

Stem cell therapy in diabetic foot patients: where are we now?

Stanley Kirana, Diethelm Tschöpe, Bernd Stratmann

Diabetes Center, Heart and Diabetes Center NRW, University Clinic of Ruhr University Bochum; Bad Oeynhausen, Germany

Abstrak

Kaki Diabetik (KD) merupakan penyakit penyerta Diabetes Mellitus (DM). DM merupakan salah satu penyebab utama kasus amputasi non-traumatik di Jerman, dengan penyakit arterial perifer berat (PAP) dengan iskemia kritis tungkai yang menjadi masalah utama. Meskipun teknik modern tersedia, intervensi perkutan dan pembedahan pemulih vaskularisasi masih terbatas. Masalah ini menyebabkan peningkatan jumlah amputasi pada pasien dengan diabetes mellitus. Proses fisiologi angiogenesis, vasculogenesis dan arteriogenesis mengarahkan ke pertumbuhan pembuluh darah kolateral pada keadaan penyakit penyumbatan pembuluh arterial penyebab iskemia tungkai. Pada praktik klinik respons angiogenik endogenik seringkali terganggu. Angiogenesis terapeutik merupakan penerapan bioteknologi untuk merangsang pembentukan pembuluh darah baru via (melalui) aplikasi lokal faktor penumbuh pro-angiogenik dalam bentuk protein rekombinan, atau terapi gen; atau dengan implantasi sel progenitor atau sel punca yang akan mensintesa sitokin angiogenik multipel. Artikel review ini merangkum fungsi endotelial dan disfungsi pada DM, mekanisme homing, metode transplantasi dan status uji klinik di bidang sel punca untuk pengobatan iskemia tungkai. (*Med J Indones 2011; 20:154-60*)

Abstract

Diabetic foot (DF) occurs as a concomitant illness of diabetes mellitus (DM). DM is one of the main causes of non-traumatic amputation in Germany with severe peripheral arterial disease (PAD) with critical limb ischemia (CLI) being of major concern. Although modern techniques are available surgical vascularisation and percutaneous intervention are limited. This problem leads increasing numbers of limb amputations in patients with diabetes mellitus. The physiological process of angiogenesis, vasculogenesis and arteriogenesis contribute to the growth of collateral vessels in response to obstructive arterial disease causing limb ischemia. In clinical practice the endogenous angiogenic response is often impaired. Therapeutic angiogenesis is an application of biotechnology to stimulate new vessel formation via local administration of pro-angiogenic growth factors in the form of recombinant protein, or gene therapy, or by implantation of progenitor cells or stem cells that will synthesize multiple angiogenic cytokines. This review summarises the endothelial function and dysfunction in DM, the mechanism of homing, the transplantation method and the status of clinical trials in stem cell field to treat limb ischemia. (*Med J Indones 2011; 20:154-60*)

Key words: diabetes mellitus, endothelial progenitor cells, peripheral arterial disease, stem cells, therapeutic angiogenesis

Diabetes mellitus (DM) is known as an emerging chronic disease world wide. DM is accepted as cardiovascular risk factor and therefore plays an important role in the pathogenesis of cardiovascular disease. Peripheral artery disease (PAD) is one of the major health problems resulting from macrovascular complication in diabetic patients. As a manifestation of atherosclerosis, PAD is characterized by atherosclerotic occlusive disease of the lower extremities and is a marker for atherothrombotic disease in other vascular beds. PAD is present in approximately one-half of all patients with foot ulcers.¹⁻⁴ Along with polyneuropathy, PAD causes foot ulceration which often leads to lower limb amputations.

DM causes almost 50% of all non traumatic lower-extremity amputations world wide. It is estimated that the life time risk for amputation in diabetic patients is 10–15%, which is 10-30 times higher in comparison to the general population.⁵⁻⁸ In Germany the number of limb amputation is increasing. According to the newest data from German Association of Angiology, which were released during the Scientific Meeting in Mannheim in September 2008, about 60.000 amputations are performed every year. Compared to European countries such

as Denmark and The Netherlands, Germany is leader in the number of limb amputation. The main factors inducing these amputations are diabetic foot (DF) or arteriosclerosis. Almost every 3rd patient suffers from diabetes mellitus. An amputation for elderly patients resembles a fatal step in their quality of life.

