revealed that the capillary density in the ischemic limb was essentially increased in the EPCs or MSCs treated diabetic mice (Figure 11) compared to the saline injected mice, indicative of an improved angiogenic response with the stem cell transplantation in these animals. However, there was no significant difference between EPCs or MSCs in the capillary density.

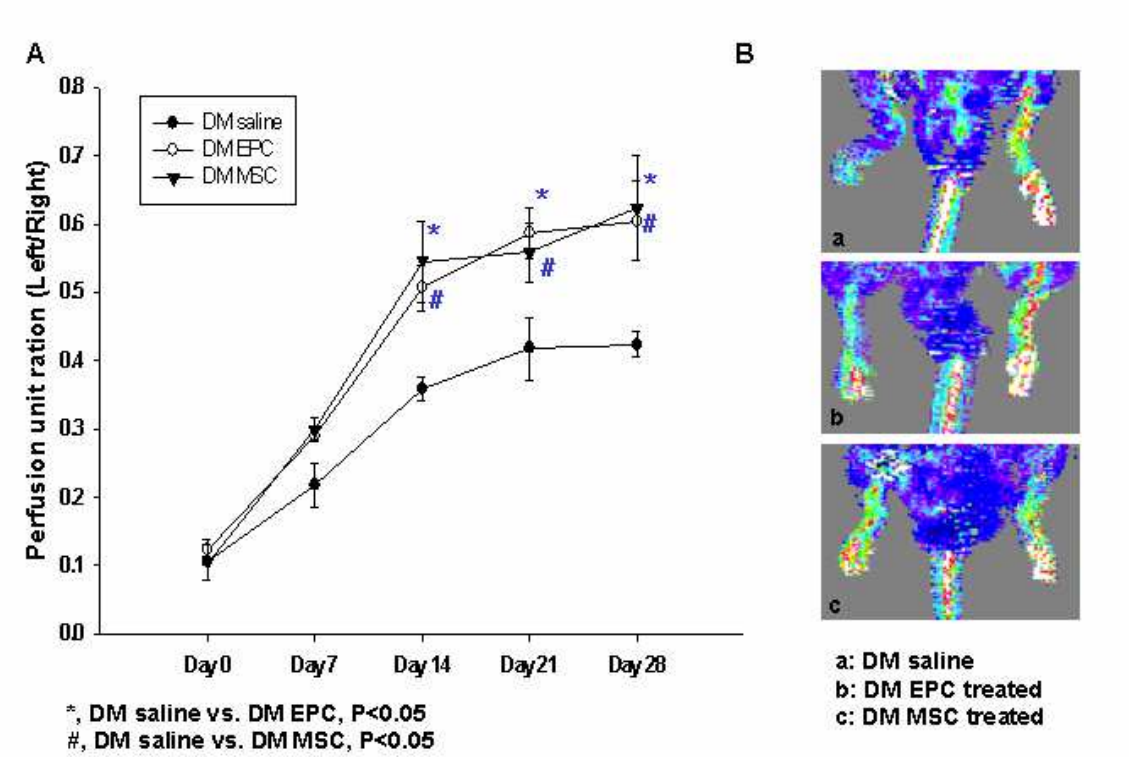

Figure 10. Ischemia-induced changes in hindlimb blood flow monitored in vivo by laser Doppler perfusion imaging performed for a month weekly after ischemia in diabetic and non-diabetic mice treated with endothelial progenitor cells (EPCs) and mesenchymal stem cells (MSCs) isolated from non-diabetic normal mice. A: Quantitative evaluation of blood flow expressed as a ratio of blood flow in ischemic foot to that in non-ischemic one. Values are means± SEM, n =7 per group. *P< 0.05 DM saline versus EPCs-injected diabetic animals. , # P < 0.05 DM saline versus MSCs injected diabetic animals. B: In color coded images, normal blood flow is depicted in red. A marked improvement of blood flow at 28 days of ischemic hindlimb in the EPCs or MSCs treated groups compared to saline injected group.

