Experimental Diabetes Research

Vascular Stem and Progenitor Cells in Diabetic Complications

Guest Editors: Gian Paolo Fadini, Paolo Fiorina, Paolo Madeddu, and Johannes Waltenberger

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Editorial Board

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Editorial

Vascular Stem and Progenitor Cells in Diabetic Complications

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Hyperglycemia and its associated biochemical abnormalities damage vascular wall cells, especially the endothelium, leading to an increased risk of cardiovascular events and disease, as well as microangiopathy and end-organ complications. In the last decade, accumulating data suggest that vascular repair mechanisms are important to maintain normal homeostasis of the arterial wall and to prevent development of pathologic processes, such as atherosclerosis, restenosis, and microvascular disease.

Diabetes mellitus, through the impairment of vascular stem and progenitor cells, entails a defective repair of the injured endothelium. The biochemical and cellular mechanisms that account for reduced or functionally impaired vascular progenitor cells in diabetes are not fully elucidated, and this is an intense area of research. Additionally, therapeutic approaches to modulate the endogenous reparative/regenerative processes are of particular interest in the setting of experimental and clinical diabetes research.

For this special issue of Experimental Diabetes Research, we invited investigators to contribute with original research articles and review articles that stimulate the continuing efforts to understand the molecular and cellular aspects underlying defective vascular repair by means of stem/progenitor cells in diabetes, as well as the development of interventions to reverse it.

The journal has received a variety of valuable submissions spanning the pathophysiological and therapeutic implications of vascular stem/progenitor cells.

The pathophysiological implications are herein described in the setting of both diabetes and the metabolic syndrome. S. Devaraj and I. Jialal report how number and/or functionality of endothelial progenitor cells (EPCs) could emerge as a novel cellular biomarker of endothelial/vascular dysfunction and cardiovascular disease (CVD) risk in patients with the metabolic syndrome. In the setting of diabetes, a focus review highlights the central contribution played by bone-marrow-derived progenitor cells in the development and progression of chronic complications. Not only are EPCs reduced and dysfunctional in diabetes, but they also appear to have a deranged differentiation capacity, which is shifted toward a procalcific phenotype that may have a negative impact on ectopic calcification and atherosclerosis. Of note, circulating progenitor cell phenotypes are not limited to EPC, but may include a variety of lineage-committed cells relevant for the pathobiology of diabetic complications. As an example, the level of pericyte progenitor cells (PPCs) in type 2 diabetes appears to be related to microangiopathy in response to glucose-lowering therapy. Among disparate complications, retinopathy has received a special attention: while G. Tremolada and colleagues provide a comprehensive analysis of

the mechanisms of neoangiogenesis in the diabetic retina, R. Longeras et al. show how pigment-epithelium-derived-factor- (PEDF-) 34 attenuates EPC mobilization from the bone marrow into the bloodstream during retinal neovascularization. This therapeutic approach can now be considered part of the armamentarium available to reverse microangiopathy, through regenerative cells. In parallel, S. Bernardi et al. provided an analysis of cell-based strategies to counter diabetic complications that have been so far devised and applied in the experimental and clinical settings. Besides cell therapies, several other pharmacologic and nonpharmacologic approaches have shown ability to reverse EPCs dysfunction in diabetes.

In conclusion, this special issue provides a series of updated reviews on vascular stem/progenitor cell defects in diabetes and on the therapeutic approaches to reverse them and counter diabetic complications. Original contributions help us to dissect the complexity of vascular stem/progenitor cell biology and trace the way for future studies in this field.

Amazingly, circulating progenitor cells are uncovering an entirely new scenario in diabetology research: it is all in the blood!

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Review Article

The Role of Angiogenesis in the Development of Proliferative Diabetic Retinopathy: Impact of Intravitreal Anti-VEGF Treatment

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Although cellular and molecular bases of proliferative diabetic retinopathy are only partially understood, it is evident that this complication of diabetes is characterized by the formation of new vessels inside the retina showing abnormal architecture and permeability. This process, if not controlled by selective laser photocoagulation, leads to irreversible retinal damages and loss of vision. Angiogenesis, that is, the condition characterized by the growth of new blood vessels originated from preexisting ones, was shown to have a major role in the pathogenesis of proliferative retinopathy and, as a consequence, intravitreal antiangiogenic injection was suggested as a feasible treatment for this disease. Here, we describe the different antiangiogenic approaches used to treat this disease along with the respective advantages and limitations when compared to laser treatment. Altogether, even though further and longer studies are still needed to clarify the best possible therapeutic protocol, the antiangiogenic treatment will reasonably have a future role in the therapy and prevention of proliferative diabetic retinopathy.

1. Introduction

As a consequence of the ongoing worldwide epidemic of type 2 diabetes [1], we expect that in few years a similar outbreak of diabetic complications, and in particular of diabetic retinopathy, will eventually follow [2, 3]. Among the complications of diabetic retinopathy, which carry an important vision impairment, there are diabetic macular edema and proliferative diabetic retinopathy. More recent data comes from study conducted in USA in which investigators estimated that the prevalence of diabetic retinopathy was 28.5%, among persons with diabetes aged 40 years and older. Approximately, 1.5% of adults with diabetes had proliferative diabetic retinopathy and 2.7% had clinically significant macular edema [4].

In particular, the proliferative stage is characterized by the formation of new leaky vessels spreading without regular orientation on the retinal surface, often invading the vitreous cavity, and finally leading to hemorrhage, fibrosis, and tractional retinal detachment.

Despite the evidence that the prevalence of proliferative diabetic retinopathy (PDR) is progressively decreasing as a consequence of the improved techniques aimed to control glucose metabolism [4–7], the overall situation is worsening once again as a consequence of the increased prevalence of type 2 diabetes. A triplication of new cases of PDR is foreseen in the next forty years [2].

2. Angiogenesis

Angiogenesis is the physiologic condition characterized by the growth of new blood vessels originated from preexisting ones.

The angiogenic process follows several steps: first of all, a number of angiogenic growth factors activate the receptors present on resident endothelial cells. Once activated, the endothelial cells begin to release specific enzymes called proteases that degrade the basement membrane, finally allowing endothelial cells to leave the original (parental) vessel wall. At this stage, endothelial cells proliferate into the surrounding matrix, taking advantage of adhesion molecules called integrins. Angiogenesis may represent a pharmacological target for combating diseases characterized by either poor vascularization or hypertrophic vasculature. Antiangiogenic therapies, in particular, are presently employed to fight cancer and other malignancies.

Concerning the eye, the angiogenic process has to be considered as a pathologic phenomenon. There are actually several conditions leading to the formation of abnormal neovascularization. Age-related macular degeneration is one of the most important diseases characterized by the formation of choroidal new vessel in the macular region finally leading; if untreated, to vision loss. The other major disease characterized by abnormal formation of retinal vessels is diabetic retinopathy, in particular the so-called proliferative stage of this disease. Regarding both retina and choroid, vascular endothelial growth factor (VEGF) was shown to be a major contributor to angiogenesis by increasing the number of new capillaries.

VEGF concentration levels, in particular, were found to be significantly increased in ocular tissues from patients with diabetes [8]. This finding raised the question of the potential role of VEGF in the pathogenesis of DR.

3. Role of Angiogenesis in the Pathogenesis of PDR

A number of evidences suggest that VEGF, and consequently angiogenesis, is involved in the pathogenesis of PDR [9]. The finding that most of VEGF production in solid tumors is due to hypoxia stimulus [10] suggested that VEGF might be an ideal candidate to mediate the hypoxia-induced intraocular neovascular response. Furthermore, VEGF is both an endothelial specific mitogen and vascular permeability factor. This finding suggests that VEGF could account for both proliferation and vasopermeability in course of proliferative diabetic retinopathy [11].

In humans, diabetic patients with established PDR have indeed high levels of VEGF in the vitreous and this dysfunction can be normalized only by laser photocoagulation [12]. Accordingly, in mouse models of ischemic retinopathy, it is also possible to prevent the development of the proliferative stage by blocking VEGF activity [13]. Finally, intravitreal injection of VEGF was shown to cause iris neovascularization in primates [14].

From a functional point of view, VEGF has been identified as a proinflammatory mediator, reasonably involved in the development of the inflammatory process that accompanies the progression of DR. VEGF actually increases the expression of the cellular adhesion molecule ICAM-1 a chemotactic factor for monocyte/macrophage lineage cells [15, 16]. Through the activation of ICAM-1, VEGF, therefore, promotes leukostasis (and vascular leakage) and increases leukocyte counts in the retinas of diabetic animals [17, 18] and in human diabetic retinas [19] Conversely, blockage of VEGF decreases retinal leukocyte counts in experimental diabetes [20]. Altogether these findings provided a robust rationale for the setting up of clinical trials to verify VEGF blockade as a therapy for DR.

4. Intravitreal Anti-VEGF Treatment as a Therapy for PDR: Comparison with Laser Treatment

Panretinal photocoagulation (PRP) is at present the only successful evidence-based treatment for PDR. PRP reduces in fact the risk of severe visual loss by 50–60% with regression of the majority of neovascularizations over a period of 3 months. In particular, it was shown that when PDR regresses within the first 3 months after PRP treatment, the visual prognosis tends to be excellent [21].

Among the proposed mechanisms underlying PRP effectiveness are reduced oxygen requirement that follows the destruction of the highly metabolically active outer retinal cells and improved retinal oxygenation derived from choroidal circulation. Several attempts have been made to modify PRP laser techniques to reduce side-effects such as decreased visual acuity, peripheral field loss, and macular edema [22]. Despite this evidence, several patients still require supplemental laser treatment, and nearly 4.5% show disease progression that finally require pars plana vitrectomy (PPV), even in presence of an adequate PRP [23].

Limits of PRP include poor response to treatment, pain, nyctalopia, loss of peripheral vision, uveal effusions, worsening of macular edema, and difficulty to treat eyes with vitreous hemorrhage.

Most patients require at least two treatment sessions and several return for multiple additional sessions in case of persistent neovascularisation.

Altogether, nondestructive approaches alternative to PRP, such as VEGF inhibition, have been recently investigated as possible new therapies for PDR [24].

The molecules currently under investigation to treat PDR are Macugen (Pegaptanib sodium), Lucentis (Ranibizumab), and Avastin (Bevacizumab). The widespread use of these molecules in clinical practice is so far limited by their short-lived effects and the lack of established protocols.

4.1. Macugen. Pegaptanib sodium (Macugen, Eyetech Inc, Cedar Knolls, NJ, USA) is a 28-nucleotide RNA aptamer that binds specifically to the VEGF-A165 isomer, the major pathological VEGF protein in the eye.

4.2. Lucentis. Ranibizumab (Lucentis; Genentech USA, Inc., San Francisco, CA, USA/Novartis ophthalmics, Basel, Switzerland) is an engineered, humanized, recombinant antibody fragment (Fab) active against all VEGF-A isoforms. As it lacks the Fc domain, it has a much shorter half-life than other anti-VEGF agents. Lucentis is presently licensed

as an intravitreal agent for the treatment of wet, age-related, macular degeneration (ARMD).

4.3. Avastin. Bevacizumab (Avastin; Genentech Inc., San Francisco, CA, USA) is a full-length recombinant humanized antibody active against all isoforms of VEGF-A. This large sized molecule (molecular weight: 148 kDa) has two times the half-life of ranibizumab, with a prolonged effect on retinal neovascularisation [25].

Bevacizumab is currently not licensed for intraocular use but is nonetheless the most used among anti-VEGF agents. Three randomized nonplacebo controlled trials on intravitreal bevacizumab for the treatment of PDR have been recently published [26–28]. Several other clinical trials are presently ongoing (http://www.clinicaltrials.org).

The standard average endpoint for evaluating the effectiveness of anti-VEGF treatments is commonly considered as the persistence of the effect of treatment for at least 6 months after the intraocular injection. Unfortunately, few clinical studies include a 6-month followup. Comparative analysis between different studies is not so simple as quantitative evaluation of the extent and severity of neovascularization differ between individuals. And this makes more difficult the translation of results of these studies into clinical practice.

In a retrospective analysis, Adamis et al. [29] demonstrated a persistent beneficial effect of intravitreal pegaptanib in patients with PDR, with 62% of the treated eyes showing regression or absence of neovascularization 6 months after injection.

A recent study from Cho et al. [30] studied the effects of intravitreal injection of Bevacizumab on VEGF expression and inflammation in fibrovascular membranes from 18 patients with PDR. An immunohistochemical staining for VEGF, CD31, and CD68 was performed in three different groups; group 1 : 4 inactive PDR eyes, group 2 : 10 active PDR eyes treated preoperatively with adjunctive intravitreal bevacizumab, group 3 : 5 active PDR eyes not treated preoperatively with bevacizumab. They found that IVB caused some reduction in VEGF expression and vascular densities in a limited number of active PDR patients, but they also demonstrated that a single injection may not be enough to induce complete blockage of VEGF and pathologic neovascularization in active PDR patients.

A possible solution to overcome these limits in efficacy could result from combined therapy consisting in laser treatment followed by intravitreal injection of anti-VEGF. This approach could have some advantage by increasing the extent of treatment, by accelerating the effect of laser photocoagulation, and by providing alternative therapeutic solutions when laser delivery by itself is difficult or impossible.

5. Bevacizumab

Concerning Bevacizumab and laser treatment, the study done by Mirshahi et al. [31] is probably the largest performed to date. Forty patients with type 2 diabetes and PDR in both eyes with high risk profile underwent scatter laser treatment following the ETDRS protocol and had a single bevacizumab injection in one eye; sham injection was performed in the controlateral eye used as control. This study demonstrated that at week six 87.5% of eyes treated with bevacizumab had complete regression of neovascularization versus 25% in the sham treated group ($P < 0.005$). At week 16, the difference between the two groups disappeared. This study provides further evidence that bevacizumab has an inhibitory effect on the formation of new vessels. This study allows to conclude that intravitreal bevacizumab is a valid treatment for early high-risk PDR.

5.1. Eyes Resistant to Panretinal Photocoagulation (PRP). The effect of intravitreal Bevacizumab in eyes with persistent, active PDR was assessed by Jorge and colleagues in a noncomparative trial [32]. One injection of bevacizumab was administered to 15 eyes that were then followed for 12 weeks. As a result, best-corrected visual acuity (BCVA) was improved significantly from baseline at all time points (1, 6, and 12 weeks), from 20/160 at baseline to approximately 20/125 at 12 weeks. The mean area of fluorescein leakage was also improved significantly at all time points. No significant adverse events could be demonstrated. If these results will be confirmed by further and larger studies, bevacizumab will be identified as an important intervention for eyes with refractory PDR.

Intravitreal bevacizumab for cases that were not responsive to traditional PRP has been evaluated in another study by Moradian and colleagues [33]. Thirty eight eyes received a bevacizumab injection at baseline, and after 6 or 12 weeks according to the research protocol. Clearance of vitreous hemorrhage and regression of active fibrovascular tissue were considered as endpoints. A tendency toward resolution of vitreous hemorrhage with a trend toward significance could be shown at 6 weeks ($P = 0.06$). No significant change in the extent of fibrovascular tissue occurred, even though several eyes could not be evaluated for this variable because of media opacity. The most remarkable finding in this study was probably the occurrence of two tractional retinal detachments (5.3% of study eyes). This finding is in line with the report by Arevalo and colleagues that 5.2% of the eyes with PDR developed TRD after an extra intravitreal bevacizumab injection performed before vitrectomy [34].

Taken together, the above-described studies suggest that intravitreal bevacizumab decreases leakage from diabetic neovascular lesions in newly diagnosed and refractory disease. Further studies are now necessary, particularly on possible long-time side effects before we will be able to translate these research findings into clinical practice.

5.2. In Case of Vitreous Hemorrhage. Persistent and recurrent vitreous hemorrhage is a common complication of vitrectomy for diabetic retinopathy with an incidence ranging from 12% to 63% [35].

Bevacizumab was shown to reduce intra- and postoperative bleeding and surgical operating times when used before the surgical removal of vitreomacular membranes [36–38] In most studies, bevacizumab has been administered for just

one week preoperatively, to avoid the occurrence of tractional retinal detachment in patients with severe PDR [33, 34].

In a study, Rizzo and colleagues randomized 22 eyes with severe PDR and TRD either to intravitreal bevacizumab 5 to 7 days before PPV or to placebo [28]. As a result, they demonstrated that difficulties in the surgical procedure, as evaluated by recording operative times, number of instrument exchanges, number and severity of intraoperative bleeds, dissection techniques, and intraoperative retinal tears were reduced in the bevacizumab group.

Similarly, Yeh and colleagues tested the effect of bevacizumab as an adjuvant therapy 1 week before vitrectomy [37]. They enrolled 41 eyes with severe PDR and active fibrovascular proliferation extended to the periphery. The authors randomized these eyes to bevacizumab or to placebo. As a result, intraoperative bleeding from proliferative tissue was significantly worse in the control group, even though intraoperative subretinal hemorrhage was more frequent in the bevacizumab group ($P = 0.004$). The authors concluded that although an increased rate of intraoperative subretinal hemorrhage occurred in the bevacizumab group, several potential benefits of the drug finally outweighed the observed adverse effects.

A large trial of bevacizumab along with vitrectomy has been recently performed by Ahmadieh and colleagues [38]. The authors randomized 68 eyes scheduled to undergo PPV for PDR to intravitreal bevacizumab, 1 week before PPV, or to sham injection. Only 34 eyes completed the study as in several cases treated with bevacizumab a significant improvement during the week after the injection could be demonstrated. The incidence of postvitrectomy haemorrhage 1 week and at 1 month after surgery was significantly lower in the group treated with bevacizumab compared to the controls ($P = 0.023$ and $P = 0.001$, resp.). Also intraoperative bleeding was significantly less in the bevacizumab group ($P = 0.035$), as was the need to use intraoperative endodiathermy.

Altogether, the above-described studies indicate that bevacizumab before vitrectomy represent a valid approach for PDR. Injection of bevacizumab 1 or 2 weeks before PPV did not cause any adverse outcomes. Further studies with a larger number of patients are now warranted to confirm these preliminary results.

5.3. In Case of Neovascular Glaucoma. Anti-VEGF agents might have role in the management of one of the most severe forms of secondary glaucoma, the so-called neovascular glaucoma (NVG).

On this regard, Chalam et al. [39] reported complete regression of neovascularization due to aggressive NVG within 3 weeks from the treatment with bevacizumab.

A trial on 26 eyes with NVG was performed by Costagliola et al. [40]. The authors demonstrated that at the end of the treatment, in all patients, it was possible to appreciate a regression of neovascularisation paralleled by a reduction of intraocular pressure (IOP). After one year of followup, however, three eyes required glaucoma valve implants and 14 patients were treated with standard glaucoma medication.

A massive regression of iris neovascularization in a 2-week period and no significant changes in IOP could be demonstrated in NVG patients treated with injection of bevacizumab by Lim et al. [41].

Finally, Eid et al. [42] recently demonstrated that combining bevacizumab with good PRP ablated the ischaemic retina and ensured good success rates in 20 patients with intractable glaucoma.

5.4. In Case of Chataract Surgery. Sixty-eight eyes with any type of DR at the end of cataract surgery were randomized to bevacizumab by Cheema and colleagues [43]. As a result, 1 month after treatment, 5 control eyes progressed in the severity of DR versus only four treated eyes ($P = 0.002$). Macular edema was also more common in control eyes.

Takamura and colleagues also injected bevacizumab at the conclusion of cataract surgery in diabetic patients [44]. During the followup the treated eyes, when compared to control eyes had a significant improvement with respect to preoperative measurements.

A similar study was performed by Lanzagorta-Aresti and colleagues [45] in patients with moderate NPDR and DME. Twenty-six eyes that underwent laser treatment followed by uncomplicated cataract surgery received bevacizumab or sham injection. As a result, the treated group showed a significant improvement in BCVA and no change in CMT. The sham group showed a worsening of visual acuity and a significant increase in CMT. Although the results look promising, further studies are now necessary to confirm these early findings.

6. Pegaptanib Sodium

The effect of intravitreal Pegaptanib (Macugen) on diabetic macular edema [46] was evaluated in retrospective analysis aimed to compare the effect of pegaptanib on ocular neovascularization to a sham group. Sixteen subjects were included in the study. Eight subjects in the intravitreal pegaptanib group ($n = 13$) showed regression of neovascularization (62%) at 36 weeks, whereas none of the eyes in sham group ($n = 3$) showed regression of neovascularization. However, in three of the eight treated eyes (37.5%), ocular neovascularisation recurred at the end of followup.

More recently, González et al. [47] performed a prospective, randomized, controlled, open label study aimed to clarify the efficacy of intravitreal pegaptanib versus PRP in the treatment of active PDR. As a result, by week 12, in all eyes receiving pegapanib, a complete regression of retinal proliferation could be demonstrated and was maintained through week 36.

7. Ranibizumab

There are no final reports on the effect of ranibizumab on PDR [48]. The Diabetic Retinopathy Clinical Research Network (DRCRnet) is presently performing a randomized prospective controlled trial to determine whether intravitreal ranibizumab or a steroid given to patients with PDR and

macular edema can reduce the risk of visual loss following PRP and provide good visual outcomes over a short term. The primary outcome measure includes visual acuity outcomes at 14 weeks. Secondary outcome measures include changes in retinal thickness, presence, and extent of new vessels on fundus photos and vitreous haemorrhage.

The study is currently closed and the scientific community is waiting for the final results. (Intravitreal Ranibizumab or Triamcinolone Acetonide as Adjunctive Treatment to Panretinal Photocoagulation for Proliferative Diabetic Retinopathy. Available at: http://drcrnet.jaeb.org/ Accessed: May 10, 2011).

8. Other Anti-VEGF Drugs

Among the others, a new and alternative way to block VEGF is represented by VEGF-trap (aflibercept). VEGF-trap is a fusion protein made of immunoglobulin domains of both VEGF-1 and -2 fused to an Fc-fragment of human IgG. VEGF-trap acts as a soluble receptor as it is able to bind every isoform of extracellular VEGF [49]. Whether this approach is really effective and may reduce the side effects of standard anti-VEGF therapy remains to be seen. A major problem with pan-isoform blockade of VEGF is indeed the decrease in physiologic revascularization, a process that is important in preventing PDR [50].

RNA interference is a classic example of basic research that has moved from bench to bedside. Intracellular transcription of VEGF can actually be shut down by means of RNA interference, finally decreasing the production of VEGF released from the retinal pigment epithelium. This kind of approach is presently studied in treatment of wet AMD [51]. As previously done with other anti-VEGF drugs, after demonstration of its safety and efficacy in neovascular AMD, RNA interference will for sure explore also patients with PDR.

Finally, another novel therapy may consist in the use of small molecules that, acting as tyrosine kinase inhibitors, become able to inhibit the intracellular signaling cascade of VEGF. These substances could be of use in the treatment of PDR [52], although the results of preliminary studies seem to suggest the exacerbation of diabetic neuropathy as a possible, not irrelevant, side effect [53].

There are presently just few studies aimed to evaluate the above-described new drugs in the treatment of diabetic retinopathy, and they are all referred to patients with diabetic macular edema.

9. Limits of Anti-VEGF Treatment

A major limit in anti VEGF treatment consists in the evidence that recurrence of retinal neovascularisation following anti-VEGF treatment is a quite common finding in a period that ranges between 2 weeks [54] to 3 months [55, 56], after injection. A reinjection 3-month after the baseline is probably a reasonable timing in most cases, especially in case of patients with high-risk PDR. Results in this field are still discrepant between different groups. Minnella reported that the effects of bevacizumab were maintained at 3 months in 15 treated eyes [57].

Conversely, Schmidinger et al. [58] reported that 62% (8 of 13) of treated eyes required retreatment with bevacizumab 3 months after baseline injection because of the appearance of new vessels.

10. Side Effects of Anti-VEGF Treatment

Along with its therapeutic effect on ocular neovascularization, Bevacizumab treatment may be accompanied by a number of side effects. Tractional retinal detachment (TRD) may sometime affect patients with severe PDR [59] treated with this drug. It has been hypothesized that bevacizumab might induce a fibrotic occlusion of new vessels. The contraction of this fibrous tissue may, therefore, result in TRD and vitreous haemorrhage [60–62]. Alternative mechanisms underlying the development of TRD could be the high fluctuations in intraocular pressure (IOP) [63] and deformation of the eye during intravitreal injection with possible intrusion of the vitreous in the sclera, resulting in vitreoretinal traction [64]. A possible explanation for the increased IOP could be the blockage of the internal trabeculae by bevacizumab itself that, being a large 148-kDa protein, may act as an additional barrier [65].

Lee and Koh [66] documented angiographically a foveal avascular zone enlargement following pars plana vitrectomy and treatment with bevacizumab. The authors attributed this finding to a total, nonselective blockage of VEGF levels, when it is well established that physiological concentrations of VEGF are thought to be essential for maintaining foveal circulation and visual acuity.

Further studies are needed to verify the systemic side-effects of anti-VEGF agents, particularly in diabetic subjects with significant vascular complications. Among the systemic side effects, the most common is hypertension (5.6%), followed by other cardiovascular complications [67, 68]. The use of bevacizumab in women of child bearing age need to be carefully monitored Kumar et al. [69].

At the moment, a large prospective trial aimed to verify the presence of short- and long-term adverse effects of bevacizumab treatment is still lacking.

The largest dataset for bevacizumab treatment is presently represented by a retrospective study [70] of 1,173 patients who received intravitreal bevacizumab and were followed for 12 months. A number of adverse effect were reported: seven cases of acute elevation of blood pressure, six strokes, five myocardial infarctions, five deaths, seven cases of bacterial endophthalmitis, seven cases of tractional retinal detachment, and four cases of uveitis.

Mason and colleagues retrospectively studied 5,233 intravitreal bevacizumab treatments and found a single case of acute postinjection endophthalmitis [71]. Safety concerns the use of bevacizumab comes from studies of the intravenous use in cancer therapy. Established side-effects in these studies include arterial thromboembolism, gastrointestinal perforation, hemorrhage, hypertensive crisis, and nephrotic

syndrome [72, 73]. Concerning other anti-VEGF treatments, the VISION trial performed in patients with neovascular AMD treated with intravitreal pegaptanib [74, 75] reported no systemic side effects that could be attributed to treatment over the course of the study. Some rare specific ocular complications, such as endophthalmitis, traumatic lens injury, or retinal detachment, were attributed to the injection procedure rather than to the medication.

The MARINA and ANCHOR studies aimed to treat neovascular AMD, reported the safety of intravitreal ranibizumab. The MARINA study (two-year observation) showed no increase in systemic adverse effects with ranibizumab [76]. By pooling together the safety data from PIER, MARINA, and ANCHOR (one-year observation) it was possible to demonstrate an increased rate of vascular events (2.1% rate of myocardial infarction and stroke) in the ranibizumab arms versus the control (1.1%) [77].

Finally, although VEGF has been implicated in the development of a number of ocular neovascular diseases, physiologic concentrations of endogenous VEGF play a strong role not only in maintaining the correct perfusion of the retina, but they also have a key role in the survival of the retinal neuron, the Muller cell, and photoreceptors [78, 79].

A recent study conducted in mouse eyes, in fact, reported a significant loss of neuronal retinal ganglions cells due to a chronic inhibition of VEGF [80]. Caution must be warranted.

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Review Article

It Is All in the Blood: The Multifaceted Contribution of Circulating Progenitor Cells in Diabetic Complications

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Diabetes mellitus (DM) is a worldwide growing disease and represents a huge social and healthcare problem owing to the burden of its complications. Micro- and macrovascular diabetic complications arise from excess damage through well-known biochemical pathways. Interestingly, microangiopathy hits the bone marrow (BM) microenvironment with features similar to retinopathy, nephropathy and neuropathy. The BM represents a reservoir of progenitor cells for multiple lineages, not limited to the hematopoietic system and including endothelial cells, smooth muscle cells, cardiomyocytes, and osteogenic cells. All these multiple progenitor cell lineages are profoundly altered in the setting of diabetes in humans and animal models. Reduction of endothelial progenitor cells (EPCs) along with excess smooth muscle progenitor (SMP) and osteoprogenitor cells creates an imbalance that promote the development of micro- and macroangiopathy. Finally, an excess generation of BM-derived fusogenic cells has been found to contribute to diabetic complications in animal models. Taken together, a growing amount of literature attributes to circulating progenitor cells a multi-faceted role in the pathophysiology of DM, setting a novel scenario that puts BM and the blood at the centre of the stage.

1. The Burden of Diabetic Complications

Diabetes mellitus (DM) has reached a worldwide growing epidemic diffusion. DM is associated with a significantly reduced quality of life and represents an important health and social problem. Most importantly, DM leads to severe complications in many organs and tissues through the induction of microangiopathy and macroangiopathy. Hyperglycemia-induced biochemical abnormalities, such as overactivation of PKC and MAPK, excess flux through the exosamine and polyol pathways, and production of advanced glycation end-products (AGEs), all stem from the high concentration of reactive oxygen species induced by the overflowing mitochondrial respiratory chain [1]. These damage pathways induce profound changes in vascular endothelial and smooth muscle cells and subsequent modifications of the extracellular matrix (ECM). DM increases 2-3-fold the risk of cardiovascular disease (CVD), owing to the widespread endothelial dysfunction, which is considered the first step in the atherogenetic process [2, 3]. Atherosclerotic vascular disease in DM is aggressive, multifocal, distal and develops earlier than in non-DM subjects. Importantly, other cardiovascular risk factors that typical associate with DM, such as hypertension, obesity and dyslipidemia, concur to the accelerated risk of CVD. Microvascular complications, including retinopathy, nephropathy, and neuropathy, develop as a consequence of structural and functional damage to the microcirculation of target organs. Typical morphological features include thickening of the basement membrane, loss of pericyte coverage, capillary rarefaction, excess deposition of stiff EMC components leading to reduced perfusion, atrophic changes, and fibrosis. All these morphological features are reflected by organ dysfunctions, including visual loss, impaired glomerular filtration or tubular resorption, reduced nerve conduction velocity. Importantly, organs that are less commonly recognized among the targets of diabetic microangiopathy are the myocardium, the lung, and the bone marrow (BM).

2. The Plasticity of Circulating Progenitor Cells

In the adult organism, the BM represents the privileged site of hematopoiesis and the reservoir of stem/progenitor cells. In the last decades, it has been recognized that the BM harbours small subsets of progenitor cells for multiple cell lineages, not limited to the hematopoietic system [4, 5]. These cells can leave the BM upon appropriate stimulation and migrate in peripheral organs through the bloodstream. The prevailing concept is that immature cells in the BM niche retain plasticity and can undergo a multilineage differentiation, recapitulating some developmental steps taking place in embryonic stem cells. The best known form of this phenomenon is endothelial differentiation of BM-derived cells, which gives rise to endothelial progenitor cells (EPCs) [6]. Cell-tracking experiments using BM chimeric mice expressing the green fluorescent protein (GFP) or other reporters found that BM-derived cells can repopulate several organs and tissues, differentiating into multiple phenotypes [7–9]. Similarly, the study of rare cases of human sex-mismatched transplantation allowed to follow the fate of BM-derived cells by looking at the signal of the sex chromosomes and showed repopulation of the myocardium, lungs, kidney, and gastrointestinal tract by donor-derived cells [10–13]. It should be noted that not all studies unequivocally confirm the ability of BM-derived cells to contribute to peripheral cellular phenotypes different from hematopoietic cells [14, 15]. This discrepancy may depend upon the use of different cell tracking methods, imaging techniques, and disease models.