In the other hand, the patients are often late to seek specialized professional care or they are not treated in a vascular center. These factors along with pathology-anatomical factors limit the possibilities for revascularisation.

The strengths and limitations of surgical revascularisation in PAD are well known. Surgical revascularisation and percutaneous interventions are limited to patients with critical limb ischemia (CLI) and those with disabling claudication (Fontaine Stadium IIb–IV) due to discrete proximal disease.^{1,9}

Endothelial functions and dysfunctions

As key elements in the maintenance of tissue homeostasis, blood vessels serve as the conduits of circulation, transporting nutrients and oxygen to organs and tissues, and removing destructive catabolites and xenobiotics from the blood flow.¹⁰

For a long time two specialized endothelial functions were accepted: gas exchange in pulmonary circulation and fenestration in hepatic and splenic circulation. Under normal homeostatic conditions, the endothelium resists vasospasm, prevents leukocyte and platelet adhesion to the vessel wall, favours fibrinolysis, combats coagulation of blood, and inhibits the proliferation of vascular smooth muscle cells.

During the last two decades, accumulating evidence has described the vascular endothelium as an active endocrine, paracrine, and autocrine organ, indispensable for the maintenance of vascular homeostasis. Altered homeostasis induced by various stimuli may cause localized alterations, or 'endothelial dysfunction', of the antihemostatic properties, vascular tone, heightened leukocyte adhesion, and increased production of cytokines and growth factors. The dramatic change of endothelial interactions with blood leukocyte occurring in inflammation provides an example of endothelial activation.^{10,12,13}

Diabetes mellitus and endothelial dysfunction

Vascular diseases, including atherosclerosis, medial calcification, and microangiopathy, are prevalent in patients with DM and are the principal causes of morbidity and mortality in these individuals. Atherosclerosis occurs earlier in patients with diabetes, frequently with greater severity and more diffuse distribution.

Hyperglycemia *per se* causes endothelial dysfunction. Healthy humans exposed to a hyperglycaemic clamp sufficient to increase forearm glucose concentration experience impaired endothelium-dependent vasodilation.^{10,14,15} Hyperglycemia also decreases flow-mediated endothelium-dependent vasodilation of the brachial artery of healthy subjects.^{14,16,17} Hyperglycemia may decrease the bioavailability of nitric oxide (NO) through multiple mechanisms (Figure 1). Additionally, hyperglycemia may increase the formation of oxygen-derived free radicals that inactivate NO or cause intracellular signalling disturbances that inhibit nitric oxide synthase (NOS) activity and thereby reduce NO production. Hyperglycemia is also associated with increased oxidative stress. Increased inactivation of NO by oxygen-derived free radicals and decreased production of NO by NOS reduce NO levels in the vascular milieu.^{14,17-20}

Rationale for stem cell therapy in diabetic foot

The essential part of normal wound healing is the formation of new blood vessels within the provisional wound matrix that is referred to as granulation tissue. Neovascularisation of the granulation tissue occurs by the processes of angiogenesis or vasculogenesis, or

both.^{21,22} Therapeutic angiogenesis has been studied many times for the treatment of patients with peripheral arterial occlusive disease who do not qualify for surgical revascularisation or radiologic intervention. Angiogenesis can be achieved by introducing growth factors as mature proteins or as complementary DNA carrying vector systems (cDNA-plasmid) or stem cells which contain endothelial progenitor cells (EPCs) and mesenchymal cells.²³⁻²⁷

The following sections discuss various methods of using stem cell therapies to induce angiogenesis in DF.

