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Promise of endothelial progenitor cell for treatment of diabetic retinopathy

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Abstract

Progressive obliteration of the retinal microvessels is a characteristic of diabetic retinopathy. The resultant retinal ischemia leads to sight-threatening neovascularization and macular edema. Bone marrow-derived endothelial progenitor cells play a critical role in vascular maintenance and repair and forms the basis of cellular therapy for revascularization of ischemic myocardium and ischemic limbs. Emerging studies show potential of these cells in revascularization of ischemic retina and this review summarizes this possibility. We also report current pharmacological options to correct diabetes-associated defects in endothelial progenitor cells for their therapeutic transfer.

Keywords

diabetic retinopathy; endothelial nitric oxide synthase; endothelial progenitor cell

Diabetic retinopathy is the leading cause of visual impairment in the Western world [1]. The majority of patients with Type 1 diabetes will develop retinopathy and approximately 20–30% will advance to the blinding stage of the disease. Greater than 60% of patients with Type 2 diabetes will have retinopathy. With the global epidemic of Type 2 diabetes, this dilemma is likely to worsen as over 360 million people are projected to suffer from diabetes and its complications by 2030. Diabetic retinopathy is thought to be a result of diabetes-induced retinal microvascular dysfunction. Diabetes causes two basic dysfunctions at the retinal capillary level: capillary leakage (loss of the functional integrity of the blood retinal barrier) or capillary closure or ischemia (the formation of acellular capillaries, with loss of blood supply to the neural retina).

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Capillary leakage causes diabetic macular edema (DME), the principal cause of vision loss in diabetes. Capillary closure in the retina leads to diabetic macular ischemia (DMI) and causes loss of reading vision. Bilateral DMI causes legal blindness and, importantly, there is no therapy for DMI. Moreover, patients commonly have a mixture of DME and DMI. Hyperglycemia damages retinal microvasculature, which results in increased permeability, blood and serum leakage to the extravascular space, and progressive decline in retinal blood flow; as well as closure of the retinal microvasculature. In addition the level of damage to the microvasculature is impacted by the duration of diabetes, the degree of blood sugar and hypertension control and, alternatively, patient-specific differences in the ability to repair damaged endothelium.

There is clear evidence that strict metabolic and blood pressure control can lower the risk of developing retinopathy and reducing disease progression. In addition, newer therapies targeted at treating either DMI or proliferative diabetic retinopathy, include corticosteroids and anti-VEGF antibodies, and represent promising alternatives to control disease progression. Currently laser therapy is the standard of care for DME, which results in modest preservation of visual acuity [2,3]. However, actual improvement in visual acuity is uncommon and this treatment can be destructive to various neurovascular layers of the retina and choroid. Moreover, laser is less effective in the presence of diffuse or chronic DME and in the presence of mixed DME and capillary closure [3].

Emerging studies show that endothelial progenitor cells (EPCs), derived from the bone marrow [4] or the vasculature itself [5], play a critical role in vascular maintenance and repair. Accumulating evidence shows that hemangioblast activity (Figure 1) persists into adult life in healthy individuals [6,7] but not individuals with diabetes. Thus, bone marrow-derived EPCs form the basis of current cellular therapy for revascularization of ischemic tissues, such as myocardium and limbs. In this review we will discuss the potential use of bone-marrow-derived EPCs for repair of leaky and occluded retinal vasculature in diabetic patients.

Both Type 1 and Type 2 diabetic individuals with vascular complications would potentially benefit from cellular therapy with autologous cells. However, in diabetes bone marrow-derived progenitors are dysfunctional, generating fewer endothelial cells and these cells demonstrate reduced proliferative and migratory function [8]. Enhanced oxidative stress in diabetes contributes to progenitor dysfunction [9]. Accumulation of reactive oxygen species (ROS) increases cellular/replicative senescence in these progenitors as does increased angiotensin II, oxidized low-density lipoprotein and homocysteine. EPCs of diabetic origin show a reduced ability to integrate into endothelial cell tubes *in vitro* compared with EPCs of nondiabetic origin [10–13]. Vascularization is depressed when EPCs from streptozotocin-induced diabetes mellitus mice are injected into normal mice [14].