3. Endothelial Progenitor Cells

EPCs are immature BM-derived cells which undergo differentiation into endothelial cells and participate in endothelial repair and neoangiogenesis [6]. EPCs are commonly defined and enumerated by flow cytometry based on the co-expression of stemness antigens (e.g., CD34 and/or CD133) and endothelial markers (e.g., KDR). EPCs can also be isolated from circulating mononuclear cells using disparate culture protocols yielding heterogeneous cell types (reviewed elsewhere [16]). Briefly, it should be taken into account that a net separation between EPCs and hematopoietic cells, either progenitor or myeloid lineage-committed cells, is not always possible. As a result, several cultured EPC phenotypes retain overlapping features with the hematopoietic system [17]. EPCs can be mobilized from the BM into the peripheral blood in response to many stimuli including tissue ischemia, cytokines, and growth factors [18]. Once in the bloodstream, EPCs home specifically to sites of vascular damage to repair the disrupted endothelium and to provide pro-angiogenic stimuli in an attempt to restore blood flow and counter shortage of oxygen and nutrients [19]. With these two seminal functions, it is easy to understand how EPCs act as an integrated component of the cardiovascular system, which is subjected to pathological changes and is also a target of therapy. Importantly, EPCs are profoundly altered in the setting of type 1 and type 2 DM [20]. Several antigenic EPC phenotypes (e.g., CD34+KDR+) are profoundly reduced in the blood of type 2 diabetic patients compared to controls, independently of concomitant risk factors [21]. Pauperization of EPCs in diabetes is thought to explain, at least in part, the high CVD risk associated with DM, as patients would be less able to repair the endothelial injury and to counter ischemia with neoangiogenesis. Indeed, there is a close negative correlation between the severity of vascular disease and the level of circulating EPCs in diabetic patients [22]. The reduction of EPCs may also intervene as a pathogenic factor in microangiopathy, as clinically significant correlations have been found in the setting of retinopathy, nephropathy, and wound healing [23–25]. Not only EPCs are reduced in the bloodstream of diabetic patients, but they also show functional defects, such as impaired adhesion, proliferation, and tubulogenesis [22, 26]. These data support the notion that an altered EPC biology in DM compromises the ability to counter the excess damage caused by hyperglycemia and the associated biochemical abnormalities [27]. Besides a pathophysiological role in diabetic complications, the level of circulating EPCs may also represent a biomarker of future risk, as progenitor cell counts independently predict the occurrence of adverse cardiovascular events in different cohorts of patients [28, 29].

4. Smooth Muscle Progenitor Cells

Circulating SMPs were originally identified by studies in which mice were transplanted with genetically labelled BM and, after vascular injury, it was found that a quote of cells within the neointima coexpressed BM-tracing markers and alpha-SMA [7, 30, 31]. While these findings were not confirmed by other investigators [32, 33], data also accumulated on the possibility to isolate SMPs from peripheral blood mononuclear cells using different culture protocols (reviewed in [34]). The exact phenotype of SMPs is unclear and residual overlapping with the hematopoietic system (such as CD14 and/or CD45 expression) may occur as for EPCs. EPCs and SMPs may also share a common ancestor and cells may undergo shift from and back each phenotype in vitro and in vivo [35]. SMPs can be obtained from the CD34+ population and/or from the CX3CR1+ myeloid population [36, 37]. The existence of SMP has important implications for tissue engineering, as SM cells are necessary to create vascular grafts, but also holds negative implications for vascular disease, in which SM cells may play detrimental roles. In the setting of DM, SM cell function and phenotype are altered and some cells are shifted from a contracting phenotype to a secreting phenotype [38]. Nguyen et al. have reported that PBMC from diabetic patients as compared to controls, when cultured in conditions that foster SM cell growth, gives rise to a higher number of SM-like progenitor cells expressing both contractile and fibrogenous markers [39]. These findings were suggestive of the fact that circulating progenitors in DM are shifted from the generation of an endothelial phenotype to a SM phenotype. As it has been shown that BM-derived cells home to sites of

glomerular damage [40], these cells may intervene in the setting of diabetic microvascular complications, contributing for instance in the progression of diabetic nephropathy. Consistently with these findings, In a mouse model of type 1 diabetes, Westerweel et al. found an accelerated generation of SMP from blood cells, probably driven by the TGF-beta signalling. In addition, GFP+ BM-derived cells coexpressing SM cell markers were recruited at sites of neointima formation after cuff arterial damage, which was increased in diabetic animals [41].

Pericyte progenitor cells (PPCs) represent a phenotype closely related to SMPs. They are typically defined by expression of pericyte markers, such as PGDFRbeta and/or NG2. PPCs can be isolated from mature blood vessels and show potent vasoregenerative potential [42], just as pericyte govern vascular stability. Circulating PPCs have been identified, although their origin is not clear [43]. We have reported that PPCs are increased in diabetic patients with microangiopathy after improvement of glucose control [44]. The clinical significance of this finding remains to be elucidated. Speculatively, increased vasoprotective PPCs may represent a beneficial effect of glucose control that translates into improved outcomes. Alternatively, the surge in PPC level may represent a consequence of microvascular lesion regression or even progression, as microangiopathy can occasionally worsen after rapid glucose control.

5. Cardiomyocyte Progenitor Cells

Early studies using GFP BM chimeric mice were able to detect an extensive repopulation of the infarcted myocardium by BM-derived cells, with initial evidence of transdifferentiation of homed cells into cardiomyocytes [8, 45]. In humans, a proof of concept of this biological phenomenon has been provided in sex mismatched heart transplants, showing high level of cardiac chimerism caused by the migration of primitive cells from the recipient to the grafted heart [10, 46, 47]. The phenotype and kinetics of BM-derived circulating cardiomyocyte progenitor cells (CPCs) have been subsequently investigated. For instance, Wojakowski et al. found that myocardial infarction induces the BM to release CD34/CXCR4+, CD34/CD117+, and c-Met+ progenitor cells, which express the cardiac genes GATA4, MEF2C, Nkx2.5/Csx [48]. The extent to which these cells engraft into the infarcted heart was not determined and the true quantitative contribution of BM-derived cells in myocardial remodelling after injury has been questioned [49, 50]. An important issue is that EPCs themselves have the potential to transdifferentiate into cardiomyocytes in vitro, when co-cultured with neonatal rat heart cells [51]. Interestingly, this phenomenon is impaired in patients with coronary artery disease and can be restored by statin therapy [52]. Whether cardiomyocyte differentiation of circulating progenitors is affected by diabetes remains to be established.

6. Osteoprogenitor Cells

Ectopic calcification is a hallmark feature of diabetic vasculopathy [53]. Calcification can develop in the medial layer or in the neointima of atherosclerotic plaques. Medial calcified arteriosclerosis leads to arterial stiffening and raises blood pressure, while neointimal calcification can destabilize the plaque and lead to rupture or hemorrhage. The mechanisms driving vascular calcification are incompletely understood, ranging from ion imbalance, loss of inhibitors, and cellular alterations [54]. The type of vascular cells giving origin to calcifying cells is also unclear, with SM cells, pericytes, and advential progenitor cells possibly being involved. Recent data show that circulating calcifying cells (osteoprogenitor cells, OPCs) contribute to intraplaque calcification [55], while a role for BM-derived cells in medial calcification has not been substantiated so far [56]. OPCs may originate from the BM hematopoietic or mesenchymal compartments. It has been shown that CD34+ cells and EPCs can express bone-related proteins, such as osteocalcin (OC) and bone alkaline phosphatase (BAP) and develop a tendency to form calcified nodules in vitro and when using in vivo assays [57]. These osteogenic EPCs, or OPCs, are increased in patients with coronary artery disease, and data in humans suggest that they are recruited from the bloodstream to the diseased coronary arteries [57, 58]. Expression of OC on EPCs correlates with arterial stiffness in humans [59], lending support to the hypothesis that OPG participates in arterial calcification. In diabetic patients with coronary artery disease, CD34+ cells show a phenotypic shift from endothelial commitment to a procalcific phenotype, as evidenced from the excess OC expression over KDR [60]. In cultured monocytic EPCs, this phenomenon may be attributable to inflammatory stimuli, as it can be recapitulated by LPS [60]. In addition, we have recently identified a subpopulation of circulating monocytes expressing OC and BAP, called myeloid calcifying cells (MCCs), that are increased in the BM, peripheral blood, and atherosclerotic lesions of diabetic patients compared to controls [61]. MCCs represent one aspect of monocyte plasticity and a novel indicator of deranged monocyte biology in the setting of DM. Finally, OPCs may also derive from the BM mesenchymal compartment and can be mobilized into the bloodstream in response to bone fractures [62]. The complex epidemiologic and pathophysiologic relationships between bone and vascular disease suggest that OPCs may be involved in the regulation of the bone vascular axis [63], through yet unidentified mechanisms. In support of this, osteogenic EPCs appear to be increased also in osteoporotic women [64].

7. Proinsulin-Expressing Cells

A few years ago, while studying gene therapy in streptozotocin (STZ) diabetic mice, a group of investigators detected expression of the insulin gene in several organs and tissues outside the endocrine pancreas [65]. Then, they identified proinsulin- (PI-) expressing cells that appear in animals after induction of hyperglycemia. These cells derive from the BM, resemble cells of the monocyte/macrophage lineageand display a proinflammatory phenotype, as evidenced by the expression of TNF-alpha. When looking at the distribution of the PI-expressing BM-derived cells (PI-BMDCs) throughout the rodent organism, authors found

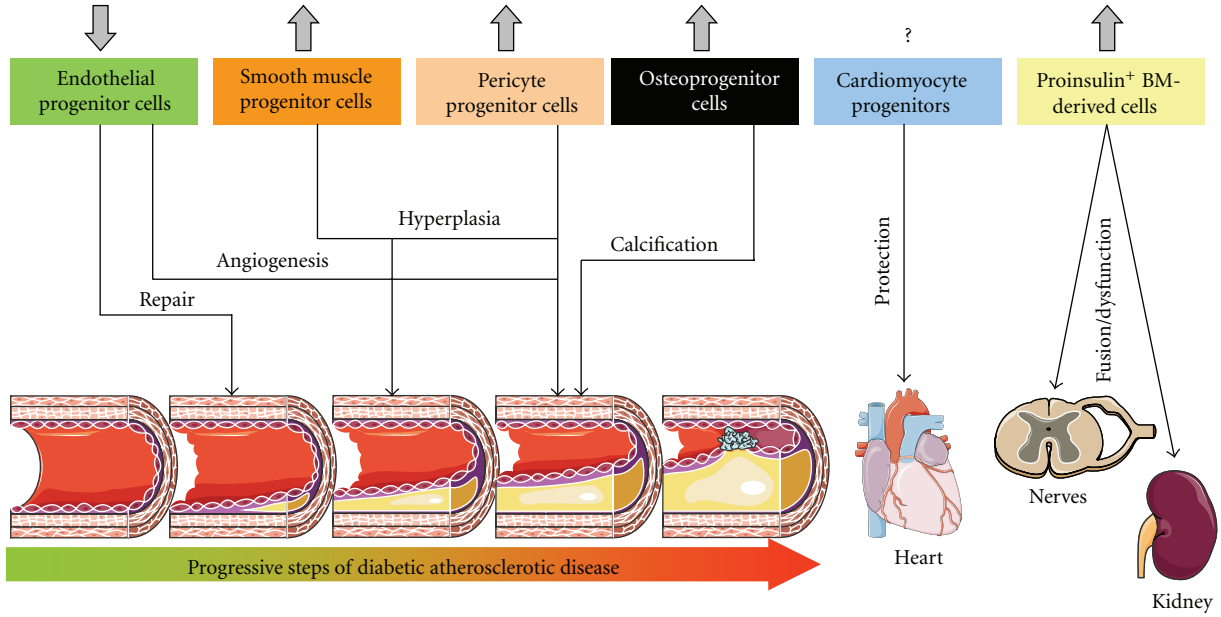

Figure 1: The multifaceted contribution of circulating progenitor cells in diabetic complications. Different lineage-committed progenitor cells are altered in the setting of diabetes and contribute to the development of diabetic complications. Grey arrows indicate the effects of diabetes of number and function of the various bone-marrow-derived cell subtypes.

these cells in multiple tissues and organs [66]. Importantly, PI-BMDCs appear to have enhanced fusogenic properties, at least in part mediated through the diabetes-specific PARP-1 pathway [67]. Fusion of PI-BMDCs with resident cells has been shown to contribute to diabetic complications [68]. For instance, fusion of hematopoietic cells with peripheral neurons impairs nerve function in a diabetic mouse model [69, 70]. Additionally, fusion of PI-BMDCs with renal tubule cells is believed to contribute to the development and/or progression of diabetic nephropathy, as the resulting polyploid cells are proinflammatory and interfere with normal tubule function [71]. The fusogenic properties of these cells are abolished when mice are transplanted with BM cells from PARP−/− donors [67]. Interestingly, an excess generation of these proinflammatory PI-expressing myeloid cells after development of diabetes may contribute to virtually all diabetic complications, by means of fusion with resident cells. This is an entirely new mechanism of action that link BM cells to distant target organs. Whether or not this mechanism is active also in humans needs to be addressed.

8. The Diabetic Bone Marrow

The profound alterations of all these circulating progenitors intuitively led investigators to hypothesize a BM defect associated with DM. In 2006, we first reported that BM mobilization of progenitor cells is impaired in diabetic animals compared to controls after stimulation by ischemia or exogenous mobilizing agents (G-CSF and SCF) [72]. The postischemic mobilization was defective in DM because ischemia was unable to upregulate the hypoxia sensing system HIF-1alpha and its downstream targets (such as SDF-1alpha), which signal the BM for the need of vasoregenerative progenitor cells, like EPCs. This pathway has been subsequently confirmed by others and defects of the HIF-1alpha pathway in DM have been better elucidated [73, 74]. On the other hand, to explain the impaired progenitor cell mobilization after direct BM stimulation, an intrinsic BM defect had to be postulated. Recently, Oikawa et al. have shown that DM induced BM microangiopathy with morphological features similar to other typical diabetic microvascular complications, including basement membrane thickening, capillary rarefaction and apoptosis [75]. As a functional consequence stem cell niche characteristics were altered, thus potentially affecting the BM response to mobilizing agents. Busik et al. have found that DM impairs autonomic bone marrow innervation, which is critical for G-CSF induced mobilization of stem/progenitor cells. This BM neuropathy, in turn, compromised the extent and timing of progenitor cell release, an event that preceded the development of distant vascular complications [76]. The early onset of bone marrow defect in the natural history of diabetes is also suggested by a study showing that CD34+ cells start to decline in prediabetes and show a first nadir in newly diagnosed type 2 DM [77]. More recently, Ferraro et al. showed that STZ diabetes in mice interrupts the dynamic anatomy of the BM stem cell niche suggesting a defect in the activation of the sympathetic nervous system with consequent impaired SDF-1alpha regulation. As a clinically relevant counterpart, they show in a retrospective case series that G-CSF stem cell mobilization in patients undergoing autologous transplantation is impaired in the presence of diabetes or hyperglycemia [78]. This issue is being explored in an ongoing prospective clinical trial

in diabetic and non diabetic patients (NCT01102699), as a proof-of-concept for the so-called diabetic stem cell "mobilopathy" [79]. However, it is fascinating that complex niche dysfunction in DM may not only impair progenitor cell mobilization, but also affect differentiation of progenitor cells, with defective generation of EPCs and CPCs and excess production of SMPs, OPCs and PI-BMDCs that exert detrimental effects on diabetic complications [80].

9. Concluding Remarks

The studies summarized so far currently attribute to circulating progenitors for multiple cell lineages important roles in the pathogenesis of diabetic complications. Progenitor cells typically originate from the BM and intrinsic BM alterations in DM begin to be characterized. Thus, the emerging scenario put the BM in the centre of a new pathophysiological model of diabetic complications, as a link between distant and disparate target organs (Figure 1). Importantly, stem cell failure is typically associated with aging and it is worth to note that, owing to the burden of complications, DM is considered a disease of accelerated aging [81].

At least some of the progenitor cell dysfunction found in DM are reversible [82–84]. For instance, glucose control with insulin therapy has been shown to increase EPCs [85] while normalization of glucose metabolism by islet transplantation in type 1 diabetes reversed EPC defects [86]. In addition, inhibition of DPP-4 with sitagliptin increased EPCs in 4 weeks in type 2 diabetic patients, possibly through an effect on SDF-1alpha [87]. Finally, the discovery of progenitor cell reduction in diabetes represents the rationale for devising cell-based therapeutic strategies [88], which show promising results for both coronary and peripheral vascular disease [89, 90].

Despite these data, several aspects of progenitor cells biology in DM still need to be extensively investigated. Among all, the monocyte plasticity and its deranged polarization [91], which is thought to account for unbalanced EPC, SMP, MCC and PI-BMDC generation, deserve a special attention.

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Review Article

Regenerative Therapies for Diabetic Microangiopathy

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Hyperglycaemia occurring in diabetes is responsible for accelerated arterial remodeling and atherosclerosis, affecting the macro- and the microcirculatory system. Vessel injury is mainly related to deregulation of glucose homeostasis and insulin/insulin-precursors production, generation of advanced glycation end-products, reduction in nitric oxide synthesis, and oxidative and reductive stress. It occurs both at extracellular level with increased calcium and matrix proteins deposition and at intracellular level, with abnormalities of intracellular pathways and increased cell death. Peripheral arterial disease, coronary heart disease, and ischemic stroke are the main causes of morbidity/mortality in diabetic patients representing a major clinical and economic issue. Pharmacological therapies, administration of growth factors, and stem cellular strategies are the most effective approaches and will be discussed in depth in this comprehensive review covering the regenerative therapies of diabetic microangiopathy.

1. Introduction

Diabetes represents one of the greatest medical and socioeconomic emergencies worldwide. Approximately 17.5 million people have been diagnosed with diabetes in the USA and their number is continuously growing by 1 million per year [1]. Hyperglycaemia, occurring in type 1 (T1D) and type 2 diabetes (T2D), is responsible for a wide number of complications, with the vascular ones representing the leading cause of morbidity and mortality in diabetic patients [2]. Accelerated arterial remodeling, atherosclerosis, and endothelial cell dysfunction, affecting the macro- and the microcirculatory system, are the main evidences and lead to progressive tissue hypoperfusion and hypoxia [3]. In diabetes, multiple actors concur in causing vascular remodeling, among them metabolic factors (e.g., hyperglycaemic and oxidative stress) which are important for chemical and biological modifications of the extracellular matrix, endothelial/vascular smooth muscle cells, and mechanical factors (e.g., wall shear and circumferential stress) due to the concomitant hypertension, which cause enhanced inward remodeling, paralleled by intima/media thickening, and attenuation of vessel dilation [4, 5]. Moreover, hyperglycaemia and advanced glycation end products (AGEs) have been shown to increase the matrix surrounding endothelial cells and vascular smooth muscle cells, increasing the deposition of proteins and the entrapment of molecules and reducing metalloproteinases activity, thus being responsible for impaired vessel dilation and wall stiffening [6, 7]. Interestingly, the UK Prospective Diabetes Study (UKPDS) and Diabetes Control of Complications Trial (DCCT) have found microvascular disease and hyperglycaemia intrinsically related [8]. Pharmacological therapies with anti-inflammatory and anti-AGE/ROS drugs, angiogenesis inhibitors, administration of growth factors, either as recombinant proteins or via gene transfer, and stem cellular strategies are the most effective approaches and will be

Table 1: Role of β-cell replacement in the treatment of diabetic microangiopathic complications divided by site of pathology. Islet and pancreas transplantation generally stabilize and in some cases improve the major diabetic complications in the long term. RAGE: advanced glycation end products receptor.

Complication	Pancreas transplantation	Islet transplantation	References
Ocular	Does not prevent diabetic retinopathy, while the reversal is still controversial	Stabilization of retinopathy, Increase in arterial and venous retinal blood flow velocity	Ramsay et al. N Engl J Med, 1988 [9]; Lee et al. Transplant Proc, 2005 [10]; Venturini et al. Transplantation 2006 [11]
Renal	Reduction in the thickness of the glomerular and tubular basement membranes, Decreased urinary albumin excretion	Retarded progression of diabetic nephropathy, Decreased urinary albumin excretion survival of the kidney graft	Fioretto et al. N Engl J Med, 1998 [12] and Lancet, 1993 [13]; Fiorina et al. J Am Soc Nephrol, 2003 [14]
Neurological	Progressive improvement of nerve conduction velocity	Positive impact on polyneuropathy, Reduce nerves' RAGE expression, Conservation of perineurium and vasa nervorum	Kennedy et al. N Engl J Med, 1990 [15]; Del Carro et al. Diabetes Care, 2007 [16]
Cardiovascular	Positive effects on atherosclerosis coronary and carotid intimal thickness reduction Peripheral vascular disease can worsen	Reduction in carotid intima media thickness Stabilization of microangiopathy in skin biopsies Improved dyastolic function Reduced hemostatic abnormalities	Fiorina et al. Diabetes Care, 2005 [17], Del Carro et al. Diabetes Care 2007 [16]; Larsen et al. Diabetes Care, 2007 [18]

discussed in depth in this comprehensive review covering the regenerative therapies of diabetic microangiopathy.

2. β-Cell Replacement

Subcutaneous daily insulin injections improve glycometabolic control and HbA1c% levels; however they are not able to entirely halt the occurrence of diabetic complications [13]. β-cell replacement, either pancreas or islet transplantation, is a life-saving intervention which can restore normoglycaemia [19].

2.1. Pancreas Transplantation. Despite being a procedure still affected by major risks, pancreas transplantation, when successful, leads to an immediate insulin withdrawal, as the newly transplanted pancreas is capable to secrete insulin immediately after the revascularization, normalizing HbA1c% levels in the long term (up to 10 years) [20]. Diabetic retinopathy is the most frequent diabetic complication with at least 75% of patients with T1D developing the disease by 10 years and 40% of them degenerating into blindness within 3 years [21]. Studies performed in diabetic patients receiving pancreas or kidney-pancreas transplantation have shown that the normoglycaemia achieved with these approaches do not prevent or reverse diabetic retinopathy [9] (Table 1). Conversely, Wang et al. reported regression of diabetic nephropathy at 1 year in 43% of simultaneous kidney-pancreas-transplanted subjects [22]. In general, it has been shown that pancreas transplantation halts the progression of diabetic nephropathy [13, 23, 24] (Table 1). Studies performed on pancreas-transplanted patients have shown that diabetic glomerular lesions affecting their own kidneys prior to transplantation were not ameliorated by 5 years of normoglycaemia, while an improvement was observed after 10 years [12, 13] (Table 1). Different studies have shown that neuropathy progression (and the associated vascular degeneration) can be halted by successful pancreas transplantation [15, 25, 26]. Martinenghi et al. reported that progressive amelioration of nerve conduction velocity was prominently related to the pancreas graft, given that in a cohort of kidney-pancreas-transplanted patients, the failure of the pancreas, that occurred at least 2 years after successful combined transplantation, was associated with a deterioration of nerve conduction velocity back to pretransplant levels [27]. Coronary intimal thickness has been reported to regress in 40% of transplanted patients, as well as carotid atherosclerotic plaques have been seen to improve within 2 years after pancreas transplantation [28].

2.2. Islet Transplantation. Islet transplantation is a new concept β-cell replacement alternatively employed to pancreas transplantation in selected groups of patients suffering from severely poor glycaemic control and recurrent hypoglycaemic episodes, especially if associated with reduced hypoglycaemic awareness [8]. Lee et al. reported a stabilization of retinopathy in islet transplanted patients in some cases even at 1 year after transplant [10]. Venturini et al. investigated through color-Doppler-imaging the blood flow of the central retinal arteries in a group of islet-transplanted patients, before and at 1 year after transplantation, and found a significant increase in arterial and venous retinal blood flow velocities in transplanted patients compared to the control group [11] (Table 1). Islet transplantation is often performed in diabetic patients who have already experienced a kidney transplant due to end-stage renal disease; however, no unique interpretation about islet transplantation role on kidney graft function has been reached so far. Our group showed that in T1D patients islet transplantation is able to improve kidney graft survival and function, also decreasing microalbuminuria [14] (Table 1). Lee et al. studied the peripheral nerve

function of islet transplanted patients demonstrating that β-cell replacement has a positive impact on polyneuropathy as well [10]. Accordingly, Del Carro et al. showed that islet transplantation may stabilize or even improve polyneuropathy by reducing AGE receptors (RAGEs) expression in formerly kidney-transplanted T1D patients [16] (Table 1). In the same work, skin biopsies from islet-transplanted patients showed a higher conservation degree of perineurium and vasa nervorum compared to end-stage renal disease and kidney-transplanted T1D subjects [16]. In a previous work, we similarly demonstrated that skin biopsies performed in successful islet transplanted patients, showed a stabilization of diabetic microangiopathy after 3 years of follow-up, with an increased expression of endothelial nitric oxide and von Willebrand factor, associated with a reduction of capillary basement membrane thickness and endothelial cellular swelling [17] (Table 1).

3. Pharmacological Therapies

3.1. Anti-Inflammatory Drugs. High glucose concentrations induce the production of the proinflammatory cytokine IL-1 in human pancreatic β-cells, contributing to impaired insulin secretion and β-cell proliferation [29]. In a randomized double-blind, trial, anakinra (a recombinant human IL-1 receptor antagonist) reduced HbA1c% and IL-6 concentration increasing C-peptide secretion in T2D patients [18]. Recent studies suggested that anti-inflammatory drugs may have an important role in diabetes therapy. The administration of salsalate (a prodrug carrying fewer side effects than aspirin) for a period of 2–4 weeks in T2D patients was able to reduce glucose blood concentrations while increasing insulin secretion [30–34]. Pentoxifylline is an anti-inflammatory methyl-xanthine derivative which is currently studied in a number of clinical trials evaluating its role in diabetic patients with nephropathy. It has been shown to significantly decrease proteinuria in both T1D and T2D patients; however given the small number of patients and the short duration of the studies, additional research is required to determine whether long-term use of pentoxifylline could be considered for the prevention or treatment of diabetic complications [35, 36]. Finally, it is becoming more and more evident that conventional therapies for diabetes are effective in reducing inflammation and improving diabetes outcomes via indirect or pleiotropic mechanisms. In fact, lifestyle modifications promoting weight loss, caloric restriction, and physical exercise, together with metformin and statin therapy, have recently been shown to reduce high C reactive protein levels in T2D patients [37, 38].

3.2. PKC Inhibitors. Hyperglycaemia is a fundamental metabolic factor involved in the development of both micro- and macrovascular complications, having numerous adverse effects such as the chronic activation of protein kinase C (PKC), a family of enzymes profoundly involved in the control of multiple cellular pathways [39]. Different PKC isoforms (PKC-α, -β1/2, and PKC-δ) have been shown to be associated with vascular alterations such as modifications of permeability, angiogenesis, synthesis of the extracellular matrix, cell growth/apoptosis, leukocytes migration, and cytokines production, thus leading to pathologies affecting the macrovasculature (atherosclerosis, cardiomyopathy) and the microvasculature (retinopathy, nephropathy, and neuropathy) [40]. Both preclinical and clinical studies using PKC-β inhibitors have been carried out, obtaining encouraging results. The PKC-β inhibitor ruboxistaurin (LY333531, RBX/Arxxant; Eli Lilly and Company, Indianapolis, IN) was employed in murine models of diabetes with virtually no effect on HbA1c%, blood glucose level, or blood pressure, however leading to normalization of glomerular filtration rate (GFR), urinary albumin, and TGF-β1 excretion and also reducing the glomerular and mesangial extracellular matrix [41, 42]. A multicenter pilot study evaluated the effect of LY333531 (32 mg/day) in T2D patients affected by diabetic nephropathy in addition to their current therapy with angiotensin-converting enzyme inhibitors and angiotensin receptor blockers, showing a 25% reduction in urinary albumin creatinine ratio (ACR) after 1 year and a conservation of the estimated GFR level [43]. The PKC-β inhibitor diabetic retinopathy study (PKC-DRS) reported a lower incidence of visual loss, need for laser treatment, and macular oedema progression in T1D and T2D patients treated with 32 mg/day LY333531 for 36–46 months [44, 45]. Inhibition of PKC-β through LY333531 is also beneficial to patients with symptomatic diabetic neuropathy, improving sensory symptoms and vibration sensation [46, 47]. A phase II multinational pilot study assessed the effect of LY333531 (32 or 64 mg/day for 1 year) in patients with diabetic neuropathy demonstrating a significant reduction of total symptoms and improvement at the vibration detection threshold [48]. Treatment with LY333531 (32 mg/day for 7 days) has been shown to prevent endothelium-dependent vasodilation abnormalities induced by hyperglycaemia as well [49].

3.3. AGEs and Oxidative Stress Strategies. Diabetes is characterized by abnormalities of mitochondrial ROS production that generate an increased oxidative stress in endothelial cells thus causing the development of diabetes complications [50]. In physiologic ageing AGEs can target proteins forming irreversible complexes that become resistant to proteolytic degradation; however, in the diabetic state the process results accelerated [51]. Aminoguanidine, scavenging intermediates in the glycation catalytic process, inhibits the formation of AGEs slowing the progression of diabetic nephropathy [52]; however the first clinical study performed in T1D patients with nephropathy and retinopathy, beside recording a consistent reduction of proteinuria, reported retinopathy worsening in some treated patients compared to placebo [53]. Vitamins of the B group represent another approach for the reduction of AGEs-related complications [54]. Preclinical studies revealed that pyridoxamine is effective in preserving kidney function in T1D and T2D murine models [54]. Consistently phase II clinical trials in proteinuric T1D and T2D patients showed a significant decrease in serum creatinine level, albumin, and TGF-β urinary excretion. An ongoing phase IIb study (http://clinicaltrials.gov/, NCT00734253) is

evaluating the safety and efficacy of pyridoxine in nephropathic T2D patients. Finally, another clinical trial (http://clinicaltrials.gov/, NCT00565318) is now assessing the effect of benfotiamine, a lipophilic analogue of thiamine (B_1 vitamin), on urinary albumin and β_2-microglobulin excretion in T2D patients. Another class of therapeutic agents useful in reducing AGEs accumulation is advanced glycation cross-link breakers. Recently TRC4186 has been shown to decrease albuminuria and improve kidney function in diabetic mice with progressive cardiac and kidney failure, but 2-week treatment with ALT-711 (alagebrium), a novel AGE breaker compound had no effects on motor/nociceptive nerve dysfunction and vascular stiffness in diabetic mice after 8 weeks of diabetes [55, 56]. A clinical trial evaluating the effect of alagebrium (200 mg twice daily) on diabetic nephropathy (http://clinicaltrials.gov/, NCT00557518) reported a decrease in arterial pulse pressure and an increase in endothelial function and artery compliance [57, 58].

3.4. Inhibitors of Angiogenesis. Vascular endothelial growth factor (VEGF) is overproduced in the diabetic retina in response to capillary loss and/or microaneurysm formation; thus inhibition of VEGF activity may play a pivotal role in the prevention of diabetes-related retinopathy [59]. Currently, there are three main anti-VEGF agents under investigation: pegaptanib sodium, a pegylated RNA aptamer that binds $VEGF_{165}$ and the longer VEGF isoforms (Macugen; Eyetech Pharmaceuticals Inc, New York, and Pfizer Inc, NY); ranibizumab, a recombinant humanized monoclonal antibody specific for all human VEFG isoforms (Lucentis; Genentech Inc, South San Francisco, CA), and bevacizumab, another full-length humanized monoclonal antibody against all isoforms of human VEGF (Avastin; Genentech, South San Francisco, CA) [60]. VEGF has a role in the pathogenesis of diabetic nephropathy as well. SU5416 by selectively blocking all VEGF receptors at the level of the tyrosine kinase has been reported to ameliorate albuminuria in an experimental model of diabetic nephropathy [61]. In preclinical studies ruboxistaurin attenuated the effect of VEGF and so the progression of diabetic nephropathy [62].

4. Cellular Therapies

Stem cells have the unique ability to potentially originate any organ or tissue, being undifferentiated and capable of self-renewal [63]. Stem cells can be obtained from embryos, umbilical cord blood, and adult tissues (as bone marrow or adipose tissue) [63].

4.1. Cord Blood Stem Cells. Cord blood stem cells (CB-SCs) are a heterogeneous population composed of (i) very small embryonic-like stem cells, (ii) mesenchymal stem cells (MSCs), (iii) hematopoietic stem cells, and (iv) endothelial progenitor cells (EPCs) [63]. CB-SCs are easily collectable from 60–80 cc of umbilical cord blood and exhibit common features like the presence of long and highly preserved telomeres, the ability to form colonies when cultured *in vivo*, and a virtually absent oncogenic potential [63, 64]. An experimental study performed by Naruse et al. highlighted the therapeutic role for *ex vivo* expanded CB-EPCs in the treatment of diabetic neuropathy, showing that hind limb EPCs intramuscular injection into streptozotocin- (STZ-) induced diabetic rats improved muscles microvascular net, sciatic nerve conduction velocity, and endoneurial nutritive blood flow [65] (Table 2). Additionally, a clinical trial evaluating safety and efficacy of allogeneic CB-MSCs injections into pathologic lower limbs of T2D patients affected by peripheral arterial disease is currently ongoing at the Stem Cell Research Center at Qingdao University, (http://clinicaltrials.gov/, NCT01216865).