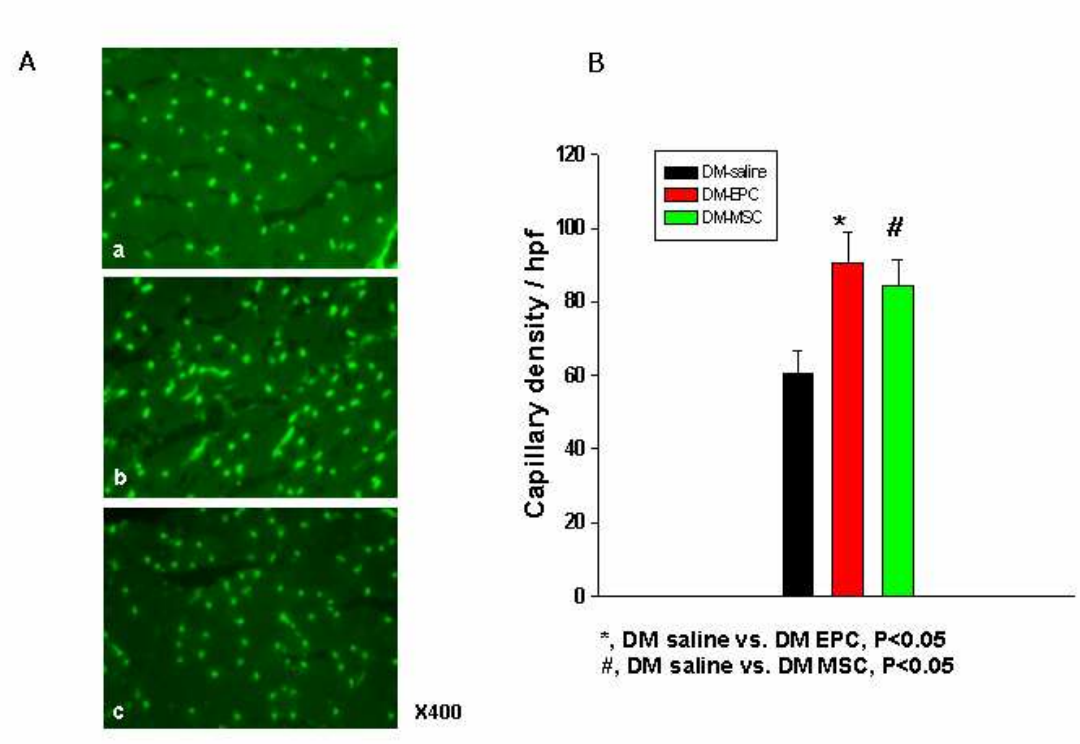

Figure 11. Capillary density of hindlimb muscle with CD31 immunofluorescence staining after stem cell transplantation. A: Representative pictures from saline (a), endothelial progenitor cells (EPCs) (b), and mesenchymal stem cell (MSCs) (c) transplantation to diabetic hindlimb ischemia. B: Quantitative analysis showed that the number of capillary was significantly higher in the EPCs or MSCs treated diabetic hindlimb compare to the saline injected groups. However, there was no significant difference between EPCs and MSCs treated groups.