Our group and others have evidence that the development of acellular capillaries may be due to failed attempts at repair of injured capillaries and persistence of ischemia [15,16]. An understanding of the basic mechanism responsible for the diabetes-associated defect in EPC function is key to correcting this defect and allowing the use of a diabetic patient's own EPCs for vascular repair. Specifically in the retina, correction of this dysfunction could treat early and intermediate stages of vasodegeneration to enhance vessel repair, reverse ischemia and prevent progression to the late stages of diabetic retinopathy. Interestingly, EPCs can also participate in pathological neovascularization [17], with these cells being a source of angiogenic factors. Moreover the diabetic patient with elevated serum levels of VEGF [18] and granulocyte-monocyte colony stimulating factor [19] could be at particular risk of activating EPCs into a pathological phenotype that could result in aberrant neovascularization rather than physiological repair. Therefore, their therapeutic use must be

thought out carefully. However, before EPCs can be used therapeutically as cellular therapy in retinopathy to re-endothelialize acellular capillaries and eliminate retinal ischemia, several key questions must be answered. What triggers this phenotypic change in diabetic cells taking them from reparative to deleterious? What is the best reparative progenitor population? Are some sub-populations more resistant to the injurious effects of diabetes? Should the bone marrow, the source of these cells, be a target for retinopathy therapy?

Identification of endothelial precursors based on culture

There exist two main approaches to isolate cells with endothelial characteristics and regenerative capacity: culturing of mononuclear cells (either bone marrow or peripheral blood-derived) or the use of a set of antibodies to various ‘identifying’ markers of these cells. Culture-selected cells can be classified into two distinct phenotypes as shown in Figure 2 [20,21].

The typical approach for the culturing of EPCs involves the growth of peripheral blood mononuclear cells (PB-MNCs) in selective medium and on dishes coated with either fibronectin or collagen [22,23]. The medium typically contains a cocktail of endothelial growth factors, such as VEGF, FGF, EGF, IGF, hydrocortisone and ascorbic acid [4,24]. Sequestration of growth factors within the fibronectin substrate may be particularly beneficial for facilitating EPC differentiation, which may not occur with collagen IV, vitronectin or fragments of fibronectin [25]. After 7–10 days in culture under the above conditions can produce early EPCs (eEPCs), which are mainly derived from monocytes, do not proliferate and typically begin to die after a few weeks in culture [26,27]. By contrast, late outgrowth endothelial cells (OECs) usually begin growing more than 2 weeks after isolation, eventually proliferate very rapidly, resemble microvascular endothelial cells (with a cobblestone morphology), and appear to expand indefinitely (Figure 3) [28,29]. The distinction between these two cell types is reinforced by their expression of different markers. Late OECs do not express CD1a or CD14 and have a low expression of CD45, the pan-leukocyte marker, while OECs form endothelial tubule on 3D extracellular matrix (Figure 4) [20]. The precise origin of the OEC is still in question; however, Ingram *et al.* showed that the endothelium is the source of this progenitor [5]. The OEC represents one cell in a million mononuclear cells found in the circulation and thus can only be identified once it has grown out in culture.

Most early studies of cell therapy in the heart and limbs employed eEPCs; thus, there is abundant evidence that these transplanted cells can enhance revascularization [30], although there is much debate regarding the mechanism of these effects. By contrast, OECs have been minimally studied *in vivo* [31]. Still their higher proliferative potential may be important when a large supply of regenerative cells is required. Thus, OECs may have great therapeutic utility as a cellular therapy [32]. Moreover, it has been shown by Yoon *et al.* that early and late outgrowth EPCs can act together to stimulate vascular repair [31].

Identification of EPCs based on FACS or magnetic bead selection

In contrast to differential culture, many groups, including ours, have characterized endothelial precursors using a panel of immuno-logical and nonimmunological markers, in freshly isolated cells and used these sorted cells directly for either *in vitro* or *in vivo* studies. This is the same approach commonly used for the isolation of cells (from a patient’s own bone marrow or peripheral blood) for therapeutic use. The original combination of CD34 and VEGFR-2 as surface markers remains the most common, but several other markers have been used to refine the identification of this therapeutic cell type. Some have described CD133 (prominin) as an additional marker [33], particularly to identify immature EPCs. Asahara’s pioneering study of endothelial precursors also showed that, coplating EPCs with

both CD34⁺ and CD34⁻ markers increased proliferative potential of the progenitor. This suggests a possible role for the CD34⁻ population (including CD14⁺ monocytes) in regulating EPC proliferative and differentiation responses. CD14⁺ monocytes can differentiate into endothelial cells after selective culture [27,34]. Interestingly, it has been suggested that CD14⁺ cells, which express low levels of CD34 that is undetectable with conventional techniques, contain the true progenitor cell population with the greatest regenerative capacity [35]. Recent reports are also emerging regarding CD14⁺ cells constituting a distinct population of EPCs, which can improve healing and vascular growth; this cell is now commonly called the 'angiogenic monocyte'. CD14⁺ cells can also serve as a reserved population for the treatment of conditions such as diabetes in which CD34⁺ ability is compromised [36].