4.2. Mesenchymal Stem Cells. Among stem cells, great clinical interest is reserved to MSCs. Beside their potential to differentiate [66, 67], MSCs are characterized by strong hypoimmunogenic features, such as low expression of MHC-I-related antigens and absence of MHC-II antigens [68], making these cells immune privileged [69–77] (e.g., MSCs neither induce $CD4^+$ activation nor are subjected to cell lysis induced by cytotoxic lymphocytes [78]). Moreover, MSCs are able to exert anti-inflammatory actions, for instance, by decreasing the secretion of TNF-α by dendritic cells [76, 77]. In a murine model of diabetic cardiomiopathy, Zhang et al. evaluated the ability of intravenous administration of BM-MSCs to either promote angiogenesis and mitigate ventricular remodeling, reporting an increased number of myocardial arterioles, an improvement of cardiac functionality, and a decrease of metalloproteinases activity, with a consequent smoothing of heart remodeling [79] (Table 2). Additionally, Wu et al. showed that allogeneic transplantation of BM-MSCs significantly promoted wound healing in diabetic *db/db* mice by accelerating reepithelialization and reconstituting capillary network density [80] (Table 2). Finally, Yang et al. showed that adipose-derived MSCs infusion in an STZ-induced murine model of diabetic retinopathy reduced blood glucose level and blood retinal barrier damage [81] (Table 2).

4.3. Endothelial Progenitor Cells. EPCs are circulating cells originating from several tissues such as bone marrow, peripheral blood, and cord blood, which are able to generate mature endothelial cells and vascular structures [82]. Thanks to their involvement in angiogenic and vasculogenic processes [82–86], EPCs represent an additional powerful tool for the treatment of diabetic microvascular complications. Interestingly, Barcelos et al. showed that human fetal aorta $CD133^+$ progenitor cells, when transplanted into a murine model of diabetic wound, are either able to promote angiogenesis and to release VEGF-A in a high-frequency fashion [87]. Fiorina et al. demonstrated that the mobilization of endogenous BM-EPCs to the site of wound in a mouse model of diabetic wound healing is improved by the targeting of CXCR4/CXCL12 axis through a CXCR4 antagonist [88]. A currently ongoing phase I clinical trial is evaluating the efficacy and safety of the administration of AMD3100 (Plerixafor) and rhPDGF-BB (Becaplermin), two novel agents able

Table 2: Overview of the experimental studies describing the role of stem cells in diabetic microangiopathy treatment and prevention. AD: adipose derived; BM: bone marrow; CB: cord blood; EPCs: endothelial progenitor cells; MSCs: mesenchymal stem cells; BRB: blood retinal barrier.

Complication	Cells	Outcomes	References
Neurological	CB-EPCs	Increased number of microvessels, Improved sciatic motor nerve conduction velocity, Increase of sciatic endoneurial nutritive blood flow	Naruse et al. Diabetes, 2005 [65]
Cardiovascular	BM-MSCs	Enhanced number of myocardial arterioles, Increase in fractional shortening, Mitigation of heart remodeling.	Zhang et al. Exp Clin Endocrinol Diabetes, 2008 [79]
Wound Healing	BM-MSCs	Acceleration of wounds healing, Increase in capillary density.	Wu et al. Stem cells, 2007 [80]
Ocular	AD-MSCs	Repair of BRB damages	Yang et al. Graefes Arch Clin Exp Ophthalmol, 2010 [81]
Wound Healing	BM-EPCs	Promotion of neovascularization.	Asai et al. Circulation, 2006 [89]
Neurological	BM-EPCs	Restoring of nerve conduction velocity, Increased blood flow in sciatic nerve, Increased nerves capillary density.	Jeong et al. Circulation, 2009 [90]

to mobilize endogenous BM-EPCs, for the treatment of peri-pheral arterial disease in T2D patients, (http://clinicaltrials.gov/, NCT01353937). Recently, Asai et al. demonstrated that Sonic Hedgehog (SHh, a protein that stimulates BM-EPCs proliferation/migration and VEGF production, promoting the neovascularization of ischemic tissues) was responsible for wound healing acceleration in *db/db* mice by enhancing angiogenesis and recruiting BM-EPCs into the wound [89] (Table 2). Jeong et al. reported that hind limb BM-EPCs infusion was able to reverse diabetic neuropathy in STZ-induced diabetic mice, improving sciatic nerve conduction velocity and blood flow, compared to saline treated controls [90] (Table 2). Finally, transplanted BM-EPCs exhibited homing attitudes to the sciatic nerve and its vasa nervorum, also reporting a paracrine activity realized through the release of either angiogenic and neurotrophic factors [90] (Table 2).

5. Gene Therapy

Growth factors administration by gene transfer is a promising approach for the treatment of diabetic microangiopathy, promoting endothelial cell proliferation, migration, and blood vessel formation.

5.1. Vascular Endothelial Growth Factor. VEGF is an endothelial-specific growth factor that promotes endothelial cells proliferation, differentiation, and survival, mediates endothelium-dependent vasodilation, induces microvascular hyperpermeability, and participates in interstitial matrix remodeling [91]. Notably, at low concentration VEGFs are mostly vasculoprotective, at high concentration have angiogenic effects, whereas at sustained high dose cause pathological angiogenesis [91]. VEGF-A, particularly its 165 isoform, plays a major role in vascular biology and is the first candidate for therapeutic applications *in vivo* [92, 93]. Isner et al. first demonstrated the tolerability of plasmid injection transferring a human $VEGF_{165}$ encoding plasmid (ph) in patients with peripheral arterial disease [94, 95]. The safety and beneficial results of $phVEGF_{165}$ were reported as well by Shyu et al. in patients with critical limb ischemia (CLI) [96]. Similarly, intramuscular $phVEGF_{165}$ was tested in ischemic neuropathic patients [97]; however, no relevant biologic effects were obtained due to the partial inefficiency of VEGF gene transfer [98]. Conversely, a phase I study demonstrated the major efficiency of adenoviral (Ad) vector in $VEGF_{121}$ transfer in severe vascular disease patients, showing an increase of lower-extremity flow reserve in response to acetylcholine [99]. Less optimistic outcomes were shown in other studies where $Ad.VEGF_{121}$ administration in patients with intermittent claudication was not associated with an improvement in exercise performance and quality of life, and similarly, treatment with $Ad.VEGF_{165}$ did not reduce the rate of amputation events [100] (Table 3).

5.2. Fibroblast Growth Factors. Fibroblast growth factor (FGFs) is a large family of proteins capable of modulating the proliferation and migration of endothelial cells, fibroblasts, and smooth muscle cells [101]. FGF-1 is a potent mitogen for vascular cells and induces the formation of mature blood vessels *in vivo*. In 2007, Sanofi-Aventis started a promising trial (currently ongoing) based on the gene transfer of FGF-1 plasmid DNA in patients with CLI, known as TAMARIS (Therapeutic Angiogenesis for the Management of Arteriopathy in a Randomized International Study) [102], aiming at evaluating the efficacy and safety of intramuscular administration of NV1FGF in CLI patients, a plasmid-based gene delivery system for the local expression of FGF-1 [103, 104] (Table 3). After 1 year of follow-up, the primary endpoint of major amputations or death occurrence did not differ between the treated and the placebo arm [105]. FGF-4 has been shown to stimulate endothelial cells proliferation, migration, and neovascularization *in vivo* by upregulating endogenous VEGF-A expression [106]. Indeed, a currently ongoing clinical trial is evaluating the therapeutical potential of $VEGF\text{-}A_{165}$/basicFGF delivery in the myocardium of refractory

Table 3: Role of gene transfer in the treatment of diabetic microangiopathy. Gene transfer promotes endothelial cell proliferation, migration, and blood vessel formation. VEGF: vascular endothelial growth factor; FGF: fibroblast growth factor; HGF: hepatocyte growth factor; HIF-1α: hypoxia inducible factor-1α.

	Study	Year	Identifier	Site	Status/recruited pts
VEGF	Vegf gene transfer for critical limb ischemia	2010	NCT00056290	Steward St. Elizabeth's Medical Center of Boston	Completed
	VEGF gene transfer for diabetic neuropathy	2010	NCT00056290	Steward St. Elizabeth's Medical Center of Boston	Completed
	Angiogenesis using VEGF-A165/bFGF plasmid delivered percutaneously in no-option CAD patients; a controlled trial (VIF-CAD)	2009	NCT00620217	Institute of Cardiology, Warsaw, Poland	Completed
FGF	Efficacy and safety study of NV1FGF in patients with severe peripheral artery occlusive disease (TALISMAN202)	2010	NCT00798005	Sanofi-Aventis	Completed
	Efficacy and safety of XRP0038/NV1FGF in critical limb ischemia patients with skin lesions (TAMARIS)	2010	NCT00566657	Sanofi-Aventis	This study is ongoing, but not recruiting participants
HGF	Study of hepatocyte growth factor (HGF) via plasmid vector to improve perfusion in critical limb ischemia patients with peripheral ischemic ulcers	2011	NCT00189540	AnGes	This study has been completed
HIF	Safety and efficacy study of Ad2/Hypoxia inducible factor-1α (HIF-1α)/VP16 gene transfer in patients with intermittent claudication (WALK)	2010	NCT00117650	Genzyme	This study has been completed

coronary artery disease patients [102]. So far, most efforts have concentrated on a single gene delivery therapy; however as multiple proteins are involved in the angiogenic process, efficiently delivering more than one gene might be a valuable approach [106].

5.3. Hepatocyte Growth Factor. Hepatocyte growth factor (HGF) is a large protein first identified as a potent hepatocyte mitogen and only lately discovered to stimulate endothelial cells growth [107]. Preclinical studies have shown that the delivery of HGF as a recombinant protein or naked plasmid induces therapeutic angiogenesis in rat or rabbit peripheral arterial disease models [108, 109]. Similarly, muscular injection of naked human HGF plasmid resulted in increased blood flow in the same models [109]. Following these preclinical results, the safety and efficacy of HGF plasmid administration was investigated in patients with CLI, evaluating limb tissue perfusion by transcutaneous oxygen tension measurement and reporting higher values of oxygen tension at 6 months as compared to controls [110] (Table 3).

5.4. Hypoxia-Inducible Factor-1. Hypoxia-inducible factor-1 (HIF-1) is a mesenchyme-derived pleiotropic transcriptional activator that acts as a regulator of oxygen homeostasis [111]. HIF-1 is a heterodimer composed of a constitutively expressed HIF-1β subunit and a O_2-regulated HIF-1α subunit, with the latter increasing at low oxygen levels [111]. The complex of HIF-1β/α regulates the expression of many genes, including VEGF [111]. The injection of plasmid DNA encoding HIF-1α stimulates the recovery of blood flow in hind limb ischemia animal models [112, 113]. Similarly, intramuscular injection of adenovirus encoding functional HIF-1α in a murine model of hind limb ischemia increased the recovery of limb perfusion [113]. Finally, intramuscular injection of escalating dose of adenoviral HIF-1α-VP16 in patients with CLI has shown tolerability and safety [114] (Table 3).

5.5. Nerve Growth Factor. Recently, nerve growth factor (NGF) has been discovered to have cardiovascular protective roles and angiogenetic capabilities [115]. For these reasons, it is becoming particularly appealing for the treatment of diabetes vascular complications. Emanueli et al. strikingly introduced the concept of neurotrophins, particularly NGF, as autocrine proangiogenetic factors, promoting the growth of new capillaries and accelerating blood flow recovery in ischemic muscles of a mouse model of limb ischemia [116]. In fact, NGF seems to induce angiogenesis by increasing the expression level of VEGF-A and VEGF receptors [116, 117] and also by promoting NO production and metalloproteinases upregulation [116, 118]. Accordingly, Meloni et al. translated these findings into a gene therapy approach for the prevention of diabetic cardiomyopathy in a murine model

of diabetes [119]. After 2 weeks of diabetes, mice were treated with NGF gene transfer via adenoassociated viral vectors [119]. Treated mice were found to be protected from myocardial microvascular rarefaction, hypoperfusion, increased deposition of interstitial fibrosis, and increased apoptosis of endothelial cells and cardiomyocytes [119].

5.6. Kallikreins. Kallikreins can be divided in tissue and plasma kallikreins differing in molecular weight, substrate specificities, and type of kinin released. Tissue kallikrein is a glycoprotein that stimulates cells to release autacoids such as nitric oxide and prostaglandins [120]. Circulating tissue kallikrein levels are increased in patients with peripheral obstructive vascular disease and in skeletal muscle after the induction of hind limb ischemia [121]. The angiogenic potential of tissue kallikrein in peripheral ischemia has been established in preclinical models using a gene-transfer approach [122]. Replication-defective adenoviral vector containing the human tissue kallikrein gene was injected into the adductor skeletal muscle of mice submitted to unilateral limb ischemia [123]. The successful transduction of the transgene resulted in ameliorated angiogenic response and haemodynamic recovery [123]. Emanueli et al. have also documented that human tissue kallikrein is able to prevent diabetic microangiopathy in STZ-induced diabetic murine models [124].

6. Conclusions

Multidisciplinary management of diabetes is of outstanding importance for the treatment and the prevention of diabetic complications, being helpful in reducing social costs and the detriment to the patient; however newer therapeutic approaches are needed. Novel mechanistic insights in the pathogenesis of endothelial dysfunction are rapidly being translated into new therapeutic opportunities; stem cells are expected to become promising therapeutic agents for diabetic patients due to their immunomodulatory characteristics, self-renewal, and differentiation ability; many clinical trials are demonstrating gene therapy as a valuable option to treat peripheral arterial disease. Nonetheless, gene therapy together with the cellular one is still waiting for randomized placebo-controlled double-blind, large-scale, clinical trials, ultimately defining their clinical role. Despite only short- and midterm follow-up data are available and long-term safety and efficacy end-points are required, some of these new strategies are close to become established therapeutic options, and some others hold in them the potential to halt diabetic complications.

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Research Article

Procalcific Phenotypic Drift of Circulating Progenitor Cells in Type 2 Diabetes with Coronary Artery Disease

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Diabetes mellitus (DM) alters circulating progenitor cells relevant for the pathophysiology of coronary artery disease (CAD). While endothelial progenitor cells (EPCs) are reduced, there is no data on procalcific polarization of circulating progenitors, which may contribute to vascular calcification in these patients. In a cohort of 107 subjects with and without DM and CAD, we analyzed the pro-calcific versus endothelial differentiation status of circulating CD34+ progenitor cells. Endothelial commitment was determined by expression of VEGFR-2 (KDR) and pro-calcific polarization by expression of osteocalcin (OC) and bone alkaline phosphatase (BAP). We found that DM patients had significantly higher expression of OC and BAP on circulating CD34+ cells than control subjects, especially in the presence of CAD. In patients with DM and CAD, the ratio of OC/KDR, BAP/KDR, and OC+BAP/KDR was about 3-fold increased than in other groups. EPCs cultured from DM patients with CAD occasionally formed structures highly suggestive of calcified nodules, and the expression of osteogenic markers by EPCs from control subjects was significantly increased in response to the toll-like receptor agonist LPS. In conclusion, circulating progenitor cells of diabetic patients show a phenotypic drift toward a pro-calcific phenotype that may be driven by inflammatory signals.

1. Introduction

Diabetes mellitus (DM) is associated with an excess risk of cardiovascular disease, which is attributable to hyperglycemia, oxidative stress, and inflammation [1]. In parallel, repair of vascular damage is compromised in DM owing to a pauperization of circulating endothelial progenitor cells (EPCs) [2]. In the bloodstream, other lineage-committed progenitor cells may have pathophysiological implications in the cardiovascular system, such as smooth muscle and cardiomyocyte progenitors [3, 4]. DM reduces circulating EPC level [5] and is also associated with an impaired generation of EPCs paralleled by enhanced differentiation of smooth muscle progenitors in vitro [6, 7]. A drift of circulating progenitors from the endothelial to smooth muscle-cell phenotype has been linked to the development of myointimal hyperplasia [8], an event that is associated with diabetes. The impaired differentiation of cultured EPCs in diabetic patients has been previously attributed to a proinflammatory status [9]. EPCs promote endothelial healing and compensatory angiogenesis, thus providing a mean of vascular repair [10]. Therefore, abnormalities of progenitor cells are considered important contributors to the development of diabetic vasculopathy, which is characterized by extensive endothelial dysfunction/damage and myointimal hyperplasia [11]. Another hallmark feature of diabetic vasculopathy is ectopic calcification. Intimal microcalcifications of atherosclerotic lesions contribute to destabilize the plaque, while medial calcification rises arterial stiffness and blood pressure [12, 13]. The mechanisms increasing vascular calcification in diabetes are incompletely understood, but cell-mediated processes are increasingly studied. In 2005 Eghbali-Fatourechi and coworkers described the existence of osteoblastic cells in the human peripheral blood [14], suggesting for the first time

that circulating cells may contribute to ectopic calcification. This hypothesis has been supported by the discovery of myeloid calcifying cells (MCCs) and by evidence of a potential role for circulating cells in vascular and valve calcification [15–17].

Based on this background, we hypothesize that circulating progenitor cells of diabetic patients may undergo a phenotypic shift from the protective endothelial commitment to a detrimental pro-calcific phenotype. To test this hypothesis, we analyzed expression of endothelial and osteogenic markers on circulating CD34+ cells and evaluated pro-calcific differentiation of EPCs in vitro.

2. Materials and Methods

2.1. Patients. The study was approved by the Ethical Committee for Clinical Experimentation of the University Hospital of Padova. Type 2 diabetic patients and controls were recruited at the outpatient clinics of the Division of Metabolic Diseases. The same exclusion criteria applied to all patients: age <18 or >80 years; recent (within 1 month) trauma, surgery, or revascularization; immunological disease, immunosuppression, or cancer; any acute disease or infection; pregnancy and lactation. Patients were divided according to the presence of diabetes mellitus (DM) and coronary artery disease (CAD). Type 2 DM was diagnosed according to American Diabetes Association criteria [18]. CAD was defined in the presence of at least one of the followings: a past documented history of myocardial infarction; angiographic evidence of one or more >70% stenosis of epicardial coronary arteries; evidence of inducible ischemia from a noninvasive stress test (either single-photon emission tomography or ultrasound Doppler examination). All patients were characterized by collection of the following data: age, sex body mass index (kg/m^2), systolic and diastolic blood pressure, diagnosis of hypertension, history of smoking, fasting plasma glucose, glycated hemoglobin (HbA1c), total cholesterol, HDL cholesterol, and triglycerides concentration. We also recorded data on diabetic complications, included retinopathy (defined by the ETDRS classification [19]), neuropathy (defined by suggestive symptoms and signs, eventually confirmed by an electromyogram), and nephropathy (defined as either a urinary albumin excretion rate >30 mg/g creatinine or an estimated glomerular filtration rate [eGFR, according to the MDRD equation [20]] $<60\ mL/min/m^2$). Peripheral arterial disease was defined as a history of claudication or rest pain in the presence of a significant stenosis of leg arteries on an ultrasound or angiographic examination. Cerebrovascular disease was defined as either a history of past stroke/transient ischemic attack, or evidence of carotid atherosclerotic plaques, determining a stenosis >20% of vessel lumen, on an ultrasound examination. Finally, we also collected data on medications.

2.2. Cell Culture. Late outgrown EPCs were cultured from peripheral blood mononuclear cells as previously described [6]. Briefly, cells were plated on six-well fibronectin-coated plates at a density of 6×10^6 cells per well and grown in supplemented endothelial cell growth medium (Clonetics) with 20% serum. The medium was changed the first time after 4 days and then each other day for a total of 2 weeks. We have previously shown that during the culture protocol these cells form clusters with a core made of rounded cells and radiating spindle-shaped cells at the periphery. At 12–14 days, these clusters dissolve and cells progressively develop as a monolayer. We characterized these cells by double immunofluorescence; cells were incubated at 37°C with 1 mg/mL DiI-AcLDL (DiIacetylated low-density lipoproteins, Molecular Probes) for 1 h, followed by dark incubation with 15 mg/mL FITC-conjugated Ulex lectin (Sigma-Aldrich) for 2 h. Nuclei were stained in blue with Hoechst 33258 (Sigma-Aldrich). In separated experiments, cells were cultured in the presence of LPS (Sigma-Aldrich) from day 7 to 14 at a final concentration of 100 nM; untreated cells served as controls, and expression level was set at 1. In parallel we also cultured human umbilical vein endothelial cells (HUVECs, Clonetics) and analyzed the expression of bone-related markers in untreated and LPS-treated cells. Experiments were performed in triplicate. Alizarin red and von Kossa stainings were not performed.

2.3. Flow Cytometry. Expression of progenitor cell antigens and differentiation markers was analyzed by multicolor flow cytometry on fresh whole peripheral blood samples. Briefly, after red blood cell lysis, cells were incubated with specific monoclonal antibodies anti-CD34 (-PE or -FITC conjugated, Becton Dickinson, BD), PE-conjugated anti-KDR (R&D Systems), or PE-conjugated anti-OC (R&D Systems) and APC-conjugated anti-BAP (R&D Systems). OC/BAP costaining with KDR was not performed. After washing, cells were analyzed by FACSCalibur instrumentation (BD) set up for analysis or rare events. We first gated CD34+ cells in the mononuclear cell fraction and then examined the resulting population for dual expression of KDR or dual/triple expression of OC and/or BAP. At least 5×10^5 events were acquired, and positive events were recorded as a fraction of the number of fated CD34+ cells. All analyses were performed by trained operators blinded to the patients status. For the analysis of cell culture, a similar gating strategy was used, with the same directly labelled monoclonal antibodies plus the PE-conjugated anti-RANKL mAb.

2.4. Statistical Analysis. Data are expressed as mean and standard error or as percentage, where appropriate. Comparison between 2 or more groups was performed using Student's t test or ANOVA, respectively. The Least Significance Difference (LSD) post hoc test was used. Comparison of categorical data was tested using the Chi square test. To test the independent association of the coexistence of DM and CAD on progenitor cell phenotypes, we run a multiple linear regression analysis in which DM+CAD+ was an independent variable together with other covariates. Covariates were selected for being different at $P < 0.05$ at the univariate comparison between patients with and without DM and CAD. Statistical significance was accepted at $P < 0.05$, and the SPSS versus 16.0 was used.

Table 1: Clinical characteristics of study patients divided according to the presence of DM and CAD. Post hoc analyses: $^{*}P < 0.05$ in DM+ versus DM−; $^{\#}P < 0.05$ in CAD+ versus CAD−.

Characteristic	DM−CAD−	DM−CAD+	DM+CAD−	DM+CAD+	ANOVA P
Number	33	19	33	22	—
Age (years)	54.3 ± 3.2	52.4 ± 3.1	61.9 ± 1.8*	67.3 ± 1.4*	<0.001
Sex male (%)	38	79#	70*	86	0.001
BMI (kg/m^2)	24.8 ± 0.4	26.8 ± 1.3	28.3 ± 0.9*	30.6 ± 0.9*	<0.001
SBP (mm Hg)	139.1 ± 4.1	120.4 ± 5.6	143.2 ± 3.2	141.7 ± 4.8	<0.001
DBP (mm Hg)	83.4 ± 2.3	75.3 ± 2.1	85.9 ± 2.1	84.5 ± 2.1	<0.001
Hypertension (%)	30	32	88*	91*	<0.001
Smoking habit (%)	16	26	6	5	0.102
HbA1c (%)	5.2 ± 0.2	5.8 ± 0.1	8.4 ± 0.3*	8.1 ± 0.3*	<0.001
FPG (mg/dL)	87.9 ± 3.9	99.3 ± 4.9	164.3 ± 11.6*	161.4 ± 10.5*	<0.001
T-CH (mg/dL)	203.1 ± 7.1	183.6 ± 11.6	178.9 ± 6.4*	158.5 ± 7.9*	0.002
HDL (mg/dL)	60.0 ± 3.7	49.2 ± 1.9#	48.1 ± 2.1*	39.6 ± 2.0#*	<0.001
LDL (mg/dL)	125.2 ± 6.7	105.7 ± 11.3	100.1 ± 5.6*	90.8 ± 6.5	0.008
Triglycerides (mg/dL)	94.7 ± 7.9	144.3 ± 27.9	150.4 ± 17.3*	141.0 ± 12.4*	0.056
Retinopathy (%)	0	0	21*	36*	<0.001
Nephropathy (%)	0	16	6	27#*	0.07
Neuropathy (%)	0	0	21*	27*	0.02
CerVD (%)	21	5	70*	50*	<0.001
PAD (%)	6	0	42*	32*	<0.001
OHA (%)	0	0	76*	68*	<0.001
Insulin (%)	0	0	42*	41*	<0.001
ACEi/ARB (%)	28	95#	76*	77	<0.001
Other anti-HT (%)	22	84#	55*	77	<0.001
Aspirin (%)	16	79#	76*	86	<0.001
Statin (%)	19	68#	58*	86	<0.001

BMI: body mass index. SDP, systolic blood pressure. DBP: diastolic blood pressure. FPG: fasting plasma glucose. T-CH: total cholesterol. HDL: high-density lipoprotein cholesterol. LDL: low density lipoprotein cholesterol. CerVD, cerebrovascular disease. PAD: peripheral arterial disease. OHA: oral antihyperglycemic drugs. ACEi: angiotensin conerting enzyme inhibitors. ARB: angiotensin receptor blockers. AntiHT, anti-hypertensive medications.

3. Results

3.1. Patients' Characteristics. A total of 107 subjects were included in the study. They were divided into 4 groups according to the presence of DM and/or CAD. Sample size was fairly balanced among groups. Among patients without DM, patients with CAD had a higher prevalence of the male gender, lower HDL cholesterol, and a much larger use of cardiovascular medications than in those without. Obvious differences were detected in patients with DM than in those without, including older age, prevalence of males, higher BMI, blood pressure, cholesterol, plasma glucose and HbA1c, comorbidities, and medications. Interestingly, among DM patients, the presence of CAD was only associated to a significantly lower HDL cholesterol and higher prevalence of nephropathy (Table 1).

3.2. Expression of Bone-Related Markers on Circulating Progenitor Cells. To detect the pro-calcific differentiation of circulating CD34+ progenitor cells, we analyzed the expression of OC and BAP. In CD34+ cells from control healthy subjects (DM−CAD−), OC was expressed on 26.7 ± 2.1% while BAP was expressed on 20.8 ± 1.7% of cells, and the coexpression of both markers was 12.9 ± 1.4%. The expression of OC and/or BAP was significantly increased in patients with DM and/or CAD. Specifically, OC expression was higher in CAD versus non-CAD patients independently of DM, and in DM versus non-DM patients independently of CAD. BAP expression was higher in DM versus non-DM patients, especially in the presence of CAD. Co-expression of OC and BAP on CD34+ cells was significantly higher in both DM and CAD patients (Figures 1(a), 1(b), and 1(c)).

3.3. Procalcific Phenotypic Drift of Circulating Progenitors. In parallel to the analysis of bone-related markers, we also examined the extent to which circulating CD34+ progenitor cells express the endothelial antigen KDR, which functionally represents type 2 VEGF receptor and is usually taken to represent endothelial differentiation [21]. This was used to determine the ratio of bone versus endothelial mark-er expression on CD34+ cells, as an indicator of a phenotypic drift of circulating progenitors toward the procalcific phenotype. We found that OC/KDR, BAP/KDR, and OC+BAP/KDR expression ratio was increased in DM+CAD+ patients versus controls by 3.6-, 2.9-, and 3.0-fold, respectively, while there were no differences among

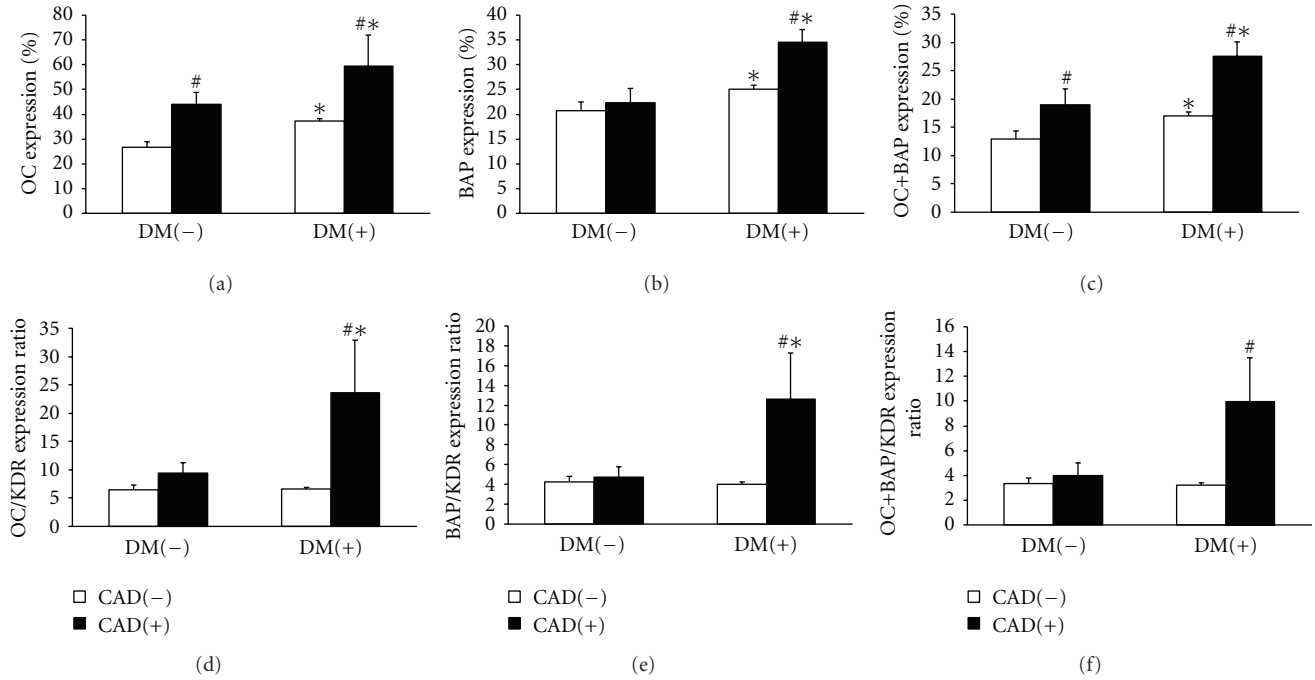

Figure 1: Expression of bone-related markers on circulating CD34+ progenitor cells. Patients were divided according to the presence/absence of type 2 diabetes mellitus (DM) and coronary artery disease (CAD). Post hoc tests: $^{*}P < 0.05$ in DM+ versus DM−; $^{\#}P < 0.05$ in CAD+ versus CAD−.

other groups (Figures 1(d), 1(c), 1(e), and 1(f)). Upon a multiple regression analysis, the coexistence of DM and CAD remained significantly associated with increased OC/KDR, BAP/KDR, and OC+BAP/KDR expression ratio versus other patients, independently of age, prevalence of hypertension, concentrations of total cholesterol, HDL and LDL, which were significantly different between the two groups (Delta ± SE 16.7 ± 6.1 for OC/KDR, $P = 0.08$; 8.7 ± 4.0 for BAP/KDR, $P = 0.030$; 6.8 ± 2.9 for OC+BAP/KDR, $P = 0.023$).

3.4. Calcification and Expression of Bone-Related Markers in Cultured EPCs. To assess whether endothelial progenitors cultured from peripheral blood mononuclear cells can undergo a pro-calcific differentiation, we isolated late EPCs from diabetic patients. Extensive characterization of these cells is reported elsewhere [6, 22]. Clusters of EPCs occasionally formed dense nodules that were highly suggestive of calcification only when cultured from DM+CAD+ patients and not from DM+CAD− patients (2/6 versus 0/7, $P = 0.05$, Figure 2(a)). As EPCs express the LPS receptors CD14 and toll-like receptor-2 (TLR-2) [23], we tested whether challenging EPCs isolated from DM-CAD subjects with LPS resulted in upregulation of bone-related markers. We found that LPS significantly increased 2.6-fold OC+BAP+ cells in the culture and upregulated BAP (3.0-fold) and RANKL (5.8-fold) on CD34+ cells. In HUVECs, which served as a control cell type, there were similar increases in OC+BAP+ cells (2.6-fold), and expression of BAP (2.9-fold), but there was no change in expression of the osteoblast marker RANKL (Figure 2(b)).

4. Discussion

In the present study, we demonstrate for the first time that circulating progenitor cells from diabetic patients with coronary artery disease undergo a pro-calcific phenotypic shift, as evidenced by increased expression of bone-related markers versus endothelial markers.