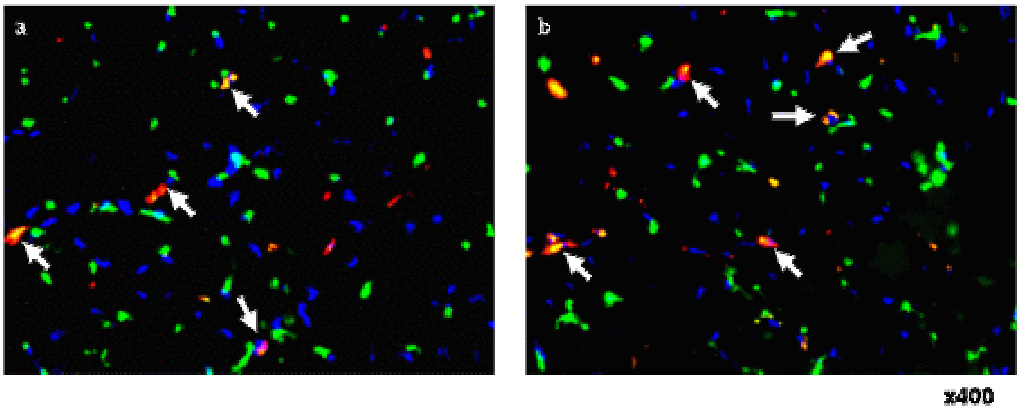

Figure 12. In vivo Fluorescein griffonia simplicifolia lectin I perfusion staining of hindlimb 2 weeks after hindlimb ischemia and cell treatment showed that cell were still alive and some of them are co-localized with lectin stained capillary (green : lectin I, red : AcDiI labeled EPC(a), MSC(b), blue : DAPI).

B. Cell fate or engraftment potential determination

To find out the survival of injected stem cells, the cells were stained with red fluorescence diI. Two weeks after cell transplantation, there are numerous injected stem cells in the hindlimb muscle which was seen as a red color in the fluorescence microscopy (Figure 12).

C. Transdifferentiation potential: co-localization with vessels

To tract the injected cells spatial placement with vessels in the tissue, we injected green fluorescence isolectin B4 intravenously to stain vessels in vivo immediately before the sacrifice two weeks after hindlimb surgery and cell transplantation. On the immunofluorescence microscopy, some of the injected red colored cells are co-localized with the green vessels which mean close proximity of stem cells in to vessels or possible transdifferentiation in to capillary (Figure 12).

D. Proliferation effect in vivo in hindlimb tissue

To compare the quantitative effect of stem cell's proliferation effect of other cells, we stained the hindlimb muscle with Ki67. It revealed higher proliferating cell numbers in the cell treated groups (EPCs, MSCs) compared to the saline injected group (Figure 13).

E. mRNA expression of various angiogenic factors or other cytokines related with cell survival, mobilization of bone marrow-derived stem cells

Two weeks after cell transplantation, to observe the changes of mRNA amount of the various angiogenic factors, mice were sacrificed and real time RT-PCR with the skeletal muscle was performed. We measured the mRNA amount of VEGF-A, fibroblast growth factor-2 (FGF-2), hypoxia inducible factor-1α (HIF-1α), platelet derived growth factor (PDGF), placenta growth

factor (PlGF), hepatocyte growth factor (HGF), angiopoietin-1, angiopoietin-2, and stromal derived factor (SDF) with the 2 weeks old hind limb tissues. The amount of mRNA of VEGF-A, HIF-1α, and FGF-2 were significantly higher in the EPC-treated hindlimb tissue compared to saline injected group. The mRNA of the cytokines of the MSCs treated group was not significantly higher compared to the saline treated group (Table 4).