When freshly isolated progenitor populations are being used the use of at least two markers is recommended. Typically CD34/VEGFR-2 or CD34/CD133 are used and the number of 'double labeled' cells in the circulation correlates with the clinical condition of the patients from which they are obtained [37–39]. The failure to reliably characterize endothelial progenitors has generated some skepticism and confusion within the field of regenerative medicine. However, a recent study [40] and an excellent review by Schatteman *et al.* [32] have suggested that despite phenotypic overlap between various bone marrow-derived or circulating cell types, the endothelial phenotype can best be characterized by three features: endothelial nitric oxide synthase (eNOS) expression, integration into tube-like structures formed by human umbilical vein endothelial cells (HUVECs), and stimulation of tube formation by HUVECs. These criteria may be the best *in vitro* method of judging endothelial phenotype, regardless of the presence or absence of particular cell surface antigens, which are often expressed transiently. This simplified interpretation is important, as it acknowledges the limitation of surface markers and affirms the importance of the identification of EPCs according to their potential to acquire functional properties of endothelial cells.

Do NO levels in progenitors predict dysfunction?

Nitric oxide (NO) generation arises from the guanidino group of L-arginine and is a NADPH-dependent reaction catalyzed by a family of NOS. Three distinct isoforms of NOS, consisting of eNOS, neuronal NOS (nNOS) and inducible NOS (iNOS), are similar in structure and function, utilizing L-arginine, oxygen and NADPH as substrates, and requiring FAD, FMN, calmodulin and tetrahydrobiopterin as cofactors.

Nitric oxide-mediated signaling pathways are essential for EPC mobilization from the bone marrow [41–43]. NO activates matrix metalloproteinase-9, releasing soluble Kit ligand, which shifts EPCs and hematopoietic stem cells from a quiescent to a proliferative niche and stimulates rapid cell mobilization to the peripheral blood [43,44]. NO regulates migration of EPCs into ischemic sites [41,45–47] and their survival [48]. We and others have shown that diabetic EPCs have decreased eNOS activity and, more importantly, that exogenous NO can correct the migratory defect in these cells [49,50]. Recently, we showed that activation of the high-density lipoprotein receptor is protective to EPCs by increasing eNOS [51], whereas activation of oxidized low-density lipoprotein receptor downregulates eNOS, supporting a key role for NO in the function of progenitors [52,53].

In Type 1 diabetes, uncoupled eNOS was found to be the major source of ROS generation [54] and, when blocked, there was reduced retinal leukostasis, blood retinal breakdown and reduced retinopathy [55]. By contrast, nNOS is highly expressed in the retina and its role in diabetic retinopathy is still being determined. Interestingly, nNOS knockout mice still develop retinal leukostasis and blood–retinal barrier breakdown, suggesting that iNOS or

eNOS may have a more central role in these particular aspects of diabetic retinopathy [55]. Diabetes-induced vascular dysfunction in rats was prevented by administration of a specific inhibitor of iNOS, 1400W [56,57].

We demonstrated in a spontaneous rat model of Type 2 diabetes that eNOS was decreased while NAD(P)H oxidase, a major source of superoxide in the vascular endothelium, iNOS and pyrooxynitrite (ONOO^-) were all increased in the retinal vasculature [58]. The shift in redox state in diabetes with increased ROS causes increased interaction of NO with O_2^- , resulting in loss of bioavailable NO and formation of ONOO^- .

The reduced NO and elevated ONOO^- can lead to microvascular dysfunction in diabetes [59–61]. ONOO^- is a potent oxidant that can attack many types of biological molecules; a high level of ONOO^- initiates lipid peroxidation, hydroperoxides, nitration of amino acids, such as tyrosine, oxidation of antioxidants, such as ascorbic acid and α -tocopherol, and direct DNA damage. The increased oxidative stress associated with diabetes can limit tetrahydrobiopterin availability [62]. Moreover, ONOO^- formation affects tetrahydrobiopterin interaction with NOS [63] leading to this ‘switch’ or uncoupling of NOS from producing NO to producing O_2^- .