In recent years, evidence accumulated in support of the existence of circulating progenitors for several lineages important for the cardiovascular system, including endothelial (EPCs), smooth muscle, and cardiomyocyte progenitor cells [3, 4, 24]. EPCs are by far the most extensively characterized of these circulating progenitors; they are defined by co-expression of immaturity (e.g., CD34) and endothelial (e.g., KDR) antigens [25]. About 10–15% of circulating CD34+ express KDR, the %KDR expression is usually taken to represent the extent to which circulating progenitors are committed to the endothelial lineage [21]. Recent data have demonstrated that circulating CD34+ progenitor cells and CD34+KDR+ EPCs can also express bone-related proteins, especially OC [26, 27]. Several preclinical studies and preliminary clinical evidence indicate that EPCs home to sites of vascular damage [28, 29]. Therefore, an osteogenic differentiation of these cells may be involved in the process of vascular calcification. Gössl et al. have found that OC expression on circulating EPCs is significantly associated with CAD in a cohort of patients with a very low prevalence of diabetes (7/72, 10%) [27].

Subsequently, they have demonstrated that OC-expressing EPCs are retained in the coronary circulation of

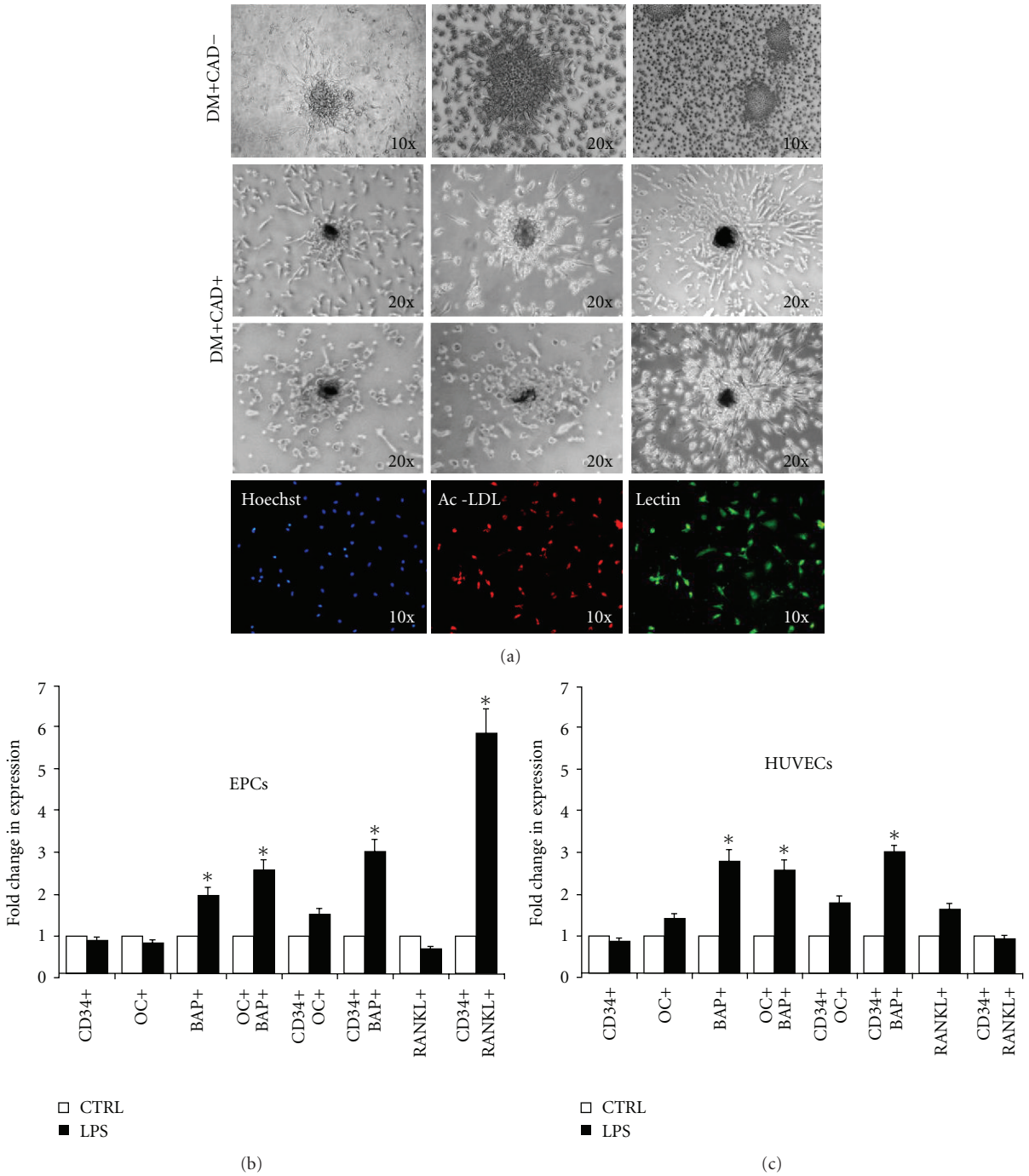

Figure 2: Pro-calcific differentiation of cultured EPCs. (a) EPCs cultured from DM+CAD+ patients developed dense nodules highly suggestive of calcification, while EPCs cultured from DM+CAD− patients did not. The lower lane shows double Lectin/AcLDL immunofluorescence analysis of cultured EPCs. ((b) and (c)) Cultured EPCs and HUVECs express bone-related antigenic markers after challenging with LPS 100 nM for 7 days. $^*P < 0.05$ versus control experiment, set at 1.0.

patients with coronary endothelial dysfunction, providing an indirect evidence in support of homing of these pro-calcific cells at sites of vascular damage [26]. We have previously shown that OC+BAP+ myeloid calcifying cells (MCCs) are increased in the bloodstream and in calcified atherosclerotic lesions of type 2 diabetic patients [15], providing the first evidence that circulating cells may contribute to ectopic vascular calcification. However, so far, there was no data on pro-calcific differentiation of circulating progenitor cells in diabetic patients. This is of paramount importance because diabetes is typically associated with an exceedingly high prevalence of vascular calcification, either medial or intimal

[30]. Herein, we show that expression of OC and BAP on CD34+ cells is increased in patients with either DM or CAD and that the coexistence of DM and CAD is associated with an almost doubled expression of these bone-related proteins. OC is a noncollagenous bone protein implicated in bone mineralization and calcium homeostasis, while BAP is a tetrameric glycoprotein found on the surface of osteoblast cells, and its function is essential to the mineralization process. If these cells are recruited to sites of vascular damage, it is easy to anticipate how they may promote the process of vascular calcification. Importantly, we have previously shown that the expression of KDR on CD34+ cells is reduced in diabetic patients with macroangiopathy, indicative of an impaired endothelial differentiation and generation of EPCs. Together with the enhanced osteogenic polarization, data consistently suggest that circulating progenitor cells of diabetic patients undergo a phenotypic drift toward the detrimental osteogenic phenotype at the expenses of the vasculoprotective endothelial phenotype. To quantitatively support this hypothesis, we examined the expression ratio of bone-related markers OC and BAP over KDR on circulating CD34+ cells. We found that OC/KDR, BAP/KDR, and OC+BAP/KDR are markedly elevated only in patients with DM and CAD and not in patients with either conditions, strengthening the association between this pro-calcific drift and diabetic vascular disease.

The degree of pro-calcific differentiation of CD34+ cells resembles the extent of OC and BAP expression on circulating monocytes and the levels of MCCs [15], suggesting that the driving force of the osteogenic program acts similarly on different cellular populations. To study the pro-calcific polarization of progenitor cells in vitro, we cultured late outgrown EPCs and found that they formed hyperdense nodular structures, highly suggestive of calcifications, only in DM+CAD+ patients and not in DM+CAD− patients. We hypothesized that EPC calcification may be driven by chronic inflammation through stimulation of innate immunity receptors, such as CD14 and TRLs [23, 31, 32], which are expressed by EPCs. Indeed, this pathway has been previously shown to be overactivated in diabetic patients and cardiovascular disease [33]. When EPCs were isolated from DM−CAD− patients and cultured with or without the TRL ligand LPS, expression of bone-related markers was significantly upregulated, also in co-expression with CD34. Of note, OC and BAP overexpression was found also in HUVECs in response to LPS, indicating that this phenotypic change occurs in endothelial cells independently of their origin. This finding should be viewed in light of the postulated cross-talk between endothelial cells and osteoblasts in the regulation of bone turnover [34]. Remarkably, induction of the osteoblast marker RANKL by LPS occurred only in EPCs and not in HUVECs, supporting that EPCs have a stronger tendency toward the osteogenic phenotype. It should be carefully noted that we did not definitely prove that EPC calcified in vitro. While it may be surprising that these cells spontaneously deposit calcium in culture without osteogenic stimuli, the relatively high serum concentration used for EPC isolation (20%) may represent a source of calcium/phosphate. Our data are supported by the recent finding of Liu et al. showing that oxidized low-density lipoprotein and β-glycerophosphate induce extensive EPC calcification in vitro [35]. However, further studies are needed to define in greater detail the calcification potential of EPCs in different culture conditions and in vivo.

5. Conclusion

Our data have important implications for the interpretation of circulating progenitor cell phenotype in relation to cardiovascular complications of diabetes. Reduced progenitor cell level and impaired endothelial differentiation are currently considered mechanisms whereby diabetes causes endothelial dysfunction and excess vascular damage [11]. Our present data indicating pro-calcific differentiation of circulating progenitors add a new plug to the puzzle and identify a hitherto unrecognized potential mechanism of vascular calcification in diabetes.

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Research Article

Amelioration of Glucose Control Mobilizes Circulating Pericyte Progenitor Cells in Type 2 Diabetic Patients with Microangiopathy

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Chronic diabetic complications result from an imbalance between vascular damage and regeneration. Several circulating lineage-committed progenitor cells have been implicated, but no data are available on pericyte progenitor cells (PPCs). Based on the evidence that PPCs increase in cancer patients after chemotherapy, we explored whether circulating PPC levels are affected by glucose control in type 2 diabetic patients, in relation to the presence of chronic complications. We enumerated peripheral blood PPCs as Syto16+CD45−CD31−CD140b+ events by flow cytometry at baseline and after 3 and 6 months of glucose control by means of add-on basal insulin therapy on top of oral agents in 38 poorly controlled type 2 diabetic patients. We found that, in patients with microangiopathy ($n = 23$), the level of circulating PPCs increased about 2 fold after 3 months and then returned to baseline at 6 months. In patients without microangiopathy (control group, $n = 15$), PPCs remained fairly stable during the whole study period. No relationship was found between change in PPCs and macroangiopathy (either peripheral, coronary, or cerebrovascular). We conclude that glucose control transiently mobilizes PPCs diabetic patients with microangiopathy. Increase in PPCs may represent a vasoregenerative event or may be a consequence of ameliorated glucose control on microvascular lesions.

1. Introduction

Chronic diabetic complications are thought to result from the detrimental effects exerted by hyperglycemia and associated metabolic abnormalities on vascular structure and function [1]. Moreover, in recent years, it became apparent that vascular regeneration is impaired in diabetes, at least in part through pauperization of bone-marrow-derived progenitors [2]. Indeed, evidences accumulated to support the existence of circulating progenitors for several phenotypes not limited to the hematopoietic lineages and potentially important for the cardiovascular system [3]. Thus, endothelial [4], smooth muscle [5], osteoblast [6] and, possibly, cardiomyocyte progenitor cells [7] from the bloodstream have been described. These cells may have various protective or detrimental effects on vascular structure and function, although their quantitative contribution to cardiovascular biology is far from being definitely elucidated [3]. In parallel, the presence of mature circulating endothelial cells (CECs) is meant to represent an epiphenomenon of the ongoing vascular damage, as these cells are passively released from the vessel wall [8]. Importantly, most these cells have been implicated in the setting of diabetes and its chronic complication, suggesting a multifaceted contribution of blood-derived cells in the complex pathophysiology of diabetic micro- and macroangiopathy. These include reduced EPCs [9] and cardiomyocyte differentiation [10], increased generation of smooth muscle progenitor cells [11] and procalcific cells [6], paralleled by a high concentration of circulating shed CECs [12]. Very recent data suggest the existence of circulating

pericyte progenitor cells (PPCs) [13]. Pericytes provide a variety of functions, such as capillary blood flow regulation, clearance and phagocytosis of cellular debris, and regulation of vascular permeability. Importantly, pericytes stabilize and monitor the maturation of endothelial cells by direct communication between the cell membrane and paracrine signaling [14]. They are recruited through the PDGF-B and PDGFR-Beta signaling, while PDGFR-Beta deficient mice display extensive vascular leakage, hemorrhage, and edema due to a defect of capillary coverage by pericytes [15, 16]. Interestingly, PPCs were found to be increased in patients and mice with malignant tumors and also increased after chemotherapy [13].

We hypothesized that PPCs play a role in the setting of diabetic microangiopathy. Based on this background and on the proposed role for PPC in response to cancer therapy, in this study we explored whether glucose control affects levels of circulating PPCs in type 2 diabetic patients, in relation to microvascular complications.

2. Materials and Methods

2.1. Patients. The study was approved by the Ethic committee of the University Hospital of Padova (protocol no. 1584P) and is registered in http://clinicaltrials.gov/ (NCT00699686). It was conducted in accordance with the Declaration of Helsinki and all patients provided written informed consent. Briefly, this was a trial of optimization of glucose control in type 2 diabetic patients poorly controlled on oral agents, with addition of basal insulin on top of their ongoing antihyperglycemic regimen. Insulin glargine and insulin detemir were compared in a randomized cross-over fashion during a 3+3 month period. The study design and clinical characteristics of the study population have been previously described [17]. The primary aim was to detect differences in the change of endothelial progenitor cells (EPCs) and circulating progenitor cells (CECs) levels in the bloodstream between the 2 insulin regimens. Out of a total of 42 patients, 21 were randomized to receive insulin glargine for 3 months and then insulin detemir for 3 months without washout, and 21 patients were randomized to the detemir-glargine treatment sequence. As a result of the study, we found that optimization of glucose control per se reduced CECs and other markers of endothelial damage, and increased EPCs, as markers of endothelial regeneration [17]. There was no difference in the effects of glargine versus detemir in terms of markers of endothelial damage and regeneration. This allowed us to consider the 2 insulin regimens and a single type of treatment. In parallel to EPCs and CECs, we also quantified circulating pericyte progenitor cells (PPCs) to evaluate the effects of glucose control on this cell type. PPC analysis was carried out in 38 patients and was unsuccessful in 4, due to technical reasons. Inclusion criteria were T2D with HbA1c >7.0% on oral agents, age 40–80 and presence of macroangiopathy (either coronary, peripheral or cerebrovascular artery disease). Exclusion criteria were T1D, acute hyperglycaemia, use of glitazones, DPP-4 inhibitors, cancer, any acute disease or infection, recent (within 3 months) surgery or cardiovascular intervention, serum creatinine >2.0 mg/dL, advanced liver disease, inability to provide informed consent, and pregnancy/lactation. All patients were characterized with anthropometric measures, evaluation of concomitant risk factors, diabetic complications and medications, as described elsewhere. Briefly, retinopathy was defined by a digital funduscopic examination as any degree of retinopathy according to the Early Treatment Diabetic Retinopathy Study (ETDRS) Research Group classification [18]. Nephropathy was defined by measuring urinary albumin/creatinine ratio on 3 different samples and the estimated glomerular filtration rate according to the MDRD equation [19]. Neuropathy was defined according to classical symptoms and signs, eventually confirmed by electromyography.

2.2. Flow Cytometry. Analysis was performed on frozen peripheral blood mononuclear cells according to a standardized protocol. PPCs were evaluated by six-color flow cytometry following an approach recently validated in our laboratory for the enumeration of CECs with some modifications [13]. PPCs were defined as Syto16+CD45−CD31−CD140b+ events. The nuclear staining Syto16 was used to discriminate between nucleated cells, platelets, and cell debris. The panel of monoclonal antibodies used included anti-CD45 (to exclude hematopoietic cells), anti-CD31 (an EC differentiation marker), and anti-CD140b (PDGFR-Beta). All antibodies were from Becton Dickinson (BD, Mountain View, CA). Cell suspensions were evaluated after cell recovery by a FACSCanto (BD). After acquisition of at least 1×10^6 cells per blood sample, analyses were considered as informative when adequate numbers of cells (i.e., >100) were collected in the PPC enumeration gates. PPCs were defined as nucleated cells, negative for the hematopoietic marker CD45 and the EC marker CD31 and positive for CD140b. The gating strategy is illustrated in Figure 1. This definition identifies circulating cells not belonging to either leukocyte populations (CD45-neg) or shed endothelial cells (CD31-neg) and expressing the pericyte marker CD140b (PDGFR-Beta).

2.3. Statistical Analysis. Data are expressed as mean ± standard error for continuous variables or as percentages for categorical variables. Comparisons between two groups were performed using two-tail Student's t-test for continuous variables or the chi-square test for categorical variables. To assess changes of PPC levels over time, we used the analysis of variance (ANOVA) for repeated measures with post-hoc paired t-tests. Statistical significance was accepted at $P < 0.05$.

3. Results

3.1. Patient Characteristics and Effects of Glucose Control. The characteristics of the 38 patients included in the study and divided by the presence/absence of microangiopathy are resumed in Table 1. Microangiopathy was defined as the presence of anyone among retinopathy, nephropathy (micro- or macroalbuminuria with or without renal failure), and neuropathy. Besides retinopathy, microalbuminuria, and

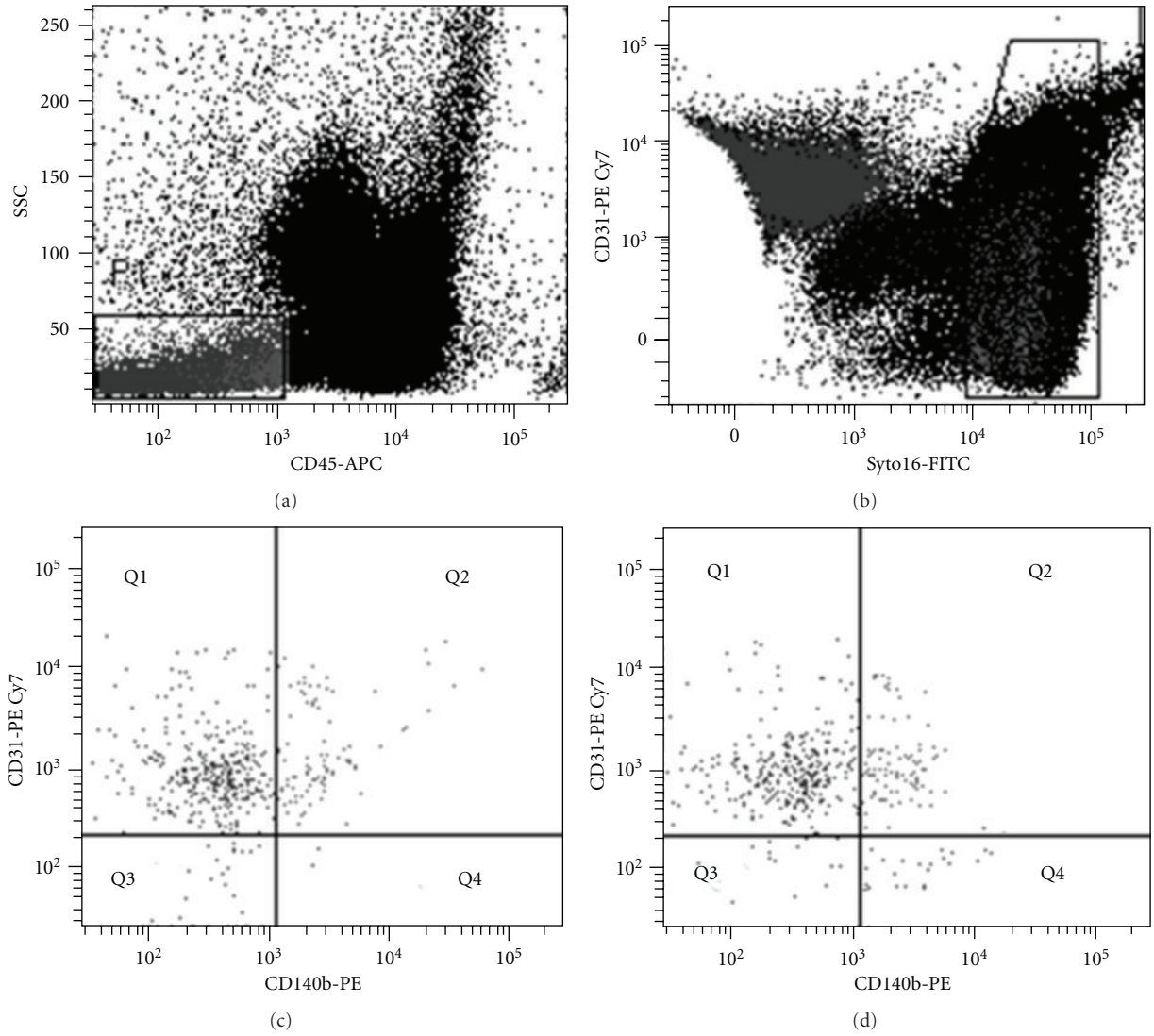

Figure 1: The gating strategy for enumeration of circulating PPCs. (a) Peripheral blood mononuclear cells were first gated into the CD45-negative fraction to exclude hematopoietic cells. (b) The total Syto16+ population of nucleated cells was selected to avoid inclusion of contaminating red cells, platelets, and debris in the analysis. (c, d) The resulting population was analyzed for expression of CD31 and CD140b. Panel (c) shows a case with low baseline PPCs (Syto16+CD45−CD31−CD140b+ cells), while (d) shows the same case 3 months after initiation of glucose control.

neuropathy, differences between the two groups regarded lower HDL cholesterol levels and higher incidence of peripheral arterial disease (PAD) in patients with microangiopathy. These patients were subjected to optimization of glucose control for 6 months by means of addition of a basal insulin therapy according to a protocol described elsewhere. As there were no differences between glargine and detemir in the effects on endothelial markers of damage and regeneration, these treatment regimens were considered altogether as a single intervention. On average, HbA1c dropped from 8.8 ± 0.2% to 7.2 ± 0.1% ($P < 0.001$) indicating good optimization of glucose control, and 17 patients (45% of total) reached a HbA1c level of 7.0% of lower. Between the two groups (w/wo microangiopathy), there were no differences in baseline HbA1c levels or achieved HbA1c during the intensification protocol at 3 months (no microangiopathy 7.4 ± 0.2; microangiopathy 7.2 ± 0.1; $P = 0.30$) and 6 months (no microangiopathy 7.3 ± 0.2; microangiopathy 7.1 ± 0.1; $P = 0.17$; Figure 2(a)).

3.2. Changes in PPC Levels during Optimization of Glucose Control. Circulating PPCs were measured at baseline, 3 months and 6 months. In the entire study population of 38 subjects, there was a trend toward increased PPC levels at 3 months versus baseline, which was not statistically significant ($P = 0.29$). There were no differences in PPC levels according to type of insulin used ($P = 0.74$ in the analysis for cross-over design). When patients were divided according to the presence or absence of microangiopathy, we found that PPC level remained unchanged during the entire course of the study

Table 1: Patients characteristics. *P* values are shown for paired Student's *t*-test or the chi-square test as appropriate. ACEi/ARB denotes angiotensin-converting enzyme inhibitors or angiotensin receptor blockers.

Characteristic	Without microangiopathy	With microangiopathy	*P*
Number	23	15	—
Age (years)	62.7 ± 2.4	67.2 ± 1.2	0.08
Sex male (%)	68.8	81.8	0.36
BMI (kg/m^2)	29.5 ± 1.3	27.2 ± 0.6	0.10
Waist (cm)	103.5 ± 3.3	99.8 ± 2.2	0.35
Baseline HbA1c (%)	9.0 ± 0.3	8.6 ± 0.2	0.19
Concomitant risk factors			
Total cholesterol (mg/dL)	183.1 ± 6.5	176.8 ± 9.7	0.61
HDL cholesterol (mg/dL)	51.3 ± 2.9	43.2 ± 2.1	0.026
LDL cholesterol (mg/dL)	105.6 ± 5.2	97.1 ± 8.5	0.43
Triglycerides (mg/dL)	131.1 ± 14.5	181.8 ± 25.2	0.11
Smoking habit (%)	0.0	13.6	0.13
Hypertension (%)	93.8	81.8	0.29
Complications			
Retinopathy (%)	0.0	45.5	<0.01
Microalbuminuria (%)	0.0	50.0	<0.01
Neuropathy (%)	0.0	40.9	<0.01
Peripheral arterial disease (%)	6.3	36.4	0.03
Coronary artery disease (%)	18.8	27.3	0.55
Cerebrovascular disease (%)	75.0	77.2	0.82
Medications			
Metformin (%)	93.8	81.8	0.29
Sulphonylureas (%)	68.8	68.2	0.97
Aspirin (%)	68.8	86.3	0.19
Statin (%)	56.3	68.2	0.46
ACEi/ARBs (%)	87.5	63.6	0.10
Other antihypertensives (%)	68.8	63.6	0.75

in patients without microangiopathy, while it significantly increased at 3 months only in patients with microangiopathy ($P = 0.01$ using post-ANOVA *t*-test; Figure 2(b)). Among the 3 different types of microangiopathy that were considered, presence of micro-/macroalbuminuria (Figure 2(c)) and neuropathy (Figure 2(d)) were associated with PPC increase at 3 months, while retinopathy was not significantly discriminative of patients that increase PPC levels during the glucose control protocol (Figure 2(e)). Interestingly, in all cases, PPC levels returned to baseline at 6 months. As a control experiment, we also divided patients according to the presence/absence of PAD, which was more prevalent in patients with microangiopathy, and found that there was no differences in the trend of PPC levels over time in the two groups of patients (Figure 2(f)). The same was for coronary and cerebrovascular disease, which showed no correlation with PPC levels over time (not shown). Concentration of HDL cholesterol was not associated with change in PPC levels during the study (not shown).

4. Discussion

In this study, we found that in type 2 diabetic patients with microangiopathy glucose control is associated with a transient increase in circulating PPC levels.

Mounting evidence suggests that multilineage circulating progenitor cells have a variety of implications in diabetes and its complications. After endothelial progenitor cells (EPCs), smooth muscle progenitors, osteoblast precursors, and cardiomyocyte progenitors [4–6, 20], recent data now suggest the existence of circulating pericyte progenitor cells (PPCs) [13]. These cells have been identified and isolated from human or murine peripheral blood and reside in the nonhematopoietic (CD45-neg) compartment, and are distinct from CECs as they lack endothelial antigens (CD31-neg), but express the typical pericyte marker CD140b (PDGFR-Beta). This antigenic phenotype supports the pericytic origin, while electron microscopy confirmed their progenitor-like morphology, with high nucleus/cytoplasm

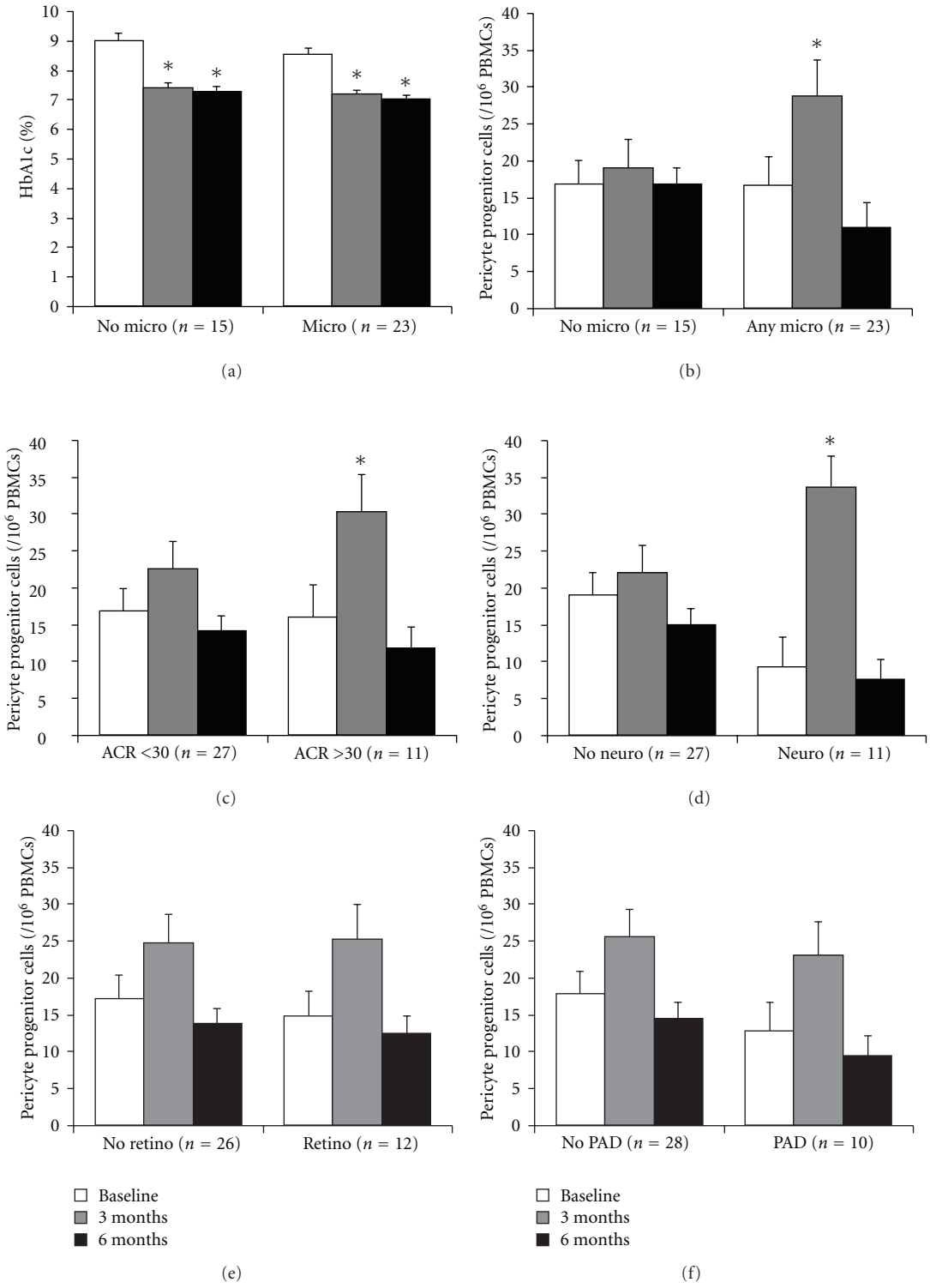

Figure 2: Effects of glucose control on HbA1c and PPCs. (a) There were no differences in HbA1c levels in patients with and without microangiopathy during time (*$P < 0.05$ versus baseline). (b) Increase in PPC levels was seen only in patients with microangiopathy (ANOVA $P < 0.05$; *post hoc $P < 0.05$). (c–f) Patients were divided according to the presence of micro-/macroalbuminuria, neuropathy, retinopathy, and peripheral arterial disease (PAD): a significant PPCs increase was detected in patients with urinary albumin-creatinine ratio (ACR) >30 mg/g and in the presence of neuropathy (ANOVA $P < 0.05$; *post hoc $P < 0.05$).

ratio, rough endoplasmic reticulum cisterns and centrioles and absence of Weibel-Palade bodies typical of CECs [13].

In the setting of diabetic complications, pericytes may play an important role. Pericytes are an important component of the neurovascular unit both in the central and peripheral nervous system and may intervene in the pathogenesis of peripheral neuropathy, through the modulation of vasa nervorum [21]. Moreover, glomerular mesangial cells, which play a central role in the pathobiology of diabetic nephropathy [22], are specialized pericytes [23]. Finally, pericyte loss is one of the earliest features of diabetic retinopathy and the consequent defective endothelial coverage of retinal capillaries favors microaneurysmatic dilation and increased permeability [24]. Therefore, the study of PPCs may have important implications in the setting of diabetic microvascular complications.

Interestingly, PPCs were found to be increased in patients and mice with malignant tumors and also increased after chemotherapy [13]. Therefore, we analyzed whether the level of circulating PPCs is influenced by optimization of glycemic control in type 2 diabetic patients in relation to the presence of microangiopathy. In a cohort of 38 patients in which HbA1c was drastically reduced by insulin therapy, a significant increase in PPC level at 3 months was detected only in the presence of microangiopathy. Of note, this increase was transient, as cell counts returned to baseline at 6 months. Importantly, the PPC increase occurred during the first 3 month period, when HbA1c dropped markedly and then stabilized for the subsequent 3 months, suggesting that glucose control was the driver of PPC increase. We found that nephropathy and neuropathy were associated with PPC mobilization, while retinopathy was not. This is probably due to the fact that most patients had mild nonproliferative retinopathy and that a stratification for retinopathy severity was impossible, as groups of patients were too small. Moreover, the systemic levels of PPCs may not reflect processes ongoing within the central nervous system.