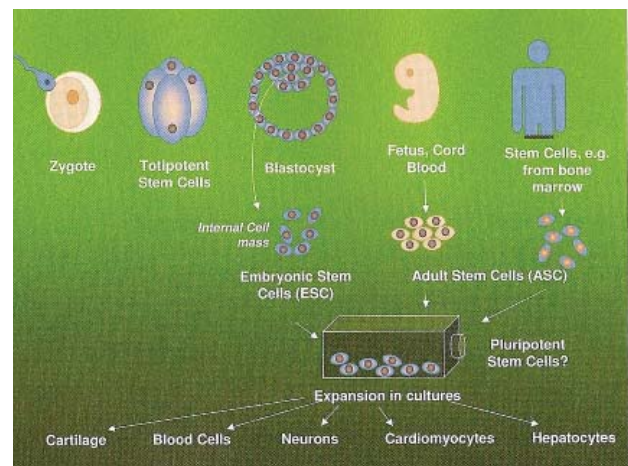


Figure 1. Sources for embryonic and adult stem cells²⁸

Definition of stem cells

All life forms begin with a stem cell, which is defined as a cell that has the dual ability to self-renew and to produce progenitors and different types of specialized cells in the organism.²⁸

Regardless of their sources, stem cells are defined by their indefinite capability of self-renewal (proliferation) and unlimited potential to generate specialized tissue cells (differentiation) (Figure 2). Currently, scientists and clinical researchers are working on two major types of stem cells, embryonic and adult, which share three characteristic properties: 1. Stem cells are premature, undifferentiated, or unspecialized. However, unspecialized stem cells can generate specialized cells, including heart muscle, blood vessels, blood cells, or nerve cells. 2. Stem cells can divide and renew themselves for long periods. Unlike other cells—which normally do not replicate by themselves—stem cells may replicate, or proliferate, many times. 3. Stem cells can respond to exogenous or endogenous signals by generating specialized cell types. When unspecialized stem cells develop into specialized cell, the process is called differentiation.²⁹