The impaired *in vivo* re-endothelialization capacity of human diabetic EPCs was restored by small interfering RNA silencing of NAD(P)H oxidase subunit p47(phox) [46]. Pretreatment of diabetic $\text{CD34}^+\text{VEGFR-2}^+$ EPCs with apocynin or gp91ds-tat decreased superoxide production and increased NO availability. Pretreatment with apocynin or gp91ds-tat reversed the impaired migration of diabetic EPCs in response to SDF-1 and VEGF *in vitro*, and enhanced their integration into ischemic retinal vasculature *in vivo* [Grant M, Unpublished Data]. Propofol, a pyrooxynitrite scavenger, inhibits NF- κ B activation, increases NO production and protects EPC from apoptosis [12,64,65]. High glucose enhances EPC senescence, impairs migration and tube formation and these effects can be ameliorated by co-incubation with the NO donor sodium nitroprusside and worsened by eNOS inhibition [66]. Diabetic EPC dysfunction was improved by the eNOS activator AVE9488 [46,67] and with PPAR- γ -agonists [68,69]. Specifically, these agonists promote differentiation, increase EPC number and migratory activity, and reduce EPC apoptotic rate [70]. Decreased NO can be due to increased oxidative degradation as well as reduced synthesis. Asymmetric dimethylarginine (ADMA), an analogue of the amino acid L-arginine, inhibits the formation of NO and augments vascular oxidative stress, partly via eNOS uncoupling, resulting in higher superoxide radical generation. ADMA levels are increased in diabetes [71] and ADMA represses EPC proliferation, differentiation and function in a concentration-dependent manner [72]. Dimethylarginine dimethylaminohydrolase inactivate ADMA and regulate NO production [73]. Overexpression of dimethylarginine dimethylaminohydrolase *in vitro* and *in vivo* increased NO production [74].

Bone marrow microenvironment is dysfunctional in diabetes

The bone marrow stem cell niche is a specialized microenvironment that nurtures and regulates the stem cell pool. We have demonstrated that diabetic bone marrow has large amounts of fat compared with bone marrow from healthy controls [75]. Adipocyte-rich marrow harbors a decreased number of progenitors and relatively quiescent stem cells. Mice that are genetically deficient in adipogenesis show accelerated hematopoietic recovery after bone marrow ablation, a phenomenon that can be reproduced pharmacologically in wild-type mice through PPAR- γ inhibition [76]. These results suggest a novel therapeutic approach to enhance hematopoietic engraftment after marrow or cord blood transplantation, or to ameliorate aplasia in genetic bone marrow failure syndromes. Furthermore, this is a

mechanism for the myelosuppression observed in patients treated with the PPAR- γ agonist rosiglitazone [77–79], a diabetes drug known to increase marrow adiposity [80].

Expert commentary

While EPCs constitute a relatively small percentage of circulating cells [81] it is established that they can specifically and effectively home to sites of retinal ischemic injury. Based on the promising results of the Reinfusion of Enriched Progenitor Cells and Infarct Remodeling in Acute Myocardial Infarction (REPAIR-AMI) trial [82,83], introduction of nondiabetic EPCs into diabetic patients is being considered to promote wound healing, which could guide their use for future treatment of diabetics with extensive retinal or macular ischemia. What exactly is the promise of cellular therapy for diabetic retinopathy? While peripheral blood or bone marrow CD34⁺ cells show promise, we believe that the *ex vivo* expanded eEPCs and OECs may represent the cell populations with the greatest therapeutic utility and may provide the basis for the most effective cell therapy for vasodegenerative disease in the retina. The eEPCs, we believe, are responsible for ‘provisional repair’, that is, transient repair, preparing the environment for more durable repair, which is the function of the OECs. In healthy retinas, both populations participate in an ordered, temporal sequence with eEPCs homing in first and then attracting the OECs later. We believe that in nonproliferative diabetic retinopathy, eEPCs have reduced function as they cannot recruit OEC into the retina to repair the acellular capillaries, while in proliferative diabetic retinopathy the eEPCs take on a proinflammatory phenotype and recruit too many OECs leading to pathological neovascularization. While this hypothesis remains to be proven, the concept of identifying the reparative cell and its time course of appearance is critically important to the derivation of cellular therapies. It is our contention that for durable repair and sustained correction of retinal ischemia the use of cells that are expanded *in vitro* (eEPC and OEC) need to be considered and that these EPCs may be better than freshly isolated cells at vascular repair. Moreover, this extended *ex vivo* period would allow more time for correction of diabetes-induced dysfunction.