There are several potential implications of our present findings. First, it is possible that glucose control induces a mobilization of bone-marrow-derived PPCs, as previously shown for EPCs [25]. These cells would then function to stabilize blood vessels and counter the progression of diabetic microangiopathy. However, the study of GFP+ bone marrow chimeric mice suggests that murine PPCs are derived from peripheral tissues and not from the bone marrow [13]. Therefore, non-bone-marrow sources of these regenerative cells should be postulated [26]. The Madeddu's laboratory has clearly demonstrated that PPCs can be isolated from the saphenous vein and display potent cardiovascular regenerative activity [27, 28]. At present, we can only speculate on the mechanisms that induce PPC mobilization: it has been previously documented that circulating progenitor cells are recruited from the bloodstream to the perivascular space through the SDF-1/CXCR4 axis, whence they are mobilized by VEGF [29]. As insulin has been reported to stimulate VEGF and to interact with PDGFR (CD140b) signaling [30, 31], these growth factors may be important. A transient release of tissue PPCs induced by glucose control may also reflect regression of pathologic vascular structures in organs hit by diabetic microangiopathy, just as it happens in cancer chemotherapy. Regression of microvascular lesions owing to lower oxidative stress and inflammation achieved by glucose control [32] may also be responsible for passive mobilization of these cells from tissues to the bloodstream. Alternatively, the transient PPCs increase may be related to the worsening of diabetic microangiopathy that is sometimes induced by rapid glucose control [33, 34]. Unfortunately, owing to the relatively short duration of our study, it is impossible to determine whether the increase in PPC was associated with a favorable or unfavorable evolution of microangiopathy.

This study has other limitations, including the relatively small sample size and, importantly, the incomplete characterization of circulating PPCs. Indeed, it must be noted that cogent demonstrations that these Syto16+CD45–CD31–CD140b+ cells truly belong to the pericyte lineage and act as progenitors are still missing for the following reasons. First, there is no surface antigen that can unequivocally identify pericyte lineage cells. Second, we found a small degree of coexpression of the pericyte marker NG2 [35] by circulating CD140b cells (not shown), suggesting that the pericytic phenotype of PPCs is incomplete. Additionally, it is not clear how PPCs are related to CD34+CD140b+ cells, which Schober et al. identified as perivascular smooth muscle progenitors cells related to the severity of cardiac allograft vasculopathy [36]. Finally, selective culture of PPCs is needed to test their phenotype, proliferative potential, and function in vitro and in vivo.

5. Conclusions

Despite these drawbacks, the interpretation of our results lends to several intriguing speculations on the pathophysiology of diabetic microangiopathy and response to therapy. The mobilization of PPCs induced by amelioration of glucose control deserves a special attention in relation to the evolution of microangiopathy over time. Further studies are required to reach a better characterization of PPCs and to understand their relationships with diabetic complications.

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Review Article

Strategies to Reverse Endothelial Progenitor Cell Dysfunction in Diabetes

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Bone-marrow-derived cells-mediated postnatal vasculogenesis has been reported as the main responsible for the regulation of vascular homeostasis in adults. Since their discovery, endothelial progenitor cells have been depicted as mediators of postnatal vasculogenesis for their peculiar phenotype (partially staminal and partially endothelial), their ability to differentiate in endothelial cell line and to be incorporated into the vessels wall during ischemia/damage. Diabetes mellitus, a condition characterized by cardiovascular disease, nephropathy, and micro- and macroangiopathy, showed a dysfunction of endothelial progenitor cells. Herein, we review the mechanisms involved in diabetes-related dysfunction of endothelial progenitor cells, highlighting how hyperglycemia affects the different steps of endothelial progenitor cells lifetime (i.e., bone marrow mobilization, trafficking into the bloodstream, differentiation in endothelial cells, and homing in damaged tissues/organs). Finally, we review preclinical and clinical strategies that aim to revert diabetes-induced dysfunction of endothelial progenitor cells as a means of finding new strategies to prevent diabetic complications.

1. Introduction

Endothelial progenitor cells (EPCs) are a subset of bone-marrow-derived cells committed to the maintenance and preservation of vascular turnover, remodeling, and homeostasis [1]. EPCs are immature cells, endowed with the capacity to be mobilized from the bone marrow into the bloodstream in response to growth factors and cytokines release [2, 3]. EPCs may differentiate into endothelial cells and finally take part in the vascular repair [2, 3]. Since 1997, when Asahara et al. published on Science the discovery of a population of circulating $CD34^+$ cells showing proliferative capacity and ability to differentiate into mature endothelial cells *in vivo* and *in vitro* [4], much debate on EPCs origin and controversies on the appropriate isolation method was generated and several acronyms have been used to refer to this cell population. Different markers have been used to describe i*n vivo* circulating EPCs, among them we should mention $CD34^+VEGFR2^+$, $CD34^+CD133^+VEGFR2^+$, $CD133^+VEGFR2^+$, and $CD133^+VeCadherin^+$ [5]. Several *in vitro* culture methods to isolate EPCs have been reported as well: colony-forming unit-endothelial cells (CFU-ECs), circulating angiogenic cells (CACs), and endothelial colony forming cells (ECFCs) [6]. Early EPCs exhibit a spindle-shaped morphology *in vitro*, have poor proliferative capacity, and produce to high extent angiogenic cytokines (e.g.; VEGF), while late EPCs show a cobblestone morphology *in vitro*, highly proliferative activity, and the ability to directly incorporate into capillary vessels [6]. However, despite the several subtypes of EPCs, there is agreement in the literature that EPCs from healthy subjects are able to repair blood vessels wall and that dysfunctional EPCs are defective

in angiogenic properties, thus contributing to vascular diseases and progression of cardiovascular syndrome [7]. Thus, healthy EPCs might represent a precondition for a functioning cardiovascular system. Indeed, EPCs number and function have been reported to be impaired in type 1 (T1D) and type 2 (T2D) diabetes [8, 9] as well as in presence of cardiovascular risk factors [10–12], while a normalization of EPC function was found in euglycemic islet-transplanted patients [13], despite immunosuppressive treatment, thus justifying the improvement of diabetic complications in these patients. We will review how diabetes interferes with EPC function and subsequently summarize potential strategies to restore/repair EPC function in diabetic patients.

2. EPC Dysfunction in Diabetes

Diabetes and hyperglycemia may affect EPC function at each step of their lifetime. In this section we provide evidence of the current knowledge on diabetes-induced damage during EPC lifespan.

2.1. Mobilization from Bone Marrow. Several studies have focused on diabetes-mediated impaired EPC recruitment in the peripheral blood. Hyperglycemia was shown to affect bone-marrow-harbored EPCs by generating a diffused endothelial damage, microvascular remodeling, and reduction in c-kit$^+$ Sca-1$^+$ cells [14] in chemically induced (STZ) diabetic mice. Moreover, in this model, EPC deficiency was associated with an increased oxidative stress, DNA damage, and cell apoptosis [14]. The molecules involved in EPC mobilization process from bone marrow are circulating molecules like SDF-1α [15, 16], VEGF [17], GM-CSF [18], IL-8 [19], and cleaving enzymes [2]. SDF-1, which interacts with CXCR-4 receptor on target cells, is released by ischemic tissues [15] (via a HIF-1α-mediated induction) and is involved in EPC mobilization [15], homing into vascular structures [15], and differentiation [16]. Similarly, the role of VEGF in EPC mobilization has been widely studied in both humans and mice showing that, following acute ischemic injury, plasma levels of VEGF increase rapidly leading to a 50-fold increase in EPC percentage in the peripheral blood [17]. Among the several mechanisms involved in the impaired bone marrow mobilization of EPCs in diabetes, endothelial nitric oxide synthase (eNOS) dysfunction has been clearly demonstrated [20, 21]. Since uncoupling of eNOS leads to superoxide anion formation instead of nitric oxide (NO), Thum et al. hypothesized that such an altered enzyme activity could have a role in the reduction of EPC number in diabetic patients because of hyperglycemia-mediated increased oxidative stress [20]. Moreover, in streptozotocin-induced diabetic rats, EPCs were 39% less than in controls and this was associated with eNOS uncoupling in the bone marrow [20]. In a model of hind-limb ischemia-reperfusion (I/R) injury, plasma levels of VEGF and SDF-1α were measured and EPCs mobilization after ischemic injury was studied in diabetic rats and compared to euglycemic rats [22]. In this study, diabetic rats proved to be unable to mobilize EPCs after ischemic injury and this evidence was associated with a reduced release of VEGF and SDF-1α from ischemic muscle [22]. Interestingly, Gallagher et al. confirmed the relationship between SDF-1α reduced production and impaired EPCs peripheral counts in a diabetic murine model of wound healing [21]. Beyond soluble molecules, cleaving enzymes have shown a relevant role in EPCs mobilization: cathepsins (in particular Cathepsin L was shown to be essential for autoimmune diabetes in mice [23]) and elastases are released by neutrophils under conditioning with G-CSF and promote the cleavage of bonds between cells and stroma and the cleavage of SDF-1α/CXCR-4 interaction, thus inducing EPCs shedding; finally, MMP-9, a proteolytic enzyme found to be activated in diabetes [24], is essential for VEGF and SDF-1α-mediated EPCs mobilization [2]; indeed eNOS knockout mice (which mediates VEGF and SDF-1α signaling) promotes a reduced MMP-9 activity and an impaired MMP-9-mediated progenitor cells release [25, 26].

2.2. Trafficking. Once EPCs have been mobilized in the bloodstream, they migrate to the sites of ischemia/damage, in a process known to be mediated by SDF-1α [15] and VEGF [27]. Segal et al. demonstrated that EPCs harvested from patients affected by T1D and T2D in presence of SDF-1α showed an impaired migration compared to healthy control subjects [28]. The isolated EPCs were also characterized by a reduced cytoskeleton plasticity [28]. Interestingly, they demonstrated that treatment with exogenous NO corrects both migration defect and deformability impairment of diabetic EPCs [28]. Moreover, glucose-dependent and protein kinase C- (PKC-) mediated eNOS uncoupling, which results in hyperproduction of ROS rather than NO production, is associated with defective migratory capacity of EPCs from diabetic patients compared to nondiabetic controls [20]. Leicht et al. observed that late EPCs isolated from patients with T2D had impaired proliferation and migratory capacity compared to cells isolated from young healthy donors or non-diabetic age-matched subjects [29]. Advanced glycation end-products (AGEs) are known to accumulate in diabetes and were proven to impair migration and enhance apoptosis in EPCs cultured from human umbilical cord blood [30]. These effects were inhibited by anti-RAGE antibodies [30]. These data were confirmed by Sun et al. that challenged EPCs with AGE-human serum albumin at different concentrations and found that it significantly decreased EPCs migration [31]. Another way in which diabetes may alter EPCs' trafficking is lipotoxicity. It is known that oxidized LDL (Ox-LDL) is associated with reduced number and increased senescence of EPCs and these effects seem to be related to Akt activation, p21 expression, and p53 accumulation [32].

2.3. Survival. EPC trafficking in the bloodstream are more susceptible to diabetes-induced apoptosis. Indeed, a lower EPCs peripheral count has been described in diabetic murine models [21, 33]. Nevertheless, several studies have associated diabetes with reduced EPCs number when cultured *ex vivo*, due to both an increased apoptosis [30, 31, 34] or diminished proliferation [29, 32, 35, 36]. In our study, the percentage

of circulating EPCs did not differ between T1D patients, islet-transplanted insulin-independent patients, and healthy controls, and no significant differences in apoptosis could be found among these subjects [13]. However, *in vitro* studies showed reduced number and increased apoptosis of diabetic-derived EPCs while a normalization of both parameters was evident in islet-transplanted patients. Lower secreting levels of IL-8 from EPCs cultured from T1D patients and a dose-dependent decrease of control EPCs number in presence of IL-8 antagonist (anti-IL-8) induced to speculate on the role of this chemokine in angiogenesis [13]. Several other investigators reported a reduced survival of EPCs cultured *ex vivo* in hyperglycemic conditions. Chen et al. cultured different subtypes (early and late) of EPCs with high glucose demonstrating a dose-dependent reduction of early EPCs number, reduced proliferation, and impaired migration ability of late EPCs compared to mannitol treatment [36]. High-glucose-mediated negative effects were restored by NO treatment and worsened by PI3K or eNOS inhibition [36]. Interestingly, it has been recently shown that treatment with adiponectin of human and murine EPCs prevents accumulation of high-glucose-induced premature senescence [33]. Other intracellular pathways have been demonstrated to be involved in EPCs survival in diabetes. The p38 MAPK pathway is activated in EPCs exposed to high glucose, inducing a dose-dependent reduction of *ex vivo* cell counts [35]. Finally, renin-angiotensin-aldosterone system has been described to be involved in EPCs survival process. Indeed, angiotensin II was shown to induce EPCs senescence [37] and aldosterone to downregulate VEGFR-2 expression leading to reduced EPCs number *ex vivo* [38]. These findings acquire interest considering that diabetes correlates with significantly higher circulating levels of angiotensin II and aldosterone [39] and that ACE inhibitors are available in clinical practice.

2.4. Homing and Differentiation. Investigators have outlined several crucial pathways involved in EPCs homing and differentiation. Interaction between SDF-1α and CXCR-4 is again fundamental, given that blockade of either the ligand or the receptor prevents recruitment to injured sites [15]. In 2007, Gallagher et al. demonstrated that the mechanism involved in diabetes-mediated EPCs dysfunctional homing is a reduced local release of SDF-1α and NO in the sites of wound and that SDF-1α exogenous administration could lead to a faster recovery of the wound [21]. Impaired capacity of EPCs to support endothelial tube formation was evidenced in T1D patients as well [8]. Marchetti et al. determined the effects of glucotoxicity on EPCs in *de novo* tube formation by culturing isolated EPCs from healthy donors with high glucose or high glucose plus benfotiamine, a scavenger of glucotoxicity [40]. While glucotoxicity led to impaired EPCs-mediated tube formation on matrigel (associated with a reduced activity of FoxO1) [40], benfotiamine could restore both FoxO1 activity and EPCs differentiation [40]. Another study showed that chronic incubation of EPCs isolated from healthy donors with high glucose levels impaired tube formation capability *in vitro* (decreasing eNOS and NO availability) [36], but could be improved by coincubation with NO [36]. Finally, the same mechanisms involved in EPC trafficking dysfunction are also relevant in homing and differentiation process.

3. Preclinical Experience in Reverting Diabetes-Mediated EPCs Damage

Several successful approaches to revert diabetes-induced EPCs dysfunction have been described in preclinical models. Herein, they are listed according to whether they have been performed *in vitro* or in animal models.

3.1. In Vitro Studies

Antioxidants. Antioxidants are relevant mediators of EPCs impairment. Indeed, Ceradini et al. demonstrated that glyoxavlase 1 overexpression, an antioxidant key factor that modifies HIF-1α, restored high glucose-induced impairment of CXCR-4 and eNOS expression in EPCs [41]. Moreover, glucose-induced impairment of human EPCs was shown to be reverted by benfotiamine administration which modulates the PI3K/Akt/FoxO1 pathway [40]. Adiponectin-based conditioning of EPCs isolated from both human peripheral blood or mouse bone marrow prevented high glucose-induced senescence that was characterized by decreased ROS accumulation [33].

Antidiabetic Drugs. Currently used antidiabetic drugs showed beneficial effects on EPCs number, and function. Liang et al. cultured EPCs from healthy donors with AGEs and rosiglitazone [34]. Indeed, rosiglitazone was able to reduce EPCs apoptosis, to increase cell number and to enhance migration capacity [34]. Interestingly, insulin was shown to increase angiogenic potential of EPCs via IGF-1 receptor signal in both healthy donors and T2D patients [42].

Gene Therapy. Several approaches aiming to restore EPCs function by knocking down or overexpressing target genes were tested in mice models. Di Stefano et al. showed that EPCs harvested from p66ShcA knockout mice were resistant to high glucose injury [43]. Diabetic EPCs in which p53 gene was deleted did not exhibit senescence and form regular vascular-like structures [32]. Finally, *ex vivo* VEGF gene transfer in EPCs enhanced EPC proliferation, adhesion, and incorporation into endothelial cell monolayers [44].

3.2. Animal Studies

Bone Marrow Mobilizing Factors. In 1999 Takahashi et al. observed that GM-CSF increased circulating EPCs in rabbits and caused an improvement in hindlimb vascularization [18]. In a model of hindlimb ischemia-reperfusion, it was shown that preconditioning with G-CSF and SDF-1α could partially recover impaired postischemic progenitor cell mobilization in diabetic rats [22]. Moreover, Gallagher et al. showed that administration of SDF-1α into wounds

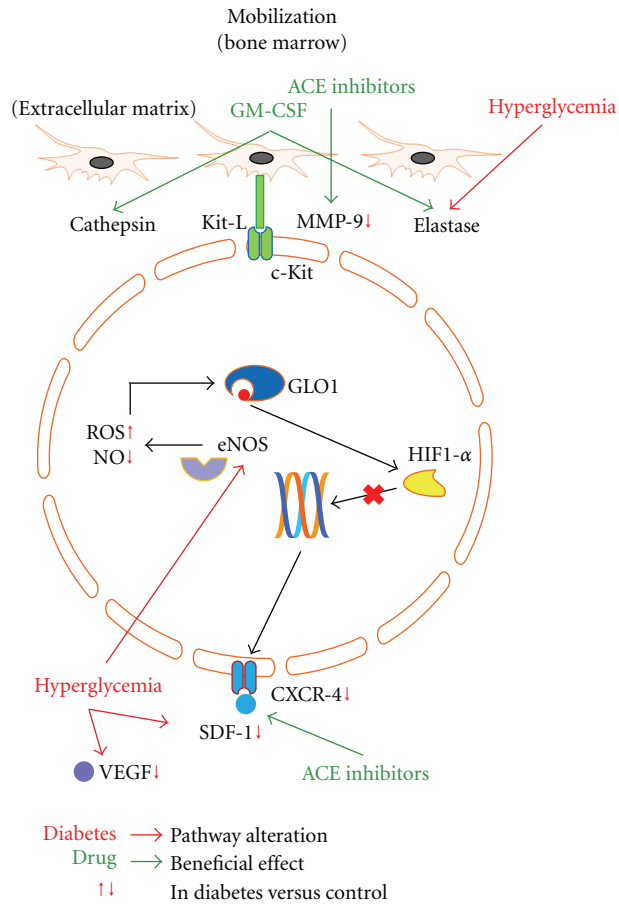

Figure 1: Pathways involved in diabetes-induced EPCs toxicity and possible strategies to reverse EPCs damage during bone marrow mobilization. EPCs recruited from bone marrow are here represented. Hyperglycemia alters CXCR-4/SDF-1α pathway, reduces VEGF levels, increases eNOS-mediated production of ROS and a reduction in cleaving enzymes activity. ACE inhibitors and GM-CSF administration improve bone marrow ability to shed EPCs in the periphery. Diabetes-specific metabolic alterations are in red, linked by red arrows to the pathways they interfere with. Red vertical arrows, next to intracellular or extracellular molecules, indicate that their concentration is diminished or increased in diabetic condition compared to nondiabetic status. Drugs with beneficial effect on EPCs are in green, linked by green arrows to the pathways they interact with. *MMP-9: matrix metalloproteinase-9; GLO-1: glyoxalase-1; ROS: reactive oxygen species; NO: nitric oxide; eNOS: endothelial nitric oxide synthase; HIF1-α: hypoxia inducible factor 1-α; SDF-1α: stem cell-derived factor-1α; CXCR-4: C-X-C chemokine receptor type 4; VEGF: vascular endothelial growth factor; Kit-L: c-Kit ligand.*

of diabetic mice reverted EPC altered homing [21]. Our group recently showed that the targeting of the CXCR4-SDF-1α axis in diabetic mice induced an increased release and engraftment of endogenous EPCs leading to neoangiogenesis and improved ability to heal diabetic wounds [45].

Cell Therapy. Tamarat et al. administered bone marrow mononuclear cells from either non-diabetic or STZ-induced diabetic mice into a mouse model of hindlimb ischemia, which in turn was either diabetic or non-diabetic [46]. Administration of diabetic bone-marrow-derived cells to non-diabetic mice improved neovascularization (compared to saline infusion) in a less extent than the infusion of non-diabetic cells, while injection of non-diabetic bone-marrow-derived cells into diabetic mice improved blood flow recovery, capillary number, and ischemic/non-ischemic angiogenic score compared to the infusion of diabetic bone-marrow-derived cells [46].

Drugs for Cardiomethabolic Control. PPAR-γ agonists were demonstrated to increase mobilization of bone-marrow-derived progenitor cells via stimulation of Akt pathway [47]. ACE or HMG-CoA reductase inhibition resulted in significant increases of EPCs levels [48]. Moreover, ACE inhibitors proved to increase bone marrow ERK phosphorylation and MMP-9 activity, while statin-based therapy led to enhancement of bone marrow VEGF levels, Akt phosphorylation, eNOS activity, and normalized ROS levels [48]. EPCs peripheral levels, during the early postmyocardial ischemia phase, were increased by ACE inhibitors or statins treatment in rats, and this effect was also associated with improved cardiac function and enhanced capillary density in the peri-ischemic area [48]. Enalapril-treated mice showed a

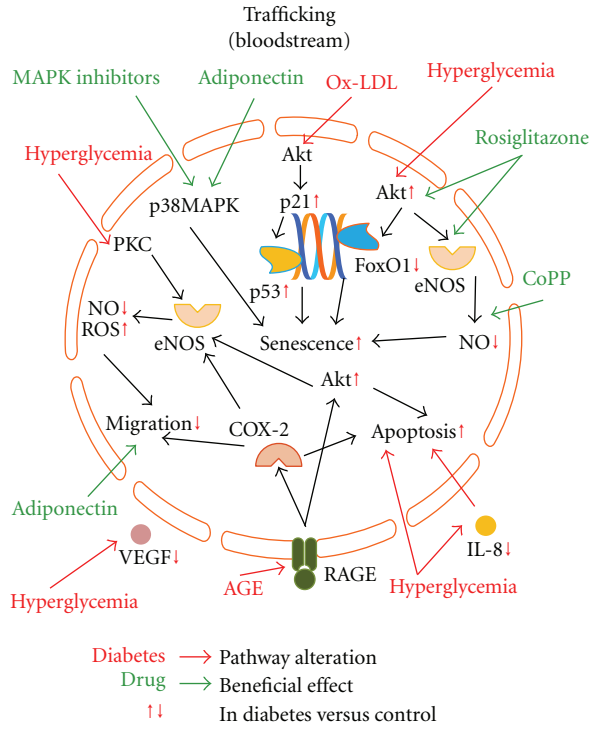

Figure 2: Pathways involved in diabetes-induced EPCs toxicity and possible strategies to reverse EPCs damage during trafficking in the peripheral blood. EPCs trafficking in the peripheral blood are here represented. Hyperglycemia, Ox-LDL, and AGEs accumulation induce an impaired migration ability and reduced cell counts by either increased senescence or increased apoptosis of EPCs in both *in vivo* and *in vitro* assays. Statins, Adiponectin, CoPP, and MAPK inhibitors are able to reverse diabetes-mediated damage on circulating EPCs. Diabetes-specific metabolic alterations are in red, linked by red arrows to the pathways they interfere with. Red vertical arrows, next to intracellular or extracellular molecules, indicate that their concentration is diminished or increased in diabetic condition compared to nondiabetic status. Drugs with beneficial effect on EPCs are in green, linked by green arrows to the pathways they interact with. *ROS: reactive oxygen species; NO: nitric oxide; eNOS: endothelial nitric oxide synthase; VEGF: vascular endothelial growth factor; Ox-LDL: oxidized low-density lipoprotein; MAPK: mitogen-activated protein kinase; CoPP: cobalt protoporphyrin; AGEs: advanced glycation end-products; RAGE: receptor for AGE; PKC: protein kinase C; IL-8: interleukin-8; COX-2: cyclooxygenase-2.*

significant enhancement in circulating progenitor cell levels and a sixfold increase in bone marrow contribution to neoangiogenesis [49]. Interestingly, recent data showed that insulin resistant rats showed an insulin-signaling defect in EPCs that reduces EPC survival and that can be reversed by knocking down NF-kB [50] (see next paragraph).

Gene Therapy. Prevention from diabetes-mediated impairment of *in vivo* angiogenesis has been shown in p66ShcA knockout mice [43]. A recent study by Desouza et al. showed that infusion of EPCs, which were knocked down for NF-kB, led to a decrease in neointimal hyperplasia after carotid angioplasty in a model of type 2 diabetes [51]. Recently, a Phase I clinical trial showed an increase in neoangiogenesis after intramuscular gene transfer of plasmid encoding human VEGF in patients with critical limb ischemia [52].

Restoration of Insulin-Producing Beta Cells Function. We have recently shown that restoration of normoglycemia by successful islet transplantation induced increased number and improved angiogenic ability of EPCs compared to T1D [13].

4. Clinical Experience and Perspectives in Reverting Diabetes-Mediated EPCs Damage

4.1. Improvement of Glycometabolic Control. Optimized glucose control is undoubtedly associated with a better outcome of macro- and microvascular complications in patients affected by diabetes [53]. We demonstrated that insulin-independent islet-transplanted patients showed a recovery of EPCs number and function [13]. Interestingly, diabetes-mediated EPCs dysfunction has been demonstrated to be reversed in obese (non-diabetic) subjects after weight loss [54] meaning that the damage does not seem to be irreversible.

4.2. ACE Inhibitors. Routinely administered drugs as ACE inhibitors and angiotensin receptor blockers proved to benefit EPCs function [55, 56], even though no randomized clinical trials are available yet, thus suggesting to capitalize

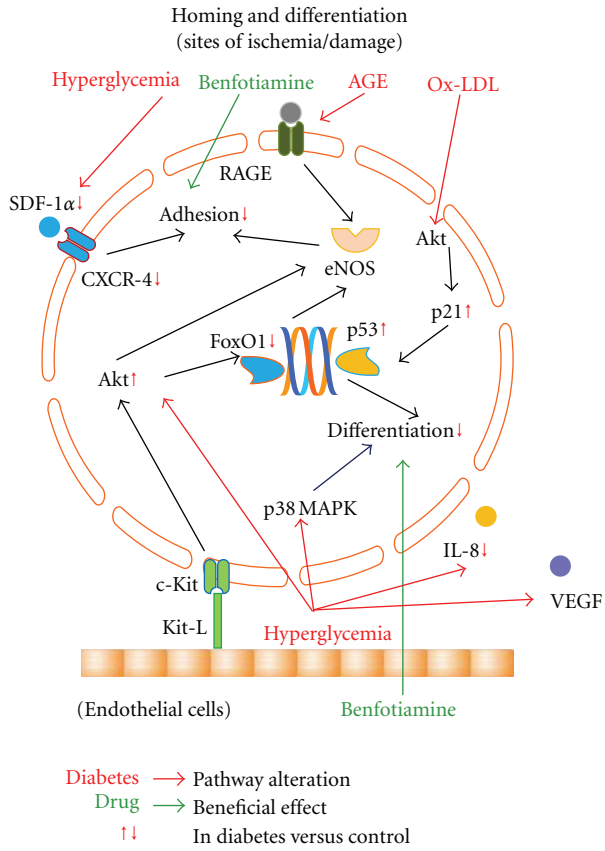

Figure 3: Pathways involved in diabetes-induced EPCs toxicity and possible strategies to reverse EPCs damage during homing. EPCs homing in the sites of ischemia/damage and differentiating into endothelial cells are here represented. Hyperglycemia, Ox-LDL, and AGEs accumulation reduce EPCs adhesion and differentiation ability in both *in vivo* and *in vitro* assays. Benfotiamine, an antioxidant molecule, is able to reverse EPCs dysfunction in homing and differentiation. Diabetes-specific metabolic alterations are in red, linked by red arrows to the pathways they interfere with. Red vertical arrows, next to intracellular or extracellular molecules, indicate that their concentration is diminished or increased in diabetic condition compared to nondiabetic status. Drugs with beneficial effect on EPCs are in green, linked by green arrows to the pathways they interact with. *MMP-9: matrix metalloproteinase-9; ROS: reactive oxygen species; NO: nitric oxide; eNOS: endothelial nitric oxide synthase; SDF-1α: stem-cell-derived factor-1α; CXCR-4: C-X-C chemokine receptor type 4; Kit-L: c-Kit ligand; Ox-LDL: oxidized low-density lipoprotein; MAPK: mitogen-activated protein kinase; AGEs: advanced glycation end-products; RAGE: receptor for AGE; IL-8: interleukin-8; VEGF: vascular endothelial growth factor.*

on this secondary effect and improve vascular function in diabetic patients. Bahlmann et al. investigated the effects of angiotensin II-receptor blockers, olmesartan and irbesartan, on EPCs in patients with T2D [56]. In both cases, ACE inhibitors increased peripheral number of EPCs compared to placebo treatment [56]. In patients with coronary artery disease, treatment with ACE-inhibitor ramipril was associated with increase in both peripheral cell count, and functional activity of EPCs, the latter being assessed by proliferation, migration, adhesion and formation of vascular structures *in vitro* [55].

4.3. Ex Vivo Conditioning. To date, several investigators explored a strategy to optimize autologous EPCs function by *ex vivo* conditioning with growth factors/chemoattractants (i.e., SDF-1α [21], VEGF [17], IL-8 [13]), antioxidants (i.e., benfotiamine [40]), hormones (i.e., adiponectin [29, 33]), gene therapy (by transfecting EPCs health-relevant genes as eNOS [20], FoxO1 [36], and HIF-1α [57]), and clinically available compounds (as p38 MAPK inhibitors [35], CoPP [58], statins [34, 59], and ACE-inhibitors [48, 49]). All these studies showed an improvement in EPCs function, but no application on humans has been tested so far. A detailed description of all molecular mechanisms involved in diabetes-mediated EPCs dysfunction and of the reported compounds potentially able to restore EPCs damage are described in Figures 1, 2, and 3.

4.4. Mobilization of EPCs to Overcome EPCs Dysfunction. Dipeptidyl-peptidase-4 (DPP-4) has been recently shown to interfere with EPC function. In a recent clinical trial, Sitagliptin increased the mobilization of EPCs in T2D patients, possibly mediated by SDF-1α upregulation [60]. Moreover, EPC mobilization is also induced by physical activity as shown in children exposed to daily exercise [61]. Other strategies, including the induction of EPCs

shedding from bone marrow via stem cell mobilizing factors (i.e., GM-CSF), have been shown to be feasible and possibly effective, but it may be argued that a nonspecific cell mobilization would occur and that autologous EPCs are anyway dysfunctional. Unfortunately, EPCs have been demonstrated to be immunogenic [62] and the attractive proposal of transplanting heterologous EPCs pooled from healthy donors would necessarily require the employment of immunosuppressive drugs.

5. Conclusions

Functional EPCs represent a prerequisite for a healthy cardiovascular system in diabetic patients. Prevention of diabetes-related macro- and microvascular complications dramatically influences the life expectance and the quality of life of diabetic patients, thus representing a crucial target for physicians. Already available drugs, currently used in clinical practice, and novel compounds should be tested in randomized clinical trials to evaluate their efficacy in normalizing or reverting diabetes-mediated EPCs damage. Moreover, *ex vivo* EPCs expansion, conditioning, and gene therapy might represent potential future strategies to reverse EPCs dysfunction, finally leading to a better cardiovascular outcome.

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Review Article

Dysfunctional Endothelial Progenitor Cells in Metabolic Syndrome

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The metabolic syndrome (MetS) is highly prevalent and confers an increased risk of diabetes and cardiovascular disease. A key early event in atherosclerosis is endothelial dysfunction. Numerous groups have reported endothelial dysfunction in MetS. However, the measurement of endothelial function is far from optimum. There has been much interest recently in a subtype of progenitor cells, termed endothelial progenitor cells (EPCs), that can circulate, proliferate, and dfferentiate into mature endothelial cells. EPCs can be characterized by the assessment of surface markers, CD34 and vascular endothelial growth factor receptor-2, VEGFR-2 (KDR). The $CD34^+KDR^+$ phenotype has been demonstrated to be an independent predictor of cardiovascular outcomes. MetS patients without diabetes or cardiovascular diseases have decreased EPC number and functionality as evidenced by decreased numbers of colony forming units, decreased adhesion and migration, and decreased tubule formation. Strategies that have been shown to upregulate and enhance EPC number and functionality include statins, angiotensin converting enzyme inhibitors, angiotensin receptor blockers, and peroxisome-proliferator-activating-receptor gamma agonists. Mechanisms by which they affect EPC number and functionality need to be studied. Thus, EPC number and/or functionality could emerge as novel cellular biomarkers of endothelial dysfunction and cardiovascular disease risk in MetS.