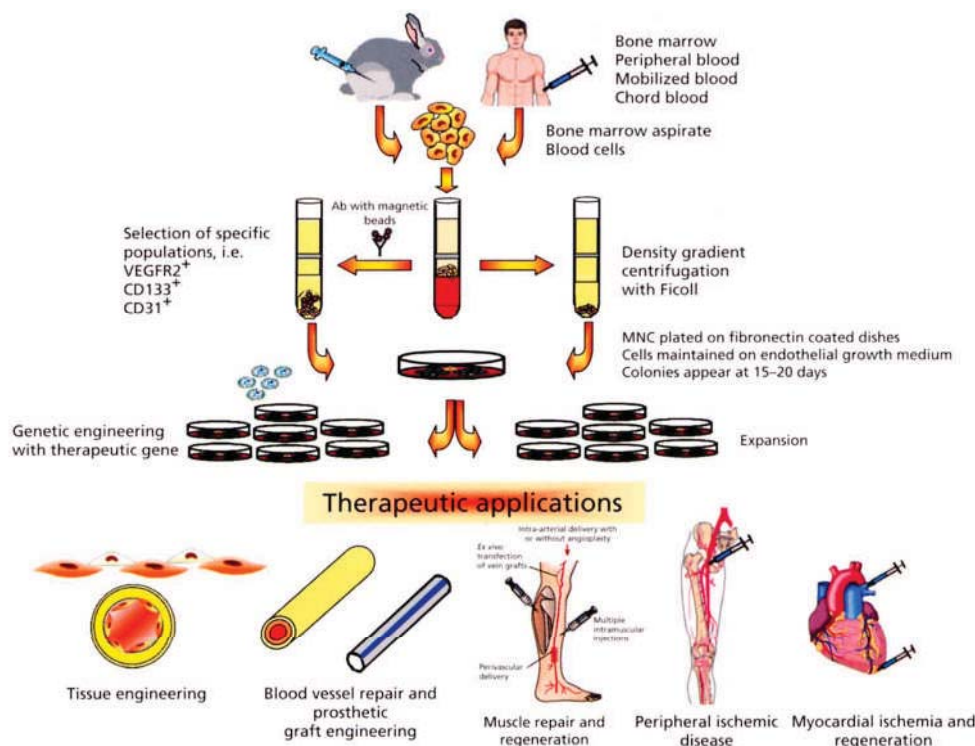


Figure 2. Isolation, cultivation and genetic engineering of endothelial progenitor cells (EPCs) for therapeutic application. EPC can be isolated from the BM-MNC, peripheral blood or umbilical chord blood with or without further selection and purification. The mononuclear cells are expanded *ex vivo* under endothelial-specific growth conditions and may be genetically modified to over express one or several therapeutic genes. The differentiated cells are then used in transplantation protocols for rescue and repair of damaged tissue such as infarcted myocardium, ischemic limb or injured muscle. The cells may also be used for endothelialisation of damaged blood vessels and vascular prosthetic grafts and in tissue engineering. (Melo LG, Pachori AS, Kong D et al. Endothelium-targeted gene and cell-based therapy for cardiovascular disease. In: De Caterina R, Libby P, eds. Endothelial Dysfunctions and Vascular Disease. Oxford: Blackwell Futura; 2007:385)

Adult and embryonic stem cells

Human embryonic stem cells (ESCs) used for research have been extracted from embryos created by *in vitro* fertilization done by the group of James Thomson in 1998 and they reported on the establishment of human ESC lines. They concluded that the ESCs have potential to form most cell types of the adult body over almost unlimited periods.^{28,30-32}

The adult body has a small number of adult or somatic stem cells in some tissues and organs. Such adult stem cells (ASCs) have been known to possess the ability to regenerate the corresponding tissue from which they are derived. Hematopoietic stem cells (HSCs), for example, continuously regenerate the circulating blood cells and cells of the immune system during the life span of the organism. Based on animal studies, many researchers have recently claimed that ASCs might exhibit developmental potentials comparable to those exhibited by ESCs. ASCs have the ability to regenerate the tissue from which they are derived over the life span of the individual, while ESCs have the potential to

form most cell types of the adult body over very long periods of *in vitro* cultivation.^{28,29,32-38} However, until now the use of human ESCs for research is prohibited in Germany. Many clinical research centers in Germany are focusing to develop the potential of ASCs.

Endothelial progenitor cells for cardiovascular regeneration

The differentiation of mesodermal cells to angioblasts and subsequent endothelial differentiation was believed to occur exclusively in embryonic development. Asahara et al. reported that purified CD34⁺ hematopoietic progenitor cells from adults can differentiate *ex vivo* to an endothelial phenotype.^{40,41} These cells were named "endothelial progenitor cells" (EPC). Shi et al. reported in 1998 the existence of 'circulating bone marrow-derived endothelial progenitor cells' (CEPC).⁵⁸ This is actually a similar finding to Asahara. EPC or CEPC were defined as cells, which express both, hematopoietic stem cell markers such as CD34 and an endothelial marker protein such as the VEGF-receptor KDR.

CD34 is not expressed exclusively on hematopoietic stem cells (HSC) but also on mature endothelial cells. Further studies used the more immature HSC marker CD133 also known as prominin or AC 133. Purified isolated CD 133⁺ cells also differentiated to endothelial cells *in vitro*.^{41,42} However, the biological function of CD133 remains unclear. It is not well known whether CD133 only represents a surface marker or has a functional activity involved in regulation of neovascularisation.⁴¹

The characterization of EPC becomes particularly difficult when cells are expanded and cultured *ex vivo*, since the culture conditions (culture supplements such as fetal calf serum and cytokines or different plastic types) rapidly change the phenotype of the cells. Moreover, continuous cultivation was shown to increase endothelial differentiation, as evidenced by elevated endothelial marker protein expression.

Smooth muscle cells (SMC) and endothelium are two essential structures for the architecture of the blood vessels. Vascular stem cells, a group of multipotent heterogeneous stem cells, largely fall into two subgroups: endothelial and SMC progenitors. EPC develop closely from CD34⁺ to bone marrow stem cells, in particular those committed to the monomyeloid cell lineage, whereas SMC progenitors may belong or originate together in mesenchymal stem cells (MSC), which are negative for CD34. Atherosclerosis causes arterial wall damage and impairs the capacity of both vascular stem cell types to regenerate neovascular tissue, or may trigger abnormal proliferation or death by apoptosis under different pathological conditions.²⁹

Mechanism of vessel formation

Angiogenesis is defined as the sprouting of new capillaries from existing vascular structure, a process that is triggered by endothelial cell migration and proliferation. Remodelling of the extra cellular matrix (ECM), tubule formation and expansion of the surrounding vascular tissue are key elements of angiogenesis.