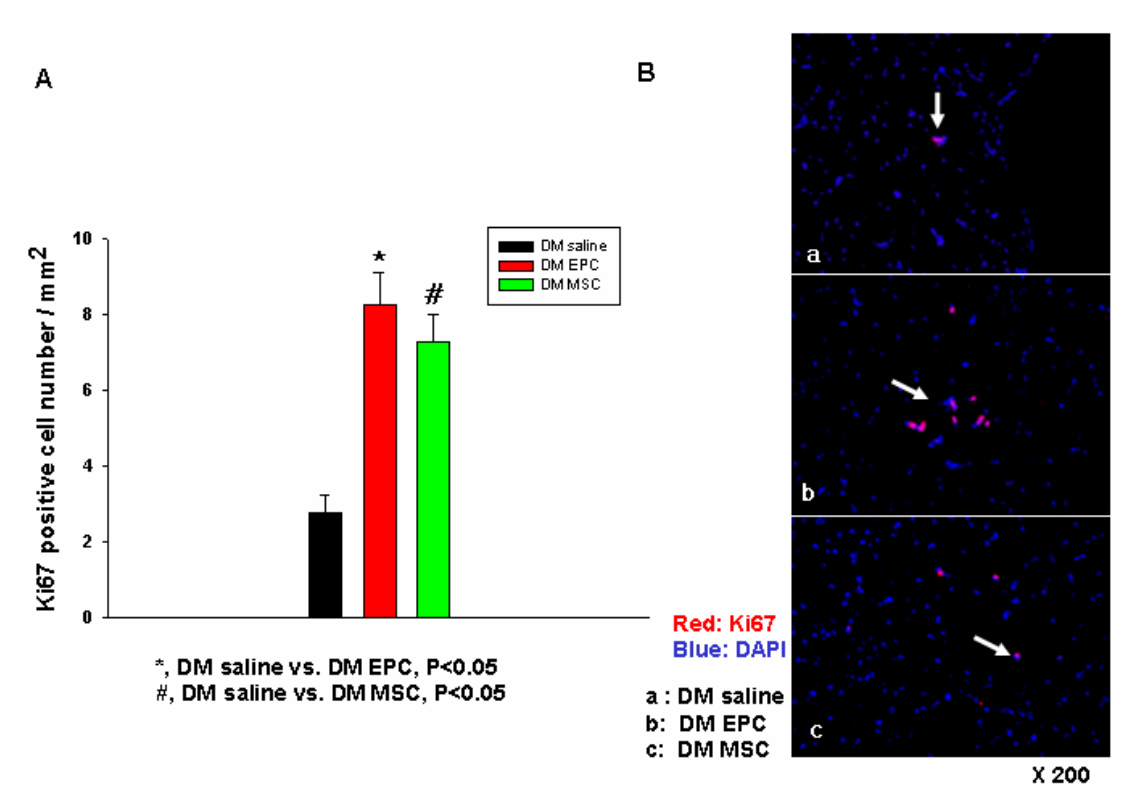

Figure 13. Ki67 immunofluorescence showed higher number of proliferating cells (arrow) in the endothelial progenitor cells (EPCs) and mesenchymal stem cells (MSCs) treated group compared to saline treated group. There was no significant difference between EPCs and MSCs group (red: Ki67, blue: DAPI).

Table 7. Real time RT-PCR data of various cytokines from diabetic hindlimb after stem cell transplantation.

	DM-saline (A)	DM-EPC (B)	DM-MSC (C)	p
VEGF-A	13.23	25.10	25.42	A vs. B <0.05 A vs. C <0.05 B vs. C = NS
HIF-1α	2.62	19.68	12.49	A vs. B <0.05 A vs. C <0.05 B vs. C = NS
FGF-2	0.26	0.47	0.31	A vs. B <0.05 A vs. C =NS B vs. C =NS
PlGF	0.05	0.32	0.25	A vs. B <0.05 A vs. C =NS B vs. C =NS
PDGF-β	0.32	0.36	0.33	NS
Ang-1	1.72	2.14	1.79	NS
Ang-2	Not measured	Not measured	Not measured	
HGF	Not measured	Not measured	Not measured	
SDF1-α	Not measured	Not measured	Not measured	

VEGF-A: vascular endothelial growth factor-A, HIF-1α: Hypoxia inducible factor -α, FGF-2: Fibroblast growth factor-2, PlGF: placenta growth factor, PDGF-β: Platelet derived growth factor-β, Ang-1: Angiopoietin-1, Ang-2: Angiopoietin-2, HGF: hepatocyte growth factor, SDF1-α: Stromal growth factor1-α