In diabetes, dysregulation of NO is central to phenotypic transitions of these cells to cells with reduced reparative function and this cellular change may be further influenced by the shifting bone marrow microenvironment associated with diabetes. *Ex vivo* correction of the diabetes-induced defects in these cells or systemic modulation of the bone marrow to correct the dysfunction of these cells will reduce the development of diabetic retinopathy.

Five-year view

The controversy surrounding EPCs will remain an area of active debate and rigorous investigation. The questions remain, what is the source of the vascular reparative cell? Is it the bone marrow or is it the endothelium itself? The field is replete with inconsistencies that have resulted in some disagreement and in differences in terminology used by different groups, such as ‘endothelial-like cells’, instead of endothelial cells, because the precursors express the leukocyte marker CD45. Others justify the outgrowth cells as *bona fide* products of EPCs based on the observation that the input cells did not express CD45. It is our feeling that we may be dealing with the same EPCs and that all this disagreement is counterproductive. The EPC may transition through many stages (phases) and may represent the same cell with different phenotypes depending on the isolation conditions or the culture conditions, as it is well established that a little VEGF in the culture medium goes a long way to making the cell appear ‘endothelial like’ and may explain, in part, the ‘appearance’ of the OEC. Another hurdle is that some investigators find that this ‘elusive precursor’ expresses the hematopoietic marker CD45 while others vehemently argue that it does not. The next big question is the therapeutic potential of the EPC. However, since the community can’t agree

on the actual cell, then optimization of its use therapeutically will probably be problematic. Careful assessment of the putative cell *in vivo* will be needed following *in vitro* characterization.

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Key issues

- It has been a decade since endothelial progenitor cells (EPCs) were discovered but considerable debate remains regarding the phenotypic nature and cellular derivation of these cells. Defining the correct population of EPCs is of salient importance for their therapeutic use.
- Pharmacological manipulation of EPCs will be necessary to correct diabetes-associated defects.
- Before clinical use of EPCs, it will be necessary to titrate their dosing regimen to avoid unwanted responses, such as accelerated proliferative retinopathy or, perhaps, even the initiation of a malignancy.

Figure 1. Fluorescence micrographs of vascular development in C57BL/6.gfp chimeric mice that underwent retinal ischemia

The mice were perfused with rhodamine-labeled dextran, and flat mounts of the retina were examined by standard and confocal fluorescence microscopy. **(A)** Typical whole retina from a treated eye. Areas with newly formed blood vessels are seen as small regions of green–yellow. Magnification 5x. **(B)** One such region is enlarged for better visualization. Magnification 20x.

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Figure 2. Common methods of precursor isolation

Culture of eEPCs includes a 5-day process wherein nonadherent mononuclear cells give rise to the EPC colony. OECs are derived from adherent mononuclear cells cultured on collagen for 21 days in endothelial growth conditions and demonstrate typical cobblestone morphology.

eEPC: Early endothelial progenitor cell; OEC: Outgrowth endothelial cell.

Figure 3. Morphology of early and late outgrowth endothelial progenitor cells (EPCs)
(A) Typical colony of early EPCs which develop into (B) spindle shaped cells by day 7. Early EPCs do not proliferate readily, which leads to the typical subconfluent appearance. By contrast, (C) late outgrowth EPCs show rapid growth as indicated by the dividing cell (black arrow) and (D) display the typical endothelial cobblestone morphology and proliferate abundantly.

Figure 4. Outgrowth endothelial cells (OECs) *in vitro* functions and characteristics
(A) OECs were placed on Matrigel™ and tube formation was studied at 24 h. OECs quickly formed endothelial tubes with a typical honeycomb-like network. (B) OECs were allowed to spread on fibronectin for 6 h and stained using rabbit anti-human FAK (phospho Y397) antibodies followed by secondary staining with goat anti-rabbit Alexa488 (green). Nuclei are stained with DAPI (blue).