The *in situ* formation of new blood vessels from EPC which differentiated into endothelial cells and fuse into luminal structures is called vasculogenesis.

Meanwhile, arteriogenesis refers to an increase in the calibre of pre-existing arteriolar collateral connections by recruitment of perivascular cells and expansion and remodelling of the extracellular matrix. Arteriogenesis increases the size and wall thickness of collateral vessels, and shear stress (rather than hypoxia) seems to be the main factor that stimulates arteriogenesis.⁹

The key steps in vessel formation—namely endothelial cell activation, migration, proliferation and reorgani-

sation—are tightly regulated in a complex balance between pro-and anti-angiogenic mechanisms (Table1).⁹

Table 1. Factors that stimulate and inhibit angiogenesis

Stimulators	Inhibitors
VEGF	Angiostatin
aFGF	Thrombospondin
bFGF	Endostatin
PDGF	Troponin-I
TGF α/β	TIMPs
TNF α	Suramin
HGF	
Angiogenin	
PIGF	
Angiopietin	
Oestrogen	
Nitric oxide	

VEGF: vascular endothelial growth factor; aFGF, bFGF: acid and basic fibroblast growth factor (FGF-1 and-2, respectively); PDGF: platelet-derived growth factor; TGF: transforming growth factor; TNF: tumour necrosis factor; HGF: hepatocyte growth factor; PIGF: placental growth factor; TIMPs: tissue inhibitors of metalloproteinases.

The most important pro-angiogenic factors are vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), also known as FGF-2. VEGF is an endothelial cell specific mitogen that is markedly up-regulated by hypoxia, and plays an important role in endothelial cell proliferation, differentiation and survival.

FGFs are non-secreted growth factors that are released only during cell death or ischemic cell injury. Clinical and biochemical factors also influence the formation of, and biological response to different angiogenic growth factors. Hypoxia, for example, is the most potent inducer of angiogenesis,⁴⁴ principally via up-regulation of VEGF, whereas diabetes mellitus and increased levels of cholesterol and lipoprotein(a) are associated with a reduced angiogenic response.⁹

As bone marrow-derived stem and progenitor cells home to sites of ischemia, this may allow the local release of factors acting in paracrine manner on the surrounding ischemic tissue. Bone marrow-derived mononuclear cells release angiogenic growth factors such as VEGF, bFGF and angiopietins, thereby enhancing the local angiogenic response.^{9,41,44} This mechanism is named 'homing' and it is the most likely theory which represents the reality in ischemic process.

During tissue injury, e.g. wound, the formation of new blood vessels is needed. Neovascularization of the wound's granulation tissue occurs by the processes mentioned above. However, in diabetic patients these angiogenic mechanisms are reduced.

The idea to induce neovascularisation in the wound area or ischemic area is now a hot topic in regenerative medicine. There are a lot of clinical trials in this area, however only few centers world wide perform these studies.

Stem cell transplantation

Until now, there are a lot of protocols, hypotheses, debates and discussions about the best methods of stem cell transplantation. Most clinical studies are performing 2 methods of stem cell transplantation: intra-arterial and intra-muscular.

The concept of intra-arterial stem cell transplantation assures that the stem cells reach all stenosis sites antegradely. We believe that the stenosis area is the ischemic area which stimulates the homing effect of the

cells. The intra-muscular application is based on the ischemic musculature which could be supplied with a high concentration of stem cells through several local depots. Whether the cells reached the ischemic area by blood flow remains unclear. In theory, it induces the homing effect and then stimulates the angiogenesis or vasculogenesis in the ischemic area.⁴⁵⁻⁴⁷

Current clinical trial

There are a lot of clinical trials running on this topic (Table 2). However, the outcomes have not been published yet. Only some studies published their results or case reports.^{25,45,46} A multi center study has not been performed to take conclusion of stem cell therapy. To what we know, the data from other centers as well as ours show positive results (Figure 3).