1. Introduction: The Metabolic Syndrome

The metabolic syndrome (MetS) comprises a cluster of abnormalities, with insulin resistance (IR) and adiposity as central features [1]. Five diagnostic criteria have been identified by the National Cholesterol Education Program Adult Treatment Panel III (NCEP-ATP III), and the presence of any three features (central obesity, dyslipidemia [high triglycerides, low HDL], hypertension, and impaired fasting glucose) is considered sufficient to diagnose the syndrome. Approximately 35% of US adults have the MetS and this appears to be a very common syndrome globally. Also, the prevalence increases with age [2]. MetS confers a two- to fourfold increased risk for cardiovascular disease (CVD) and fivefold increased risk of diabetes [3].

2. Endothelial Dysfunction and MetS

A key early event in atherosclerosis is endothelial cell dysfunction, which is precipitated by several noxious insults including obesity, hypertension, and dyslipidemia hyperglycemia, all features of MetS. Numerous groups have reported endothelial dysfunction in patients with MetS. Esposito et al. showed that compared with 60 control subjects matched for age and sex, patients with the metabolic syndrome had decreased endothelial function [4]. In the Framingham Offspring participants, Hamburg et al. [5] showed, in age and gender adjusted models, that MetS was associated with decreased flow-mediated dilation (FMD). There was progressively lower vasodilator function with increasing number of MetS components. Lteif et al., using leg blood flow

measurements, showed that patients with MetS had worse endothelial function [6]. Also, in the Prospective Study of the Vasculature in Uppsala Seniors (PIVUS) study, using different techniques to assess vasodilation in conduit and resistance arteries in MetS, the authors showed decreased flow-mediated vasodilation (FMD) [7]. In the Northern Manhatten study (NOMA), Suzuki et al. reported that MetS was associated with decreased flow-mediated dilatation (FMD) and increased CVD over 81 months [8]. Thus, it is clear that MetS patients have impaired endothelial function. This has major implications with regards to subsequent CVD.

However, despite being used in several studies, the measurement of endothelial function by flow-mediated dilation is far from optimum and there is much variability in the studies reported above such as the NOMA and Framingham studies in which the mean FMD in controls were 6.3 and 3.3%, respectively.

3. Endothelial Progenitor Cells (EPC)

There has been much interest recently in a sub-type of progenitor cells, isolated from bone marrow, umbilical vessels, and peripheral blood of adults that have the capacity to circulate, proliferate, and differentiate into mature endothelial cells, termed endothelial progenitor cells (EPCs). EPCs circulate in the blood and appear to home preferentially to sites of vascular or tissue injury, contributing significantly to both reendothelialization and neoangiogenesis. It needs to be stated at the outset that there is much controversy with respect to the correct definition of EPCs [9–11]. Generally, it is accepted that EPCs are characterized by the assessment of surface markers such as CD34 and vascular endothelial growth factor receptor-2, VEGFR-2 (KDR) [11]. Importantly, $CD34^+KDR^+$ combination is the only putative EPC phenotype that has been demonstrated repeatedly and convincingly to be an independent predictor of cardiovascular outcomes [12, 13].

4. EPC and Cardiovascular Events

In a 10-month follow-up study, Schmidt-Lucke et al. [14] showed that the level of $CD34^+KDR^+$ cells independently predicted cardiovascular events and progression of atherosclerosis in a mixed population of healthy subjects and cardiovascular patients. In a larger study, Werner et al. [15] have reported that $CD34^+KDR^+$ cell count predicted cardiovascular events and cardiovascular death during a 12-month followup in 519 patients with coronary artery disease (CAD). Also, in a subset, colony forming units (CFUs) predicted cardiovascular events. Furthermore, Hill et al. [16] reported a strong correlation between the number of circulating endothelial progenitor cells (measured as colony forming units (CFUs)) and the subjects' combined Framingham risk factor score. Also, the measurement of flow-mediated brachial-artery reactivity revealed a significant relation between endothelial function and the number of progenitor cells. Indeed, levels of circulating EPC were a better predictor of vascular reactivity than was the presence or absence of conventional risk factors. Fadini et al. showed that a low CD34 count, a measure of progenitor cells, in addition to metabolic syndrome was associated with increased cardiovascular events (CVEs) [17]. Fadini's group have also shown an association between EPC reduction and increased carotid intima media thickness (c-IMT), as a marker of early atherosclerotic remodeling in healthy subjects [18].

In addition to flow cytometric quantitation of CD34/KDR predicting CVE, also functional assays such as CFU and EPC migration have been shown to correlate significantly with CAD risk factors, severity, and events [12–16]. Thus, the measurement of EPCs may be a surrogate biologic marker for vascular function and cumulative cardiovascular risk, suggesting further that endothelial injury in the absence of sufficient circulating progenitor cells may unfavorably affect the progression of CVD.

Additionally, various risk factors for CVD have been shown to impair EPCs in terms of functional features: proliferation (important for amplifying the cellular pool), migration (critical for homing of circulating EPCs), and survival [19]. Furthermore, decrease in circulating EPCs contributes to impaired angiogenesis as well as progression of atherosclerosis and patients at risk for CAD have decreased number of circulating EPCs with impaired activity. Thus, it seems important that both the number and functional activity of EPCs should be investigated. The individual components of the MetS are associated with impairment of EPCs number and function [20].

5. EPC and MetS

With regards to the MetS, there is sparse data on EPC number and functionality [21]. There appears to be two studies that have directly looked at EPC number in MetS patients (without other confounding, comorbidities such as diabetes or cardiovascular disease) and matched controls. In the study by Westerweel et al., they show that circulating $CD34^+KDR^+$ EPC levels were reduced by nearly 40% in obese men with MetS compared to nonobese men [22]. Although this was a small study that included 19 patients with MetS, it is important to emphasize that in this study, they excluded patients with overt clinical CVD or diabetes. They did not study EPC functionality.

In the study by Jialal et al. [23], they reported on EPC number and functionality in a larger sample size of subjects with MetS ($n = 46$) of which 77% were female and matched controls ($n = 31$). In accord with the study in obese males, they showed a significant decrease in EPC number, also defined by CD34/KDR dual positivity. Furthermore, these investigators also looked at functionality of EPCs such as colony forming units, migration, and tubule formation [23]. In addition to the reduction in numbers, they showed that there were significant impaired clonogenic capacity and also an impaired capacity to incorporate into tubule structures. Whilst there was a decrease in migration of the EPCs in MetS this did not attain significance. However, it needs to be emphasized that none of the subjects were diabetic or had CVD in the above 2 studies and none were on medications

that affect EPCs suggesting that the defect in EPCs manifest early in nascent MetS prior to the development of diabetes or CVD. Fadini et al. have reported in a study decreased circulating EPCs and progenitor cells in diabetic patients with peripheral vascular disease [24]. In this paper, they did a subgroup analysis of MetS patients versus non-MetS patients. However, not much detail is provided with respect to coexistent diseases and morbidity such as diabetes and peripheral vascular disease or concomitant medications in these two subgroups. Since, this was a study with the primary aim to look at EPC status in diabetic patients with peripheral vascular disease the data in patients in MetS is not as detailed as reported in the 2 studies that focused on MetS alone [22, 23]. In a subsequent report by Fadini et al., they showed that in patients with MetS, there was a decrease in progenitor cells ($CD34^+$ cells) [17]. It appears that many of these patients also could have diabetes, and be on medications such as statins, angiotensin converting enzyme Inhibitors (ACE-I), angiotensin receptor blockers (ARBs), and antidiabetic therapy such as pioglitazone, which could have influenced the data [24]. The reported decrease in progenitor cells in these 2 studies was confirmed in the study by Jialal et al. [23]. Previously, Satoh et al. [25] have reported increased EPC number in CAD patients with MetS and without MetS. They did not compare patients with MetS with controls and their sample sizes were small ($n = 15$ for acute myocardial infarction and $n = 16$ for patients with stable angina angina pectoris and MetS, resp.). Interestingly, they also showed increased oxidative DNA damage, decreased telomerase activity, and decreased telomere length, a marker of increased senescence in EPCs of CAD patients with MetS than the CAD patients without MetS. This suggests that the increase in EPC with CVD was a dysfunctional population since EPCs are generally well endowed with antioxidant defenses. Other functional measures of EPC activity such as tubule formation or colony forming units or adhesion was not investigated in this study. Thus, this needs to be investigated further.

Recently, Vignera et al. [26] reported increased EPCs in patients with arterial erectile dysfunction and MetS compared to controls. It is possible that in ED, where there is profound vascular dysfunction, a particular subtype of EPC (CD45 negative, CD34 positive, and CD144 positive) are increased due to a compensatory increase in mobilizing factors and could depict repair mechanisms. It is however important to point out that these investigators did not use the classical CD34/KDR criteria. Furthermore, the increased EPC in their subjects correlated with endothelial microparticles (EMPs) and IMT suggesting that this is a dysfunctional population. However since they did not study EPC functionality unlike the study by Satoh et al. [25], one cannot critically appraise this report. Also, limited data is provided with respect to medications that can affect EPC numbers and comorbidities such as diabetes and CVD, which are common accompaniments of erectile dysfunction and could further influence their findings. Indeed, previous studies have also reported decreased EPCs in such patients and a significant correlation of the decreased EPCs to increased cardiovascular risk.

There is very limited data that specifically looked at EPC status either number and/or functionality in patients with MetS without the complications of diabetes and CVD. In the two studies which specifically address this, both have shown, decreased number of EPC, they are at variance with respect to a decrease in progenitor cells since levels were not significantly lower in the study in obese males. However if the data from the studies by Fadini are considered one could conclude that progenitor cell exhaustion can be advanced as one mechanism resulting in decrease in EPC number [27]. In addition, in the Jialal et al. [23] study, they showed significant correlation of CRP levels in MetS with decreased EPC number and functionality, pointing to the role of inflammation in this process.

There is limited data with regards to mobilizing factors in patients with MetS. Egan et al. [28] have reported the profound reduction in EPCs due to impaired mobilization from bone marrow because of the lower expression of CXC Chemokine Receptor 4 (CXCR4+)/CD34+ cells in diabetics versus controls. Importantly, CXCR4, CD117, and KDR are defined as the mobilizing receptors for progenitor cells (PCs) [29, 30]. Thus, it appears that the measurement of the respective circulating ligands; vascular endothelial growth factor (VEGF) for KDR, soluble c-kit ligand (KitL) for CD117, and Stromal derived factor 1 (SDF-1) for CXCR4 is also important. Some of the accepted mobilizing factors include VEGF, stromal derived factor-1 (SDF-1), and c-kit ligand [30]. In a small study that has examined mobilizing factors in MetS, the investigators showed no significant differences in VEGF levels, but showed that progenitor cell mobilizing stromal cell-mobilizing factor (SCF) and the soluble form of SCF receptor c-kit were both reduced in patients with MetS [22]. Since this is a limited study in a small number of patients, these findings need to be confirmed in a larger study. Jialal et al. [31] recently showed in subjects with MetS ($n = 36$) compared to age- and gender-matched controls ($n = 38$) that there was a significant reduction of 83% in granulocyte colony-stimulating factor levels in patients with MetS. Also, there were decreases in SCF and SCF soluble receptor levels. However, there was no significant difference in stromal cell-derived factor-1 levels, and paradoxically, vascular endothelial growth factor levels were increased, consistent with VEGF resistance, which has been reported previously with insulin resistant states such as diabetes and MetS [32]. Data on matrix metalloproteinase (MMP)-9 levels in patients in MetS is sparse. Previously, MMP-9 levels have been shown to be increased in subjects with MetS using immunoassay [33]. Since MMP-9 levels are critical for homing of EPC [34], we examined levels of MMP-9. In accord with the previous report we show an increase in MMP 9 levels by immunoassay (Figure 1). However the relevance of this finding is questionable since we did not assay enzyme activity by zymography which is the superior measure of MMP-9 activity.

Thus, whilst there is much controversy with regards to the nomenclature and definition of EPCs, it needs to be emphasized that EPC number, EPC migration, and colony forming units which appear to connote early EPCs have clearly been shown to correlate with risk factors, CVD

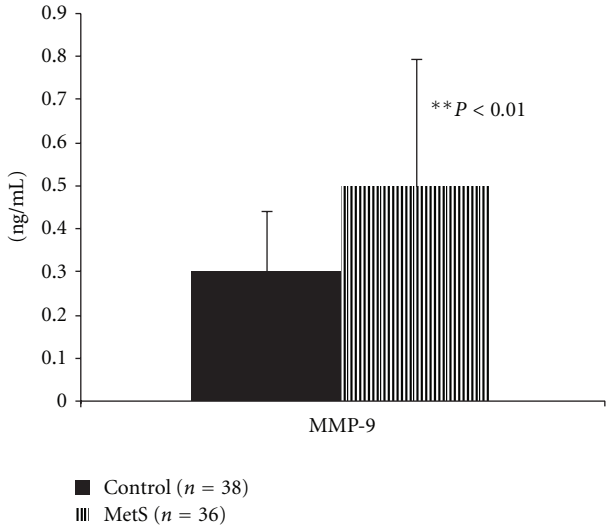

Figure 1: MMP-9 Levels in Patients with MetS compared to matched controls. Plasma MMP-9 levels were measured in patients with MetS compared to matched healthy controls ($n = 38$ and 36/group, resp.) using a sandwich ELISA (R&D Biosystems). Data are expressed as mean ± S.D in ng/mL. $^{**}P < 0.01$ compared to controls.

severity and predict CVD events to date. However, whilst it is claimed that the late EPCs are more likely to become endothelial cells, it needs to be emphasized that to date no studies have reported that late EPCs predict CVE in patients [30].

Thus, the published studies have shown that EPC numbers and functionality is impaired in MetS. The potential mechanisms that have been advanced so far include decreased progenitor cells and dysregulation of EPC mobilizing factors. Longevity of EPC in MetS has not been reported and thus studies directed at telomere biology and apoptosis are urgently needed in patients with MetS without comorbidities.

In conclusion, EPC number and functionality could serve as an additional novel cellular biomarker of endothelial integrity and impaired neoangiogenesis in patients with MetS who clearly have manifest endothelial dysfunction. Prospective studies should demonstrate that they predict CVD. Strategies that have been shown to upregulate and enhance EPC number and functionality such as statins, ACE-I, ARBs, PPAR-gamma agonist, and INCRETIN-based therapies, need to be studied more carefully with respect to both number and functionality of EPCs since this could inform us of their direct beneficial effects on the vulnerable vasculature of Mets. Thus, EPC number and/or functionality could emerge as a novel cellular biomarker of CVD risk and could better inform clinicians about potential pharmacotherapy for patients with MetS.

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Research Article

A PEDF-Derived Peptide Inhibits Retinal Neovascularization and Blocks Mobilization of Bone Marrow-Derived Endothelial Progenitor Cells

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Proliferative diabetic retinopathy is characterized by pathological retinal neovascularization, mediated by both angiogenesis (involving mature endothelial cells) and vasculogenesis (involving bone marrow-derived circulating endothelial progenitor cells (EPCs)). Pigment epithelium-derived factor (PEDF) contains an N-terminal 34-amino acid peptide (PEDF-34) that has antiangiogenic properties. Herein, we present a novel finding that PEDF-34 also possesses antivasculogenic activity. In the oxygen-induced retinopathy (OIR) model using transgenic mice that have Tie2 promoter-driven GFP expression, we quantified Tie2GFP$^+$ cells in bone marrow and peripheral blood by fluorescence-activated cell sorting (FACS). OIR significantly increased the number of circulating Tie2-GFP$^+$ at P16, correlating with the peak progression of neovascularization. Daily intraperitoneal injections of PEDF-34 into OIR mice decreased the number of Tie2-GFP$^+$ cells in the circulation at P16 by 65% but did not affect the number of Tie2-GFP$^+$ cells in the bone marrow. These studies suggest that PEDF-34 attenuates EPC mobilization from the bone marrow into the blood circulation during retinal neovascularization.

1. Introduction

Vascular development is regulated by a tight and complex balance between pro- and anti-angiogenic factors such as vascular endothelial growth factor (VEGF) and pigment epithelium-derived factor (PEDF), respectively [1–4]. This balance is important to maintain homeostasis of blood vessel formation. Under certain pathological conditions, such as proliferative diabetic retinopathy, age-related macular degeneration, tumorigenesis, and rheumatoid arthritis, this balance is disrupted, leading to pathological neovascularization [1, 3]. Pathological neovascularization involves two distinct mechanisms, vasculogenesis, in which bone marrow-derived endothelial progenitor cells (EPCs) contribute to new blood vessel formation [5–8], and angiogenesis, in which existing mature endothelial cells proliferate and migrate to form new blood vessels [9, 10]. Several angiogenic inhibitors have been identified which effectively inhibit pathological neovascularization, but the effect of such antiangiogenic factors on vasculogenesis has not been established.

PEDF is a 50-kDa secreted glycoprotein bearing multiple biological activities [11–15], including potent antiangiogenic activity, which has been shown to inhibit pathological neovascularization, such as retinal neovascularization, which occurs during proliferative diabetic retinopathy [12, 14, 15]. However, the effect of PEDF on vasculogenesis has not been documented. Recently, a 34-amino acid peptide derived from the N-terminus of PEDF (PEDF-34) was found to possess intact *ex vivo* antiangiogenic activity and inhibit choroidal neovascularization in rats [16, 17]. In the present study, we investigated the direct effects of PEDF-34 on the proliferation and viability of primary endothelial cell cultures and on *in vivo* blood vessel development using the chicken chorioallantoic membrane (CAM) assay. We also used the oxygen-induced retinopathy (OIR) mouse model to

assess the ability of the PEDF-34 to inhibit retinal neovascularization. Using transgenic mice that express GFP under the control of the endothelial cell-lineage specific promoter Tie2 (Tie2-GFP mice), we quantified bone marrow-derived EPCs and circulating endothelial cells by fluorescence-activated cell sorting (FACS) of Tie2-GFP$^+$ cells. These studies are the first to demonstrate that systemic administration of PEDF-34 peptide is sufficient to inhibit retinal neovascularization. We also demonstrate for the first time that retinal neovascularization in the OIR model coincides with a spike in the number of circulating endothelial cells and EPCs. Furthermore, PEDF-34 blocks the spike in circulating endothelial cells and EPCs during OIR. These data suggest that in addition to its localized anti-angiogenic effects on neovascular lesions, PEDF may also have a systemic activity that blocks the release of EPCs from bone marrow to reduce EPC-mediated vasculogenesis during retinal neovascularization.

2. Materials and Methods

2.1. PEDF-34 Peptide. The PEDF-34 peptide, spanning from amino acids 44 to 77 of the N-terminus of the PEDF protein, was chemically synthesized by Proteintech lab (Chicago, IL) and purified by HPLC. Proper synthesis of the peptide was controlled by mass spectrometry.

2.2. Bovine Retinal Capillary Endothelial Cell (BRCEC) Isolation and Culture. BRCEC were isolated from whole retinas of cows younger than 18 months in accordance with USDA regulations. Briefly, retinas were carefully singled out from eyecups, washed, homogenized, and digested prior to being applied to a series of filters. Cells were grown in collagen-coated flask in the presence of 10% human serum in Dulbecco's modified eagle medium (DMEM) containing low glucose (1 g/L) until confluency. The endothelial cell identity of the BRCEC cultures was confirmed by their ability to uptake acetylated low-density lipoprotein (LDL) labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (Dil-Ac-LDL) (Harbor Bio-Products). Following a 1 hr incubation with Dil-Ac-LDL, cells were fixed in 4% paraformaldehyde, counterstained with DAPI included in mounting media (Vectorlabs, Burlingame), and examined by fluorescence microscopy. Only BRCEC preparations with more than 95% purity were used in this study.

2.3. Cell Viability Assays. A cell line derived from rat Müller cells, rMC-1, was kindly provided by Dr. Sarthy at Northwestern University. BRCECs between passages 3 and 8 were seeded in gelatin-coated 48 well plates. BRCECs and rMC-1 cells were maintained in regular DMEM growth media until the assay. Then, the growth media were replaced with DMEM containing 1% FBS, low glucose (1 g/L), and 1% antibiotic/antimycotic, and the cells were treated with PEDF-34 in various concentrations for 72 h. At the end of the treatment, an MTT assay (Roche, Nonnenwald Germany) was performed according to the manufacturer's recommendation. Data were collected on a Victor plate reader.

2.4. Detection of Apoptosis by FACS Analysis. Adherent BRCECs were trypsinized for 2 min, and the trypsinization was stopped with PBS/10% fetal calf serum. Then cells were washed twice with annexin-binding buffer and incubated with 2.5 μL annexin-PE and 2.5 μL 7AAD for 20 min according to the instructions of the manufacturer (Pharmingen, Annexin V-Pe Apoptosis Kit). The cells were analyzed by fluorescence-activated cell sorting (FACS) using a FACSCalibur flow cytometer and Cell Quest software (BD Biosciences, Rockville, MD).

2.5. Chicken Chorioallantoic Membrane (CAM) Assays. CAM assays were used to assess the antiangiogenic potential of PEDF-34 *in vivo*. Briefly, fertilized eggs were incubated at 37°C and 65% relative humidity for three days with a rocking mechanism. The embryos were then removed from the incubator, washed with a 1 : 100 solution of benzalkonium chloride (Research Chemicals, Heyshan Lanes, UK) in distilled water, and cracked out into 100-mm Petri dishes (BD Falcon, Franklin Lakes, NJ). A circular section (1.2 mm diameter) of nitrocellulose was saturated with either PEDF-34 or control peptide at the required concentration. An identical nitrocellulose section was saturated with sterile PBS and used as internal control in each CAM. The nitrocellulose disks were placed onto the surface of the embryo in an area of active vascularization. Each peptide and the control PBS were reapplied onto the disks every 24 h. After 72 h incubation, the disks were carefully removed from the surface of the embryo. Images were taken of the area in the vicinity of the removed disks. Blood vessel density was evaluated by densitometric analysis of the images of the disk areas using ImageJ (NIH).

2.6. Induction of Retinal Neovascularization in Mice and Quantification of Retinal Nuclei. Tie2-GFP mice, transgenic mice that express GFP under the control of the Tie-2 promoter were a kind gift from Dr. Sanai Sato. The Tie2-GFP mice at postnatal day 7 (P7) were exposed to hyperoxia (75% O_2) for 5 days. They were brought back to normoxic room air at P12 and were thereafter maintained at normoxia to induce retinal neovascularization. At P12 the OIR mice were separated into 2 groups: one group was injected intraperitoneally with PEDF-34, once a day from P12 to P17, and the other group was injected with BSA in a similar fashion. At P18, the eyes were enucleated, fixed in 10% paraformaldehyde, and embedded in paraffin. Sagittal sections of 5 μm thickness were made using a microtome (Microm HM 325). Noncontinuous sections were mounted on slides and stained with hematoxylin and eosin as described by Smith et al. [18]. Light microscopy was used to count nuclei of vascular cells present on the vitreal side of the retina. A total of 8 sagittal sections from each eye were examined, and cell numbers were averaged for each group. The average number of preretinal nuclei was compared to the control group by Student's t-test.

2.7. EPC Isolation. Tie2-GFP mice in FVB background were used in this study. For bone marrow cell isolation, tibias were collected, extensively flushed with PBS and crushed.

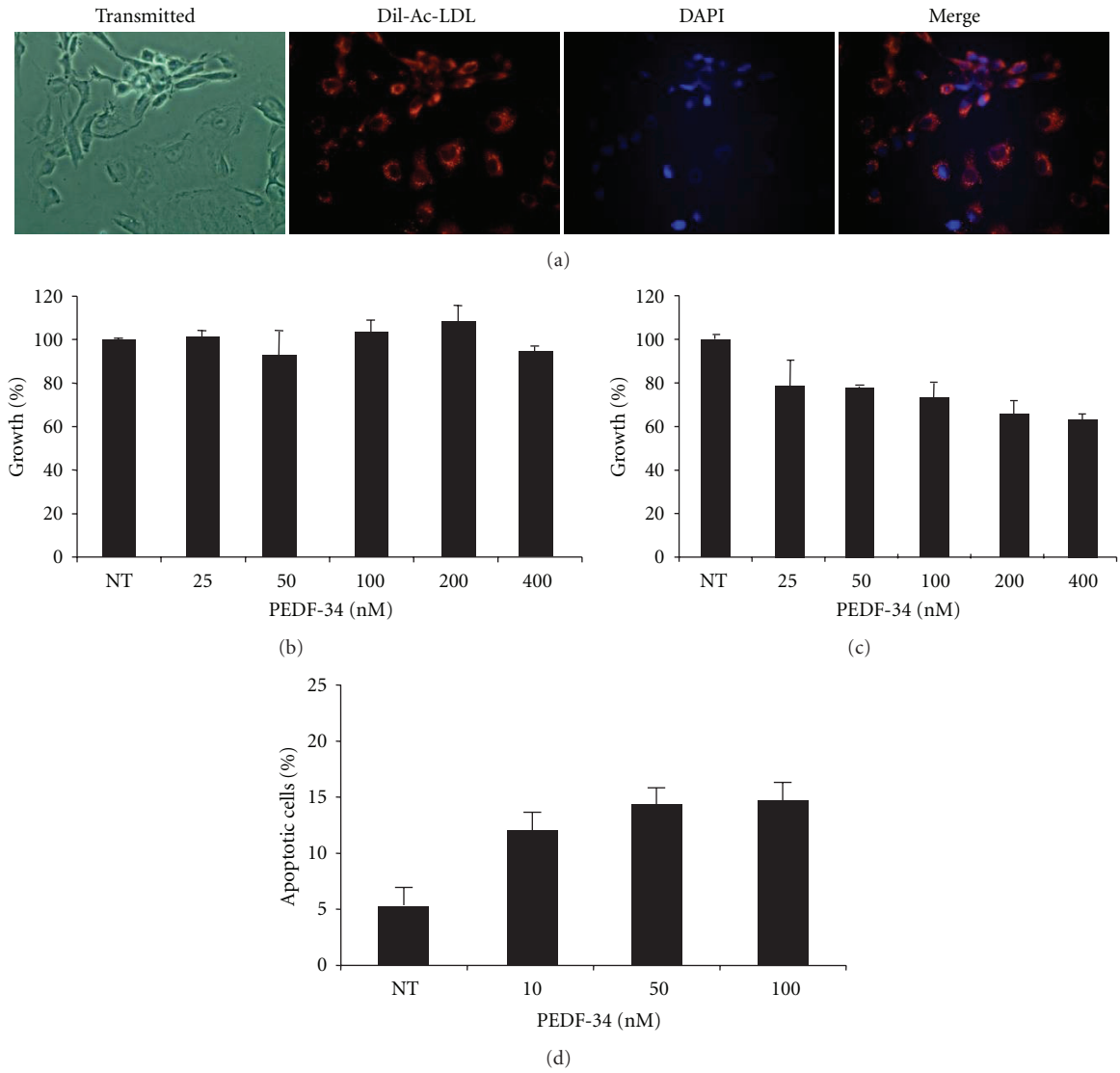


Figure 1: PEDF-34 acts in a concentration-dependent manner to inhibit cell proliferation and induce apoptosis specifically in endothelial cells. (a) The purity of primary BRCEC was examined using Dil-Ac-LDL uptake assays. The cells were counter-stained with DAPI, and exhibited a >99% purity based on positive Dil-Ac-LDL uptake. Both rat Müller cells (b) and BRCEC (c) were treated with increasing concentrations of PEDF 34-mer ranging from 25 to 400 nM. Viable cells were quantified after 72 h by MTT assay and expressed as % of the cells in control treated with the BSA only (denoted as NT on graph). (d) BRCECs were treated with increasing concentrations of PEDF 34-mer for 24 h. Apoptotic cells were quantified by counting Annexin V positive cells using FACS and expressed as % in total cells (mean + SD, $n = 3$).

Bone fragments and cells in suspension were applied to a 100 μM filer and washed with 30 mL of ice-cold PBS. Cells which passed through the filter were centrifuged for 10 min at 1,500 rpm, and the supernatant was discarded. Cells were resuspended in 4% paraformaldehyde and placed on ice shielded from the light. For peripheral blood cell isolation, blood was collected by cardiac puncture of the right ventricle, transferred to a tube with heparin salt, and placed on ice. The blood was then layered onto a histopaque 1083 (Sigma, Saint Louis, MO) and centrifuged for 30 min at 3000 rpm. The mononuclear cell fraction was collected and rinsed with ice-cold PBS. Cells were resuspended in 4% paraformaldehyde and placed on ice shielded from the light.

2.8. FACS Analysis of Tie2-GFP$^+$ Cells. Cell were fixed for 1 h in 4% paraformaldehyde, and then washed three times in cold PBS. The fixed cells were applied to a FACSCalibur flow cytometer and analyzed using Cell Quest software (BD Biosciences) using a 530 nm filter. In each sample, 1,000,000 events and 30,000 events were counted for bone marrow and circulating mononucleated cells, respectively. Collected data was analyzed by FlowJo software (Tree Star, Ashland, OR, USA).

2.9. Immunostaining and Confocal Microscopy of Tie2-GFP$^+$ Cells. Tie2-GFP$^+$ sorted cells were washed in PBS and adhered to charged histology slides for immunostaining.

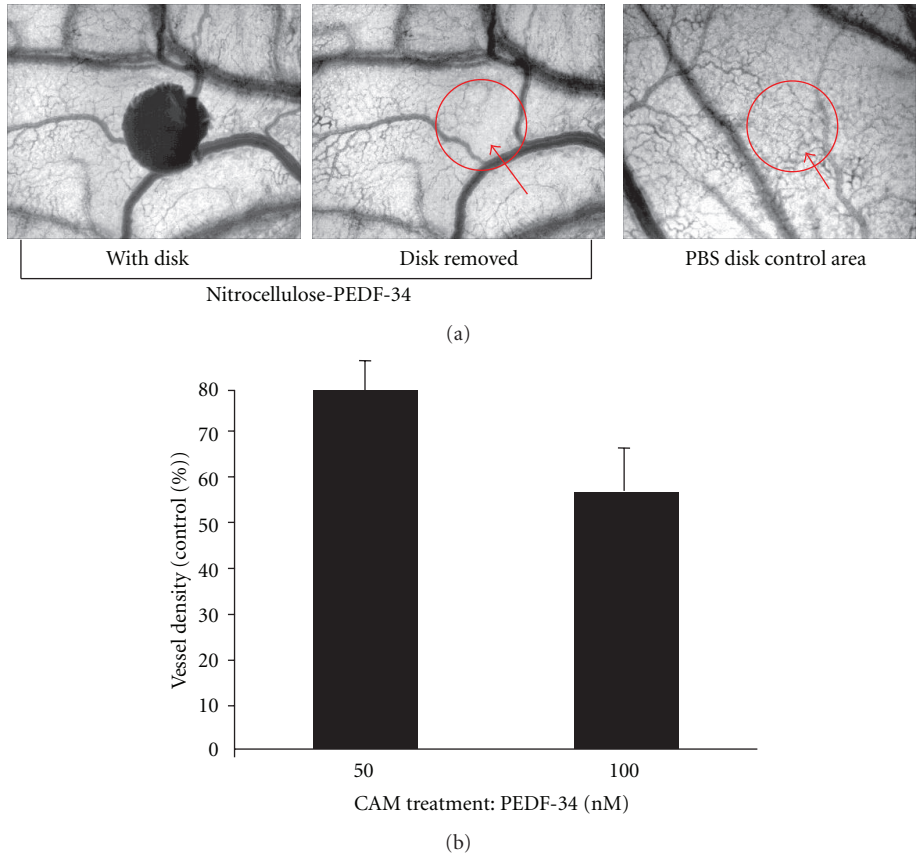


Figure 2: Antiangiogenic activity of PEDF 34-mer during *in vivo* blood vessel formation. CAMs were separately treated with either 50 or 100 nM PEDF-34. (a) Left: close-up image of a chicken embryo CAM with a nitrocellulose disk containing PEDF-34 (100 nM). Middle: the disk area after removal of PEDF-34 nitrocellulose disk. Right: image of a PBS-treated-nitrocellulose control area on the same CAM with the disk removed. Note considerable reduction in the overall number of small newly formed blood vessels, when compared with the PBS-treated control. (b) Blood vessel density in the CAMs was assessed by software analysis. Vascular densities in the disk areas of CAMs treated with 50 and 100 nM were quantified and averaged. The graph represents the percent of vascular density found under PEDF-34-treated areas compared to the PBS-treated control area, which was set at 100%. Vascular density in the CAM treated with PEDF-34 is significantly lower than the control (mean + SD, $n = 5$).