IV. DISCUSSION

Diabetic mice showed different pathophysiological findings compared to the normal in their features of neovascularization, stem cell functions, and response to the ischemia. We firstly assumed that diabetes has decreased neovascularization in the hindlimb muscle in not only with the ischemia injury but also in absence of ischemic injury. We compared the basal level of capillaries in the diabetic and normal mice and found that diabetic mice had the fewer number of capillary. The blood flow recovery measured with laser Doppler after ischemia showed impaired response. This is very true when we see the human cases. Diabetes patients have the delayed wound healing process. This is probably related the decreased the vascularity in the diabetic condition. When we count the capillary density in the diabetic hindlimb, we found the unusual findings in terms of counting of capillary in the microscopy because of the morphological change of diabetic skeletal muscle. As showed in the figure 1, there was atrophy of skeletal muscle. Therefore, if one counts the total number of capillary in the high power field such as 400 magnifications routinely, it would reveal the increased capillary number. Hence, in this case, capillary density per single myofiber should be counted. Figure 2 showed that the number of capillary per single myofiber is fewer in the diabetic muscle compare to normal. These findings were observed in other papers. Computer-assisted morphometric analysis of myofiber size by fiber type indicated a significant difference in myofiber size for the type 2b fibers in muscles from diabetic mice. Similarly, there was a shift in the fiber size distribution to include a greater number of small type 2a, 2b myofibers when compared to controls. Skeletal muscle from diabetic mice exhibited a significant change in the percentage of fiber types, with an increase in the number of type 2a fibers, a fiber type grouping[38]. In another report, morphometric analysis revealed that muscle fiber cross-sectional area was reduced 39% in diabetic rats, which, despite a lower capillary-to-fiber ratio, resulted in a 27% increase in

capillary density in diabetic rats[39]. This finding was observed in our experiment as showed in figure 2. Therefore, it is quite sure that capillary density should count not on a whole section but on a single myofiber when used as a therapeutic indicator in the diabetic mice.

To reveal the reasons of the decreased neovascularization in the diabetes, we performed various kinds of experiments. When we compared the VEGF-A mRNA content from the hindlimb, it was significantly lower in the skeletal muscle of the diabetic mice. These findings were also observed in the previous study with human[40] and mouse skeletal muscle [32]. It revealed that when vascular injury occurs in the limbs, the recovery potential might be limited.

As a consequence of defectiveness of the skeletal muscle itself, they need some important helpers. We also hypothesized that the decreased neovascularization probably related some defect of the bone marrow stem cells. One of them is the circulating EPCs. As we expected, the number of circulating EPCs was much less in the diabetic mice as showed in these experiments. These have been reported in human study[27, 41]. Therefore, they need other supporters. It has been quite true that the bone marrow derived stem cells are recruited into injury site and participate the reparative process[15-18].

However, as our data showed, diabetic bone marrow derived stem cells has the defect of paracrine function, cell adhesion, and cell migration, as well as tube formation. The Microarray data showed that a total of 12 cytokines from112 tested genes including Akt-1, Npr1, Nrb1, and TGFb-3 were less in the diabetic BM-MNCs compared to those of normal (Table 3). These findings were also observed with EPCs, MSCs Microarray data. Diabetic EPCs had a total of 11 cytokines reduced such as PDGFb, TFGb3 (Table 4). Diabetic MSCs showed reduced Akt1, PDGFa (Table 5). These results were also confirmed with real time RT-PCR. Bone marrow derived MNCs of diabetic mice have less amount of mRNA of significant angiogenic cytokines such as, angiopietin-1, and VEGF-A, IGF-1 compared to the normal cells

as the results showed above on the figure 4. EPCs and MSCs cultured from the bone marrow-derived MNCs also had the lower level of VEGF-A compared to the normal cells (figure 5).

When it comes to say paracrine effect, which means the stem cells secrete some important cytokines into the local tissue after homing to the needed area, decreased content of the genes or mRNA of the important cytokines related to angiogenesis might have resulted in the abnormal paracrine function, Therefore, it is convinced that the role of bone marrow in the regenerative process are defective in the diabetic mice.