Table 2. List of stem cell clinical trials in critical limb ischemia as listed in Clinicaltrial.gov from September 2007

Location	Status	Trial name
USA	Recruiting	ALDHbr Cells for Critical Limb Ischemia
USA	Recruiting	Safety Study of Using Stem Cells to Stimulate Development of New Blood Vessels in Peripheral Vascular Disease
USA	Recruiting	Combination Stem Cell Therapy for the Treatment of Severe Leg Ischemia
USA	Recruiting	Vascular Repair Cells (VRC) Treatment of Patients With Peripheral Arterial Disease Related Critical Limb Ischemia (RESTORE-CLI)
USA	Recruiting	Study of Autologous Bone Marrow Concentrate for the Treatment of CLI
USA	Recruiting	StemCells for Treating Critical Ischemia
Japan	Recruiting	Stem Cell Study for Patients With Leg Ulcer / Gangrene
Japan	Recruiting	TACT-NAGOYA: Therapeutic Angiogenesis Using Cell Transplantation
Korea	Recruiting	Study for Safety and Efficiency of Therapeutic Angiogenesis for Patients With Limb Ischemia by Transplantation of Human Cord Blood Mononuclear Cell
The Netherlands	Recruiting	Intra-Arterial Stem Cell Therapy for Patients With Chronic Limb Ischemia (CLI)
The Netherlands	Recruiting	Autologous Bone Marrow-Derived Mononuclear Cells for Therapeutic Arteriogenesis in Patients With Limb Ischemia
Italy	Recruiting	Autologous Bone Marrow Cell Treatment in Peripheral Atherosclerosis
France	Recruiting	Cell Therapy in Chronic Limb Ischemia
Denmark	Recruiting	Treatment of Severe Limb Ischemia With Autologous Bone Marrow Derived Mononuclear Cells
Germany	Finished	Novel Therapy of PAD by Combined Transplantation of BMCs (TAM-PAD)
Germany	Recruiting	Autologous Bone Marrow Transplantation for Critical Limb-Threatening Ischemia



Figure 3. Bone marrow stem cell therapy in diabetic foot (Bad Oeynhausen Working Group, Germany). This patient had a chronic limb ischemia with forefoot necrosis. Distal stenosis A. tibialis anterior and A. dorsalis pedis without collateralisation was verified by angiography. Discussion between vascular surgeon, intervention angiologist and internist revealed no surgical or interventional therapeutic option. Due to the stenosis location and vascular structure, no possibilities to perform bypass or other intervention were seen. The patient agreed with the autologous bone marrow stem cell therapy. Left picture shows the wound status before intra muscular stem cell transplantation. Right picture shows the complete wound healing 20 weeks after stem cell transplantation. Small calibre new vessel formation was proved by angiography. (With permission from Kirana, Stratmann et al. Wound therapy with autologous bone marrow stem cells in diabetic patients with ischaemia-induced tissue ulcers affecting the lower limbs. *Int J Clin Pract* 2007;61:690-2)

In conclusion, bone marrow derived EPCs are the newest cellular target that may be used to influence post-natal neovascularisation. The formation of new blood vessels, including collaterals, is a complex physiological process that occurs in adults in response to tissue injury or ischemia.

The stem cell transplantation to induce vessel formation is promising and could be a new therapeutic option in the future to treat limb ischemia without options of revascularization. However, there is still much to learn about the optimum treatment modality, dosing frequency and route of administration, especially in patients with diabetes mellitus. We know that these patients have a reduced capability of vascular self-renewing.

Until now, there are only some reports and clinical trials which have already been finished with the whole evaluations and they show an improvement of blood perfusion in ischemic area. Moreover, multicenter studies with large numbers of patients are expected to give more information about stem cell therapy.

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