Cells were preincubated with the appropriate antibody isotype to prevent nonspecific binding of the primary antibody. We then used the following antibodies: anti-CD117 labeled with allophycocyanin (APC) (BD Pharmingen) and rat antimouse CD133 (Chemicon). For CD133, we used an antirat IgG secondary antibody labeled with Alexa 648 (BD Pharmigen). All slides were mounted in Vectorlabs-DAPI. The cells were imaged and analyzed on a Nikon TE2000-E.

3. Results

3.1. PEDF-34 Inhibits Cell Proliferation and Induces Apoptosis in BRCEC. Full-length PEDF protein is known to target endothelial cells to inhibit proliferation and induce apoptosis [12], and PEDF-34 has previously been shown to inhibit blood vessel sprouting *ex vivo* and inhibit the progression of laser-induced choroidal neovascularization in rats [16]. To evaluate the direct and endothelial cell-specific effects of the PEDF-34 on cell proliferation and viability, both BRCEC and rMC-1 cells were treated with increasing concentrations of PEDF-34. Beforehand, the identity and purity of primary BRCEC cultures were confirmed by evaluating cellular uptake of fluorescently-labeled acetylated LDL (Dil-Ac-LDL), which is exclusively taken up by endothelial cells which express the LDL receptor. More than 99% of the isolated BRCEC were positive for Dil-Ac-LDL uptake (Figure 1(a)). MTT cell proliferation assays showed that rMC-1 cell proliferation was unaffected by up to 400 nM of PEDF-34 (Figure 1(b)). In contrast, the PEDF-34 inhibited BRCEC proliferation in a concentration-dependent manner, with as low as 50 nM causing a 20% decrease in cell proliferation and with 400 nM reducing cell proliferation by more than 30% (Figure 1(c)). These data show that PEDF-34 selectively inhibits cell proliferation in endothelial cells.

To determine whether the PEDF-34 induces apoptosis in BRCEC, Annexin V staining and subsequent FACS analysis was used to quantify apoptotic cells. PEDF-34 increased the percentage of apoptotic cells in a concentration-dependent manner (Figure 1(d)), providing the first evidence of

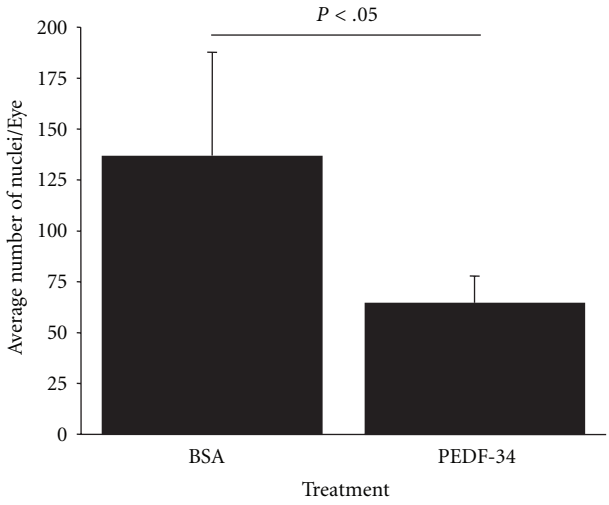


Figure 3: Systemic injection of PEDF-34 reduced ischemia-induced retinal neovascularization. The graph represents the average number of preretinal (vitreous) vascular cells in OIR mice treated with BSA or PEDF-34. (mean ± SEM, BSA $n = 4$. PEDF-34 $n = 5$).

the direct and specific proapoptotic activity of the PEDF-34 in primary endothelial cells.

3.2. Locally Delivered PEDF-34 Inhibits New Blood Vessel Formation In Vivo. The chorioallantoic membrane (CAM) of a growing chicken egg was used as a model system to test the effects of PEDF-34 on *in vivo* blood vessel formation [19]. CAMs were treated with small disks of nitrocellulose saturated with PEDF-34. Nitrocellulose saturated with PBS alone was included in every CAM as an internal negative control. After 72 h of treatment, the areas of CAMs covered by disks containing PEDF-34 showed fewer blood vessels compared to the areas covered by disks containing PBS alone (Figure 2(a)). The vessel density in the area covered by the disk was quantified by computer analysis of digital images and averaged. The results showed that 50 nM of PEDF-34 decreased vessel density by up to 20%, and 100 nM PEDF-34 decreased vessel density by 45%, compared to the average vascular density of the control (Figure 2(b)), suggesting a dose-dependent inhibitory effect of PEDF-34 on *in vivo* blood vessel formation.

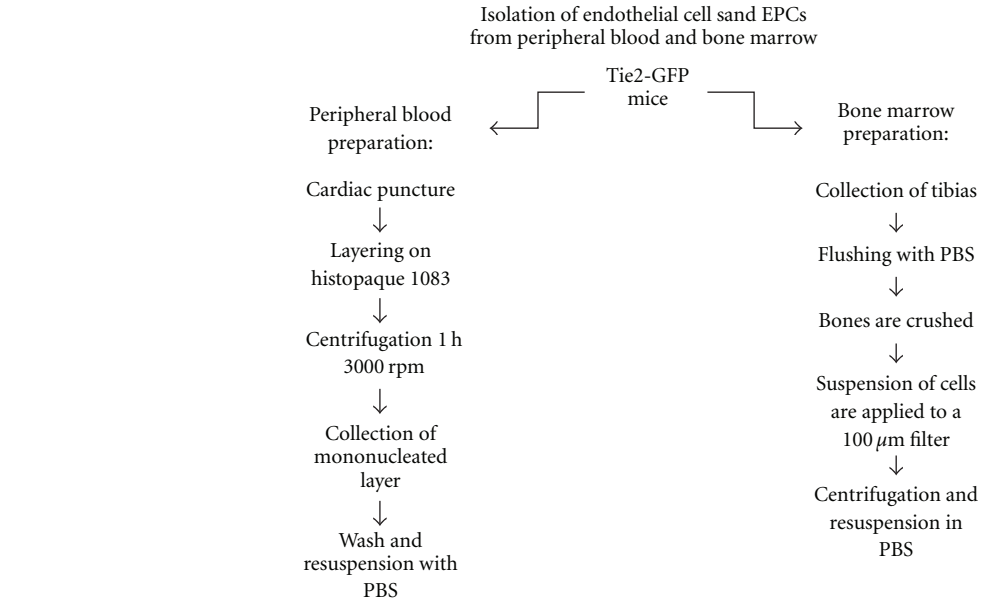
3.3. Systemic Administration of PEDF-34 Inhibits Pathological Retinal Neovascularization in the OIR Model. To evaluate the effects of PEDF-34 on vasculogenesis during pathogenic neovascularization, we used the OIR mouse model, which initiates the pathogenesis of retinal neovascularization beginning at P12 [18]. OIR mice received daily intraperitoneal injections of either PEDF-34 (5 mg/kg of body weight) or the same amount of BSA as control from age P12 to P17. At P18, neovascularization was quantified by counting preretinal nuclei in 8 discontinuous sections per eye (Figure 3). The mean preretinal neovascular cell number in the BSA treated group was 136 ± 50 (mean ± SD, $n = 4$) per section. Systemic treatment with PEDF-34 reduced the number of preretinal neovascular cells by approximately 50% to only 64 ± 13 (mean ± SD, $n = 5$) per section, significantly lower than that in the BSA-treated group ($P < .05$, $n = 5$) (Figure 3). These results demonstrate that systemic injection of PEDF-34 prevented the progression of ischemia-induced retinal neovascularization.

3.4. Isolation and Characterization of Tie2-GFP$^+$ Cells. In order to study circulating endothelial cells during OIR, we used Tie2-GFP transgenic mice, which have GFP expression exclusively in cells of endothelial cell lineage, including mature endothelial cells and bone marrow-derived endothelial progenitor cells. Prior to commencing OIR studies, we performed preliminary analyses to detect and characterize Tie2-GFP$^+$ cells from bone marrow and peripheral blood. Bone marrow cells and peripheral blood mononuclear cells were harvested separately as described in Section 2 and depicted in Figure 4(a), and then placed on ice and shielded from direct light. Cells were then fixed in 4% paraformaldehyde and subjected to FACS to sort Tie2-GFP$^+$ cells. Bone marrow cells collected from wild type C57bl/6 mice were used as a negative control. Cells collected from Tie2-GFP mice exhibited a distinctly shifted population of Tie2-GFP$^+$ cells that was absent in cells isolated from wild-type mice (Figures 4(b) and 4(c)). The efficacy of FACS-mediated separation of Tie2-GFP$^-$ and Tie2-GFP$^+$ cells was evaluated by collecting the FACS-separated cell populations and performing postanalysis using fluorescence microscopy. After staining both cell populations with DAPI, GFP fluorescence is only visible in the population of cells identified as Tie2GFP$^+$ by FACS analysis (Figure 4(d)). Thus, FACS analysis is a reliable method for isolating and quantifying Tie2GFP$^+$ cells.

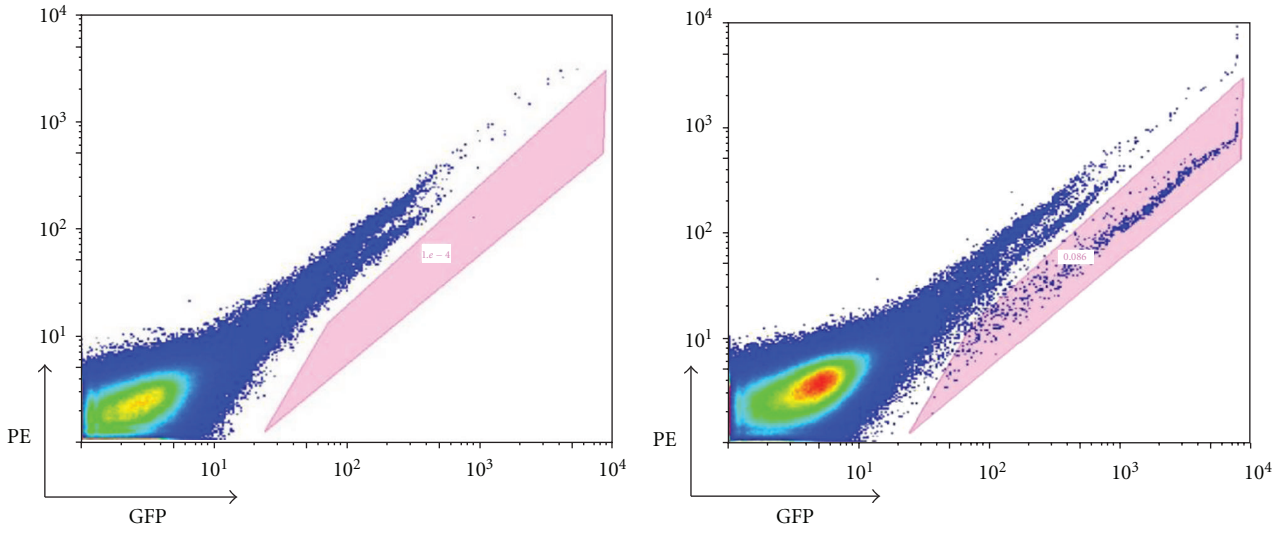
To confirm the endothelial identity of FACS-isolated Tie2-GFP$^+$ cells, cell populations designated as Tie2-GFP$^-$ and Tie2-GFP$^+$ cells were placed separately into 96-well plates for Dil-Ac-LDL uptake assays. As expected, Dil-Ac-LDL uptake only occurred in the designated Tie2-GFP$^+$ cells (Figure 4(e)). This confirmed the endothelial identity of Tie2-GFP$^+$ cells and also the reliability of the FACS-based isolation and quantification methods.

To confirm that the Tie2-GFP$^+$ cells represent EPCs in addition to circulating mature endothelial cells, the Tie2-GFP$^+$ cells were immunostained for CD117 and CD133, two established markers of EPCs [20–23]. CD117 and CD133 immunostaining of Tie2-GFP$^+$ cells was easily observed (Figure 5(a)), and quantification by FACS revealed that at least 90% of sorted Tie2-GFP$^+$ cells exhibited significant staining for CD117 (Figure 5(b)). These results show that a significant portion of the Tie2-GFP$^+$ cells are EPCs.

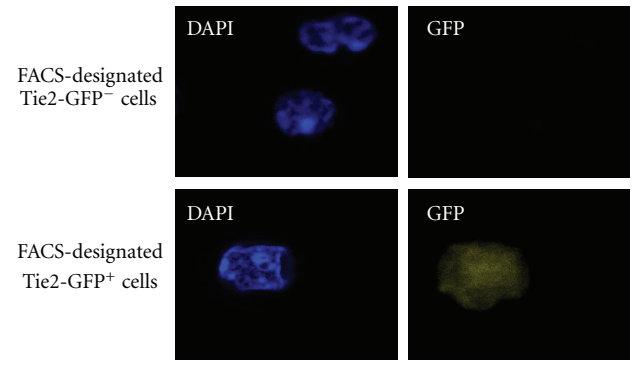
3.5. The Number of Circulating Tie2-GFP$^+$ Cells Correlates with the Progression of Retinal Neovascularization in the OIR Model. In order to characterize the correlation between circulating endothelial cells (including both EPCs and mature endothelial cells) and retinal neovascularization in the OIR model, Tie-2-GFP mice were exposed to 75% oxygen from age P7 to P12 and then returned to room air



(a)

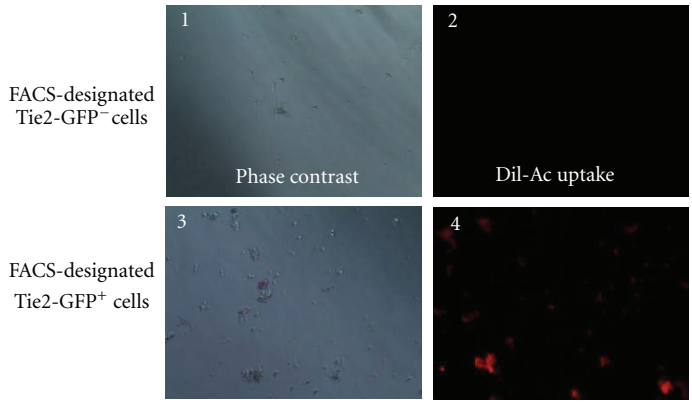


(b) (c)



(d)

Figure 4: Continued.



(e)

Figure 4: Isolation of Tie2-GFP$^+$ cells from bone marrow and peripheral blood in mice. (a) Isolation of bone marrow and peripheral blood cells was performed in parallel as depicted. Washed cells were resuspended in ice-cold PBS and shielded from the light to prevent photobleaching. (b) and (c) Flow cytometric analysis of isolated cells. Resuspended cells were subjected to FACS on an Influx cell sorter. The profile obtained from Tie2-GFP mice (c) exhibited an additional population when compared to the wild-type C57BL/6 (b) (denoted in the shaded pink box). Therefore, this exclusive population was characteristic of the Tie2-GFP$^+$ cells. (d) Tie2-GFP$^-$ and Tie2-GFP$^+$ cells as identified by FACS analysis were collected separately, fixed onto slides, counter-stained with DAPI, and visualized by fluorescence microscopy. Only cells identified by prior FACS analysis as Tie2-GFP$^+$ exhibited significant levels of green fluorescence by microscopic (100x) analysis. (e) Tie2-GFP$^+$ cells which were isolated by FACS from Tie2-GFP mice were viable and of the endothelial cell lineage, as demonstrated by their ability to uptake Dil-Ac-LDL. Cells sorted by FACS as Tie2-GFP$^-$ did not have the ability to uptake Dil-Ac-LDL.

to induce retinal neovascularization [18]. Tie2-GFP$^+$ cells from both bone marrow and peripheral blood were quantified by FACS. Age-matched Tie2-GFP mice maintained in constant room air were used as non-OIR controls. In bone marrow, there was no significant increase in Tie2-GFP$^+$ cells at P12, P16 and P20, compared to age-matched non-OIR controls (Figures 6(a), 6(c), and 6(e)) suggesting the oxygen treatment does not influence the percentage of Tie2-GFP$^+$ cells present in the bone marrow. In contrast, in peripheral blood, there was a significant increase in the number of circulating Tie2-GFP$^+$ cells at age P16, but not at P12 and P20, when compared to age-matched controls (Figures 6(b), 6(d), and 6(f)). P16 correlates with the most aggressive stage of retinal neovascularization which occurs from P16–P18 [18]. Thus, the peak in the number of circulating endothelial cells coincides with the peak of retinal neovascularization, which strongly suggests circulating endothelial cells contribute to the pathogenesis of retinal neovascularization.

3.6. PEDF-34 Blocks the Increase in Circulating Tie2-GFP$^+$ Cells during Retinal Neovacularization in the OIR Model. To evaluate the potential for PEDF-34 to act on circulating endothelial cells, OIR was induced in Tie2-GFP mice, and once mice were returned to normoxic room air at P12 to induce retinal neovascularization, mice received daily i.p. injections of PEDF-34 from P12 up to P17. Control OIR mice received an equivalent quantity of BSA. Tie2-GFP$^+$ cells from peripheral blood and bone marrow were quantified by FACS at P16. The injection of PEDF-34 (5 mg/kg body weight) resulted in a 55% reduction in the number of circulating Tie2-GFP$^+$ cells in the peripheral blood (Figure 7), but did not affect the number of Tie2-GFP$^+$ cells in the bone marrow (see Supplemental Figure 1 in Supplementary Material available online at doi: 10.1155/2012/518426). A lower dose of PEDF-34 (1 mg/kg body weight) did not result in a significant decrease in the number of circulating Tie2-GFP$^+$ cells (Figure 7). These results suggest that PEDF-34-mediated inhibition of Tie2-GFP$^+$ cells in circulation is both highly specific and dose-dependent.

3.7. The Effect of PEDF-34 on Circulating Tie2-GFP$^+$ Cells Is Not via Regulation of VEGF. The regulation of EPC release from the bone marrow is not well understood. However, VEGF has previously been shown to enhance pathological neovascularization partially by increasing the release of EPCs from bone marrow into the blood circulation [6]. Furthermore, we have previously demonstrated that PEDF competes with VEGF for binding to VEGF receptor 2 on endothelial cells [24], which is suggested to be a mechanism for the antiangiogenic activity of PEDF. Thus, to determine if PEDF-34 reduces circulating endothelial cells during retinal neovascularization by targeting VEGF-mediated EPC release from bone marrow, we tested the effect of PEDF-34 on plasma VEGF levels. Tie2-GFP mice with OIR received daily i.p. injections of PEDF-34 or BSA (5 mg/kg body weight) from P12 to P15. VEGF concentrations in the plasma were measured by ELISA at P16. PEDF-34 did not affect VEGF plasma levels, compared to the group treated with BSA (Supplemental Figure 2).

4. Discussion

Pathological neovascularization is a common cause of vision loss in diabetic retinopathy. With the number of patients affected by diabetes growing rapidly, it has become a

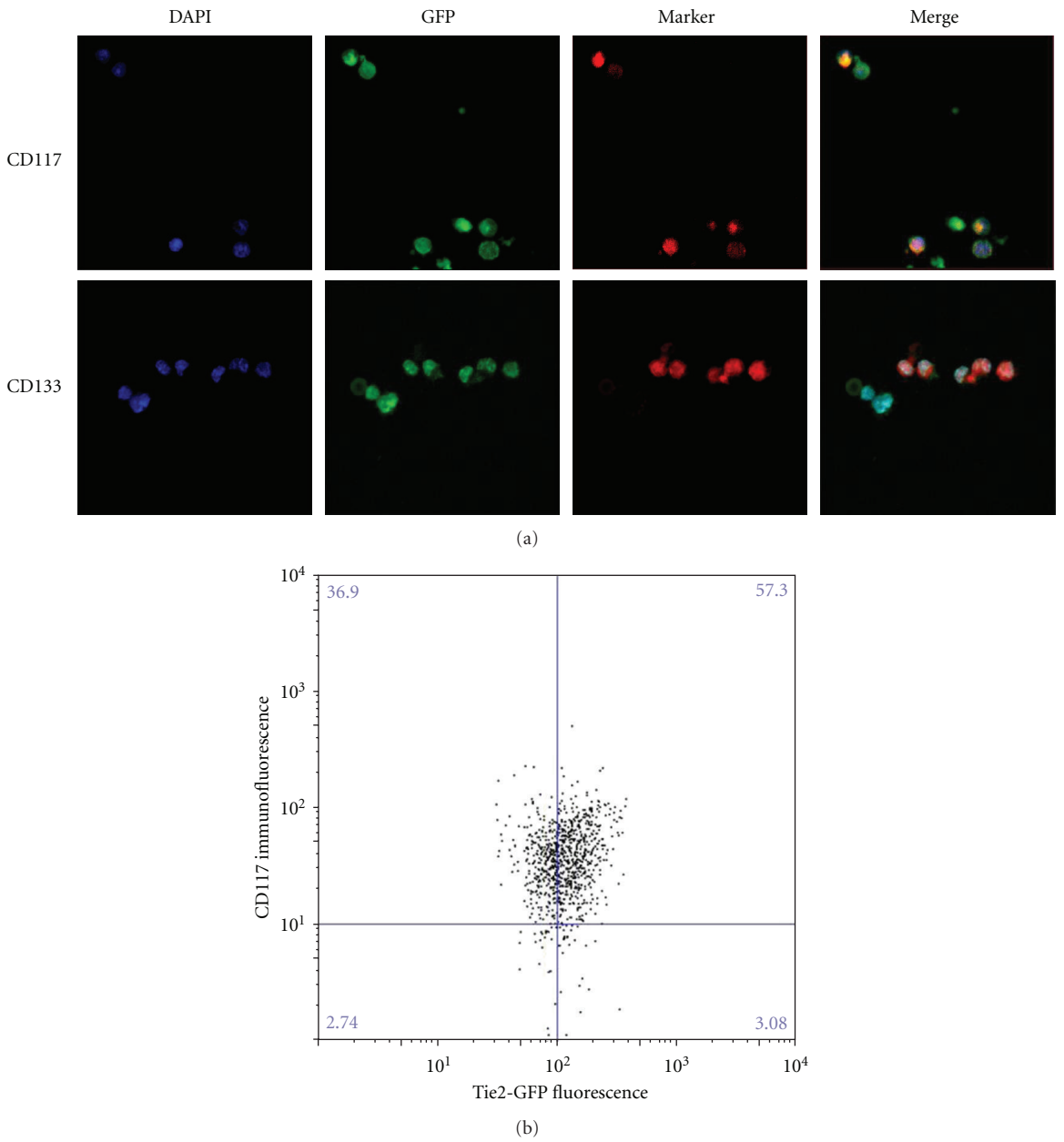


Figure 5: Characterization of circulating Tie2-GFP$^+$ cells as EPCs. (a) Tie2-GFP$^+$ cells from Tie2-GFP mice were sorted onto slides and immunostained for the EPC-specific markers CD117 and CD133 and counterstained with DAPI. Confocal microscopy was used to capture images at 40x magnification; (b) EPCs were fixed, sorted for GFP, then immunostained for CD117, and subjected to FACS. Most Tie2-GFP$^+$ cells stained positive for CD117, indicating the majority of circulating endothelial cells are EPCs.

major public health quest to arrest and prevent neovascular complications associated with diabetes. Although several anti-VEGF therapies have displayed beneficial effects for the treatment of diabetic retinopathy, patients often become refractive to anti-VEGF therapy. Thus, more drugs with different molecular mechanisms need to be developed. Vasculogenesis was previously considered to occur primarily during physiological development of vasculature, but recent evidence indicates that EPC-mediated vasculogenesis also contributes to pathological neovascularization, including retinal neovascularization [4, 5, 7, 20]. Thus, blockade of the release of EPCs from the bone marrow into the circulation represents a new target for pharmacological intervention of pathological neovascularization.

Endogenous antiangiogenic proteins have been intensively studied over the past few years due to their therapeutic potential in the treatment of neovascular disorders, including proliferative diabetic retinopathy [25]. Their regulatory roles in angiogenesis have been well established *in vitro* and *in vivo* [12, 15, 26, 27], although their effects on circulating endothelial cells and EPC-mediated vasculogenesis have not been investigated previously. The data presented herein

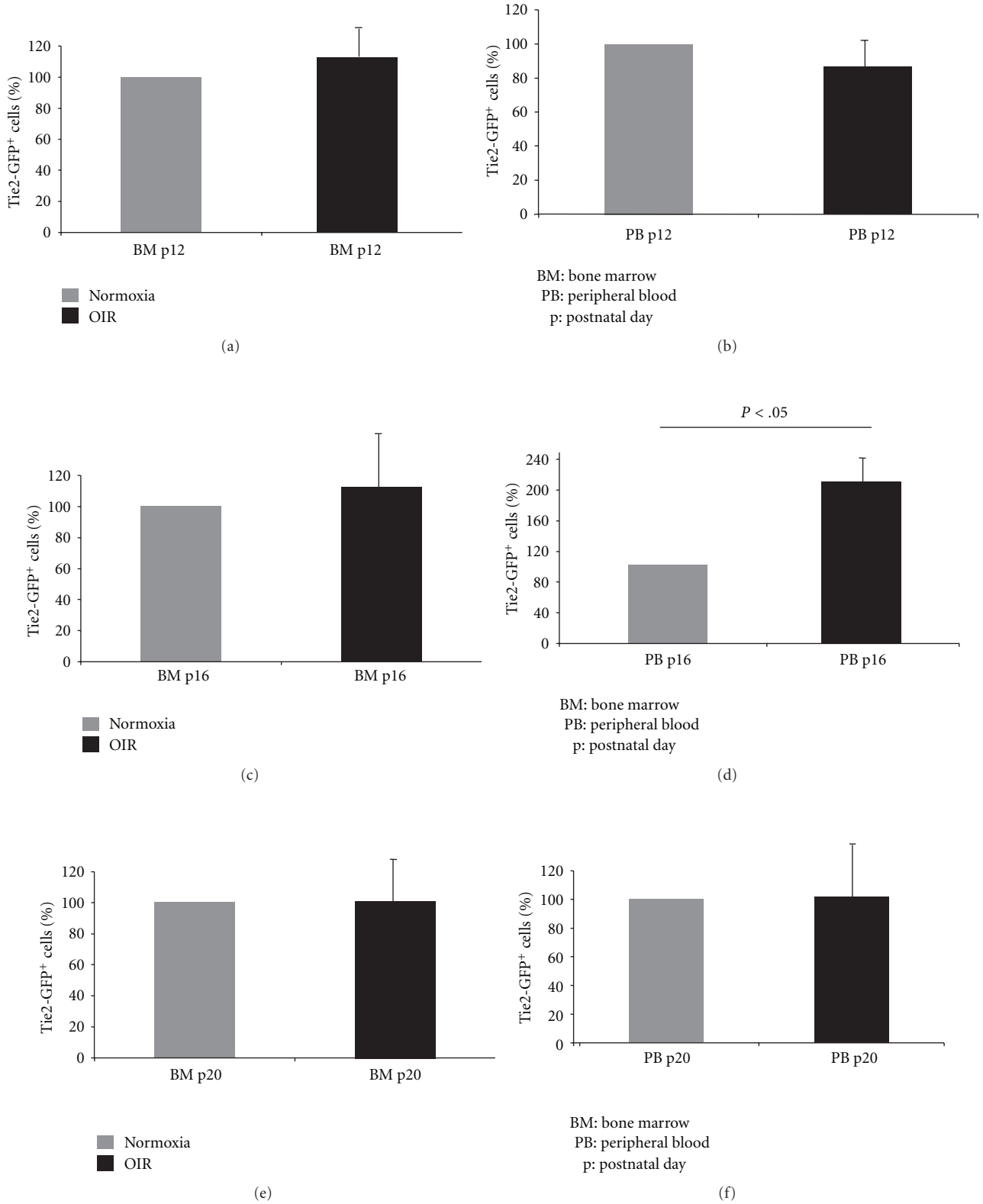


Figure 6: The number of circulating endothelial cells and EPCs increases in OIR mice at P16. Tie2-GFP$^+$ cells in both the bone marrow (BM) and peripheral blood (PB) from Tie2-GFP mice were quantified by FACS at p12, p16 and p20 under normoxic rearing conditions (grey), or in the OIR model (black). Graphs represent the percent of Tie2-GFP$^+$ cells based on 100% being set to the average number of Tie2-GFP$^+$ cells from normoxic mice.

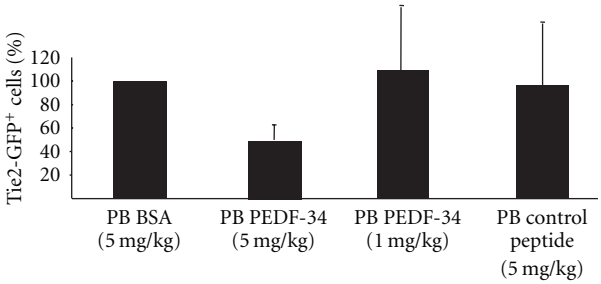


Figure 7: PEDF-34 reduces the number of circulating endothelial cells and EPCs in OIR mice. In the OIR model, Tie2-GFP pups received a daily i.p. injection of either PEDF-34, BSA, or a control peptide in PBS at a dose of 5 mg/kg of body weight from P12 to P16. At P16, Tie2-GFP$^+$ circulating endothelial cells and EPCs in peripheral blood were quantified by FACS. The graph represents the percentage of Tie2-GFP$^+$ cells in peripheral blood, based on 100% being set to the average number of Tie2-GFP$^+$ cells from OIR mice injected with BSA alone.

demonstrates that a 34-amino acid peptide fragment derived from the N-terminus of the angiogenic inhibitor PEDF inhibits circulating endothelial cells and EPCs during the pathogenesis of retinal neovascularization in the OIR model.

The OIR model is commonly used to study retinal neovascularization [18], because it has a well-characterized and highly reproducible course of retinal neovascularization. In the OIR model, transient exposure to hyperoxia from P7 to P11, followed by return to normoxia at P12 causes ischemia-induced retinal neovascularization that involves increased VEGF expression, decreased PEDF expression, and increased vascular leakage [3, 15]. Previous studies have shown that these features of retinal neovascularization are transient and peak at P16–P18. The present study is the first to demonstrate that the number of circulating endothelial cells and EPCs also peaks at P16 during OIR, correlating with the peak in VEGF expression and the most aggressive stage of retinal neovascularization. This correlation provides strong evidence that the increase in circulating endothelial cells and EPCs contributes to the retinal neovascularization in the OIR model. In contrast, EPC abundance in bone marrow is not changed in OIR mice at any of the time points analyzed. However, it is likely that bone marrow-derived EPCs are mobilized and contribute to the spike in circulating endothelial cells and EPCs, and that their numbers are so quickly replenished in the bone marrow that no mobilization-induced dip in bone marrow EPC numbers is observed. This theory is supported by our data which shows that most circulating endothelial cells also express EPC markers (Figure 5), and thus, are likely to have recently entered the circulation from the bone marrow.

Similar to previous studies of full-length PEDF, the systemic injections of PEDF-34 significantly reduced the progression of retinal neovascularization in the OIR mouse model. Furthermore, PEDF-34 blocked the OIR-induced spike in circulating endothelial cells at P16. This strongly suggests that PEDF-34 inhibits retinal neovascularization by targeting and reducing circulating endothelial cells, although the mechanism by which PEDF-34 reduces circulating endothelial cells and EPCs is unclear. Our finding that PEDF-34 inhibits cell proliferation and induces apoptosis in primary endothelial cell culture suggests PEDF-34 may directly target circulating endothelial cells and EPCs to induce apoptosis or inhibit cell proliferation. Another possibility is that PEDF-34 may block the release of EPCs from the bone marrow into the circulation. Regulation of EPC release is not well understood. However, VEGF is known to play an important role [6]. We measured VEGF levels in the plasma following treatment with PEDF-34 and found that VEGF levels in the plasma were not affected by systemic administration of PEDF-34 (Supplemental Figure 2). However, our previous studies have shown that PEDF competes with VEGF for binding to VEGF receptor 2 (VEGFR2) [24], which accounts for some of the antiangiogenic activity of PEDF. Thus, it is possible that PEDF-34 could bind to VEGFR2 on EPCs and impede VEGF signaling to reduce the VEGF-induced stimulation of EPC release without reducing VEGF levels. Alternatively, PEDF-34 may primarily target the existing vasculature and neovascular lesions to reduce the expression of cell adhesion molecules and soluble signaling molecules, such as $\alpha_5\beta_3$ and $\alpha_5\beta_5$ integrins, intercellular adhesion molecule 1 (ICAM1), and vascular cell adhesion molecule 1 (VCAM1), which act as recruitment signals for circulating endothelial cells and EPCs. In this case, the PEDF-34-mediated reduction in circulating endothelial cells and EPCs could be an indirect result of PEDF-34 targeting the vasculature to decrease expression of recruitment factors.