Furthermore, in terms of bone marrow derived stem cell function, adhesion assay of EPCs to vitronectin and lamine showed decreased attached cell number in the diabetic stem cells compared to the normal stem cells (Table 6). These findings were also observed in the other paper with some different findings [19]. They described that EPCs adhesion to Fibronectin, collagen was not reduced but reduced in the adhesion to human umbilical vein endothelial cells activated by tumor necrosis factor-alpha (TNF-alpha). The tube formation assay of EPCs also showed much less organized tube in the diabetic cells (figure 6). Usually in the tube formation assay one count the number of the attached stem cells such as EPCs to the human umbilical vein endothelia cells (HUVEC). However, we only cultured EPCs in Matrigel for 4 days without HUVEC and observed distinct tube morphology from normal EPCs. When MSCs were exposed to Matrigel to form the tube, they did not make tube morphology. The migration assay of the EPCs to the VEGF-A showed the similar result from other reports that diabetic cells are less migrated than normal cells. In these experiments, we firstly did the migration assay with diabetic MSCs and showed decreased migration ability of the diabetic MSCs. From all these functional assays, we can assume that, when injury occurs to the diabetic mice, the ability of homing of the bone marrow derived stem cell to the injury sites and participation to the regeneration would be hampered.

The reasons why diabetic stem cells have a various functional impairment were proposed from some researchers as below. Adverse metabolic factors, including oxidized small and dense low density lipoprotein (ox-dmLDL) can contribute to the reduced number and the impaired functions of EPCs in diabetic patients[42]. Increased oxidative stress could play a role in the development of EPC dysfunction in type 1 DM[43].

To improve the regeneration capability in the diabetes, we hypothesized that normal non-diabetic stem cell transplantation could be the way out of this whole scenario. Therefore, we treated bone marrow derived EPCs and MSCs collected from the normal mice in to the diabetic mice hindlimb injury site. There have been some reports about EPCs or MSCs cell treatment in to non-diabetic hindlimb injury models. However, there are only one report of EPCs treatment for the diabetic hindlimb ischemia[26] and no data with MSCs so far. Two weeks after cell treatment, the laser Doppler blood flow showed the significantly better results compared to the saline injected groups even though they did not show the same good findings as showed in the normal mice recovery process after ischemia. The therapeutic effect of EPCs and MSCs were comparable.

To delineate the mechanism of this favorable therapeutic effect of the normal stem cell treatment to the diabetic hindlimb ischemia, we did several experiments. Firstly, we observed the pathology, if they increased the capillary density in the diabetic hindlimb muscle after ischemia and cell transplantation. The number of capillary density of cell treated groups showed higher than saline injected group. We proposed that the mechanism of this improved recovery and capillary density is probably from the paracrine effect of the normal injected stem cells. This is because the diabetic cells had decreased amount of important angiogenic cytokines as documented in this experiments. The normal stem cells can overcome these flaws and contribute fully in the regeneration when injected into the ischemic sites. Our hypothesis was

documented by increased mRNA amount of VEGF-A, HIF-1a, FGF-2, PlGF in the hind limb tissue after cell therapy compared to the saline injected group. The other positive effect of stem cell treatment was the increased cell proliferation number as showed on the result. Furthermore, the injected EPCs and MSCs were still alive in the mice until 2 weeks after treatment and also there were some co-localized stem cells with the capillary of the hindlimb muscle which suggest of the possibility of the transdifferentiation of the injected stem cell in to one of the structures of vessels.

In the comparison of stem cell's therapeutic effects, there is one data that compared BM-MNC and MSCs[17]. They reported that MSCs are better that BM-MNC in terms of capillary density and laser Doppler blood flow. In our study, we compared the characteristics and therapeutic effect of the EPCs and MSCs. In the Microarray data, we found that diabetic EPCs had 12 cytokines reduced compared to normal EPCs, but diabetic MSCs showed 3 cytokines that reduced compared normal MSCs. We assumed that it probably related the different characteristics between EPCs and MSCs as well as the recovery of the cell function during cell culture in the case of MSCs. The therapeutic effect observed by capillary density and laser Doppler blood flow after transplantation did not show difference between MSCs and EPCs.