5. Conclusion

This study is the first to demonstrate that an antiangiogenic peptide, PEDF-34, reduces circulating endothelial cells during ischemia-induced neovascularization. This strongly suggests that the PEDF-34 peptide combines antivasculogenic activity and antiangiogenic activity in one peptide. Thus, the PEDF-34 peptide could be a superior biological therapeutic for the treatment of pathological neovascularization, such as proliferative diabetic retinopathy. Since PEDF-34 is a fragment from an endogenous human protein which exists in normal human tissues, and because the small PEDF-34 peptide can be generated in large quantity with high purity by solid phase synthesis, PEDF-34 has great potential for large-scale pharmaceutical development.

Acknowledgments

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Review Article

Cell-Based Therapies for Diabetic Complications

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In recent years, accumulating experimental evidence supports the notion that diabetic patients may greatly benefit from cell-based therapies, which include the use of adult stem and/or progenitor cells. In particular, mesenchymal stem cells and the circulating pool of endothelial progenitor cells have so far been the most studied populations of cells proposed for the treatment of vascular complications affecting diabetic patients. We review the evidence supporting their use in this setting, the therapeutic benefits that these cells have shown so far as well as the challenges that cell-based therapies in diabetic complications put out.

1. Introduction

The worldwide increase in the prevalence of diabetes mellitus reinforces the search for solutions to prevent it as well as to oppose the development and the progression of its complications. Particularly, the increasing prevalence of diabetes mellitus (DM) now affects adolescents and younger adults, thus promoting an earlier development of invalidating chronic diseases [1]. Experimental evidence suggests that cell-based therapies might represent a new and promising strategy for the treatment of diabetic vascular complications, and growing interest has recently been focused on mesenchymal stem cells and endothelial progenitor cells. Both cells types not only act against the mechanisms underlying diabetic complications but also rescue the abnormalities that stem cells present in diabetic patients, which contribute to the vascular complications. Notably, these cells avoid the ethical issues relating to the use of the embryonic cells. However, there are concerns about how the diabetic environment affects these cells. So, additional challenges for these cells include making them resistant to the diabetic environment and thus increasing their clinical efficacy [2].

On these premises, we will here review the evidence suggesting why adult stem/progenitor cells should be used in diabetic patients, the therapeutic benefits that these cells seem to offer for treating macrovascular and microvascular complications, and the challenges that cell-based therapies in DM present.

2. Stem Cells

Adult stem cells comprise of roughly 3 different groups: the bone marrow stem cells (BM-SC), the circulating pool of stem/progenitor cells (which are also derived from the bone marrow), and the tissue-resident stem cells. BM-SC can be further categorized into multipotent adult progenitor cells, mesenchymal stem cells (MSC), and hematopoietic stem cells. The circulating pool of stem/progenitor cells includes different types of cells, among which the most studied for the setting of vascular complications are the endothelial progenitor cells (EPC). EPC were identified by Asahara et al. [3] in the search for circulating angiogenic cells. They observed that these cells were able to form new blood vessels and promote neovascularisation after ischemia. Therefore, these cells seem to be the most promising in the setting of DM because of their potential utility in therapeutic neovascularisation and vascular repair. This paper will be focused on MSC and EPC, since these subsets of cells are the most studied in the field of the cell-based therapies for DM and for diabetic complications.

MSC are a subset of cells that express on their surface CD54/CD102 (intracellular adhesion molecule), CD166

(vascular cell adhesion molecule), CD49 (α-integrin) as well as CD73 (5′ ribonucleotide phosphohydrolase) and CD90 which also regulate cell-to-cell interactions. They also express CD44 (receptor for hyaluronic acid), CD105 (modulator of cellular responses to TGF-β), and MHC1, whereas they do not express CD34, CD14, CD45, CD11a/LFA-1, and CD31, which are surface markers featuring hematopoietic cells and/or EPC instead [4]. MSC are present in the bone marrow, but can also be found in many other fetal and adult tissues. Indeed, they are generally isolated from bone marrow, adipose tissue, umbilical cord blood, and compact bone. MSC display a great therapeutic potential because, beyond their capability to differentiate into muscle, neural precursors, cardiomyocytes, and other cells types, they are able to migrate and home in injured sites, where they act both by regenerating tissues and by secreting trophic factors and paracrine mediators. Moreover, these cells interact with the immune system, particularly with dendritic cells, T cells and NK cells and therefore they modulate the outcome of immune cells responses, apparently by inhibiting TNF-α and INF-γ and by increasing IL-10 [5]. Therefore, their unique immunomodulatory properties make these cells appropriate for both autologous and allogenic transplants, since they avoid and/or actively suppress the immunological responses that cause rejection of transplants. For the same reason, they are now being studied for the treatment of immunological diseases, among which is type 1 DM [6]. Indeed, in the non obese diabetic mice "NOD mice", the injection of MSC reduced the capacity of diabetogenic T cells to infiltrate pancreatic islets, thus preventing β-cell destruction [7]. Another model of type 1 DM is injecting mice with streptozotocin, which is a drug destroying the β-cells [8]. Also in this model, MSC were able to differentiate into insulin-producing cells releasing insulin in a glucose-dependent manner and improving the natural history of diabetes [9, 10]. Moreover, it has been demonstrated that, when cotransplanted with islets, MSC improved graft morphology and function by the promotion of revascularization [11].

EPC are adult hemangioblast-derived cells [12], which are characterized by the expression of CD34, vascular endothelial growth factor receptor 2 (VEGFR-2), and CD133, which has been included as marker expressed on primitive cells but not on differentiated ones. In fact, as the hemangioblasts destined to become endothelial cells differentiate, they downregulate the hematopoietic cells marker $CD133^+$ (AC133) expression [12]. EPC can be isolated from human peripheral or umbilical cord blood and can also be found in bone marrow niches. The interest in EPC comes from the fact that these cells have been shown to have direct angiogenic actions and/or to be able to support angiogenesis. Particularly, like for MSC, part of their therapeutic potential could be related to their ability to secrete paracrine mediators. In this respect, several studies have shown that these cells release interleukins, growth factors, and chemokines that altogether regulate $CD14^+$ cells, accelerate vascular network formation, and enhance healing processes [2]. Therefore, they are a promising therapeutic tool in the setting of diabetic complications, which are a consequence of dysfunctional vascular responses.

3. Rationale for the Use of Adult Stem/Progenitor Cells for Diabetic Complications

Diabetic patients exhibit impaired mobilization of adult stem cells from the bone marrow [13] and dysfunctional circulating progenitor cells [14, 15]. A growing body of evidence has demonstrated that DM is associated with a generalized reduction in circulating EPC and that this decline is linearly correlated with the severity of DM, in terms of HbA1c and blood glucose, whereas it is inversely related to glucose control [16–18]. Busik and colleagues suggested that diabetic neuropathy, altering the circadian rhythm of bone marrow cells release, could be one of the factors accounting for the defective mobilization of stem/progenitor cells coupled to an increased number of cells trapped in the bone marrow [19]. Apart from diabetic neuropathy, the factors that have been classically related to impaired stem/progenitor cells mobilization are the direct and/or indirect effects of hyperglycemia. Fadini and colleagues have demonstrated that the bone marrow mobilization of cells is sensitive to hyperglycemia [13]. Using a model of hind limbs ischemia-reperfusion (I/R) injury for the study of EPC mobilisation in type 1 DM, they observed that diabetic rats were completely unable to mobilise EPC after I/R injury, compared to the control rats showing a mobilisation curve within 7 days after injury. However, after insulin administration and premedication with granulocyte-colony stimulating factor (G-CSF) and other stem cells factors, they achieved a partial recovery in postischemic EPC mobilisation [13]. This study suggests that mobilization mechanism is sensitive to chronic hyperglycemia and early on remains reversible.

One of the mechanisms involved in the toxic effects of hyperglycemia on BM-SC seems to be the unbalance between nitric oxide (NO) and reactive oxygen species (ROS) [20]. It is known that hyperglycemia increases ROS formation which, by reacting with NO, lead to a reduction in NO bioavailability, therefore impairing NO signalling. Moreover, diabetic BM-SC display uncoupled endothelial NO synthase (eNOS) activity, promoting the production of ROS and so increasing the unbalance between ROS and NO [20]. Any reduction in NO bioavailability is believed crucial for BM-SC mobilization since NO-mediated signalling is essential for activation of MMP-9 which, in turn, shifts resident cells from a quiescent to a proliferative state and stimulates their rapid mobilization into the circulation [21]. Consistent with this concept, Segal and colleagues showed that incubating diabetic $CD34^+$ cells with NO donors corrected their migratory defect, proving that impaired NO signalling in DM significantly contributes to bone marrow dysfunctional responses [22]. It is reasonable to suggest that MSC migratory properties could also be affected in DM. Diabetic patients display increased circulating levels of osteoprotegerin (OPG) [23], which is a soluble TNF-receptor with atherogenic [24] and diabetogenic [25] actions. Notably this peptide is the decoy receptor for the TNF-related apoptosis-inducing ligand (TRAIL) and displays antiatherosclerotic and antidiabetogenic properties. Our group has recently shown that TRAIL

is able to promote the migration of BM-MSC *in vitro* [26]. OPG dose dependently neutralizes the promigratory activity of TRAIL [27], so the high levels of OPG observed in diabetic patients might impair the pro-migratory signalling driven by TRAIL, accounting for the abnormalities of BM-SC in DM.

Several *in vitro* works have pointed out that the diabetic *milieu* does not only impair BM-SC mobilization, but it also affects the lifespan and the functions of adult stem cells which may account for the reduction in circulating EPC. Particularly, hyperglycemia has been shown on its own to accelerate the senescence of EPC by the activation of p38/MAPK [28] and Akt/p53/p21 [29] pathways or by downregulation of sirtuin 1 [30]. In this setting, the senescence of EPC could also be due either to the NO reduced bioavailability mentioned previously, since it has been demonstrated that NO delays endothelial cells senescence through the activation of telomerase [31], or to the increased apoptosis induced by ROS. It has indeed been demonstrated that the deletion of p66ShcA, which is a gene regulating the apoptotic responses to oxidative stress, rescues the EPC defects induced by hyperglycemia [32]. However, in a work aimed at defining cross-sectionally the time course of EPC alterations in type 2 DM and to identify potential mechanisms of progenitor cells reduction, Fadini and colleagues found that the lower the count of $CD34^+$ cells the higher their apoptotic rate but also that there was no difference in the apoptotic rate between patients with and without DM and that the percentage of EPC apoptosis was too low to fully explain a decreased cell count [33]. Thus, *in vivo* studies have not confirmed yet if diabetic EPC have a shortened lifespan, and other mechanisms, apart from the reduced lifespan, may account for the reduction of these cells in DM. Likewise, when cultured in hyperglycemic conditions, MSC increase the production of intracellular ROS which reduce hypoxia-induced factor1α (HIF1α) expression and consequently attenuate hypoxia-induced vascular endothelial growth factor (VEGF)-A and platelet-derived growth factor (PDGF)-B transcription [34]. Moreover, it is well known that hyperglycemia leads to nonenzymatic glycosylation of proteins and subsequent formation of advanced glycation end products (AGEs) that interacting with their own receptor, RAGE, then activate several intracellular pathways ultimately leading to tissue damage [1]. In this setting, AGEs directly impair the reparative function of both EPC and MSC, and several works have evaluated AGEs deleterious effects on EPC [35–37] as well as on MSC. After isolation of MSC from rats with type 1 DM, Stolzing and colleagues studied their *ex vivo* ability to proliferate and differentiate into the fibroblastic colony-forming unit. They reported that colony size and number were significantly reduced in diabetic rats, mainly because of the induction of cell apoptosis and senescence by AGEs [38]. Consistent with this, when treated with glyceraldehydes and glycolaldehydes, MSC showed reduced cell proliferation, increased cell apoptosis, and impaired differentiation into adipogenic, chondrogenic, and osteogenic clones. These effects were partially prevented by the antiserum against RAGE [35, 39].

Altogether these experimental works demonstrate that DM affects the mobilization and the functions of adult stem cells; therefore they provide the rationale for the use of adult stem cells for diabetic complications.

4. Adult Stem/Progenitor Cells for the Treatment of Macrovascular Complications and Diabetic Cardiomyopathy

4.1. Macrovascular Complications. Both type 1 and type 2 DM increase the incidence and progression of atherosclerosis [40] into large arteries and the development of macrovascular complications. Their major clinical manifestations are coronary artery disease (CAD), peripheral artery disease (PAD), and stroke. In particular, patients with DM have a 2–4 fold increased risk of fatal myocardial infarction, PAD and stroke, together with poorer long-term outcomes [40, 41]. The evidence supporting the utility of cell-based therapies in this setting, and particularly EPC-based therapies, comes from clinical studies showing an inverse relation between the number of EPC and the occurrence of cardiovascular diseases (CVD). Consistent with the reduction of $CD133^+$cells observed in patients with CVD, $CD34^+/VEGFR\text{-}2^+$ and $CD133^+$ cells counts have indeed been shown to predict the occurrence of CVD in one-year follow-up studies [42, 43], whilst $CD34^+$ and $CD34^+/KDR^+$ cells counts might be helpful in stratifying the cardiovascular risk of the patients [44]. As expected, in patients with DM and metabolic syndrome, circulating $CD34^+$ cell numbers were also found to be an independent risk marker of CVD [45], leading to the hypothesis that the reduction in circulating progenitors is not only a marker but also a causative factor for the increase in cardiovascular events. Interestingly, significantly lower numbers of EPC were observed in diabetic patients when PAD had developed [46].

Although a study by Ma and colleagues showed that the treatment with EPC reduced the stenosis obtained after denudation of the common carotid artery in rabbits [47], data on the utility of cell-based therapies to prevent atherosclerosis are indeed conflicting. Silvestre and colleagues have demonstrated that transplantation of BM-SC in ischemic Apolipoprotein E-knockout mice, which is the most largely used animal model for the study of atherosclerosis [41], disappointingly accelerated atherosclerosis without altering the plaque composition [48]. Moreover, smooth muscle progenitor cells have been shown to contribute to the exaggerated intimal hyperplasia found in DM [49]. Consistent with this, in the clinical trials evaluating cell-based therapies after myocardial infarction, one of the major side effects that have been observed was the aggravation of the restenosis [50]. In this setting, another issue that needs to be further investigated is whether arrhythmias are a real safety concern, given that a higher number of arrhythmic events have been reported after intramyocardial delivery of cells, particularly skeletal myoblasts [51]. However, the trials aimed at myocardial repair in patients with acute myocardial infarction have also proven that the intracoronary infusion of BM-SC or $CD133^+$or MSC is associated with an improvement in the global left ventricular ejection fraction, a reduction in the end-systolic left ventricular volumes, and

a better perfusion in the areas of infarction [52, 53]. These effects are supposed to be due, at least in part, to the ability of these cells to stimulate myocardial repair/regeneration and neovascularisation (Figure 1).

Cell-based therapies appear promising also in the setting of PAD. A growing body of evidence strongly suggests the utility and effectiveness of adult stem cells for therapeutic neovascularisation both in absence [54–57] and in presence [58–60] of DM. Diabetic PAD is a systemic disease characterized by occlusion of peripheral arteries together with a severe impairment in the development of collateral vessels believed to be caused by endothelial dysfunction and the lack of growth factors, such as VEGF, both driven by glucotoxicity [1, 40]. The ability of EPC and MSC to produce angiogenic factors (by restoring the physiological levels of VEGF and HIF1α) and to differentiate into vascular cells in the periphery [61] has been implicated in the recovery of the native blood flow in ischemic hind limbs after their use. Recently, the transplantation of MSC for therapeutic neovascularisation has also been proven beneficial in type 1 diabetic patients with bilateral upper extremity digital gangrene, demonstrating improved arterial perfusion, good healing of all amputation sites, and cessation of pain [62].

Furthermore, in the context of macrovascular complications, intravenous autologous MSC transplantation has been shown to be able to reduce the mortality rate in patients with ischemic stroke [63].

4.2. Diabetic Cardiomyopathy. Diabetic cardiomyopathy should be considered separately from the so-called macrovascular complications of DM, since it corresponds to the stage characterized by the development of ventricular dysfunction in patients affected by DM, in the absence of CAD, valvular heart disease, or hypertension [64]. Its features, which are heterogeneous, are mainly due to cell apoptosis [64] associated with a dramatic reduction in tissue-resident stem cells [65], extensive myocardial fibrosis, and capillary rarefaction [66]. In particular, it has been shown that the abnormal myocardial matrix deposition associated with DM relies on increased collagen synthesis and on its reduced degradation, whose main effectors are the metalloproteases (MMP). Consistent with this, the diabetic myocardium is characterized by decreased activity of MMP-2, leading to increased collagen accumulation, and increased activity of the apoptotic factor MMP-9 which is responsible for apoptosis of endothelial cells, reduction of capillary density, and poor myocardial perfusion instead. In a study on rats with type 1 DM, the intravenous infusion of MSC improved cardiac function through increased angiogenesis and attenuated cardiac remodelling. Eight weeks after the induction of DM, rats were infused with MSC, which then homed into the myocardium and led to increased myocardial arteriolar density and decreased collagen content in the diabetic myocardium. Interestingly, increased MMP-2 activity and decreased transcriptional level of MMP9 were also reported [67]. However, even more fascinating is the possibility of developing noninvasive cell-based therapies relying on the trophic activities of MSC (Figure 1). A recent study with a hamster heart failure model has demonstrated that an intramuscular delivery of MSC would be sufficient to significantly improve ventricular function, enhancing capillary and myocyte densities, attenuating apoptosis, and reducing fibrosis. This was reported to be due to a trophic cross-talk among the injected MSC, the bone marrow, and the heart [68].

5. Adult Stem/Progenitor Cells for the Treatment of Microvascular Complications and Wound Healing

5.1. Diabetic Nephropathy. Diabetic nephropathy is now the most common cause of end-stage renal failure in the Western societies. The arterial damages and the changes to the glomerular ultrastructure, mainly mesangial expansion and glomerular membrane thickening, are the principal mechanisms causing diabetic nephropathy [1]. These effects are both driven by hyperglycemia, and thus it is not surprising that one of the most important interventions in preventing diabetic nephropathy, or attenuating it, can be achieved by tight glycemic control [69]. In this setting, it has been shown that EPC mobilize into damaged glomeruli [70], possibly participating in glomerular capillary regeneration. More recently, a subset of hematopoietic stem cells, featured by the expression of the surface molecules $CD24^+/CD133^+$, has been shown promising as it was able to regenerate both tubular cells and podocytes. This is quite significant because the depletion of these cells plays a crucial role in the development of glomerulopathies which are now believed to be podocytopathies [71]. However, in the context of cell-therapy approaches for diabetic nephropathy, the most attractive candidates seem to be the MSC. So far, several works have shown that MSC administration can both prevent and treat diabetic nephropathy. In mice with type 1 DM [72], MSC had the ability to induce β pancreatic islets regeneration with consequent achievement of a better glycemic control that, in turn, prevented the development of diabetic nephropathy. MSC also had the ability to slow the progression of diabetic nephropathy through mechanisms independent from glycemic control [73] (Figure 2). Indeed, after an infusion of MSC, 11% of these cells engrafted into the kidneys, where they differentiated into endothelial cells and possibly mesangial cells. This was associated with a significant decrease in mesangial thickening, extracellular matrix deposition, and macrophages infiltration [74].

5.2. Diabetic Neuropathy. Diabetic neuropathy is estimated to affect over half of the patients with DM. It is a form of neuropathy that affects the somatic and autonomic divisions of the peripheral nervous system, but the spinal cord and the higher central nervous system can also be damaged. The main underlying cause is glucotoxicity and its downstream effects [1]. High glucose levels, oxidative stress, and AGEs reduce nerve blood flow and impair neurotrophic support, altogether leading to neural cells degeneration. Cell-based approaches promoting endogenous production of neurotrophic factors, such as nerve growth factor (NGF),

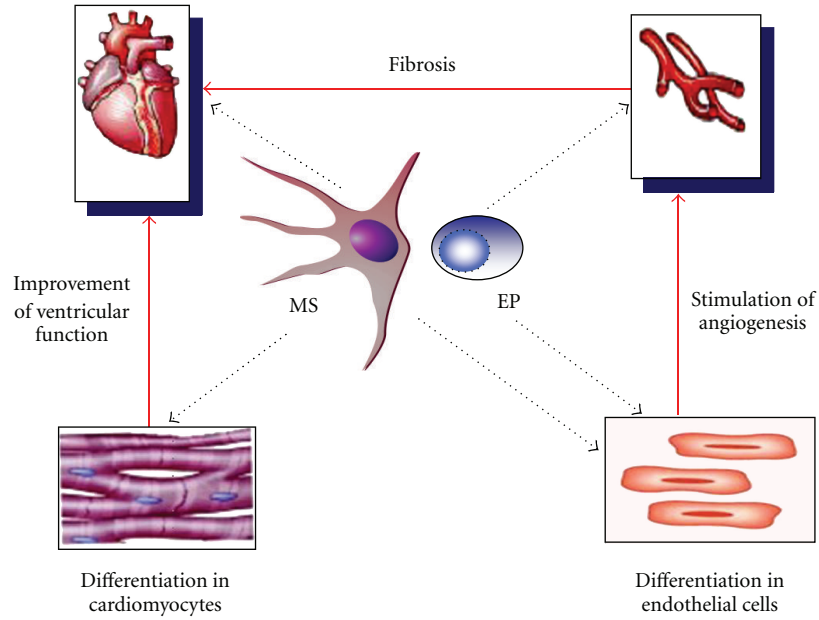

Figure 1: Effects of MSC and EPC on myocardial repair/regeneration and angiogenesis. The activities of MSC and EPC may derive from their differentiative ability (into cardiomyocytes and/or endothelial cells) as well as from secretion of paracrine mediators promoting myogenesis, angiogenesis, and heart functionality, in direct and/or indirect manners.

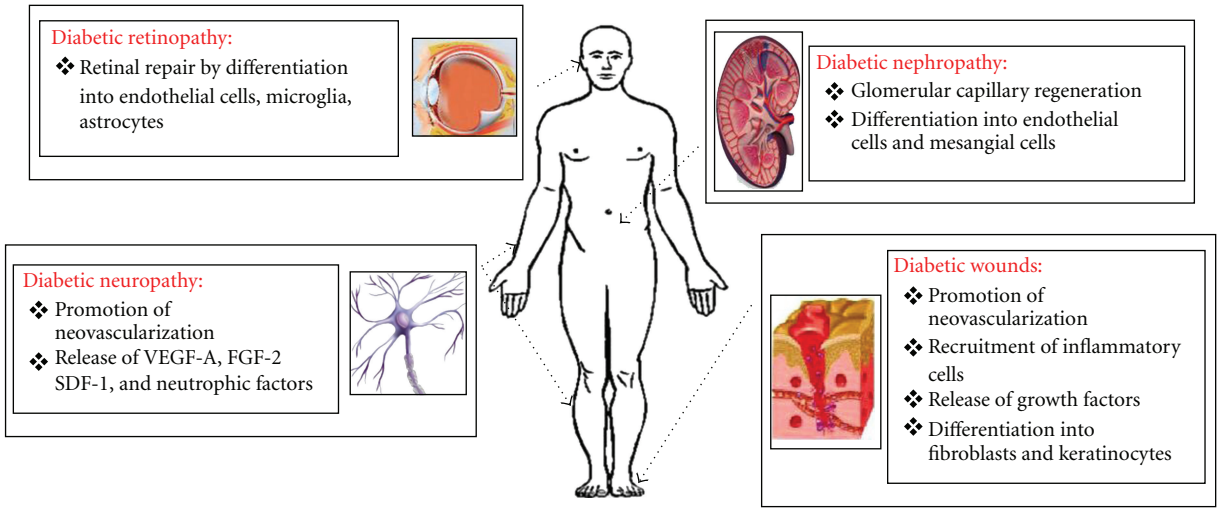

Figure 2: Potential role of EPC and MSC in the control of diabetic microvascular complications and wound healing. Diabetes mellitus is characterized by microvascular complications (retinopathy, nephropathy, and neuropathy) and prolonged/incomplete wound healing. Cell-based therapies may control these complications by different potential mechanisms.

hepatocyte growth factor (HGF), neurotrophin 3, or VEGF, have recently shown some success [1] (Figure 2).

In the setting of diabetic neuropathy, both EPC and MSC have been investigated. Many studies have shown that transplantation of EPC ameliorates the blood flow to peripheral ischemic tissues. Naruse and colleagues investigated whether a unilateral intramuscular injection of EPC into the hind limb skeletal muscles could ameliorate diabetic neuropathy. After such transplantation, more vessels were observed in the injected sites, and this was associated with an amelioration of sciatic nerve blood flow and motor nerve conduction velocity. These data suggest that the ability of EPC to treat diabetic neuropathy is due to the promotion of therapeutic neovascularisation [75] (Figure 2). In addition, in another study, intramuscularly injected EPC not only increased nerve blood flow but also increased the number of vasa nervorum ameliorating the microvascular insufficiency typical of diabetic neuropathy [76]. As a matter of interest, in the same study, EPC were also found to increase the production of VEGF-A, brain-derived neurotrophic factor, fibroblast growth factor (bFGF)-2, stroma-derived factor-1 (SDF-1), and other neurotrophic factors, therefore indicating

that EPC may reverse various manifestations of diabetic neuropathy through both angiogenic and neurotrophic properties (Figure 2). Consistent with this, *in vitro* studies proved that EPC were able to make Schwann and endothelial cells proliferate and to reduce the number of apoptotic cells [76].

Also MSC have displayed angiogenic and neurotrophic properties. Four weeks after their intramuscular injection, MSC started producing bFGF and VEGF, and this was associated with an increased ratio between capillaries and muscular fibers, an increased blood flow to the sciatic nerve, an improvement in motor nerve conduction velocity, and a reduced hyperalgesia [77]. Although one of the most exciting properties of MSC, from a therapeutic perspective, is their ability to differentiate into multiple cellular phenotypes, any differentiation into neural cells, such as astrocytes, oligodendrocytes, and Schwann cells, has not yet been observed [77] (Figure 2).

5.3. Diabetic Retinopathy. DM is the leading cause of cases of blindness among adults. Diabetic retinopathy is characterized by a complex of vascular and retinal lesions, all ultimately due to hyperglycemia. This disorder can be categorized into the non proliferative diabetic retinopathy (NPDR) and the proliferative diabetic retinopathy (PDR) [1]. NPRD is characterized by vascular changes leading to retinal ischemia, whereas PDR is the result of an aberrant reactive neovascularisation. Most works carried out in this field have featured EPC, reporting a reduced number of EPC in patients with NPDR [78, 79] but an increased number of EPC in those with PDR. This is consistent with the hypothesis that, since EPC can rescue and maintain the existing retinal capillary bed in healthy patients, the reduced number of EPC observed in DM might predispose to NPDR [2]. Once the damage is initiated, an inflammatory reaction will take place and the bone marrow will respond by increasing the mobilization of EPC, which will eventually result in an abnormal neovascularisation leading to PDR. To date, the studies carried out on ischemic retinal injury have documented the participation of adult stem cells in the retinal repair, showing their ability to home into damaged areas and to differentiate into endothelial cells, microglia, and astrocytes [80–82] (Figure 2). However, these studies were all conducted in animal models of ischemic retinal injury and so concern still remains about the viability of these therapeutic options in the long run, since they could worsen the aberrant reactive neovascularisation featuring the PDR that follows any ischemical retinal injuries. Therefore, PDR may represent a contraindication for angiogenic cell-based therapies.

5.4. Wound Healing. Another common complication of DM is represented by the prolonged and incomplete wound healing, caused by compromised angiogenesis, diminished cells recruitment, lack of growth factors, and impaired formation of collagen matrix. It has been demonstrated that generally the number of MSC increases considerably in the site of an injury, and that after a vascular trauma a rapid mobilization to the injured site of EPC also takes place. Wound healing normally results from a combined effort of inflammatory and noninflammatory cells recruited to the injured site. Recent studies suggest that MSC and EPC are a significant proportion of the noninflammatory cells that migrate to the skin. In DM, the number of EPC within the granulation tissue has been found to be significantly reduced with respect to non diabetic controls [83] and locally increased apoptosis and decreased proliferation of these cells have also been reported. Several works have shown that MSC accelerate wound closure by differentiating into fibroblasts and keratinocytes, and promoting neovascularisation and regeneration of appendages and recruiting inflammatory cells into wounds [84, 85] (Figure 2). Transplantation of EPC has also been shown to enhance wound healing in mice [86], and this seems to rely on the release of paracrine mediators, such as the release of VEGF, HGF, G-CSF, and PDGF [84, 87]. As expected, in the setting of DM, the same mechanisms, mentioned above for MSC and EPC, have been shown to enhance wound healing [88, 89] and to also be an effective treatment of foot ulcerations [90–92] (Figure 2).

6. Genetic Manipulation and Pharmacological Strategies Aimed at Reversing the Alterations of Adult Stem/Progenitor Cells in Diabetes

6.1. Genetic Manipulation. The evidence obtained so far makes for a compelling argument for the use of MSC and/or EPC in the setting of DM [2]. Because of the broadly dysfunctional cell functions found in DM, it is believed that cells to be used for treatment of diabetic complications should be equipped with cellular and molecular tools to make them withstand the *in vivo* diabetic *milieu*. Thus, studies into the genetic modification and/or manipulation of diabetic cells have commenced as approaches in overcoming this issue. In recent work, Marrotte and colleagues [93] transfected EPC with the gene of manganese superoxide dismutase, in order to correct its decreased expression found in diabetic EPC. They found that, after this *ex vivo* manipulation, the EPC transplanted contributed significantly to the accelerated wound healing in a type 2 DM animal model. So far, several molecules have been targeted, such as human telomerase reverse transcriptase (hTERT), which was shown to delay EPC senescence [94], and the glycogen synthase kinase 3-β, which enhanced the EPC vasoregenerative potential [95]. In MSC, the overexpression of GATA-4, CXCR4, and Akt-1 led, respectively, to increased cell survival and angiogenesis [96], enhanced *in vivo* mobilization into ischemic areas [97], and better functional repair in a mouse infarct model [98]. Although the genetic manipulation of adult stem cells dysfunctions in DM has shown promising results, one should be very cautious when adopting this approach because of its potential side effects. For instance, targeting senescence/survival regulatory pathways warrants greater understanding given the risk of malignant transformation of the cells.

6.2. Pharmacological Strategies. Other approaches have pharmacologically targeted the intracellular dysfunctions that

take place in DM. For example, the effects of AVE9488 [99], GH [100], both stimulating eNOS, and those of rosiglitazone, which has antioxidant properties [101] have been studied as treatments for the reduced NO bioavailability [102]. Interestingly, Sorrentino and colleagues showed that the effect of rosiglitazone treatment was comparable to that of small-interfering RNA silencing NADPH oxidase subunit p47. Both approaches reduced NADPH oxidase activity, restoring NO bioavailability, and improved *in vivo* reendothelization capacity of EPC isolated from diabetic patients. However, whether increasing NO production and bioavailability may result in higher production of reactive oxygen species that will further increase oxidative stress leading to vascular damage is unknown yet. The blockade of p38/MAPK pathway, using its specific inhibitor SB203580, has also been assayed. Seeger and colleagues demonstrated that the *ex vivo* treatment of EPC with SB203580 was able to significantly ameliorate their revascularisation properties, possibly through the regulation of their proliferation and differentiation [103]. *Ex vivo* treatment of MSC with IGF-1 and IGF-2 made MSC regain the functions affected by DM [104]. Finally, antagonists of CXCR4 (such as AMD3100 and SDF-1βP2G), which disrupt the interaction between the CXCR4 receptor (on hematopoietic cells) and the CXCL12 (expressed by stromal cells), have already been shown promising in accelerating blood flow restoration in diabetic mice [105].

7. Conclusions

The past decade has provided new and fascinating *in vitro* and *in vivo* data supporting the use of MSC and EPC for the treatment of diabetic complications. However, among the issues raised, the possible contribution of these cells to lesion formation, in terms of atherogenesis, neointimal hyperplasia, and retinal aberrant angiogenesis, as well as the potential risk of their malignant transformation will certainly require further long-term analysis. Also, it is yet to define the best way to make these cells withstand the diabetic *milieu* in the long run. Therefore, a greater understanding of MSC and EPC biology, both in *in vitro* and *in vivo* studies, is needed to establish the safety of their use as a novel and efficient therapeutic agents in the treatment of complications of DM.

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