We concluded that diabetes mellitus is the condition of impaired neovascularization and this is berceuse of the decrease the angiogenic cytokines in the hindlimb itself and reduced number and function of the bone marrow derived EPCs or MSCs in the process of regeneration process. The normal bone marrow-derived stem cells may overcome these defects when transplanted in to the diabetic hindlimb ischemia. These findings can be applied to the clinical field of peripheral artery disease in the diabetes mellitus.

V. CONCLUSION

Diabetes mellitus has the impaired angiogenic process after hindlimb ischemia. It might be from the change of the skeletal muscle characteristics and dysfunction of the circulating and bone marrow derived stem cells. However, normal bone marrow derived stem cells transplantation showed the favorable effects in the recovery of diabetic hindlimb ischemia.

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Abstract (In Korean)

당뇨병성 하지 혈관 질환 모델에서의 골수 줄기 세포 치료

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이 주 용

당뇨병은 심장 혈관 질환의 가장 중요한 위험 요소 중에 하나이며 최근 그 유병율과 발병율이 증가하여 중요한 보건학적 사회문제로 부각되고 있다. 당뇨병 환자에서 상처 회복이 느리고 조직 손상 시 회복이 느린 것은 경험적으로 알려져 있다. 본 연구의 목적은 쥐를 이용하여 당뇨병에서 하지 허혈 시 재생이 느린 이유를 밝히고 혈관 전구세포나 간엽 줄기 세포와 같은 골수에서 유래한 줄기 세포의 기능을 평가하며 골수 줄기세포를 당뇨병이 유발된 쥐의 급성 하지 허혈 모델에 사용 했을 때의 치료 효과를 보기 위함이다.

당뇨병에서 하지 혈관 손상 후 정상으로 회복이 지연되는 것은 골격근 내에 VEGF-A 가 적고, 말초 혈액 내에 있는 혈관 전구세포의 수가 적으며 골수에서 유래하는 혈관 전구세포 그리고 간엽 줄기 세포의 혈관 재생에 관여하는 세포 기능이 떨어진 것 때문인 것으로 사료된다. 또한 당뇨병 쥐에서 얻은 골수 유래 전구세포는 VEGF-A 등의 중요한 혈관 재생에 필요한 cytokine 의 mRNA 양이 정상 쥐에서 얻은 세포에 비해 적었다. 정상 쥐에서 채취한 혈관 전구 세포와 간엽 줄기 세포를 당뇨병 쥐의 하지 허혈 수술 후 주사 한 결과 대조군에 비해 현저한 혈류 개선 효과를 보여 주었다. 주사한 세포는 2 주 관찰 하는 동안 조직 내에서 살아 있었으며 골격근 내에서 미세 혈관 및 증식 중인 세포의 개수가 증가된 것을 보여주었다. 이는 투여한 세포에서 분비되는 VEGF-A, FGF-2, PlGF 등의 혈관 조성에 관여하는 단백질의 증가 효과와 줄기 세포가 미세 혈관을 구성하는 일부 세포로 분화되었기 때문으로 본 연구 결과 추론할 수 있었다.

결론적으로 당뇨병에서 하지 허혈에 대한 회복이 느린 것은 당뇨병에 의한 하지 조직 자체의 변화와 골수 및 말초 혈액에서 줄기 세포의 기능이 떨어진 것에 기인한 것으로 사료되며 정상 골수 줄기 세포를 이용하면 허혈 손상 시 치료 효과를 얻을 수 있을 것으로 사료 된다.

핵심 되는 말 : 당뇨병, 줄기 세포, 하지 허혈, 혈관전구 세포, 골수 간엽 줄기 세포